STRUCTURE ACTIVITY RELATIONSHIP STUDIES OF OCHRATOXIN A ANALOGUES.

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

SUMMARY

Mycotoxins have assumed worldwide importance due to the ubiquitous occurrence of toxigenic fungi, their infestation of plant-based foods and feeds and the subsequent economical and health impact it because of their contamination of commercial products. Ochratoxin A (OA) is a nephrotoxic mycotoxin produced by isolates of *Aspergillus ochraceus* and *Penicillium verrucosum* and occurs frequently in nature. The major target for toxicity of OA in mammalian species is the kidneys and it has been the major cause of Danish Porcine Nephropathy. OA has also been extensively implicated in the aetiology of Balkan Endemic Nephropathy and Chronic Interstitial Nephropathy in Northern-Africa. Furthermore, OA has been identified as a carcinogen, an immunosuppressant and a teratogen with respect to the foetal central nervous system.

Although a large amount of research has been conducted into the chemical nature of the toxicity of OA, the exact molecular mechanism of action of OA is not yet conclusive. Numerous structure activity relationship studies have suggested that the toxicity of OA may be assigned to three major processes: (i) inhibition of ATP production; (ii) inhibition of protein synthesis; and (iii) the disruption of hepatic microsomal calcium homeostasis through the promotion of membrane lipid peroxidation. It is the aim of this thesis to gain a better understanding, through the synthesis of OA analogues, of the chemical structure responsible for the toxic function of the ochratoxins.

The halogen-group has extensively been implicated in the toxicity of the ochratoxins. This is evident in ochratoxin B (OB), the dechloro analogue of OA, which is approximately ten times less toxic than OA. Preliminary tests have indicated that bromo-ochratoxin B(BrOB), the bromo analogue of OA, is more toxic than ochratoxin A to renal cells. Fluoro-ochratoxin B and other analogues of OA, where other amino acids are incorporated, should provide invaluable information on the structure-activity relationships and the mode of action of the ochratoxins. Our research effort addresses both these aspects (i) fluorination of the dihydroisocoumarin moiety and (ii) the

coupling of different amino acids and dipeptides to the non-toxic hydrolysed product of OA, ochratoxin α.

Chapter one includes a review of the important biological aspects of OA that has served as a guideline to the synthesis of effective OA analogues. An overview of the relevant chemistry involved in the modification of OA will conclude the chapter.

Chapter two entails a discussion of fluorine in bio-organic chemistry. This includes an overview of the impact that fluorine substitution has on the biological reactivity of molecules. A review on the synthesis of organofluorine compounds, which forms the emphasis of this study, concludes the chapter.

Chapter three elaborates on the different methodologies used in our attempts to synthesise fluoro-ochratoxin B and other analogues. These included the direct electrophilic fluorination of OB and different analogous aromatic model compounds by xenon difluoride, *N*-fluorobenzenesulfonimides and Selectfluor™ as fluorinating agents. Also involved is an investigation into an alternative route for the synthesis of fluoro aromatic compounds from bromo and chloro analogues by means of palladium catalysed trimethyl- and tributylstannyl and trimethylsilylation which in turn may be substituted with fluorine by means of xenon difluoride. Efforts towards the direct catalytic fluorosubstitution of aryl halides are also investigated. The synthesis of a key intermediate, fluoroacetoacetaldehyde, in a *de nova* synthetic route to fluoro-ochratoxin B is also discussed.

Furthermore, the synthesis of novel OA analogues with respect to the replacement of the L-phenylalanine moiety is addressed. This includes the conversion of OA to Oα, by acid hydrolysis, followed by the coupling of *ortho*-, *meta*- and *para*- substituted DL-fluorophenylalanine to the lactone acid. This is followed by the synthesis of histidylhistidine methyl ester and attempted coupling to Oα. The coupling of halosalicylic acids and salicylic acid to L-phenylalanine, for use as model aromatic substrates for fluorination, is discussed. Peptide coupling by dicyclohexylcarbodiimide carboxyl activation, with reference to the protection of the phenolic hydroxyl group in 5-chlorosalicylic acid for application to Oα, concludes this work.

OPSOMMING

Mikotoksiene is van wêreld-wye belang as gevolg van die alomteenwoordige voorkoms van toksigeniese fungi, hul besmetting van plantaardige kossoorte en voerstowwe en die gevolglike ekonomiese en gesondheidsimpak deur die besoedeling van kommersiële produkte. Ochratoksien A (OA) is 'n nefrotoksiese mikotoksien wat geproduseer word deur isolate van *Aspergillus ochraceus* en *Penicillium verrucosum* en kom algemeen in die natuur voor. Die niere is die hoof teiken vir vergifiting deur OA in soogdierspesies en is as die vername oorsaak van "Danish Porcine Nephropathy" aangewys. OA word verder aangedui as die oorsaak vir "Balkan Endemic Nephropathy" en "Chronic Interstitial Nephropathy" in Noord-Afrika. OA is verder geïdentifiseer as 'n karsinogeen, immuno-onderdrukker en is teratogenies ten opsigte van die sentrale senuweestelsel van fetusse.

Alhoewel aansienlike navorsing alreeds gewei is aan die chemiese natuur van die toksisiteit van OA, is die presiese molekulêre meganisme van OA reaktiwiteit onbeslis. Verskeie struktuur-aktiwitweit verwantskaps studies dui daarop dat die toksisiteit van OA hoofsaaklik toegeskryf kan word aan drie hoof prosesse: (i) inhibisie van ATP produksie; (ii) inhibisie van proteïen sintese; en (iii) die ontwrigting van hepatiese mikrosomale kalsiumhomeostase deur die bevordering van membraanlipiedperoksidasie. Hierdie tesis het ten doel, deur die sintese van OA analoë, om 'n beter insig oor die chemiese struktuur wat verantwoordelik is vir die toksiese funksionaliteit van ochratoksiene te verkry.

Die halogeen substituent is grootliks geïmpliseer in die toksisiteit van OA. 'n Bewys hiervan is ochratoksien B (OB), die dechlooranaloog van OA, wat ongeveer tien maal minder toksies is as OA. Voorlopige ondersoeke het aangetoon dat bromo-ochratoksien B (BrOB), die broomanaloog van OA, meer toksies is vir nierselle as OA. Fluoorochratoksien B en ander analoë van OA, waar ander aminosure geïnkorporeer word, behoort waardevolle inligting te voorsien met betrekking tot die struktuur-aktiwiteitsverwantskappe en die wyse waarop ochratoksiene funksioneer. Hierdie navorsingspoging spreek beide aspekte aan; (i) die fluorering van die

dihidroïsokumarien gedeelte en, (ii) die koppeling van verskillende aminosure en dipeptiede aan die nie-toksiese hidrolieseproduk van OA, nl. ochratoksien α.

Hoofstuk een vervat 'n oorsig van die belangrike biologiese aspekte van OA wat dien as riglyn vir die sintese van doeltreffende OA analoë. Die hoofstuk word afgesluit met 'n oorsig van die relevante chemie betrokke by die modifisering van die struktuur van OA.

Hoofstuk twee bevat 'n bespreking van die aanwending van fluoor in bio-organiese chemie. Dit bevat 'n oorsig van die impak wat fluoorsubstitusie het op die biologiese reaktiwiteit van molekules. 'n Opsomming oor die sintese van organofluoorverbindings, wat die essensie van hierdie studie is, beëindig die hoofstuk.

Hoofstuk drie handel oor die veskillende metodes wat toegepas is in pogings om fluoorochratoksien B en ander analoë te sintetiseer. Dit sluit in die direkte elektrofiliese fluorering van OB en ander verwante aromatiese modelverbindings deur gebruik te maak van xenondifluoried, *N*-fluoorbenseensulfonimied en Selectfluor™ as fluoreringsreagense. Dit behels verder ook 'n ondersoek na 'n alternatiewe roete tot die sintese van fluooraromatiese verbindings vanaf broom- en chlooranaloë. Vir die doel word palladiumgekataliseerde trimetiel- en tributielstannilering, en trimetielsililering wat vervolgens deur middel van xenondifluoried met fluoor gesubstitueer kan word, aangewend. Pogings tot die direkte katalitiese fluoorsubstitusie van arielhaliede word ook bespreek. Die sintese van 'n sleutel-intermediêr, fluoroasetoasetaldehied, in 'n *de nova* sintese roete tot fluoorochratoksien B word bespreek.

Die sintese van nuwe OA analoë, met betrekking to die vervanging van die L-fenielalanien (L-Phe) groep word ondersoek. Dit bevat die omsetting van OA na Oα, deur suurhidrolise, gevolg deur die koppeling van *orto*-, *meta*- en *para*-gesubstitueerde DL-fluoorfenielalanien aan die laktoonsuur, Oα. Daarna word die sintese van histidielhistidienmetielester en die verdere pogings aangaande koppeling met Oα bespreek. Die koppeling van halosalisielsure en salisielsuur aan L-Phe wat dien as model aromatiese verbindings vir fluorering, word behandel. Peptiedkoppeling met behulp van disikloheksielkarbodiimied-karboksielaktivering, met inbegrip van die

beskerming van die fenoliese hidroksiel groep in 5-chloorsalisielsuur vir die toepassing op Oα, beëindig hierdie werk.

ACKNOWLEDGEMENTS

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LIST OF ACRONYMS AND ABBREVIATIONS

Ala	Alanine
Asp	Aspartic Acid
Boc	*tert*-Butoxycarbonyl
BrOB	Bromo-Ochratoxin B
BrSAPhe	*N*-(5-bromo-2-hydroxyphenylcarbonyl)-L-phenylalanine
CPA	Carboxypeptidase
CDI	*N*,*N*-carbonyldiimidazole
CIN	Chronic interstitial nephritis
ClSAPhe	*N*-(5-chloro-2-hydroxyphenylcarbonyl)-L-phenylalanine
Cys	Cysteine
DBU	1,8-diazobicyclo[5.4.0]undec-7-ene
DCC	Dicyclohexylcarbodiimide
DIPCDI	Diisopropylcarbodiimide
DMF	Dimethylformamide
D-OA	D-Phenylalanine Oα
d-OA	Decarboxylated OA
DPN	Danish porcine nephropathy
EI-MS	Electron-impact mass spectrometry
ES-MS	Electrospray mass spectrometry
Fmoc	9-fluorenylmethoxycarbonyl
FSAPhe	*N*-(5-fluoro-2-hydroxyphenyl)carbonyl-L-phenylalanine
F-TEDA-BF_4	Selectfluor™
GABA	Gamma aminobutyric acid
Glu	Glutamic acid
HPLC	High performance liquid chromatography
IOB	Iodo-Ochtatoxin B
IR	Infrared spectroscopy
Leu	Leucine
L-Phe	L-Phenylalanine
Met	Methionine
NFBS	*N*-fluorobenzenesulfonimide
NFOBS	*N*-fluoro-*o*-benzenedisulfonimide
NHS	*N*-hydroxysuccinimide
NMR	Nuclear magnetic resonance
OA	Ochratoxin A
Oα	Ochratoxin α
Oα-Cl	Acid chloride of Oα
OA-NHS	*N*-hydroxysuccinimide ester of Oα
OB	Ochratoxin B
Oβ	Ochratoxin β
OD	Ochratoxin B ethyl ester
OH-Pro	Hydroxyproline
10-OH-OA	10-OH-Ochratoxin A
4-OH-OB	4(*R*,*S*)-OH-Ochratoxin B
OM-OA	Ochratoxin A-*O*-methyl ether
OE-OA	*N*-ethyl ochratoxin A-amide

OP-OA	Lactone opened ring OA
PPB	Pyridinium hydrobromide perbromide.
PheRS	Phenylalanyl-tRNA synthetase
SAPhe	*N*-(2-hydroxyphenylcarbonyl)-L-phenylalanine
Ser	Serine
SOD	Superoxide dismutase
TBAF	Tetrabutylammonium fluoride
TEA	Triethylamine
Thr	Threonine
Tryp	Tryptophan
Tyr	Tyrosine
TLC	Thin layer chromatography
UV	Ultra violet
UST	Urinary system tumours
Val	Valine

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CHAPTER 1

SECTION A: BIOLOGICAL ASPECTS OF OCHRATOXIN A AND OTHER MYCOTOXINS.

1.1 Introduction

All forms of life, including fungi, require exogenous material to incorporate into their biomass. Certain heterotrophs* such as molds require simple organic compounds for both the synthesis of cell constituents and to produce energy for these synthetic processes. These facets, which encompass a complex network of enzyme-catalysed reactions, are known as primary metabolism. In addition to the reactions of primary metabolism, reactions frequently occur which are non-essential to life, often characteristic to genera, species or strain and which may be considered as an expression of the uniqueness of the organism. These reactions are grouped under the term secondary metabolism and the products associated with it as secondary metabolites.

Secondary metabolites are distinguished from primary metabolites by their constrained distribution in plants and other organisms, there specificity to its producing organism and to the biochemical pathways it is derived of. An important characteristic of secondary metabolites is that they may be derived from primary metabolism and may be regarded as excretions, which have no vital importance as an energy source or as a mechanism of storage. The significance of secondary metabolites to their producers has been the source of much debate and numerous theories have been proposed.

Weinberg, 1971, rationalized the broad spectrum of pharmacological activity exhibited by secondary metabolites in lower to highly specialized organisms as factors participating in an ecological relationship between the producer and interacting organisms. Bu'Lock, 1965, proposed a maintenance hypothesis that gave emphasis to the regulatory role of secondary metabolites in metabolism, thus accentuating the greater importance of secondary metabolism than its products.

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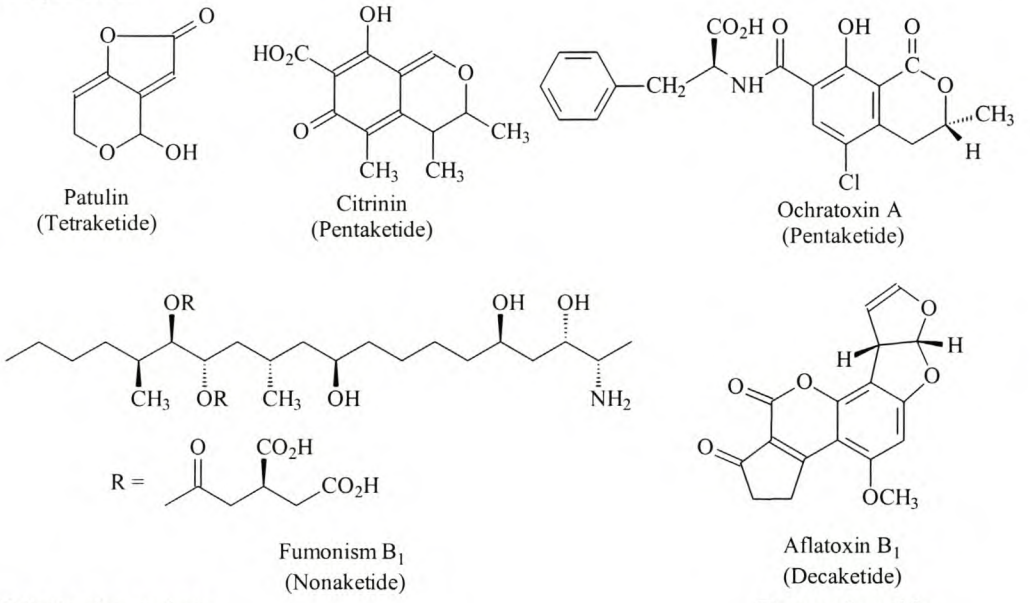
* Organisms that require an organic source of carbon.

Bu'Lock, 1980, reported on the possible relationship between the production of secondary metabolites and differentiation of the producing organism. This was illustrated in cultivated strains of *Aspergillus fumigatus* producing the antibiotic trypacidin in surface cultures. It was found that the production of metabolites in the individual cultivation vessel was directly proportional to the sporulation of the cultures. It was demonstrated by Parker and Jenner, 1968, that trypacidin was only isolated from spores and could not be found in the vegetative mycelium or growth medium.

One such class of secondary metabolites is the Mycotoxins. These naturally occurring toxicants are produced by a variety of fungal species and originate from different biosynthetic pathways. This results in a heterogeneous group of chemically diverse compounds (see Figure 1.1), manifested in living cells by a broad spectrum of biochemical effects.

Polyketides



Diketopiperazines **Tetramic acids**

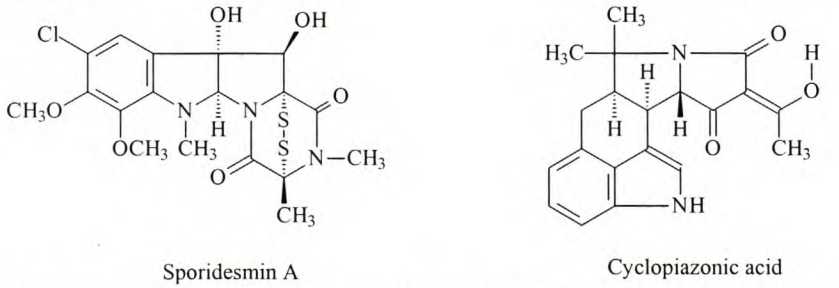
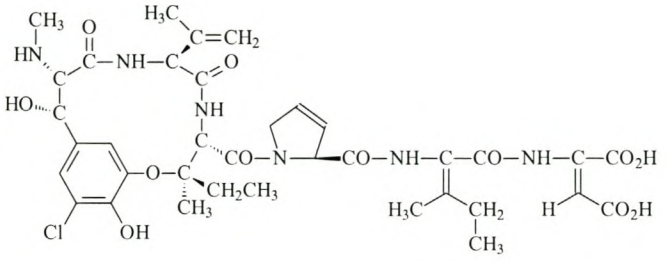
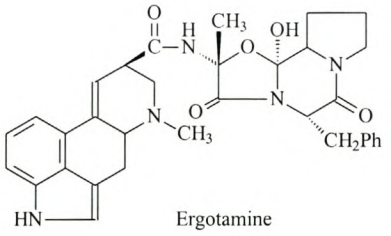


Figure 1.1 Mycotoxins as representatives of the main biosynthetic categories of secondary metabolites.

Peptides

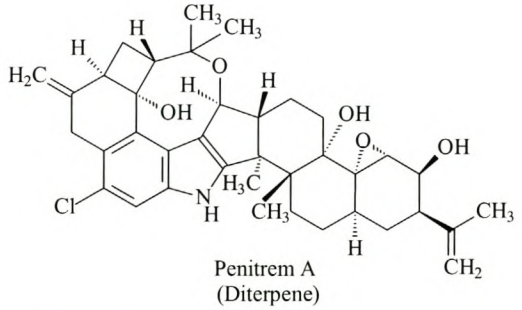


Phomopsin A

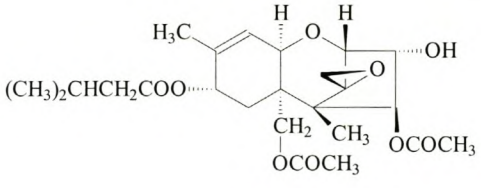


Ergotamine

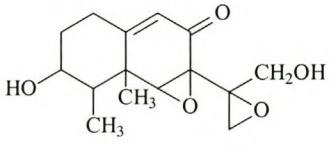
Terpenes



Penitrem A
(Diterpene)



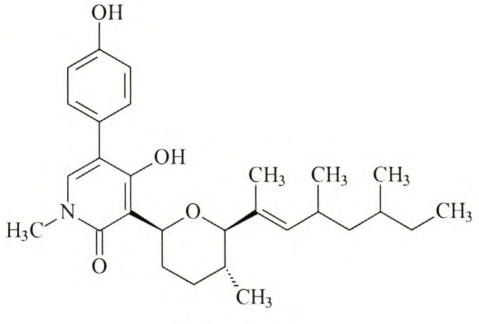
T2-toxin



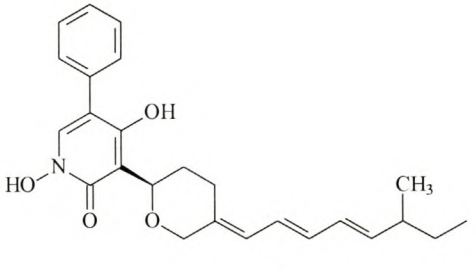
Phaseolinone

(Sesquiterpenes)

Pyranyl/pyridone



Sambutoxin



TMC-69

Figure 1.1 *Continued*

Mycotoxins have attained worldwide importance due to the ubiquitous occurrence of toxigenic fungi and their subsequent infestation of plant-based foods and feeds. More importantly though is the health concerns of affected consumers, such as mammals, fish and poultry. The diseases caused by the consumption of mycotoxins by humans and animals are called mycotoxicoses and is not only derived from contaminated plant-based foods, but also from their metabolites in animal-derived foods such as, milk, cheese and meat.

The magnitude of this problem is evident from the numerous diseases associated with mycotoxins (see Table 1.1). The chemical diversity and complexity of the mycotoxins and the associated pathological manifestations in living tissue pose a difficult task in the diagnosis of affected species. Kuiper-Goodman, 1989; Kuiper-Goodman and Scott, 1989, and Kuiper-Goodman *et al.*, 1996, contributed greatly to the risk assessment of mycotoxins. The human health concerns depend on the amount and toxicity of the mycotoxin consumed, the body weight and physical status of the affected individual, the simultaneous presence of other mycotoxins and other dietary factors. The occurrence of animal mycotoxicoses is mainly governed by climatic conditions in a region and the agricultural practices employed in growing and harvesting crops and feeds.

Hsieh, 1990, correlated the role of a mycotoxin in the aetiology of a form of human disease along the following criteria:

a) Occurrence of the mycotoxin(s) in food supplies;
b) Human exposure to the mycotoxins;
c) Correlation between exposure and incidence;
d) Reproducibility of characteristic symptoms in experimental animals;
e) Similar mode of action in human and animal models.

Of these criteria, the most important evidence in the assignment of a mycotoxin as the causative substance would be the incidence of the disease in response to the exposure to the mycotoxin.

Table 1.1 Diverse biological activity displayed by some representative mycotoxins (Source: modified from Steyn and Stander, 2000).

Mycotoxin	Biological activity	Associated Diseases	Producing Genus	References
Patulin	Mutagenicity, neurotoxin, antibacterial	Tremorgenic toxin ("staggers syndrome")	*Penicillium*	Engel and Teuber (1984)
Citrinin	Nephrotoxicity	Yellow rice toxicoses	*Aspergillus, Penicillium*	Betina (1984)
Ochratoxin A	Carcinogenicity nephrotoxicity	Balkan endemic nephropathy, Cronic interstitial nephropathy in North Africa. Nephropathy in pigs (Danish porcine nephropathy) and poultry	*Aspergillus, Penicillium*	Van der Merwe *et al.* (1965) Petkova-Bocharova *et al.* (1988) Pohland *et al.* (1992) Creppy *et al.* (1999a)
Fumonisin B_1	Carcinogenicity, neurotoxicity	Equine leuko-encephalomalacia, Porcine pulmonary oedema, oesophageal cancer	*Fusarium*	Bezuidenhout *et al.* (1988) Gelderblom *et al.* (1988) Jackson *et al.* (1996)
Aflatoxin B_1	Carcinogenicity, teratogenicity	Human primary liver cancer, turkey X disease	*Aspergillus*	Büchi *et al.* (1966) Van Rensburg (1986) Bressac *et al.* (1991) Groopman *et al.* (1992)
SporidesminA	Hepatotoxicity, photosensitivity	Facial eczema in New Zealand sheep	*Pithomyces*	Mortimer *et al.* (1978)
α-Cyclopiazonic acid	Neurotoxicity, carcinogenicity	Acute toxicity, liver necrosis in rats	*Aspergillus, Penicillium*	Holzapfel (1968) Purchase (1971)
Phomopsin A	Hepatotoxicity	Lupinosis in sheep	*Phomopsis*	Culvenor *et al.* (1989)
Ergotamine	Vasoconstriction, neurotoxicity	Ergotism, St Anthony's Fire in humans	*Claviceps*	Stoll (1952) Scott *et al.* (1992)
Penitrem A	Neurotoxicity	Tremorgenic toxins	*Penicillium*	De Jesus *et al.* (1983) Steyn and Vleggaar (1985)
Trichothecenes (T-2 toxin)	Dermatoxicity, haematopoietic effects	Alimentary toxic aleukia	*Fusarium*	Plattner *et al.* (1989) Wannemacher *et al.* (1991) Grove (1996)
Phaseolinone	Mutagenic, anti-leishmanial and antiplasmodial	Antifertility in rats	*Aspergillus, Macrophomina*	Bhattacharya (1990) Isaka *et al.* (2000)
Sambutoxin	Hemorrhagic	-	*Fusarium*	Kim *et al.* (1995) Williams and Turske (2000)
TMC-69	Antitumor antibiotic	-	*Chrysosporium*	Kohno *et al.* (2001)

Kuiper-Goodman, 1991, emphasized that based on the aforementioned approach of risk assessment, especially with respect to carcinogenicity data, the extrapolation of toxicological data from animal models to humans is subject to numerous variables. The differences in the pharmacokinetics between the species, which include absorption, distribution and metabolism, affect the assessment of the toxicity of the compound.

Kuiper-Goodman, 1991, concluded that the overall risk extrapolation from animal data to humans involves:

a) Extrapolation from high doses within the experimental range of animal experiments to low doses, usually outside the experimental range, to which humans might be exposed

b) Extrapolation from test species to humans; and

c) Extrapolation to the most sensitive subgroup of humans.

A study of a specific mycotoxicosis, specifically ochratoxicosis, would require the isolation and identification of the producing fungus. Followed by the chemical characterization of the mycotoxin, which will allow the monitoring of the toxin in nature and regulation of the toxin in biological systems.

In our research effort, the study into the structure activity relationship of different ochratoxin analogues becomes of fundamental importance in elucidating the role of ochratoxins as causative agents in diseases.

In the following sections attention will be directed to the different aspects of ochratoxin metabolism, which will include production, natural occurrence, chemical characteristics, molecular mechanisms, pharmacokinetics, toxicology, prevention and treatment. The knowledge gained will be employed in the synthesis of relevant ochratoxin A (OA) **3**, analogues.

1.2 Production of Ochratoxin A.

The most frequent fungal contaminants of cereal grains and other plant-based foods in storage are the genera *Penicillium* and *Aspergillus*, several of which produce OA (Frisvad and Samson, 1991). These fungi have been termed as storage fungi.

OA was first reported as a naturally occurring mycotoxin, produced as toxic secondary metabolites of *Aspergillus ochraceus* Wilh. by Van der Merwe *et al.*, 1965. In recent years a wide range of other *Aspergillus* and *Penicillium* species has been identified as producers of OA (Frisvad and Samson, 1991; Marquardt and Frohlich, 1992; Abarca *et al.*, 1994 and Varga *et al.*, 1996) (see Table 1.2).

Table 1.2 OA-producing fungal species.

Aspergillus	Penicillium
Main producing species	
A. ochraceus (A. alutaceus)	*P. viridicatum (P. verrucosum)*
Other producing species	
A. melleus (A. quercins)	*P. commune*
A. alliaceus	*P. cyclopium*
A. ostianus	*P. palitans*
A. sclerotiorum	*P. purpurescens*
A. albertensis	*P. variabile*
A. wentii	
A. auricomus	
A. niger var.niger	
A. sulphureus (A. fresenii)	

The ochratoxins are contaminants of a wide range of plant and animal products, the extent of which are governed by various factors. These include water activity (a_w), temperature, type of substrate, presence of competitive microflora, fungal species, and the integrity of the seed (Marquardt and Frohlich, 1992).

The minimum a_w values for OA production by *A. alutaceus* and *P. verrucosum* observed for stored grains varied between 0.83 and 0.90. At optimum a_w levels the temperature range for OA production by *A. alutaceus* and *P. verrucosum*, was 12 to 37°C and 4 to 31°C, respectively. This is evident in countries with colder climates such as Scandinavia and Canada, where *P. verrucosum* is the key producer of OA in cereals. In contrast, in the warmer climatic zones such as Australia and Yugoslavia, *A. alutaceus* is the foremost producer of OA in stored crops (Krogh, 1987a).

Madhyastha *et al.* 1990, in an *in vitro* study, followed the production in time of OA, ochratoxin B (OB) and citrinin by *A. alutaceus* and *P. verrucosum,* when inoculated in grain (wheat and corn) and oilseeds (soybean, peanuts and rapeseed). The results indicated that the *A. alutaceus* inoculated oilseeds (peanuts and soybean) achieved higher OA and OB production than the grain substrate (wheat and corn). The opposite applied for the *P. verrucosum* inoculated grain crops (wheat and corn), supporting OA and citrinin production more effectively than the oilseed substrates.

Chelack *et al.*, 1991a, found that the production of OA by *A. alutaceus* on sterilized barley was comparatively higher than the production of OA on unsterilized barley. It would thus be possible to regulate the production of OA by varying the amount of non-OA producing microflora in inoculated storage grains.

Röschenthaler *et al.*, 1984, found that subdivided and subcultures of *A. alutaceus* produced variable amounts of OA. Chelack *et al.*, 1991b, reported on the inconsistent OA production by variants of *A. alutaceus* (isolated from the fungus after gamma irradiation) from inoculated barley.

1.3 Natural Occurrence of OA

OA contamination of commodities worldwide has become a serious problem, not only from a health perspective but also from an economical point of view. High-energy plant-based products such as cereal grains, oil seeds, nuts, dried fruit and other feedstuffs are all susceptible to contamination by OA due to the widespread distribution of toxigenic fungi. The consumption of contaminated feeds by non-ruminant animals such as poultry and pigs poses the risk of contamination of meat and

meat products, which inevitably affects humans (Van Egmond and Speijers, 1994). This is manifested further by the detection of OA in milk of lactating women, posing the threat of possible spread to infants.

OA occurrence is most prevalent in European and North American countries. Apart from climatic conditions the agricultural techniques employed during harvesting and pre-harvesting play an equally important role in the prevention of contamination. Van Egmond and Speijers, 1994, reported the highest levels of OA contamination to be in cereals (maize between 10 and 500 μg kg^{-1}, wheat between 5 and 135 μg kg^{-1} and barley between 10 and 500 μg kg^{-1}).

In meat products the highest incidence is found in kidneys from pigs (which range between 2 and 100 μg kg^{-1}) and poultry (average of 19 μg kg^{-1}). Lower levels of OA are also found in other tissue such as liver, muscle, adipose and blood of these animals.

The natural occurrence of OA in commercial feedstuffs, such as coffee beans has been reported by several authors (Joosten *et a*l., 2001; Mantle and Chow, 2000). Concentrations of OA in green coffee beans ranges between 0.2 and 360 μg kg^{-1}. Blanc *et al.*, 1998, found that more than 80% of OA found in contaminated beans are destroyed during the roasting and brewing processes.

Reports on the occurrence of OA in wine have shown a considerable level of contamination (Visconti *et al.*, 1999; Abrunhosa *et al.*, 2001). The degree of contamination shows prevalence for both the type of wine and the geographical origin of the grapes. The highest concentrations of OA (up to 7.0 ng/ml) and incidence (92%) were found in red wine samples originating from southern and warmer regions of Europe (Ottender and Majerus, 2000). Stander and Steyn, 2001, in a comprehensive survey of OA in South African wines, reported that no regional influence could be observed and found that both red and white wines contained similar levels of OA.

1.4 Chemical Structure.

The structure of OA was derived from its physical characteristics, several degradation experiments (Van der Merwe, 1965), the total synthesis (Steyn and Holzapfel, 1967), and by biosynthetic studies employing both radioactive isotopes (Steyn *et al.*, 1970) and stable isotopes (de Jesus *et al.*, 1980). Bredenkamp *et al.*, 1989, determined the crystal structure for both OA and OB in an attempt to rationalize the relationship between structure and function (see Figure 1.2).

OA is comprised of a pentaketide derived, 7-carboxy-5-chloro-8-hydroxy-3*R*-methyl-3, 4-dihydroisocoumarin (the hydrolysed product ochratoxin α, Oα) which is linked through its 12-carboxyl group *via* an amide bond to L-phenylalanine (L-Phe) (Van der Merwe *et al.*, 1965).

OA is a colourless, crystalline compound, obtained readily by crystallisation from benzene. The crystals have a melting point (m.p.) of *ca* 90°C (containing approximately 1 mol of benzene). Recrystallisation from xylene yields crystals with a m.p. 171°C (Van der Merwe *et al.*, 1965). The compound is soluble in polar organic solvents, slightly soluble in water, and soluble in a dilute alkaline solution of sodium hydrogen carbonate (Steyn, 1967).

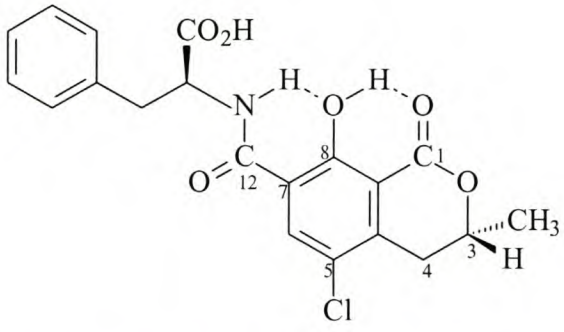
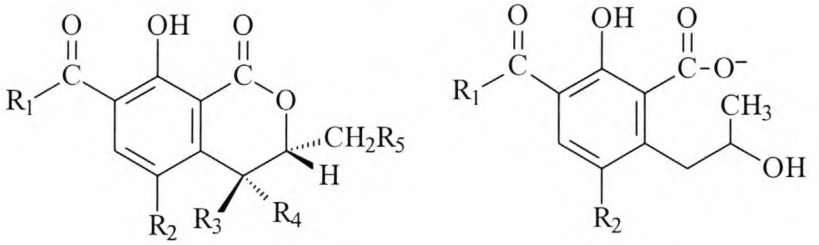


Figure 1.2 Ochratoxin A, β-form (Bredenkamp *et al.*, 1989)

1.4.1 Naturally occurring analogues of OA.

The dechloro analogue OB, is a colourless crystaline compound spectroscopically closely related to OA but differing vastly in biological activity. OB is approximately 10 times less toxic than OA and displays no immunosuppressive activity (Luster *et*

al., 1987). OB can be obtained by quantitative catalytic dechlorination of OA (10% palladium-charcoal and ammonium formate in methanol) (Bredenkamp *et al.*, 1989).



Tabel 1.3 Naturally occurring ochratoxins.

OA analogues	R_1	R_2	R_3	R_4	R_5	References
Ochratoxin B (OB)	Phe	H	H	H	H	Van der Merwe (1965)
Ochratoxin C (OC)	Phe-ethyl ester	Cl	H	H	H	Steyn and Holzapfel(1967) Chu (1974)
Ochratoxin A methyl ester (OE)	Phe-methyl ester	Cl	H	H	H	Steyn and Holzapfel(1967) Chu (1974)
Ochratoxin B methyl ester (OF)	Phe-methyl ester	H	H	H	H	Steyn and Holzapfel(1967) Chu (1974)
Ochratoxin B ethyl ester (OD)	Phe-ethyl ester	H	H	H	H	Steyn and Holzapfel(1967) Chu (1974)
Ochratoxin α (Oα)	OH	Cl	H	H	H	Suzuki *et al.* (1977) Hansen *et al.* (1982)
Ochratoxin β (Oβ)	OH	H	H	H	H	Hutchison *et al.* (1971)
4*R*-OH-Ochratoxin A (4-OH-OA)	Phe	Cl	H	OH	H	Hutchison *et al.* (1971) Størmer *et al.* (1983) Xiao *et al.* (1996a)
4*S*-OH-Ochratoxin A (4-OH-OA)	Phe	Cl	OH	H	H	Scott *et al.* (1971) Størmer *et al.* (1983)
4*R*-OH-Ochratoxin B (4-OH-OB)	Phe	H	H	OH	H	Xiao *et al.* (1996a)
10-OH-Ochratoxin A (10-OH-OA)	Phe	Cl	H	H	OH	Størmer *et al.* (1983) Xiao *et al.* (1996a)
Lactone opened ring OA (OP-OA)	Phe	Cl	-	-	-	Xiao *et al.* (1996b)
Ochratoxin A, hydroxyproline	OH-Pro	Cl	H	H	H	Hadidane *et al.* (1991)
Ochratoxin A, lysine,serine,tyrosine	Lys,Ser,Tyr	Cl	H	H	H	Hadidane *et al.* (1991) Creppy *et al.* (1990)

Both the ethyl and methyl esters of OA and OB have been isolated from *A. ochraceus* cultures (Steyn and Holzapfel, 1967). Stander *et al.*, 2001a, reported that OA methyl ester was not hydrolysed by carboxipeptidase A (CPA). This was ascribed to the obstruction, brought about by the methyl group, of the ionic interaction between the carboxyl group and the active site of carboxypeptidase A. The observed similarity in *in vivo* toxicity of OA and OE can be attributed to the facile ester hydrolysis of OE by intestinal esterases. In a similar manner the respective esters of OB was found to be resistant to hydrolysis and as expected, non-toxic.

The non-toxic cleavage product, Oα, a residue from the protozoan and bacterial enzyme hydrolysis of OA, is found in various organs in rats. This includes mostly the caecum, duodenum, ileum and the pancreas, whereas in the kidneys and liver low to no activity has been found, respectively (Suzuki *et al.*, 1977; Hansen *et al.*, 1982 and Størmer *et al.*, 1983). Oα can be obtained synthetically by acid hydrolysis (6M HCl), treatment with carboxypeptidase A or α-trypsin, rendering the dihydroisocoumarin (Oα) and corresponding amino acid (L-Phe).

In a similar manner the hydrolysis product of OB, Oβ, occurs in nature. Patterson *et al.*, 1976, studied the comparative rates of hydrolysis of OA and OB in pregnant sows. OA was found to be partially hydrolysed in the intestines, whereas the presence of higher levels of Oβ in the faeces of sows fed only OB indicated a higher rate of hydrolysis. It was further found that OB relative to OA has a lower absorption from the intestines. This suggested a more rapid enzyme hydrolysis of OB in the liver. Extracts of rat hepatocytes and intestinal tissue confirmed a higher hydrolysis of the non-toxic analogue (Hult *et al.*, 1977). Doster and Sinnhuber, 1972, in an *in vitro* kinetic study of bovine carboxypeptidase A, showed that OB hydrolyses up to two hundred times faster than OA. Stander *et al.*, 2001b; found that although CPA had a lower relative affinity for OB than OA, the turnover for OB was ten times higher than for OA (see also section 1.5).

Naturally occurring analogues with modification to the isocoumarin moiety have been isolated by Hutchison *et al.*, 1971, from *P. viridicatum* grown from YES medium. The intraperitoneal injection of male Wistar rats with OA caused the excretion of OA, Oα

and 4-hydroxyochratoxin in the urine. With the OA proving fatal in all of the test cases, the hydroxyochratoxin was non-toxic. Størmer and Pederson (1980) implicated cytochrome P-450 in the hydroxylation, and subsequent detoxification, by rat liver microsomes.

Størmer *et al.*, 1981, concluded that under the influence of cytochrome P-450 the (4*R*)-OH-OA epimer is formed as the major product in human and rat liver microsomes, while the (4*S*)-OH-OA epimer is more readily formed in pig liver microsomes. Oster *et al.*, 1991, contributed by purification and indentification of porcine hepatic cytochrome P-450 fractions and suggested this enzyme to have an important role in OA oxidation.

The 10-OH analogue to OA was isolated from rabbit liver microsomes (Størmer *et al.*, 1983) and shown to be less toxic than OA. Xiao *et al.*, 1996b, reported the isolation of an opened lactone ring analogue of OA in the bile of rats after administering OA. OP-OA was shown to be highly toxic when injected intravenously but not intraperitonealy in rats, with the toxicity of intravenously administered OP-OA being even greater than that for OA.

1.4.2 Synthetic analogues of OA.

Several derivatives of OA have been prepared by the substitution of the L-Phe side chain with various amino acids (see Table 1.4). Creppy *et al.*, 1983a, found that for tyrosine, valine, serine and alanine analogues the toxicity is the greatest, the methionine, tryptophan, and glutamic acid analogues have on intermediate toxic effect, and the glutamate and proline substituted OA has the lowest toxicity. The relative toxicity of these analogues may be correlated to some extent to the presence of an aromatic ring substituent on the respective amino acids. Creppy *et al.*, 1990 also showed that OA could be hydroxylated by phenylalanine hydroxylase to form the tyrosine analogue of OA.

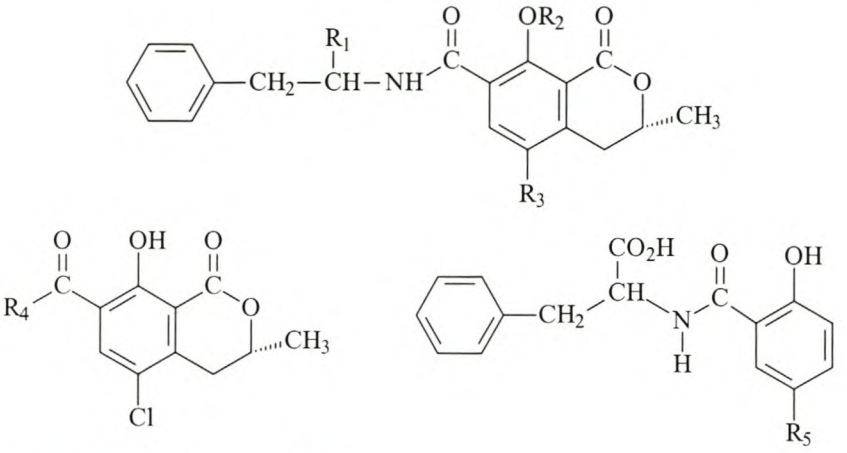


Table 1.4 Synthetic analogues of OA.

Analogue	R_1	R_2	R_3	R_4	R_5	References
OA-*O*-methyl ether (OM-OA)	COOH	CH_3	Cl	-	-	Xiao *et al.* (1995)
OA-*O*-methyl-methyl ester	$COOCH_3$	CH_3	Cl	-	-	Van der Merwe *et al.* (1965)
OA-ethylamide (OE-OA)	$CONHC_2H_5$	H	Cl	-	-	Xiao *et al.* (1995)
Decarboxylated (d-OA)	H	H	Cl	-	-	Xiao *et al.* (1995)
Bromo-OB (BrOB)	COOH	H	Br	-	-	Steyn and Payne (1999)
Iodo-OB (IOB)	COOH	H	I	-	-	Steyn and Payne (1999)
Oα-methyl ester (M-Oα)	-	-	-	OCH_3	-	Xiao *et al.* (1995)
OA-D-Phe (D-OA)	-	-	-	D-Phe	-	Xiao *et al.* (1995)
OA-L-Tryp, Ala, Tyr, Cys, Glu, OH-Pro, Leu, Met, Val, Ser, Asp, Thr	-	-	-	L-Trp, Ala, Tyr, Cys, Glu OH-Pro, Leu, Met, Val,Ser, Asp, Thr	-	Steyn *et al.* (1975) Creppy *et al.* (1983)
Salicyl-Phe (SAPhe)	-	-	-	-	H	Stander *et al.* (2001a)
Fluorosalicyl-Phe (5FSAPhe)	-	-	-	-	F	Stander *et al.* (2001a)
Chlorosalicyl-Phe (5ClSAPhe)	-	-	-	-	Cl	Stander *et al.* (2001a)
Bromosalicyl-Phe (5BrSAPhe)	-	-	-	-	Br	Stander *et al.* (2001a)

In a study to ascertain the role of the halogen group in the toxicity of OA, Steyn and Payne, 1999, synthesised the bromo and iodo analogues of OA. This was followed by the first biological production of bromo ochratoxin B by *A. ochraceus* Wilh. (Stander *et al.*, 2000). In a comparitive study Stander *et al.*, 2001a, found that OA and BrOB exhibited similar enzyme affinity (k_{cat}) and turnover values (K_m) upon treatment with CPA (Tabel 1.5). For the specific case of the chloro and bromo compounds the halogen group has no apparent influence on substrate binding or catalytic function. The fact that preliminary results have revealed an increase in toxicity of BrOB to renal cells compared to OA, shows that the rate of hydrolysis is not solely responsible for the degree of toxicity of the ochratoxins (Creppy, 1999b).

Table 1.5 Hydrolysis of Ochratoxins and analogues by Carboxypeptidase A. (Source: Stander *et al.*, 2001a).

Toxin	K_m **(μM)**	k_{cat} **(min^{-1})**	k_{cat}/K_m **($min^{-1}\mu M^{-1}$)**
OA	5.6	266	47
OB	36.8	2717	74
BrOB	6.6	283	43
SAPhe	0.8	2372	3163
5FSAPhe	16.0	4276	267
5ClSAPhe	5.5	2869	521
5BrSAPhe	12.9	3724	289

Stander *et al.*, 2001a, further studied the hydrolysis of various halosalicylic acid and salicylic acid coupled L-Phe analogues by CPA. It was found that for 5BrSAPhe and 5FSAPhe, higher catalytic efficiencies (k_{cat}/K_m) were obtained compared to the corresponding chloro substrate. In agreement with the ochratoxins, the non-halogenated salicylic acid coupled L-Phe showed both increased substrate binding and hydrolysis by CPA compared to the halogenated analogues. The comparison of OA and L-Phe coupled analogues hydrolysis data further reveals an increased catalytic efficiency of CPA for the L-Phe coupled substrates. This finding implies the presence of the lactone moiety directly in the toxicity of the ochratoxins. In the following section these and other functionalities implicated in the toxicity of OA will be discussed in more detail.

1.5 Molecular mechanisms of toxicity.

Although extensive research has been conducted into the chemical nature of the toxicity of OA, the exact molecular mechanism of action of OA is not yet conclusive. The toxicity of OA may be the result of three major effects: (i) inhibition of ATP production; (ii) inhibition of protein synthesis; and (iii) the disruption of hepatic microsomal calcium homeostasis, through the promotion of membrane lipid peroxidation (Creppy *et al.*, 1990). Recent studies have suggested that apart from the direct role in toxicity several indirect roles do exist in living systems (Xiao *et al.*, 1996b).

The isolation of covalent DNA adducts in OA-treated mice and from urine of patients suffering from urinary tract tumors has suggested a possible relationship between OA toxicity and macromolecule formation (Pfohl-Leszkowicz *et al.*, 1993a, 1993b). Malaveille *et al.*, 1994, have implicated the formation of a genotoxic OA phenoxide radical and a cytotoxic thiol conjugate of OA as reactive metabolites in *E. coli* strains and causative agent of toxicity.

Meisner and Chan, 1974; and Wei *et al.*, 1985, reported in an *in vitro* study of rat liver mitochondria, that OA inhibited mitochondrial respiration (see section 1.8.3) by the competitive inhibition of carrier proteins located in the inner mitochondrial membrane. Meisner, 1976, further elaborated on the effect of OA uptake, an energy consuming process with the depletion of intramitochondrial ATP and the subsequent inhibition of phosphate transport across the mitochondrial membrane.

Chu, 1971, first implicated the role of the phenolic hydroxyl group in the toxicity of OA, other natural analogues OB, Oα and OC and synthetic analogue OM-OA. Chu, 1974, showed that the dissociation constants (pKa) of the phenolic hydroxyl group for the different analogues was related to the toxicity of the analogue, and that dissociation of the hydroxyl group was required for its toxicity. This is further substantiated by the reports of higher toxicity of BrOB to kidney cells compared to OA (Steyn and Payne, 1999).

In physiological systems a significant proportion of OA, OC and possibly BrOB is in the dissociated form, and the resultant phenoxide ion could be may to react with biological constituents. A correlation between the pKa values and the observed toxicities of the ochratoxins is shown in Table 1.6.

In support of this approach, Doster *et al.*, 1974, suggested that increasing the molecular mass while still maintaining the dissociative properties of the phenolic hydroxyl group could enhance the toxicity of the ochratoxins. Doster *et al.*, 1974, found that OC is almost twice as toxic to trout than OA, while both compounds share the same pKa value.

Table 1.6 A correlation between the comparitive cytotoxicity and antimicrobial activity of OA and its dissociation- and iron chelation-potential (Source: Xiao *et al.*, 1996b).

OA and analogues	**pKa[a]**	**Fe^{2+} chelation[b]**	**HeLa cell LC_{50}[c] (mM)**	***B.brevis* MTD[d] (nmol/disc)**
OA	7.0	++	0.005	8.7
OB	7.8	+	0.054	54
OC	7.2	+++	0.009	2.0
D-OA	7.2	++	0.163	5.5
Oα	11.6	-	0.56	390
OM-OA	NA[e]	-	0.83	NI[g]
d-OA	7.9	-	7.6	NI[g]
OE-OA	6.7	+++	10.1	1.1
M-Oα	8.2	-	ND[f]	90
OP-OA	ND[f]	+++	ND[f]	NI[g]

[a]The pKa values are for the dissociation of phenolic hydroxyl group and not for the carboxyl groups. [b]Iron-chelation of OA and its analogues at pH 4.5 as determined visually by colour change, strong (+++), moderate (++), weak (+) and none (-). [c]LC_{50}, the concentration of toxin required to reduce number of cells by 50% as determined by linear regression. [d]MTD, minimum toxic dose for measurable inhibition of *B. brevis* growth in a medium at pH 6.5. [e]NA, not applicable as the phenolic group is methylated. [f]ND, not determined [g]NI, no inhibition attained at 350 nmol/disc.

As mentioned before (section 1.4.2) the relative rates of hydrolysis of the ochratoxins, and subsequent detoxification, in living tissue could be a parameter in establishing its toxicity. Bredenkamp *et al.*, 1989, speculated that apart from the direct role that the aromatic halogen group has to play in the dissociation of the phenolic hydroxyl group, the steric nature of the halogen substituent may play an important role in the ability of the ochratoxin to react with enzymes. *In vitro* studies with the enzyme, bovine carboxypeptidase A showed OB to be hydrolysed almost hundred times faster than

OA (Doster and Sinnhuber, 1972). *In vivo* studies in rat tissue extracts (liver and intestines) agreed with these findings. The *in vitro* hydrolysis by HCl revealed a reversal in the relative rates of hydrolysis for OA and OB. It was postulated that the bulkiness of the chloro-group of OA might hinder the approach of the toxin to active sites of carboxypeptidase A.

Stander *et al.*, 2001a, reported that the toxicity of BrOB could not be attributed solely to its rate of hydrolysis. When compared to OA, CPA had relatively similar catalytic efficiency values for the bromo analogue (k_{cat}/K_m = 43) and OA (k_{cat}/K_m = 47). The fact that BrOB showed a much elevated toxicity to renal cells as compared to OA suggests that other factors may be involved in the toxicity of OA. In agreement to the ochratoxins, the halosalicylic acid coupled phenylalanine analogues showed little disparity to the affinity of CPA. Efficiency levels for CSAPhe (k_{cat}/K_m = 521) were comparable to that of BrSAPhe (k_{cat}/K_m = 289) and FSAPhe (k_{cat}/K_m = 267).

Rahimtula *et al.*, 1988 and Omar *et al.*, 1990, proposed that the iron-chelating ability of OA in lipid peroxidation (see section 1.8.1) might play an important role in rendering the molecule toxic. The presence of the phenolic hydroxyl group in the formation of the OA-Fe^{3+} chelate is essential (see Table 1.6). The complexation of OA with iron (an essential cofactor in lipid peroxidation) facilitates the subsequent reduction of iron in both NADPH-dependant microsomal and ascorbate-dependant lipid peroxidation. This was demonstrated by the increase in lipid peroxidation in liver and kidney microsomes treated with OA, and in the *in vivo* lipid peroxidation assessment in OA inoculated rats. Hasinoff *et al.*, 1990, postulated that the OA-iron complex, in the presence of the NADPH-cytochrome P-450 reductase system produces an extremely toxic hydroxyl radical, which may be responsible for the toxicity of OA.

Xiao *et al.*, 1996b, in a study assayed the toxicity of different analogues in prokaryotic (*Bacillus brevis*), eukaryotic (HeLa) systems (see Table 1.6) and animals (mice, see Table 1.7). They found no direct correlation between the dissociation of the phenolic hydroxyl group and the iron-chelating ability of the analogues and their observed toxicity. The methylated-OA analogue (OM-OA) does not contain a free phenolic

hydroxyl group, and thus cannot participate in the chelation of Fe^{2+}, yet still exerts a marked toxicity to HeLa cells.

Creppy *et al.*, 1983a, 1984 and 1990, suggested that OA toxicity is derived from its phenylalanine moiety. This was established by the ability of OA, as a phenylalanine analogue, to competitively inhibit phenylalanine tRNA synthase and thus inhibit protein synthesis (see section 1.8.2). This was also observed for other phenylalanine metabolizing enzymes (Pitout and Nel, 1969; Parker *et al.*, 1982). The findings of Xiao *et al.*, 1996b, (Table 1.6, 1.7) supports this proposal as the toxicity of D-OA is approximately 30-fold less toxic to HeLa cells than OA, while it is essentially non-toxic to mice. In contrast OA shows a high toxicity to mice. The differences in metabolic constituents, e.g. the composition of the cell wall, may explain the observed differences in the relative toxicity of OE-OA to *B. brevis* and HeLa cells compared to that of OA.

Table 1.7 Toxicity of OA and its analogues to mice (Source: Xiao *et al.*, 1996b).

Analogues	Dosage (mg/kg BW)[a]	Death Rate[b]	Analogues	Dosage (mg/kg BW)[a]	Death Rate[b]
Control	0	0/10	OP-OA	500	0/10
OA	20	3/10	OM-OA	200	0/10
	50	9/10		500	10/10
D-OA	50	0/10	OB	200	0/10
	200	0/10		500	10/10
OE-OA	200	0/10	Oα	200	0/10
	500	10/10		500	10/10
d-OA	200	0/10	M-Oα	200	0/10
	500	10/10		500	10/10

[a] Dosage, the amount of toxin (mg) injected (ip) into mice per kg body weight (BW)
[b] Death rate, numbers of dead mice/10 mice dosed 72 h after injection.

Xiao *et al.*, 1996b, postulated that the toxicity of the analogues is more closely associated with the isocoumarin moiety, and with the lactone carbonyl specifically. This was substantiated in the isolation of the OP-OA in the bile of rats injected with OA, and by the subsequent high toxicity observed for the intravenous administration of OP-OA in the same rats. In contrast, intraperitoneally administered OP-OA in mice showed much lower toxicity compared to OA. It was postulated that the difference in toxicity might be due to the lower rate of delivery of OP-OA to target organs (kidney

and liver) relative to OA. The latter is in agreement with the findings of Kumagai, 1988, in that the rate of absorption across the intestinal epithelium for OA is much slower when it exists in its more polar form. OP-OA was found to be stable at physiological pH, but converted back to OA at low pH.

Xiao *et al.*, 1996b, concluded that OA could react in accordance with naturally occurring lactones, by covalently modifying vital biomacromolecules such as enzymes (Borgstrom, 1988; Saltz *et al.*, 1993). In an example of the inhibition of serine proteases by certain isocoumarin compounds, the hydroxyl group of serine in the active site of the enzyme and the lactone carbonyl group of the isocoumarin is involved in the formation of an acyl enzyme complex which leads to the inhibition of the enzyme (Kam *et al.*, 1992; Hernandez *et al.*, 1992). This concept is supported by the isolation of ochratoxin A metabolites and adducts to DNA extracted from monkey kidney cells. In a similar fashion the toxicity of OA may be the result of the covalent modification of hydrolytic enzymes by a reaction involving the lactone carbonyl group (Grosse *et al.*, 1995).

In conclusion the toxicity of OA and its analogues can be ascribed to the presence of an array of functional groups, which may act in a direct or indirect manner and in either an exclusive or in collaborative capacity with other functional groups.

1.6 Pharmacokinetics of OA.

Several toxicokinetic studies have been performed with OA in a number of different species of animals. A broad spectrum of results and toxigenic profiles obtained have shown that several factors influence absorption, distribution, metabolism (biotranformation), and excretion that ultimately affect the toxicity of OA (Kuiper-Goodman and Scott, 1989; Marquardt and Frolich, 1992).

The distribution of OA was found to be in two kinetically distinct compartments with variations among different animals (Chang and Chu, 1977; Galtier *et al.*, 1979 and 1981). The first is a central metabolic and excretion compartment, which includes the plasma, and the other the peripheral compartment, which includes the large intestines, skin, muscle and other organs able to retain the toxin (Galtier *et al.* 1981).

OA is mainly absorbed in a passive manner from the upper gastrointestinal tract i.e. oesophagus, stomach, and the small intestines (jejunum) (Lee, 1984; Galtier, 1978; Kumagai and Aibara, 1982; Roth *et al.*, 1988), although a large amount of OA is absorbed across the mucosal membrane in the nonionized or partially ionized form rather than the ionized form. As mentioned previously, the phenolic hydroxyl group (pKa 7.1) at physiological pH (7.1) will only be partially ionized. The role of the carboxyl group in absorption is secondary to the phenolic hydroxyl group, since it exists mostly in the ionized form at digestive pH (≈ 2.6) (Metzler, 1977). Xiao, 1991, found that the diet (hay or grain) of sheep plays an important role in the bioavailability of OA. The pH of the digestive substance affects the rate and amount of OA absorption in these animals. In ruminants, the low pH of the rumen facilitates the absorption of OA into the systemic circulation.

The presence of microorganisms in the rumen, caecum and large intestine alleviates the toxic effects of OA by its enzymatic hydrolysis to Oα. Madhyastha *et al.*, 1992, found that micro-organisms in the digesta from the caecum and large intestines of the rat were capable of hydrolysing OA *in vitro*. Madhyastha *et al.*, 1992, further reported that the excretion in urine and faeces of Oα and OA decreased and increased respectively, in rats treated with a broad spectrum antibiotic, e.g. neomycin. These findings illustrate that the intestinal microorganisms in non-ruminants affect the disposition of OA in its biotransformation of OA to the hydrolysed metabolite, Oα.

The affinity of OA to bind certain plasma proteins in the blood plays an important role in its distribution and ultimate toxicity. The OA-protein complex not only allows for the passive adsorption of ionized forms of OA but also an extended half-life in the systemic circulation (Hagelberg *et al.*, 1989). This essentially delays the elimination of OA from the systemic circulation to the renal and hepatic cells. This was illustrated by Kumagai, 1985, in the increased clearing of OA from system circulation by albumin deficient rats.

Stojkoic *et al.*, 1984, reported that apart from albumin, as plasma binding protein, a smaller plasma constituent is present with a superior binding affinity to OA. It was proposed by the authors that the ability of such aggregates to be transported across

glomelular membrane in the kidneys could be the causative agent of the accumulation of OA and ultimate nephrotoxic effects observed. This concept was given substance by the study of Ychiyama and Saito, 1987, who found that OA had lower affinity for the soluble tissue proteins of kidneys and liver than for plasma albumin. Hagelberg *et al*, 1989, established a correlation between the binding affinity and the half-life of OA in the circulatory system, by toxicokinetic studies in a variety of animals (See Table 1.8).

Table 1.8 Toxicokinetic parameters and binding Constants of OA. (Source: Hagelberg *et al.*, 1989)

Species	**$K_D(M^{-1})^a$**	**$t_{1/2}(h)^b$**	**$f_u(\%)^c$**
Fish	0.07	8.3	22
Quail	5	12	0.2
Mouse	1	48	0.1
Rat	40	170	0.02
Monkey	0.7	840	0.08
Pig	2	150	0.1
Man	1	-	0.02

[a]K_D, affinity constants [b]Biological half-life of OA after intra venous administration.[c]Unbound or free fraction of the toxin

Hagelberg *et al.*, 1989, concluded that the exact relationship between the strong-protein binding of OA, the low rate of systemic circulatory clearance and resultant toxicity has not been established. Screemannarayana *et al.*, 1988, in a toxicokinetic study of orally administered OA in calves, found that absorption occurred in two distinct phases. The distribution followed two first-order processes, which varied in rate. It was proposed that OA was rapidly absorbed from the rumen and then followed by enterohepatic recycling.

Stander *et al.*, 2001b, reported that the markedly high elimination half-lives observed for non-human primates (rhesus and vervet monkeys) compared to other species (mammals, fish and birds) could be ascribed to the differences in the degree of binding to serum proteins, including albumin. Furthermore, the long retention of OA in the blood can be ascribed not only to the high affinities for plasma proteins but also the recirculation of the toxin in the liver.

Roth *et al.*, 1989, in the elucidation of secondary distribution peaks further substantiated this approach by reports that large amounts of OA were secreted in the bile as conjugated OA (glucoronides and sulfates). The billary recycling and secretion will be followed by the reabsorption of the OA from the intestines into the blood. This will further facilitate the redistribution of OA into the peripheral compartments, ultimately leading to an overexposure of OA to organs, such as the kidneys and the liver.

Apart from the liver, the kidneys also play an important role in the recycling of OA. Stein *et al.*, 1985, reported that the reabsorption of OA from the renal proximal tubules back into the blood promotes the residual persistence of OA in the peripheral tissues and may be implicated in the degeneration of renal tubules found in rats (Albassam *et al.*, 1987) pigs (Szczech *et al.*, 1973) and poultry (Elling *et al.*, 1979). The pharmacokinetic data for OA and its metabolites in different animal species are given in Table 1.9.

Table 1.9 Distribution and elimination half-lives of different animal species (Source: modified from Steyn and Stander, 2000).

Test animal	$t_{1/2\alpha}$(min)[a]	$t_{1/2\beta}$(h)[b]	Test animal	$t_{1/2\alpha}$(min)	$t_{1/2\beta}$(h)
OA			**OB**		
Rats(iv)	160±17[c] 126[d]	103±16[c] 120[e]	Rats(iv)	14±4[c]	4.2±1.2[c]
Rats(p)	-	170[e]	**OC**		
Rats(o/iv)	-	55[d]	Rats(iv)	6±1.2[c]	0.6±0.2[c]
Rabbits	114[f]	8.2[f]	**Oα**		
Chickens	30[f]	4.1[f]	Rats(iv)	31±5[c]	9.6±2.3[c]
Cattle	108[g]	-			
Pig	-	72-150[e,f]	**OA-OH**		
Pre-ruminant calf	-	77[h]	Rats(iv)	19±4.7[c]	6±0.9[c]
Mouse	-	24-48[e]			
Rhesus monkey (iv)	-	840[e]	**OP-OA**		
Vervet monkey (iv)	58-72[i]	462-484[i]	Rats(iv)	163±5[c]	50.5±2.8[c]

[a]$t_{1/2\alpha}$ = Distribution half-life; [b]$t_{1/2\beta}$ = Elimination half-life; p = peripherally; iv = intravenous; o = orally. [c]Marquardt and Frohlich, 1992; [d]Galtier *et al.*, 1979; [e]Hagelberg *et al.*, 1989; [f]Galtier *et al.*, 1981; [g]Sreemannarayana *et al.*, 1988; [h]Fukui *et al.*, 1987; [i]Stander *et al.*, 2001b.

The biotransformation of OA extends further than the enzymatic hydrolysis rendering Oα. Størmer and Pederson, 1980, reported that liver microsomes from humans, pigs

and rats are able to hydroxylate OA in the presence of NADPH to (4*R*)- and (4*S*)-hydroxyochratoxin A. They further observed that hydroxylation occurs in the cytochrome P-450 system. In addition to the 4-hydroxyochratoxin, Størmer *et al.*, 1983, reported on the isolation of 10-hydroxyochratoxin from rabbit liver microsomes. Creppy *et al.*, 1983b, reported that the (4*R*)-hydroxy epimer was as toxic to yeast cells as OA, in that they both inhibit phenylalanine-tRNA synthase. These results together with the findings by Hutchison *et al.*, 1971, that the 4-hydroxy epimers are non-toxic to rats, suggest that the lower toxicity of the hydroxy analogues in mammals is due to their more rapid clearance in the faeces and urine.

1.7 Toxicology

1.7.1 OA linked to nephrotoxicity

The nephrotoxicity, together with the carcinogenicity of OA, are the most threatening effects of ochratoxicoses. This is evident in all animal species studied so far (Krogh, 1974; Krogh *et al.*, 1977; Bernt and Hayes 1979; Krogh, 1987). Histopathological profiles include the degeneration and atrophy of proximal tubules, the interstitial fibrosis of the renal cortex and the hyalination of the glomeruli.

First discovered in Denmark in 1928, Danish Porcine Nephropathy (DPN) is characterized by "mottled or pale enlarged kidneys" of pigs fed mouldy cereal feeds. The prevalence of the disease in European countries is especially high after a wet harvesting period. The unmarked clinical changes including polyurea, polydispia and a depression of growth, provides for difficult diagnosis. Haematological and biochemical studies have to be conducted on blood and urine samples of suspected pigs in order to determine infection.

Balkan endemic nephropathy (BEN), a human chronic interstitial nephropathy, is a strictly endemic disease in the rural population of Bulgaria, Romania and Yugoslavia. Although the disease is of unknown aetiology, OA has been extensively implicated.

Based on the similarities in structural and physiological kidney impairment between Danish porcine nephropathy and BEN, OA has been implicated as the causative agent of the disease (Stoev, 1993). Hult, 1982; Petkova-Boharova and Castegnaro, 1985;

Petkova-Bocharova *et al.*, 1988, reported on the simultaneous occurrence of OA in human blood, human food and animal feed in a high endemic area of the disease and compared this with the lower levels of OA found in non-endemic areas.

Mantle, 1998, reported that the renal atrophy in BEN might involve the loss of tubular epithelium by apoptosis. This observation implicates *Penicillium aurantiogriseum*, a nephrotoxic fungal species, which exerts a more potent and target specific effect than OA. Mantle *et al*, 1991, reported that the nephrotoxin causes severe histopathological changes in the proximal tubules of rats, which better correlates with the experimental model for the human disease (BEN) than ochratoxicosis.

OA is also implicated in Chronic Interstitial Nephritis (CIN) in North Africa (Bacha, 1993; Maaroufi, 1995) which is invariably associated with urinary system tumours (UST). Petkova-Bocharova *et al.*, 1988, reported that 95% of Tunisians suffering from urinary tract tumours were found to contain high levels of OA in their blood.

1.7.2 Carcinogenicity

Kanisawa and Suzuki, 1978, reported that the exposure of DDY mice to 40 mg/kg OA in their feed over a 50-week period caused renal and hepatic tumours. The renal tumours diagnosed were a benign cystic papillary adenoma and a malignant solid type renal cell tumour.

Bendele *et al.*, 1985, reported that renal adenomas and carcinoma were prevalent in mice fed a diet, which included high dosages of OA (34 mg/kg of feed). Boorman, 1989, showed in a similar two-year study of male Fisher 344/N rats that there was a high incidence of renal tubular cell adenomas and carcinomas.

It was further found that at increased levels of OA exposure the renal tumours were multiple and bilateral. Of importance was the high prevalence of metastasis found in the pulmonary and lymphoid cells. An increase in the multiplicity of fibroadenomas in the mammary glands of female rats was also of significance.

OA exposure also caused non-neoplastic renal changes including tubular cell hyperplasia, tubular cell proliferation, cytoplasmic alterations, karyomegali and degeneration of the tubular epithelium (Boorman, 1989).

1.7.3 Immunotoxicity

OA is immunotoxic to both humoural and cellular immune systems. A decrease in lymphoid cell production, proceeding from low amounts of OA ingestion, in mainly the thymus, bursa of Fabricius, spleen, and the Peers patches, has been reported for different animal species (Szczech *et al.*, 1973; Dwivedi and Burns, 1984 and 1985). The depression of thymus proliferation occurs in conjunction with a cell-mediated delayed hypersensitivity, implicating OA in both immune responses.

Luster *et al.*, 1987, reported that OA decreases natural killer cell activity by the inhibition of endogenous interferon levels. Polyinosinic:polycytidylic acid-induced interferon was markedly reduced in OA inoculated mice. The subsequent injection of polyinosinic:polycytidylic acid enhanced the natural killer cell activity in the presence of OA. The fact that the level of enhancement was slightly lower than the level induced by the acid in the absence of OA, suggests that the presence of the toxin inhibited the production of basal interferon. The ability of OA to regulate the activity of these cells contributes to the impact that OA can have on the proliferation of renal and hepatic carcinomas.

Lea *et al.*, 1989; conducted an *in vitro* study on the effect of OA on highly purified subpopulations of T lymphocytes expressing the CD4 (helper/inducer) and CD8 (suppressor/cytotoxic) phenotypic markers. OA revealed no selectivity in the inhibition of the phytohaemaglutinin-induced proliferative ability of the varied T-cell populations. The proliferation of T-cell is regulated by the binding of interleukin-2 (IL-2) to its receptors, which delivers the mitogenic signal that initiates cell division of the specific T-cell population. The absence of IL-2 induced cell proliferation observed for OA treated cell cultures could be due to the decrease of IL-2 receptor expression or production. It was suggested that the inhibitory role that OA has on protein synthesis, could provide the basis of this effect. This was supported by the

findings of Størmer and Lea, 1995 that OA had a pronounced effect on the protein synthesis of dormant T-cells.

The subchronic exposure of Balb/c mice to OA decreased the anti-body production of plaque-forming cells and suppressed the total thymocyte cell count, which included the proportion of mature thymic lymphocyte (CD^{4+} or CD^{8+}) cells. A decrease in the mitogenic responsiveness of thymocytes and splenocytes to concanavalin A (Con A) in the presence of OA is also significant. In contrast, the IL-2 production of Con A induced lymphocytes, the natural killer cell activity and the concentrations of humoral antibodies to viral antigens are not affected by the OA poisoning (Thuvander *et al.*, 1995).

Thuvander *et al.*, 1997, reported on the immunosuppression of Balb/c mice and Sprague-Dawley rats after the prenatal exposure of OA. The subchronic exposure from dietary OA resulted in the suppression of proliferation in lymphoid organs but did not affect the immune response in the offspring.

Numerous discrepancies have been reported in immunotoxicity studies of OA where the dietary intake of phenylalanine, as well as the route of administration had not been taken into consideration. Haubeck *et al.*, 1981, reported on the inhibition by OA to the immune response of Balb/c mice to sheep red blood cells in the absence and presence of phenylalanine. It was found that when the concentration of phenylalanine was approximately twice that of OA (w/w) immunosuppression was either not observed or extensively attenuated.

Creppy *et al.*, 1979a, showed that the addition of phenylalanine to OA inoculated hepatoma tissue culture cells could alleviate the inhibition of growth and protein synthesis. Creppy *et al.*, 1980, illustrated that acute poisoning by OA of mice could be prevented by the simultaneous intraperitoneal injection of phenylalanine. Furthermore, aspartame, a structural analogue to OA and phenylalanine, could be used as a preventive agent of the chronic toxic effects of OA in rats (Creppy *et al.*, 1996).

1.7.4 Teratogenicity

OA administered orally or intraperitoneally during the period of gestation had severe embryotoxic effects in rats, mice, hamsters and pigs (Brown *et al.*, 1976; Hayes *et al.*, 1974; Hood *et al.*, 1975 and Schreeve *et al.*, 1977). The degree of effects depends, among other factors, on the route of exposure and the time of the gestation period when the toxin is administered.

These effects included an increase in the number of dead and resorbed foetuses, a decrease in the foetal body weight as well as the hemorrhages and oedema in certain foetuses of rats. In conjunction with these effects, numerous anomalies were observed in the foetuses of animals treated with OA. This includes multiple gross, visceral and skeletal malformations in rats (Brown *et al.*, 1976) and hamsters (Hood *et al.*, 1975). Skeletal anomalies, specifically in the ribs, vertebrae and the skull, were accompanied by necrotic eye and brain cells of foetal mice exposed early or later in the gestation period.

1.7.5 Genotoxicity and Mutagenicity

OA is a non-mutagenic carcinogen, evident from the numerous negative gene mutation assays conducted on both micro-organisms and mammalian cells (Boorman, 1989; Würgler *et al.*, 1991; Bendele *et al.*, 1995 and Sakai *et al.*, 1992).

Manalova *et al.*, 1990, reported that OA was found to induce various numerical and structural aberrations on X-chromosomes of similar types to those detected in lymphocytes from patients suffering from endemic nephropathy. Dirheimer, 1996, reported that OA induced mutations in the modified Ames assay, sister chromatid exchange in human peripheral lymphocytes *in vitro*, and the SOS DNA repair in *E. coli* (Hennig *et al.*, 1991). De Groene *et al.*, 1996, emphasized that OA required the additional metabolic processing, by cytochrome P450 enzymes, to its metabolites (4-OH-OA) in order to exert a mutagenic effect on living tissue.

Pfohl-Leszkowics *et al.*, 1993a, identified DNA adducts *in vivo* in the liver, kidney and spleen of rodents and humans suffering from urinary tract tumours. Grosse *et al.*, 1995, reported that monkey cells were able to produce DNA-adducts when incubated

with OA. The adducts observed were similar to those found in human kidney and bladder tumours from people suffering from BEN. Furthermore, the presence of DNA adducts in cytochrome P450-expressing cells, implied their formation *via* an oxidative process involving cytochrome P450 synthase. Grosse *et al.*, 1995, further found that at elevated dosages of OA (100 μM) some adducts were completely absent. An explanation for this may be due to the inhibition of protein synthesis by OA which would lead to the impairment of certain cytochromes P450-synthase and the decrease of reactive metabolites responsible for DNA adduct formation.

1.8 Mechanisms of action.

1.8.1 Lipid peroxidation

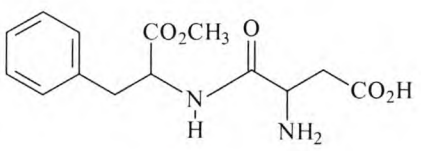
Membrane lipid peroxidation is an important part of oxidative tissue damage and can be an effect as well as a cause of the reactions culminating in cytotoxicity (Omar *et al.*, 1990). Omar *et al.*, 1991, reported that OA disrupts hepatic microsomal calcium homeostasis by impairment of the endoplasmic reticulum membrane, *via* the induction of lipid peroxidation. It was found that OA greatly enhanced the rate of NADPH- or ascorbate-dependant lipid peroxidation both *in vivo* (rats) and *in vitro* (liver or kidney microsomes) as measured by the formation of malondialdehyde. Rahimtula *et al.*, 1988 and 1989, implicated the reactivity of the phenolic hydroxyl group of the different ochratoxins in the efficiency of lipid peroxidation induction.

OA facilitates the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) *via* chelation. The subsequent reoxidation is accompanied by O_2 consumption (Omar *et al.*, 1990). Hasinoff *et al.*, 1990, reported that the Fe^{3+}-OA chelation complex produces extremely toxic hydroxyl radicals in the presence of the NADPH-cytochrome P450 reductase system and NADPH. In the presence of oxygen, the resultant OA-Fe^{2+}-Fe^{2+}-OA complex provides the active species, which induces lipid peroxidation. This provides for the offset in cellular constituents, containing polyunsaturated fatty acids and oxygen, of an array of degenerative radical reactions producing chemically reactive metabolites (Baudrimont *et al.*, 1997).

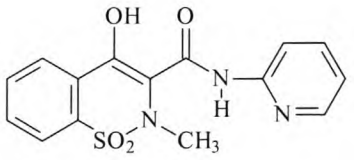
The presence of iron in the tubular lumen, due to the glomerular leak of iron bound transferrin, provides for the formation of hydroxyl radicals. The dissociation of iron

from transferrin in the acidic tubular fluid, allows for the catalysis of hydroxyl radical formation, which leads to the lipid peroxidation of the tubular membranes (Størmer *et al.*, 1996).

Baudrimont *et al.*, 1997, found that the lipid peroxidation caused by the free oxygen species induced by OA can be limited in monkey kidney cells by the addition of superoxide dismutase (SOD) and catalase, piroxicam or aspartame to the culture medium prior to OA inoculation (Figure 1.3). SOD and catalase were found to prevent lipid peroxidation more efficiently than piroxicam (at a ten-fold higher concentration than OA) and aspartame (at equimolar concentration).



Aspartame



Piroxicam

Figure 1.3 Chemical structures of aspartame and piroxicam.

1.8.2 Inhibition of phenylalanine-tRNA formation.

Creppy *et al.*, 1984, reported that OA, as a phenylalanine analogue, suppresses protein synthesis by the competitive inhibition of phenylalanine in the Phe-tRNA aminoacylation reaction catalysed by phenylalanyl-tRNA synthase (PheRS). This was illustrated by *in vitro* studies conducted on both bacterial and eukaryotic systems (Konrad and Röschenthaler, 1977; Bunge *et al.*, 1978 and Creppy *et al.*, 1979b). Apart from the effect on protein synthesis, OA also inhibits the synthesis of DNA and RNA.

Creppy *et al.*, 1979a and 1984, reported that the inhibitory effect of OA could be alleviated by the addition of phenylalanine substrate to hepatoma cells and in mice *in vivo*. Mayura *et al.*, 1984, illustrated that the administration of phenylalanine alleviated the teratogenic effects induced by the prenatal exposure of rats to OA.

Creppy *et al.*, 1983, conducted a study on the effects of OA metabolites on yeast phenylalanyl-tRNA synthetase and on the growth and *in vivo* protein synthesis of hepatoma cells. It was found that both OA and the metabolite (4*R*)-OH-OA was toxic

to the cell cultures. It was postulated that the phenylalanine moiety, acting not in isolation, could be linked to the toxicity of OA and (4*R*)-OH-OA due to the fact that Oα had no effect on the growth of cell cultures and by the reversal of the inhibition of cell growth by the addition of phenylalanine.

Creppy *et al.*, 1983a, further illustrated the direct effect of OA and (4*R*)-OH-OA on the catalytic action of PheRS. It was established that both compounds inhibited the reaction catalysed *in vitro* by this enzyme and that their toxicity could be partly ascribed to this ability. Creppy *et al*, 1983a, extended the study to include OA analogues with respect to modification to the L-Phe. The OA-phenylalanine-tRNA synthetase system exhibited the highest affinity to OA compared to other amino acid analogues (K_I ~1.3 mM). This was followed by Tyr-Oα, Ala-Oα, Trp-Oα and Ser-Oα expressing K_I values of 1.5, 1.6, 2.3 and 6.0 mM respectively. Val-Oα, Met-Oα, Glu-Oα and Pro-Oα had markedly lower affinity with K_I values of 11.0, 13.0, 14.5, and 19.0; respectively. All of the amino acid OA analogues inhibited the growth of hepatoma cells at a concentration of 90 μM. Tyr-Oα, Ala-Oα proved the most cytotoxic with the first 24 hours, followed by Ser-Oα and Val-Oα after 48 hours. The remaining analogues seem to be less pronounced and exerted cytotoxicity within 72 hours.

Creppy *et al.*, 1983a, concluded by reporting on the inhibition of protein synthesis in hepatoma cells by OA analogues. Ala-Oα was shown to have a similar inhibition to OA, with Val-Oα and Ser-Oα exerting a slightly lower inhibition. Met-Oα and Trp-Oα had a slower offset but were more pronounced after 90 minutes. Glu-Oα had a momentary inhibitory effect after an hour, with protein synthesis returning to normal after 4 hours. Pro-Oα had little effect. It was emphasised that no apparent correlation existed between the inhibition constants of aminocyl-tRNA synthetase for the various amino acid analogues and the effect observed on the cell growth or protein synthesis.

1.8.3 Inhibition of mitochondrial ATP synthesis

Moore and Truelove, 1970; reported that OA inhibits mitochondrial state 3 and 4 respiration, by the competitive inhibition of mitochondrial transport carrier proteins located in the inner mitochondrial membrane. In addition OA also alters the

mitochondrial morphology after *in vivo* administration to rats (Suzuki *et al.*, 1975; Brown *et al.*, 1986).

Meisner, 1976, implicated OA in the disruption of the intramitochondrial phosphate transport systems, as the major cause of mitochondrial deterioration. The maintenance of structural integrity of the mitochondria is essential for the optimum production of ATP. The disruption of inner mitochondrial membrane leads to the inability to transport H^+ ions from and to the matrix *via* the F_0 channel in the F_0F_1 synthase complex and to subsequently generate ATP from ADP by catalytic phosphorylation.

This effect is further amplified by the fact that the uptake of OA is an energy consuming process. The depletion of intramitochondrial ATP was most pronounced in the middle and terminal segments of the proximal tubule (Jung and Endou, 1989). The direct correlation between the inhibition of mitochondrial ATP synthesis and the toxicity of OA is not known, as can be seen by the ability of Oα, predominantly non-toxic, to inhibit mitochondrial respiration in rat liver (Moore and Truelove, 1970).

1.9 Prevention and Treatment

Several different approaches can be followed in order to minimize the exposure of humans to OA and to alleviate the toxic effect in animals. Some of the factors that are involved in the growth of storage fungi are moisture, temperature, time of storage, the integrity of the seed, O_2 and CO_2 concentrations, composition of the substrate, fungal abundance and the proportion toxigenic strains present, inter microbial interaction and routes of transmittance (Marquardt and Frohlich, 1992; Abramson and Mills, 1985).

The regulation of moisture content together with the storage temperature of feeds and foodstuffs is essential in the prevention of OA production (see section 1.2). Post harvest treatment of crops by gamma and electron beam irradiation has shown to be effective against mycotoxin producing fungi such as *A. aluteus* (Chelack *et al.*, 1991b). Tong and Draughan, 1985, reported that addition of antimicrobial food additives such as methyl paraben and potassium sorbate was effective in regulating the growth of *Aspergillus* and *Penicilium* toxigenic fungal species.

Several dietary additives have been employed to reduce OA absorption and bioavailability. Madhyastha *et al.*, 1992, reported that cholestyramine, a commercial anion exchange resin, has been shown to be an effective absorbent of OA in the gastrointestinal tract of non-ruminant animals. It was shown that 0.5% cholestyramine in the diet of a rat containing 1 mg/kg of OA, reduced the concentration of OA in the blood by 50%, and further reduced the urinary excretion and enhanced the total faecal excretion of OA.

The impact of phenylalanine administration in alleviating teratogenic and immunosuppressive effects of OA poisoning in animals has been discussed (section.1.7.3 and 1.7.4.). The action of superoxide dismutase and catalase together with aspartame and piroxicam in the prevention of the nephrotoxic effects of OA has been discussed (section 1.8.1).

Haazele, 1992, reported that the ascorbic acid (Vitamin C) addition to the diets of laying hens markedly reduced the toxic affects, including a decrease reduction of egg mass, of OA as compared to OA poisoned laying hens not treated with ascorbic acid. It has been suggested that the anti-oxidant could suppress the offset of lipid peroxidation. Gastrointestinal microorganisms in ruminants play an important role in the disposition of OA because they promote the hydrolysis of the toxin to non-toxic Oα. This is also of consequence to non-ruminant animals as found by the role that microbial populations play in the caecum and large intestines (Madayastha *et al.*, 1992) (Section 1.6).

1.10 Conclusions

From the discussion on the metabolism of OA in bacterial and animal cells, it is evident that an array of structural functionalities is implicated in the toxicity of the ochratoxins. The implicated OA functionalities include:

The halogen group: The chlorine group is evidently important in the toxicity of OA since the dechloro analogue OB is ten times less toxic. The chloro group functions in a direct role in allowing for the interaction of OA and phenylalanyl RNA-synthetase. This leads to the inhibition of protein synthesis as well as the synthesis of RNA and

DNA. OB has no interaction with phenylalanine tRNA-synthetase. The presence of the halogen group could further be implicated indirectly in the relative rates of hydrolysis of OA and OB.

In addition the chloro group may function in an indirect role, i.e. by affecting the dissociation of the phenolic hydroxyl group. In coherence to this notion, the bromo-analogue reveals an increased toxicity to renal cells (Creppy, 1999b). Recent reports showed that the methyl ester of iodo-ochratoxin is approximately twice as toxic as the bromo-analogue.

It is assumed that the dissociation of the phenolic hydroxyl group will decrease as the electronegativity of the aromatic halogen group decreases which could be a plausible explanation for the toxicities observed.

The phenolic hydroxyl group: The hydroxyl group, in its dissociative form has clearly been implicated in lipid peroxidation. This is evident in that the toxicities of the different analogues correlate with the dissociation constants of the phenolic group. This is evident in the high toxicity of OA and OC compared to the much lower toxicity of the *O*-methylated analogue. The hydroxyl group has also been implicated in the formation of DNA adducts and the bioavailability of OA by binding to serum albumin.

Amide bond: The peptide bond is readily cleaved by proteolytic enzymes carboxypeptidase A, trypsin and α-trypsin. It was found that OA had a much greater affinity for CPA than for trypsin or α-trypsin. The stability of the amide bond was first implicated in the toxicity of OA by the isolation of natural occurring, non-toxic Oα.

It was proposed that the rate of hydrolysis, a detoxification process rendering non-toxic metabolites Oα and Phe, of OA was to play an important role in the half-life of the toxin and ultimately its toxic effect *in vivo*. *In vitro* kinetic studies with the enzyme bovine carboxypeptidase A revealed that OB was hydrolysed up to two hundred times times faster than OA. It was suggested that the lesser toxicity of OB

was due to the more rapid detoxification by hydrolysis. This was challenged by reports that OB is inherently non-toxic as was shown by the inability to inhibit protein synthesis.

The lactone moiety: The lactone functionality has been the centre of biotransformation of OA to lesser toxic hydroxylated analogues (4*S*,4*R*)-OH-OA and 10-OH-OA in fungal and animal systems. The lesser toxicity may be ascribed to the increased rate of excretion into the bile and urine.

The isolation of the opened lactone form (OP-OA) analogue has implicated the lactone carbonyl group in the possible covalent modification of certain biomacromolecules. In the case of the animal models, a lower rate of delivery to target organs (kidney and liver) may explain the lower toxicity of OP-OA relative to OA.

Amino acid substituent: The natural occurrence of amino acid modified OA analogues, lysine, serine and hydroxyproline emphasized the role of the amino acid moiety in the toxicity of OA. An attempt to correlate the binding affinity of the analogues to aminoacyl-tRNA-synthetase to the inhibition of hepatoma cell growth and the inhibition of protein synthesis was inconclusive.

Ala-Oα and Tyr-Oα proved to be the most toxic with regard to the inhibition of protein synthesis and hepatoma cell growth.

No apparent correlation could be established between the chirality of the amino acid and the toxicity of OA in bacterial cells. This was shown in the similar toxicities of D-OA and OA in *B. brevis*. The study was inconclusive owing to the inability of bacterial cells, unlike eucaryotic cells, to distinguish between the L and D isomers. However, a decrease in toxicity of D-OA to eukaryotic (HeLa cells) systems (30 fold less toxic) revealed that the chirality of amino acid substituent may play an important role in the toxicity of OA.

The carboxylic acid group: The ethyl esters of both OA and OB showed greater toxicity than the corresponding parent molecules. It was proposed that the increase in toxicity was due to an increase in the molecular weight of the toxin. Holding true to this approach, the *N*-ethylamide analogue OE-OA, was found to be four times more toxic to *B. brevis* than OA. It was further suggested that the increase in lipophilicity, and subsequent cell permeation, of the ethyl ester analogue relative to OA might play a more important role than the increase in molecular weight. The carboxylic acid moiety has also been implicated in the bonding of protein.

SECTION B: THE SYNTHESIS OF THE OCHRATOXINS AND ANALOGUES.

1.11 Introduction

In this chapter the chemistry that serves as the necessary background to our research effort is highlighted. A sound understanding of this chemistry is essential for the preparation of ochratoxin analogues. The synthesis of these compounds can be accomplished by the modification of either OA or Oα, which if viable, would be a more elegant route than the alternative of a *de nova* synthesis. Aspects of both these distinct synthetic routes will be discussed starting with the total synthesis of OA, which in part encompasses the synthesis of dihydroisocoumarins. A proposed method for the *de nova* synthesis of fluoro-ochratoxin will be derived from these.

An overview of the synthesis of ochratoxin analogues by direct modification of OA or Oα will include, a) the substitution of the phenylalanine side chain, b) modification of the phenolic hydroxyl group, and c) the substitution of the chloro group. A discussion on the methods available for the synthesis of fluoroaromatic compounds will conclude the chapter.

1.12 The synthesis of OA and OB

The acid hydrolysis of OA furnishes a chlorolactone acid (Oα), formulated as 7-carboxy-5-chloro-3,4-dihydro-8-hydroxy-3-methylisocoumarin, and L-phenylalanine. From a synthesis design point of view the first step towards the synthesis of OA must be the preparation of the dihydroisocoumarin, which could then be coupled *via* an amide bond at its 7-carboxyl group to L-phenylalanine.

1.12.1 The synthesis of dihydroisocoumarins

The importance of the isocoumarins as discrete natural products was confirmed in early biosynthetic studies. It was established, through a range of labeling experiments, that the

isocoumarin moiety of OA is formed *via* the acetate-polymalonate pathway (Fereira and Pitout, 1969; Steyn *et al.*, 1970). The incorporation of the carbonyl carbon atom (derived from the C_1 pool) allowed for coupling to phenylalanine (derived from the shikimic acid pathway). The different approaches to the synthesis of OA have varied little from the established biosynthetic route.

1.12.1.1 Steyn and Holzapfel, 1967.

The synthetic approach to the lactone acid **VII**, was based on the fact that the 3,4-dihydro-3-methylisocoumarins can be prepared by the oxidation of the corresponding isochroman, 5-chloro-7-hydroxymethyl-8-methoxy-3-methylisochromans **VI** (Figure 1.4).

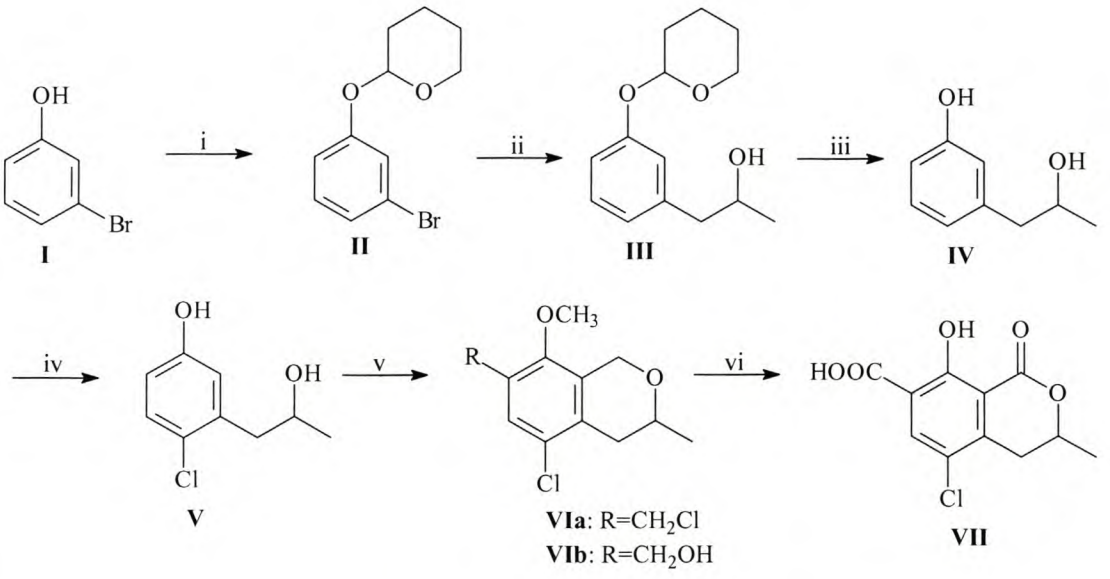


Figure 1.4 Synthesis of Oα by Steyn and Holzapfel, 1967.

Reagents: i) Dihydropyran, H^+, ii) (a) Mg, cat. EtBr, in THF (b) propylene oxide, iii) HCl, iv) Cl_2, v) $ZnCl_2$, $ClCH_2OCH_3$, vi) CrO_3.

The intermediate isochroman, **VIb**, was prepared from the protected 3-bromophenol, **II**, which was converted to the deprotected 1-(3-hydroxyphenyl)propan-2-ol, **IV**, by means of a Grignard reaction. This was followed by the non-specific chlorination of **IV** that produced the chloro analogue, **V**, in low yield. **V** was subsequently chloromethylated to **VIa**, which in turn was hydrolysed to afford the intermediate isocroman, **VIb**. Finally, the

oxidation of **VIb** yielded the desired Oα, **VII**. The low overall yield of 10% was ascribed mainly to the non-selective chlorination step.

1.12.1.2 Roberts and Woollven, 1970.

Roberts and Woollven proposed the following method for the synthesis of Oα starting from 4-chloro-7-hydroxyindanone, in which the phenolic hydroxyl group and the chlorine function were already present and correctly orientated. The dihydroisocoumarin carboxylic acid **X** was synthesized from **I** in nine steps with an overall yield of 0.65% (Figure 1.5).

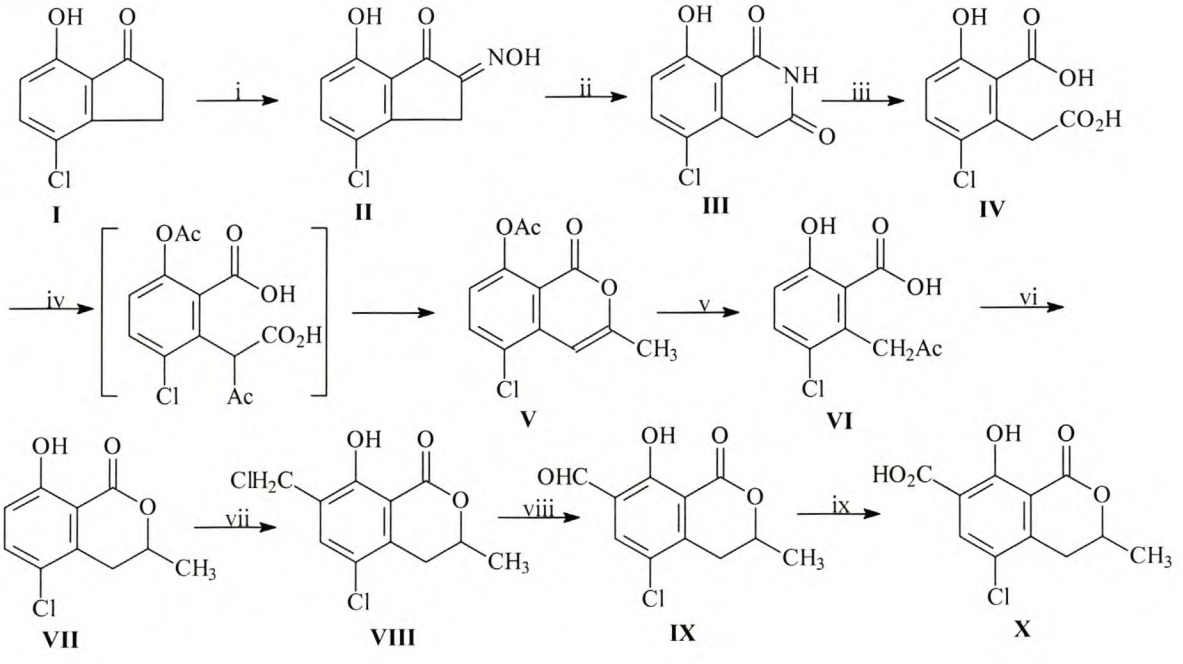


Figure 1.5 Synthesis of Oα by Roberts and Woollven, 1970.

Reagents: i) Isopentyl nitrite, HCl, ii) Toluene-*p*-sulphonyl chloride, iii) (a) NaOH (b) HCl, iv) $AcONa\text{-}Ac_2O$, v) (a) NaOH (b) HCl, vi) (a) $NaBH_4$ (b) HCl, vii) $TiCl_4$, $ClCH_2OCH_3$, viii) (a) $(CH_2)_6N_4$ (b) aq. AcOH, ix) Ag_2O.

1.12.1.3 Sibi *et al.*, 1985.

Sibi and coworkers reported on the combined use of carbamate- and amide-directed *ortho*-metalation reactions to achieve rapid access to multifunctional aromatic systems, which was extended to the synthesis of isocoumarins, Oα and Oβ.

In the synthetic design, involving comprehensive use of aromatic metalation, the first electrophile E introduced into the *ortho*-lithiated carbamate **I** is chosen to be compatible with subsequent metalation strategies and as a weaker *ortho*-director than the carbamate. This forces the second metalation into the alternative *ortho* site, allowing the 1,3-carbamoyl rearrangement (step ii). Following phenol protection, the third metalation is directed by the migrated amide, leading to the regioselective introduction of the second electrophilic substituent (step iv)(Figure 1.6).

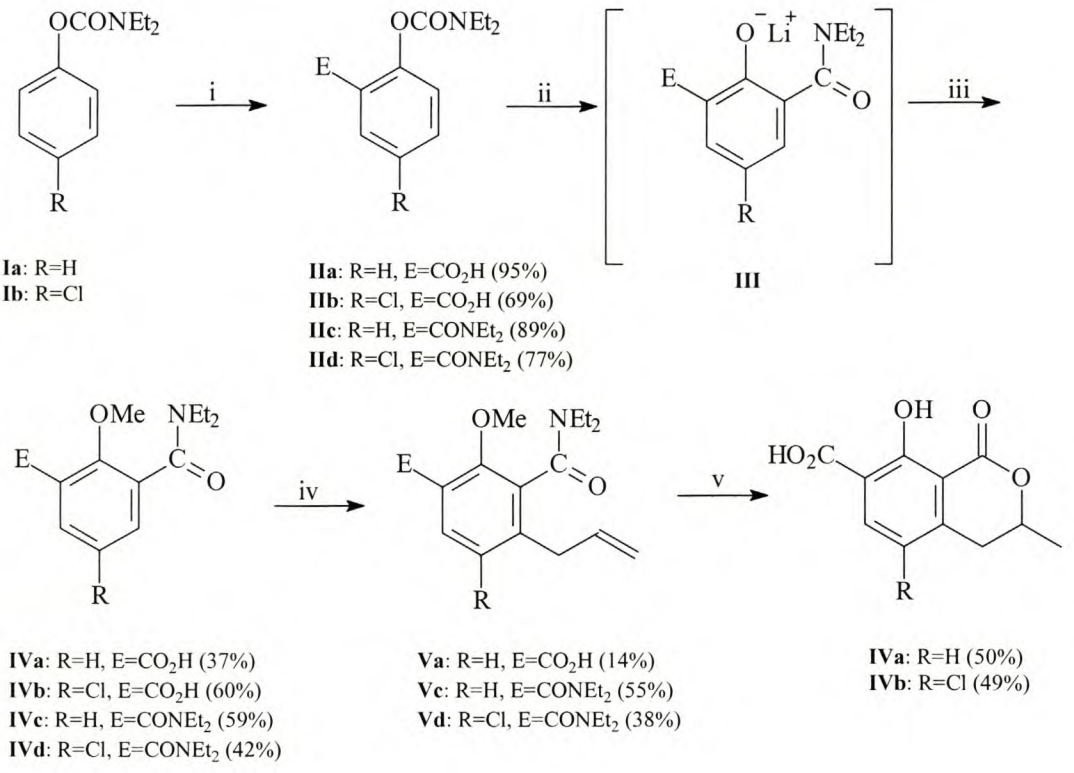


Figure 1.6 Synthesis of Oα and Oβ by Sibi *et al*, 1985.

Reagents: i) (a) *sec*-BuLi, TMEDA in THF, –78°C (b) $CO_{2(s)}$ (IIa,b), $ClCONEt_2$ (IIc,d), ii) *sec*-BuLi, TMEDA, iii) MeI, iv) (a) *sec*-BuLi, TMEDA (b) $MgBr_2.Et_2O$ (c) Allyl bromide, v) HCl

Apart from the relative modest overall yields obtained for Oα and Oβ, 14% and 6% respectively, a few drawbacks exist on the synthetic approach. In spite of considerable experimentation, improvement in the yield of the transformation **IVa-Va** was not achieved. Furthermore, the chloro benzamide **IVb** precursor for Oα, obtained in good yield by the analogous sequence **IIb→IIIb→IVb**, failed to undergo the transmetallation-allylation reaction corresponding to the conversion **IVb→Vb**.

1.12.1.4 Kraus, 1981.

From an efficiency point of view the synthetic route applied by Kraus is the most successful up to date, generating a total overall yield of 20% for Oα (Figure 1.7).

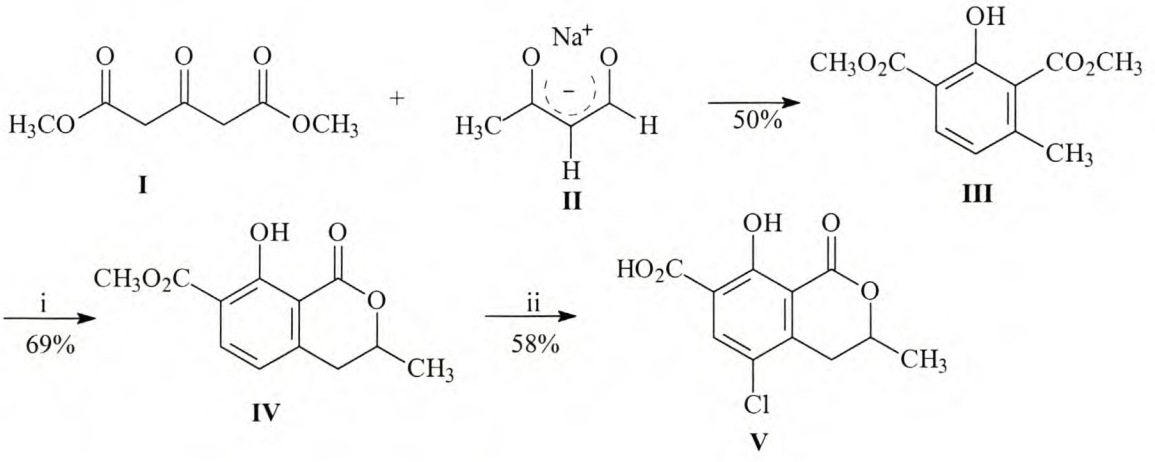


Figure 1.7 Synthesis of Oα Kraus, 1981.

Reagents: i) (a) 2 *i*-Pr_2NLi, THF-HMPA, -78°C (b) CH_3CHO, ii) (a) SO_2Cl_2, CH_2Cl_2 (b) LiOH, H_2O, CH_3OH

The diester **III** was prepared in one step from dimethyl 3-oxopentanedicarboxylate **I** and the sodium salt of hydroxymethyleneacetone **II**. **III** was subsequently deprotonated with 2 equivalents of lithium diisopropylamide in THF-hexamethylphosphortriamide at –78°C. The addition of acetaldehyde and aqueous acid workup produced lactone **IV** in good yield. Chlorination of **IV** with sulfuryl chloride in methylene chloride afforded a chlorolactone that was immediately suspended in methanol and saponified with aqueous lithium hydroxide. Apart from the relative low yield of III, this route is by far the most

efficient preparation of ochratoxin intermediates and should be amenable to considerable variation.

1.12.1.5 A proposed method for the synthesis of 5-fluorodihydroisocoumarin.

In terms of electrophilic aromatic substitution the efficient incorporation of a fluoro substituent into a multifunctional aromatic ring will most likely be encountered with difficulty. The fluorination of the aromatic nucleus will thus be confined to the first steps of a multi-step synthesis of the substituted fluoro benzene. Furthermore the lability or reactivity of the fluoro substituent to further strategies needs to be taken into consideration.

The successful bromination of sodium acetoacetaldehyde to produce 2-bromoaceto acetaldehyde has prompted the question whether this method could be extended to fluorination (Lipinsky *et al.*, 1984). Selective fluorination of dicarbonyl compounds has been reported by Banks *et al.*, 1996, using Selectfluor^{TM} and Chambers *et al.*, 1999, using F_2. The synthesis of the fluorinated analogue to the intermediate used by Kraus, 1981(Figure 2.4) will allow for the incorporation of the fluorine substituent at an early stage. The sodium salt of 2-fluoroacetoacetaldehyde, when reacted with dimethyl 3-oxopentanedicarboxylate I, can furnish the fluorinated aromatic diester (Figure 1.8).

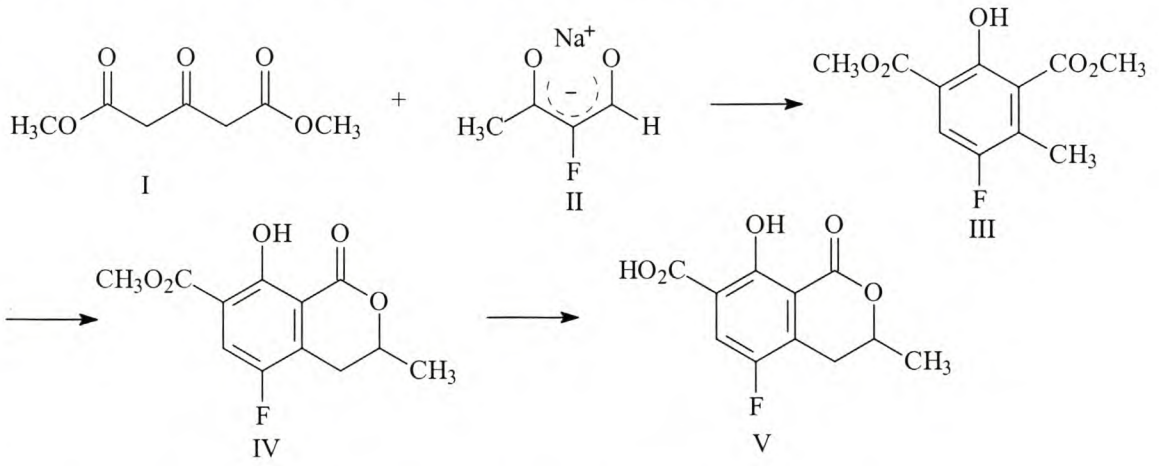


Figure 1.8 A proposed route to 5-fluorodihydroisocoumarin *via* a fluorinated diester analogous to the synthesis by Kraus, 1981, of the chloro equivalent.

In the synthetic routes taken by both Sibi *et al.*, 1985, and Roberts and Woollven, 1970, the chlorine substituent was incorporated into the aromatic ring at the start of the sequence. This approach evades the non-specific chlorination step. Sibi and coworkers showed, in the synthesis of Oα and Oβ, that the chlorine substituent has an inferior role to play in the *ortho*-directed metallation of the aromatic nucleus.

Of greater importance will be the choice of starting compounds. Sibi *et al.*, 1985, could not achieve the transmetallation-allylation of the chlorobenzamide (**IVb**, Figure 1.6), and the corresponding dechloro benzamide **IVa**, Figure 1.6) could only be afforded in the lowest yield for the allylic species **Va** (14%)(Figure 1.6). If it is assumed that the fluoro substituent will act analogously to the chloro group in activating the *ortho*-position (secondary to the amide activation) the use of **Ib** and **IIb** will be the desired starting compound and intermediate of choice (Figure 1.9).

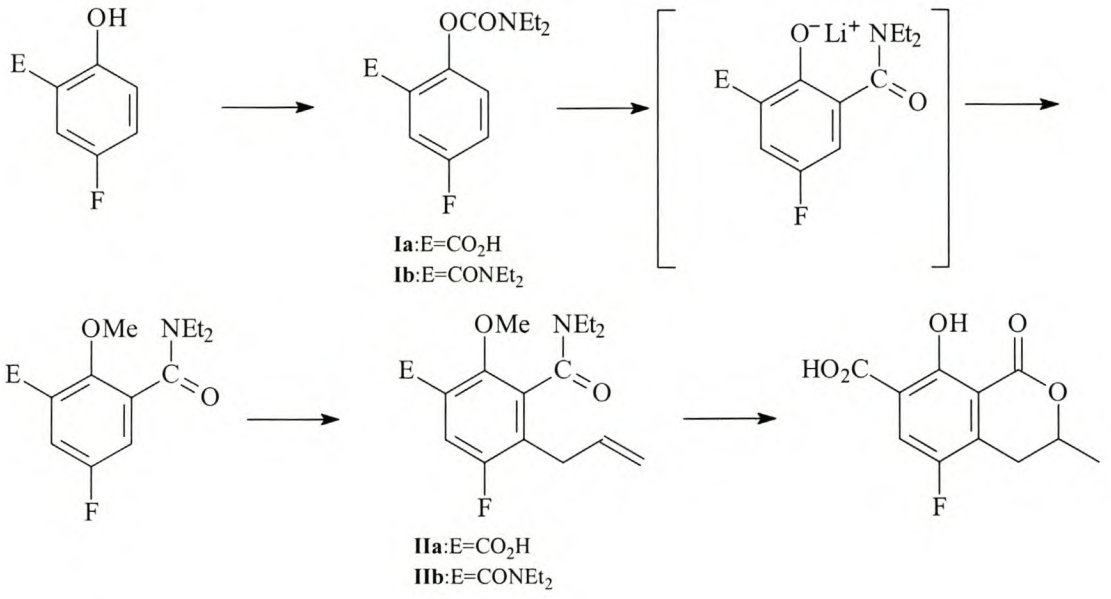


Figure 1.9 A proposed synthesis of 5-fluorodihydroisocoumarin from 5-fluorosalicylic acid *via* a *ortho*-directed metallation approach.

1.13 Synthesis of ochratoxin A analogues.

Most of the functional groups that constitute OA have been implicated in its toxicity. In order to probe the activity of a substituent, modified analogues of OA are synthesized and their toxicity measured relative to OA. High efficiency, single step conversion reactions form the ideal to this study from a cost and time point of view. Functional groups that have been probed for reactivity include a) the phenylalanine side chain b) phenolic hydroxyl group and c) the halogen group.

1.13.1 Substitution of L-phenylalanine.

Probing this reactivity, by substitution of the L-phenylalanine by other amino acids, requires a thorough understanding of peptide synthesis methodologies. A first aspect to consider is the acid hydrolysis of OA for preparation of the starting material, Oα. This will be followed by an overview of the most suitable coupling techniques available for the synthesis of analogous peptide side chains.

1.13.1.1 Acid hydrolysis of OA.

The availability of Oα, directly from the acid hydrolysis of OA, forms an easy access route to the coupling of novel amino acids to the lactone acid. *N*-Substituted amides (RCONHR′), such as OA, can by hydrolysed by either acidic or basic catalysis, with the products being the free acid and the primary amine. The mechanism entails cleavage of the peptide bond brought about by the protonation of the acyl oxygen in acid catalysis and the nucleophilic substitution of the amino group by water or a hydroxyl group in base catalysis. In acidic medium, a water molecule reacts with the resultant oxonium ion at the carbonyl carbon, allowing for the formation of the free acid. Prolonged heating is often required for complete hydrolysis of the amide.

Steyn, 1967, employed acid hydrolysis for the formation of Oα from OA. The method described required the treatment of OA with 6M HCl for 30 h under reflux. The chloroform extract furnished the lactone acid in excellent yield. Xiao *et al.*, 1995, employed a modification of this procedure in prolonging the reaction period (72 h) at

room temperature. The hydrolysed Oα crystallised from the 6M HCl solution over a 12 h period and was isolated by filtration. The relative harsh conditions necessary for the hydrolysis of the amide bond of OA compared to aliphatic amides can be ascribed to several factors. Firstly, the electron rich aromatic ring will lower the electrophilicity of the carbonyl carbon and thus retard the hydrolysis reaction. Furthermore, the electron donating *o*-hydroxyl group will increase the electron density at the *ipso* position, rendering the carbonyl carbon less electrophilic. Thirdly, and to a lesser extent, from the structure determined by Bredenkamp *et al.*, 1989 (Figure 1.2), it could be envisioned that the relatively stable six-membered ring will demand more drastic hydrolysis conditions for the cleavage of the amide bond as is used for other amides.

1.13.1.2 Peptide Synthesis

The creation of a peptide bond usually entails the coupling of two amino acid derivatives. More specifically, it requires the nucleophilic attack of a free amine **I** of one amino acid on the carboxyl carbonyl carbon **II** of the other. The subsequent elimination of the carboxylic heteroatomic group renders the amide bond **III** (Figure 1.10).

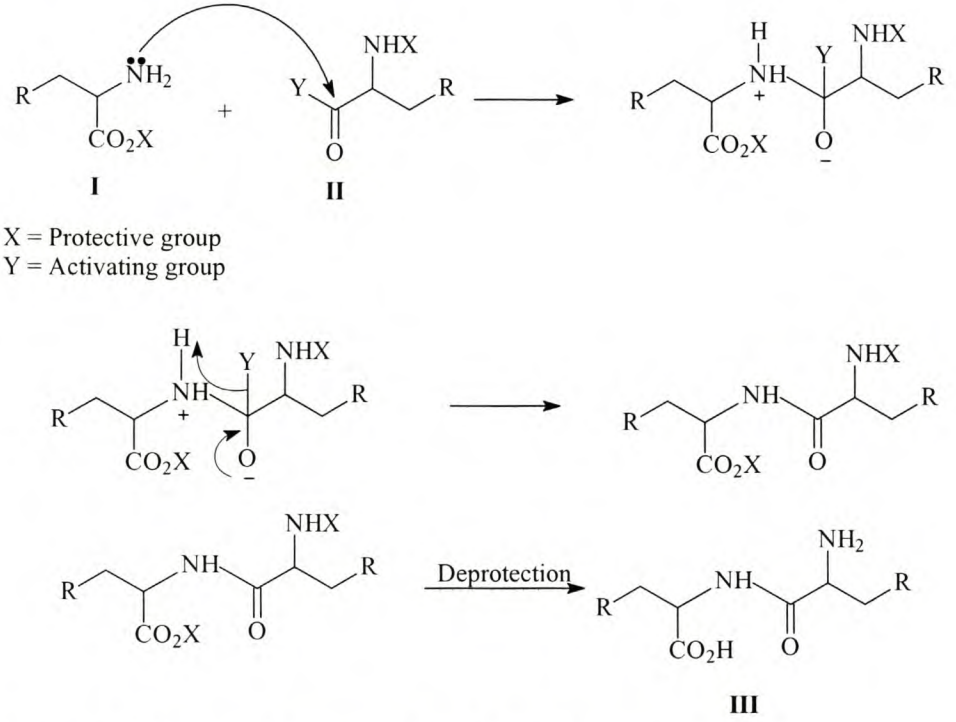


Figure 1.10 The mechanism of peptide bond formation.

However, in order to achieve effective coupling of the reactive groups, the functional groups that are not directly involved in the amide bond-forming reaction must be protected or blocked. The reactivity of the N^{α}-amino group of one of the amino acids and the *C*-terminal carboxyl group of the other are both blocked with suitable protecting groups. This is followed by the activation of the free carboxylic acid towards nucleophilic attack by the free amine group.

The amine N^{α}-protecting groups normally used are urethanes (alkoxycarbonyl derivatives). These groups can be easily introduced, as well as removed by simple acyl cleavage. The resultant carbamic acids decarboxylate spontaneously generating the free amine with minimal racemisation of the stereogenic centre. Examples of urethane-based N^{α}-amino-protecting groups include benzyloxycarbonyl groups, *tert*-butoxycarbonyl group (Boc), the 9-fluorenylmethoxycarbonyl group (Fmoc) (Lloyd-Williams *et al.*, 1997). The benzyloxy- and butoxycarbonyl groups are acid labile, whereas the Fmoc group can be removed by treatment with a solution of secondary amine. Carboxylic acids are usually protected by conversion to the corresponding methyl or ethyl esters.

Activation of a carboxyl group of an amino acid (in the case of Oα, a benzoic acid) can be achieved by the derivatisation of the acyl carbon with an electron-withdrawing leaving group. The increase in the electrophilic nature of the carboxyl carbonyl carbon as well as the decrease in bond strength to the activating group, allows for the nucleophilic substitution by the amine group. The activated states that have been employed in the coupling of Oα to L-Phe and other novel amino acids include acyl chlorides (Xiao *et al.*, 1995), acyl azides (Steyn *et al.*, 1967), and coupling agents such as dicyclohexylcarbodiimide (DCC) (Steyn, 1965), *N*-hydroxysuccinimide (NHS) (Rousseau *et al.*, 1984) and *N,N*-carbonyldiimidazole (CDI) (Xiao *et al.*, 1995). The reactivity of these groups towards nucleophilic substitution corresponds to their electron withdrawing capability (hydroxyl < secondary amine < azide < activated ester < anhydride < chloride).

The reactions described can be carried out in two phases including reactions in liquid phase or solid phase synthesis (Merrifield, 1959). The latter approach allows for a sustained control over the protection, deprotection and isolation steps in the sequence. For the creation of a single peptide bond, as in the coupling of amino acids to Oα, these reactions are normally conducted with great success by solution phase synthesis.

1.13.1.2.1 Activation as acyl chloride.

The acyl chloride functionality provides the most reactive acyl species for amide bond formation. However, the drastic conditions necessary to generate them, such as the treatment with thionyl chloride or phosphorous pentachloride, has prevented its application in the presence of sensitive amino acid derivatives. Although several milder methods for its preparation have been proposed, these methods have not been shown to tolerate sensitive *tert*-butyl or trityl protective groups (Schmidt *et al.*, 1988).

The formation of the acid chloride of Oα (Oα-Cl) and the subsequent coupling with the methyl ester of L-phenylalanine could readily be achieved, however the product isolated (in 90% yield) was predominantly the racemic product (Steyn and Holzapfel, 1967). The process entails the treatment of the lactone acid with thionyl chloride to produce the acyl chloride which is then treated with the methyl ester of L-phenylalanine in dry pyridine.

Xioa *et al.*, 1995, employed an alternative procedure to produce the acyl chloride intermediate, and reported that no racemization took place in the coupling of D-phenylalanine methyl ester to Oα. This was accompanied by excellent yields (85%) of D-OA. The procedure entails the treatment of Oα with thionyl chloride in anhydrous chloroform at reduced pressure. The hydrochloride salt of the methyl ester of phenylalanine was deprotonated by fine dry sodium metal powder in anhydrous chloroform and reacted with freshly prepared Oα-Cl. The efficiency of the reaction is greatly enhanced by the use of the sodium metal, which deprotonates the amino group which, if protonated retards the reaction. The subsequent hydrolysis of the methyl ester of OA could lead to the opening of the lactone ring. Thus the reaction mixture should be acidified to pH 1.0 and left for 12 hours to ensure the restoration of the lactone ring.

1.13.1.2.2 Activation as acyl azide

In contrast to the acyl chloride method, the use of acyl azides as an activated intermediate provides minimal racemisation but is prone to low yields. This was illustrated by Steyn and Holzapfel, 1967, in the coupling of both 5-chlorosalicylic acid and Oα to L-phe (Figure 1.11). The advantage of this method lies in the stability of the corresponding azide. Payne, 1998, in an attempt to improve the yield of the coupled product, investigated the stability of 5-chlorosalicyl azide and the coupling of 5-chlorosalicylazide to L-phe in solution and in the presence of bases: Na_2CO_3, triethylamine (TEA) and 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU). Although no method could be established for increasing yields above 50%, which will make the application to Oα viable, it was found that in the use of 2 eq TEA in the coupling process the reaction time could be accelerated from 1-4 days to 2 hours.

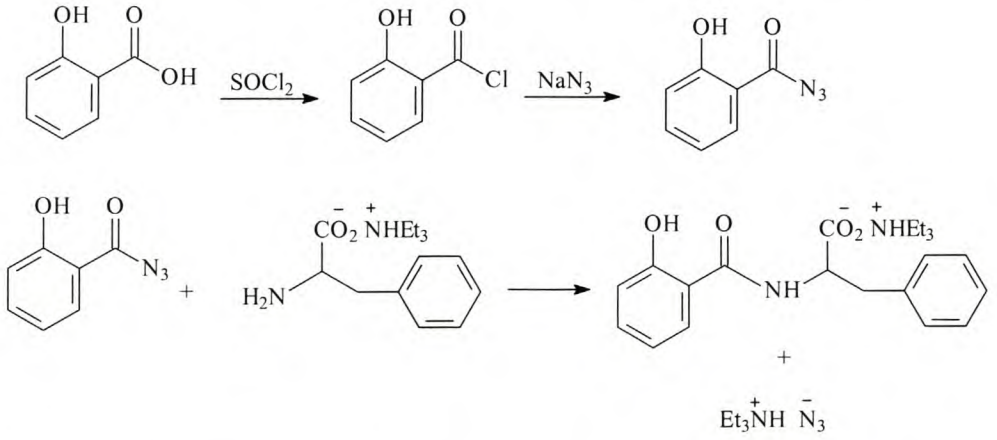


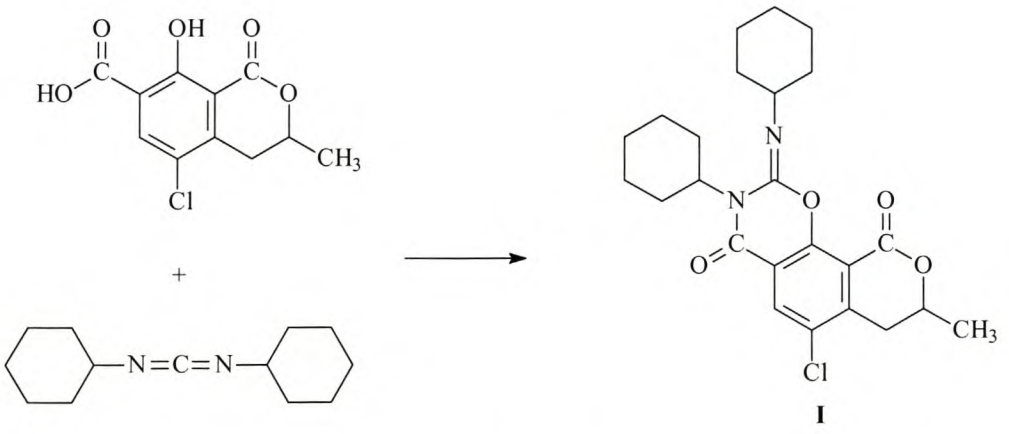
Figure 1.11 The coupling of salicylic acid to L-Phe by acyl azide activation (Payne, 1998).

1.13.1.2.3 Activation by coupling reagents

The use of coupling reagents as carboxyl activating agents is the most prevalent technique in modern peptide synthesis, of these the carbodiimides are used most often. The process entails the reaction of the coupling reagent with the free carboxylic acid group of the amino acid. The reactive intermediate, an *O*-acylisourea, will allow for amide bond formation to occur at room temperature, upon treatment with the amino component.

Dicyclohexylcarbodiimide (DCC) has been used with great success on a broad selection of substrates (Sheenan and Hess, 1955). The major advantage of this method lies in the fact that peptide coupling can be achieved at room temperature and in a short period of time.

Steyn and Holzapfel, 1967, used DCC in the coupling of L-phenylalanine methyl ester to Oα. This approach rendered very poor yields (<5%) of the desired coupled product. It was established that the majority of the lactone acid reacted rapidly with DCC to furnish a crystalline adduct **I** (Figure 1.12). Similar reactions with 5-chlorosalicylic acid showed that a similar adduct formed and that the free phenolic hydroxyl group was involved in the formation of the adduct.



I

Figure 1.12 Reaction of Oα with DCC to form adduct **I**.

In order to overcome this problem the hydroxyl group was acetylated, to form 2-acetoxy 5-chlorobenzoic acid, and reacted under similar conditions. Altough the desired product could only be obtained in 50% yield, it was shown that by the addition of p-nitrophenol to the acid and coupling reagent, prior to the addition of the L-phenylalanine methyl ester, the amide could be obtained in quantitative yield.

1.13.1.2.4 Other coupling techniques

Xiao *et al.*, 1995, reported that the use of CDI **I** as activating agent of Oα, resulted in slow reaction rates and low yield of the coupled product (Figure 1.13). The activated intermediate in this process is the acyl imidazolide **II**. Rousseau *et al.*, 1984; employed NHS **III** as coupling reagent in the synthesis of ^{14}C-labeled OA from Oα and [^{14}C]-phenylalanine (Figure 1.13). The reactive intermediate is the N-hydroxysuccinimide ester of the acid moiety **IV**. The product using this method of activation was isolated with difficulty, but in higher yield compared to the CDI-method and with maintenance of chirality.

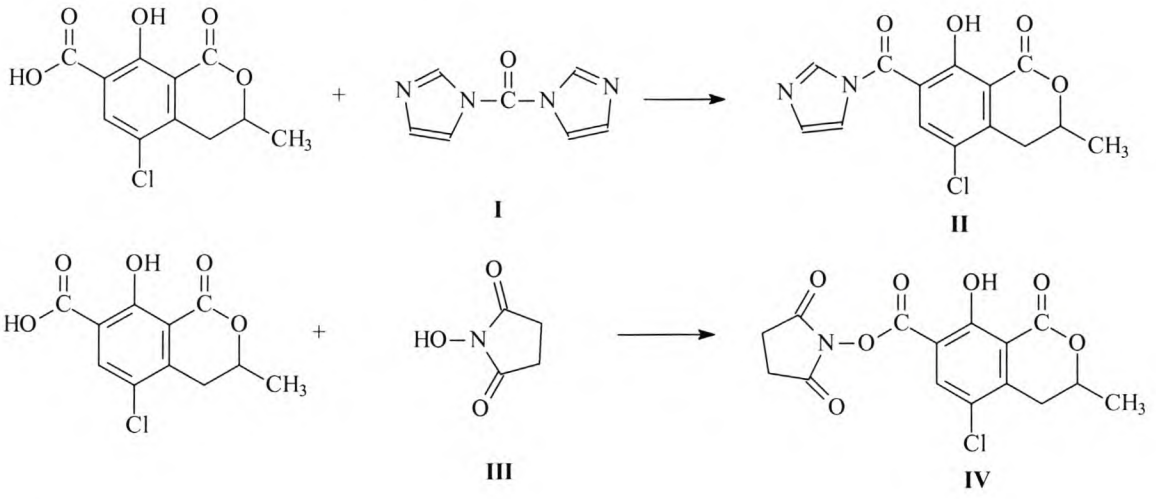


Figure 1.13 The carboxyl carbonyl activation of Oα by CDI **I** and NHS **III**.

1.13.2 Substitution of the carboxylic acid moiety of L-phenylalanine.

The free carboxylic acid functionality of the L-phenylalanine in OA has been extensively probed for its role in toxicity (Figure 1.14). Xiao *et al.*, 1995; used NHS for the synthesis of a reactive acyl intermediate, the NHS-ester of OA, which could be substituted by ethylamine to furnish the ethylamide of OA **I** without forfeiting steric integrity. Alternatively, the decarboxylated form of L-phenylalanine, phenylethylamine **II**, reacted readily with Oα-Cl, to form the phenylethylamide of Oα **III**.

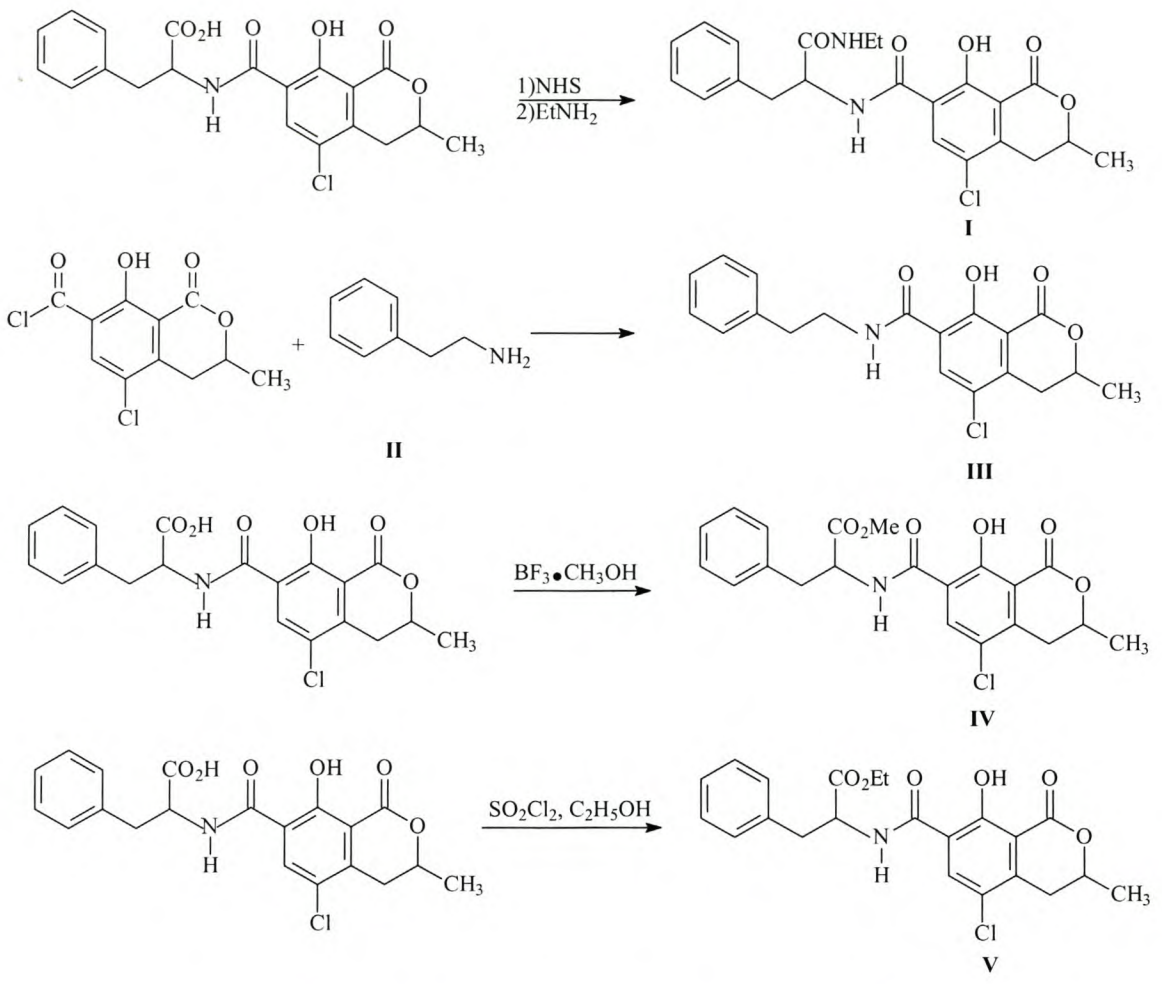


Figure 1.14 The synthesis of OA analogues with modification to the carboxylic acid group.

The methyl ester of OA **IV** can easily be obtained by the rapid treatment of the acid with a borontrifluoride-methanol solution (Figure 1.14). This method is widely used for the confirmation of the presence of OA in high performance liquid chromatography (HPLC) assays. Alternatively, the OA methyl and ethyl esters **V** can be attained by treatment of OA with sulphuryl chloride in methanol and ethanol solutions, respectively (Steyn *et al.*, 1967)(Figure 1.14).

1.13.3 Substitution of the phenolic hydroxyl

The *O*-methylochratoxin A methyl ester can be obtained by the treatment of OA with ethereal diazomethane (van der Merwe *et al.*, 1965). Furthermore, *O*-methylochratoxin A, can be obtained upon hydrolysis with 0.5M NaOH (Xiao *et al.*, 1995)(Figure 1.15).

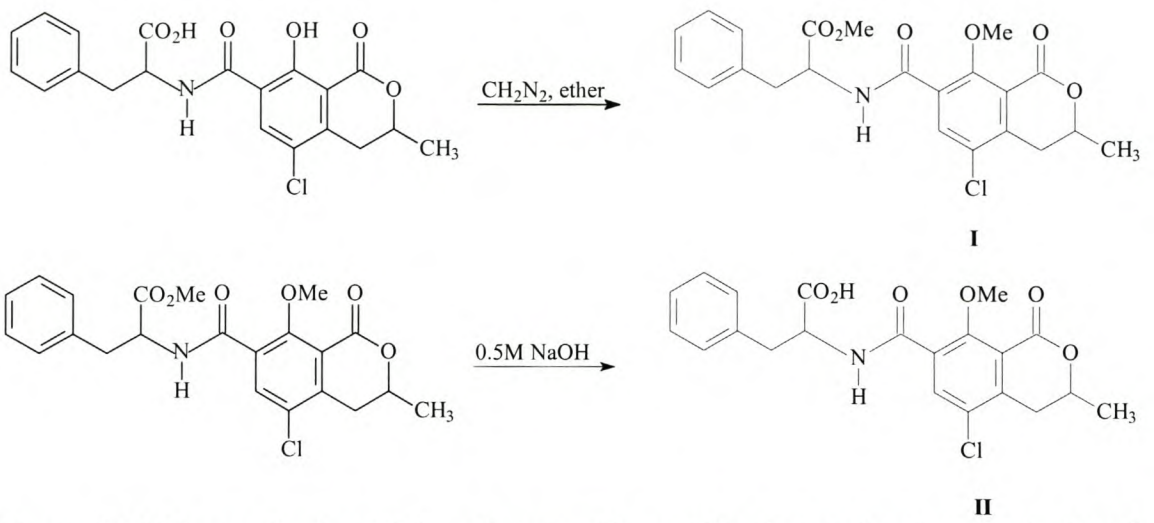


Figure 1.15 The synthesis of OA analogues with modification to the phenolic hydroxyl group.

1.13.4 Substitution of the chloro group.

OB, the dechloro analogue of OA, provides the substrate for aromatic halogenation. Alternatively, halogenation could occur *via* a halogen-exchange route, where the chloro substituent is directly replaced by another halogen (see section 2.6.1). These reactions only proceed at an acceptable rate if the leaving chloro group is activated by *ortho*- and/or *para*- electron withdrawing substituents. It is clear that this will not be feasible in the case of OA (Figure 1.16).

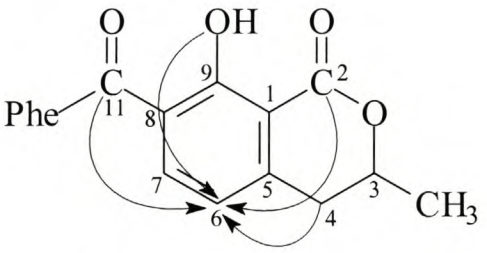


Figure 1.16 Directing effects of activating (OH, C_4) and deactivating (C_2, C_{11}) substituents towards electrophilic substitution of OB.

1.13.4.1 Hydrogenolysis of OA (as a route to OB).

Replacement of aromatic halogens by hydrogen can be accomplished by catalytic hydrogenation and reduction with complex hydrides such as $LiAlH_4$ (Beckwith and Goh, 1983), $NaAlH_4$ (Zakharin *et al.*, 1972) with inorganic (Zn-Ag couple Chung *et al.*, 1988) or organic reducing agents (*p*-toluenesulfonylhydrazine in chloroform, Sakamoto *et al.*, 1984).

Hydrogenolysis of aromatic halides using catalytic hydrogenation readily takes place with rates of replacement of halogen decreasing in the order I > Br > Cl > F (March, 1992). A general method for the replacement of aromatic chlorine by hydrogen involves catalytic hydrogenation over palladium on carbon, calcium carbonate or silica gel (Roth and Schleyer, 1983). Dehalogenation can also be achieved over platinum oxide (Adams' catalyst) and over Raney nickel. Reduction, especially those using Raney nickel, has to be carried out in the presence of alcoholic potassium hydroxide to neutralize the liberated hydrogen chloride (March, 1992).

In catalytic hydrogenation, chlorine is replaced by hydrogen in chlorinated aromatic hydrocarbons, phenols (Anwer and Spatola, 1985) and nitro benzenes (Akita *et al.*, 1986). Hydrogenolysis of chlorine in multifunctional aromatic compounds such as chloro -nitro and -carbonyl compounds, takes precedence over the reduction of nitro or carbonyl groups, provided that the halogen free compound is not over exposed to the reducing agent. The reaction is achieved using palladium on carbon or tetrakis(triphenylphosphine)palladium. Hydrogen donors, sodium hyposphite (Boyer *et al.*, 1985) or ammonium formate (Anwer and Spatola, 1985) can be employed in catalytic hydrogen transfer.

In a similar, more complex manner OA is highly functionalised containing carboxylic acid, lactone and amide carbonyl groups, which would easily be reduced by most of the mentioned reducing agents. Steyn and Holzapfel, 1967, employed Raney nickel in an alkaline solution in the dehalogenation of Oα to Oβ. The method described by Bredenkamp *et al.*, 1989, in which OA was dehalogenated over Pd/C in the presence of

ammonium formate and methanol proved to be fast and almost quantitative in yield (Figure 1.17).

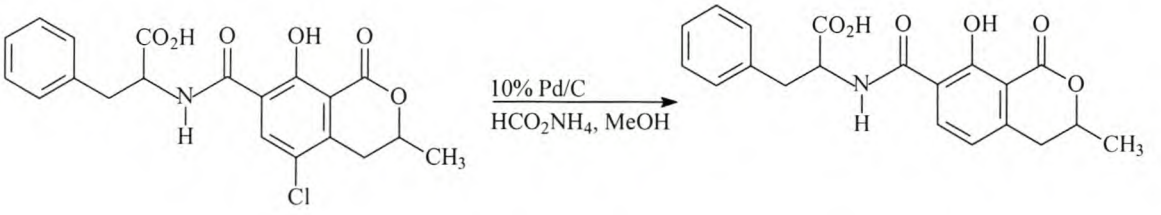


Figure 1.17 The catalytic hydrogenolysis of OA (Bredenkamp *et al.*, 1989).

1.13.4.2 Bromination of OB

The bromination of aromatic compounds is usually achieved by treatment with bromine in the presence of a catalyst. For aromatic compounds activated towards electrophilic bromination such as anilines, phenols and naphthalenes, bromination proceeds very rapidly and in the absence of a catalyst (Baciocchi and Illuminati, 1967). Phenols can be brominated exclusively in the *ortho* position by treatment with bromine at –70 °C in the presence of *t*-butylamine or triethylenediamine, which conveniently precipitates the liberated HBr (Pearson *et al.*, 1967). Certain alkylated phenols can also be brominated in the *meta* position by bromine in a superacid solution of SbF_5-HF.

The conversion of the phenol group by the strong acid to a OH_2^+ group results in the activation of the *meta* position (deactivation of *ortho* and *para* position) to bromination (Jacquesy *et al.*, 1980). *Para* bromination of phenols can be achieved by bromine in the presence of *S*-chlorodimethylsulfonium chloride, $Me_2S^+Cl\ Cl^-$ (Olah *et al.*, 1986). Bromine thus provides an effective source of electrophilic bromide (Br^+) for the treatment of phenols. However, for the bromination of small amounts of OA, free bromine does provide difficulty in handling. Fieser and Fieser, 1967, reported on pyridinium hydrobromide perbromide (PPB) as a stable, crystalline, easily preparable source of bromine, which with respect to reactivity, is comparable to bromine. The controlled reactivity of PPB is ideal for monobromination of activated aromatic ethers and phenols such as pyrogallol (Steenkamp *et al.*, 1985). Steyn and Payne, 1999,

employed PPB in the bromination of salicylic acid which afforded 5-bromo salicylic acid in 19% yield. The very efficient bromination of OB followed which produced BrOB almost in quantitative yield (Figure 1.18).

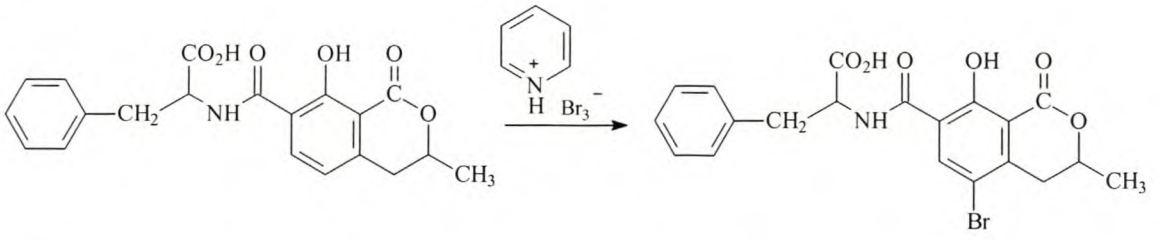


Figure 1.18 The bromination of OB (Steyn and Payne, 1999)

Furthermore, the first biosynthetic production of BrOB by *Aspergillus ochraceus* Wilh. was reported by Stander *et al.*, 2000.

1.13.4.3 Iodination of OB

Elemental iodine is the least reactive halogen towards electrophilic aromatic substitution (Merkushev, 1988). In most cases iodine needs to be oxidised by an oxidising agent e.g. HNO_3, HIO_3, SO_3, peracetic acid or H_2O_2, to render it more electrophilic. This, however allows for limited use in sensitive multifunctional compounds.

ICl is a better iodonating agent than iodine due to the net polarisation of the molecule. The more electronegative chlorine atom (δ–) increases the partial positive charge on the lesser electronegative iodine atom (δ+). Woollet and Johnson, 1943, have shown that salicylic acid upon treatment with ICl could easily be iodonated to 3,5- diiodosalicylic acid. Payne, 1999, extended the method to iodonation of OB, but with little success. Iodo-ochratoxin B was eventually isolated in good yield, employing the method used by Venter and Langenhoven, 1996 (Steyn and Payne, 1999). This method entails the use of HgO and iodine in a methanol:ethanol (1:1) solution (Figure 1.19). Schmiedova *et al.*, 1989, had previously reported on the synthesis of ^{125}IOB from OB by the use of chloramines and N^{125}I. The ^{125}IOB was used in the radioimmunoanalysis of OA.

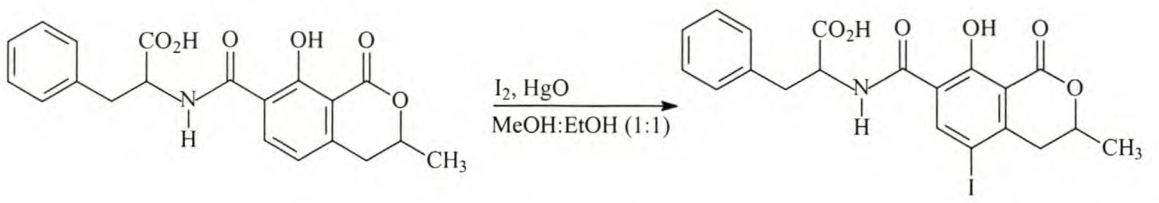


Figure 1.19 The iodination of OB (Steyn and Payne, 1999).

In order to rationalise the role of the halogen substituent in OA toxicity it is important to relate the electronic nature of the halogen (and its effect on the aromatic ring substituents) to the observed toxicity. Preliminary tests have shown that the toxicity of the ochratoxins increases in the order of OA < BrOB < IOB. The electronegativity of the halogens increases in the order I < Br < Cl. Thus the smaller the electronegativity of the halogen the higher the toxicity of the ochratoxin. OB, with a proton substituent less electronegative than any of the halogen substituents, is less toxic than OA and is not in agreement with this analogy. The electronegativity of the halogen group has a direct bearing on the dissociative property of the phenolic hydroxyl group (Chu, 1974) and has been implicated in lipid peroxidation (Rahimtula *et al.*, 1988). It could thus be hypothesised that fluoro-ochratoxin B, with the greatest electronegativity, could have the lowest toxicity. More importantly though, would be the relative observed toxicity of fluoro-ochratoxin B to OB. It was proposed that the toxicity of OA *in vivo*, was defined by the rate of hydrolysis by carboxypeptidase to the non-toxic Oα (Bredenkamp *et al.*, 1989) (section 1.5). In this mechanism the sterical bulk of the C-6 substituent should be taken into consideration. The difference in size of chlorine and hydrogen atoms is much greater than that of fluorine relative to the hydrogen atom. The syntheses of fluoro-OB will thus not only provide a probe for the reactivity of the halogen substituent but also ascertain by which mechanism the substituent exerts its role in toxicity.

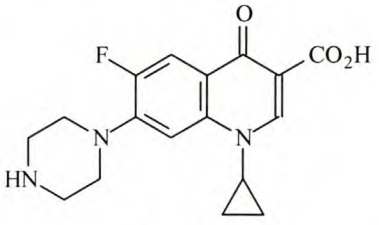
An attempted synthesis of fluoro-OB demands a thorough understanding of the chemistry involved in introducing a fluorine atom into an organic substrate. As the main focus of our research effort, the biological aspects and synthesis of organofluorine compounds will be dealt with in detail in chapter 2.

CHAPTER 2

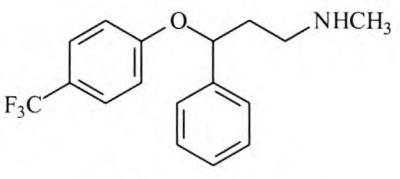
SECTION A: FLUORINE IN BIO-ORGANIC CHEMISTRY

2.1 Introduction

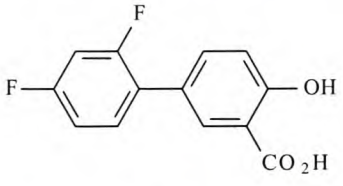
Since the discovery of hydrogen fluoride by Scheele in 1771, and the more importantly the isolation of elemental fluorine by Henri Moissan in 1886, synthetic fluorine chemistry has seen an enormous and diverse growth. But it was the contribution of Swarts in 1892 that accelerated the growth of organofluorine chemistry with his work on the preparation of fluorinated materials by metal fluoride promoted halogen-fluoride exchange reactions. Industrial incentives manifested in the commercial utility of organofluorine compounds as coolants, developed by Midgley and Henne in 1930, led to the further development of synthetic fluorine chemistry (Banks and Tatlow, 1994, and references cited). Fluorine containing compounds find application not only in the inorganic but also in the polymer, pharmaceutical, and organic chemical industry with organofluorine compounds being the most prevalent. The vast differences in the number of industrial applications can solely be ascribed to the dramatic changes in the physical and chemical properties of compounds upon fluorination. These properties are manifested in commercial products which range from agrochemicals, medical and health care products to surfactants, textiles, dyes and polymers (Figure 2.1).



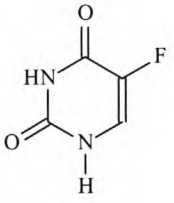
Ciprofloxacin
Broad-spectrum fluoroquinolone antibacterial drug



Fluoxetine
Anti -depressant drug

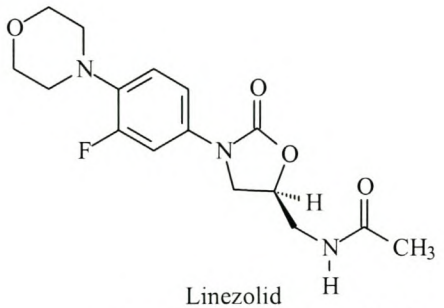


Diflunisal
Analgesic, anti-inflammatory, anti-pyretic drug.



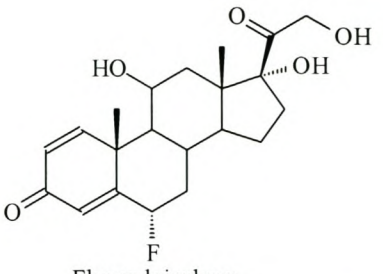
5-Fluorouracil
Anti-cancer agent

Figure 2.1 Representative commercial organofluorine compounds.



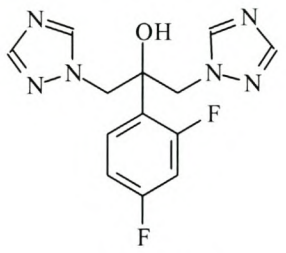
Linezolid

Oxazolidinone type antibiotic



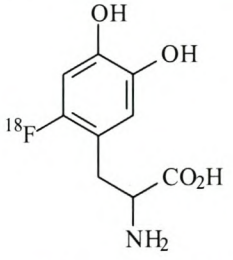
Fluprednisolone

Glucocorticoids; anti-inflammatory



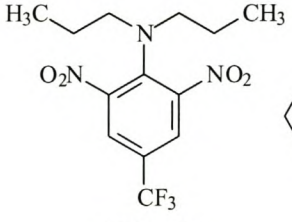
Fluconazole

Anti-fungal agent

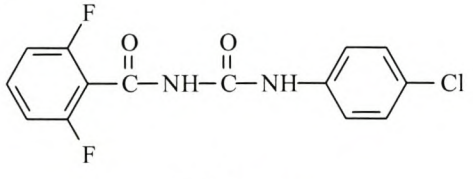


6-[18F] Fluoro-L-DOPA

Radiolabelled anti-Parkinsonian drug



Trifluralin



Diflubezuron

Agricultural products

$CF_3CHClBr$	$CHFClCF_2OCHF_2$	$CF_3CHClOCHF_2$
Halothane	Enflurane	Isoflurane

Anaesthetics

$-[-CF_2-CF_2-]_n-$	$-[-CH_2-CF_2-]_n-$	CF_3CH_2F
(PTFE)	(PVDF)	**Refrigerants**

Polymers

Figure 2.1 *Continued*

The profound and often unexpected effects that fluorine can have on the biological activity of compounds are well-documented (Fried and Sabo, 1954; Welch, 1987). Selective fluorination has been an extremely effective synthetic tool for modifying and probing reactivity. By substituting a hydrogen or hydroxyl group for a fluorine group in biologically relevant molecules often yields a derivative with increased selectivity or a modified spectrum of activity not in coherence to other halogens (Kirk *et al.*, 1971)

It was the pioneering work of Fried and Sabo, 1954, on the synthesis of 9α-fluoro-hydrocortisone acetate that led to the first successful application of selective fluorination for the purpose of modifying biological activity (Figure 2.2).

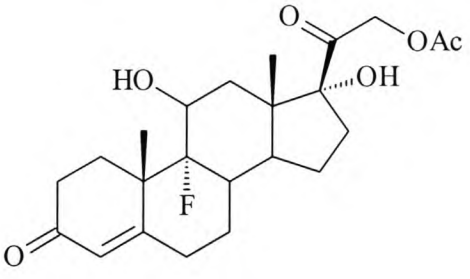


Figure 2.2 9α-Fluorohydrocortisone acetate

Recognition of these properties has induced extensive interest in the synthesis and biological assaying of fluorinated analogues to biologically significant compounds.

2.2 Structural and bonding characteristics of fluorine.

Fluorine contains nine protons in its nucleus which interacts strongly with the same number of electrons due to the occupancy of both protons and electrons of the inner shell. Thus the Van der Waals radius of fluorine (r_F ~1.35) is contracted to such an extent that it closely resembles that of an hydrogen atom (r_F ~1.10), allowing fluorine to be the only element to replace hydrogen without notable steric consequences (Schlosser, 1977). In contrast to their similarities in size, fluorine and hydrogen are quite different in their reactivity.

The high effective nuclear density and the tendency to complete its valence orbital renders fluorine strongly electronegative and reactive. Attached to a reactive centre it proves to be a moderately active leaving group. Placed in the vicinity of a reaction center it proves to substantially influence reaction mechanisms *via* its strong inductive electron-withdrawing effect. This is illustrated in the example of fluoroacetic acid, that acts as an anti-metabolite to acetic acid, which mimics acetic acid in the entering of the tricarboxylic cycle (Krebs cycle).

Structural and bonding characteristics of organofluorine compounds are thus derived from three essential factors: (1) the extreme electronegativity of fluorine (2) the strength of the C-F bond (3) the small size of the bound fluorine.

Excluding unreactive neon, fluorine is the most electronegative element with a Pauling electronegativity of 4 as compared to 3.5 for oxygen, 3.0 for chlorine or 2.8 for bromine. It is this property that is the apparent source of the marked differences that are observed if fluorinated and non-fluorinated molecules are compared.

Of all the halogens bound to carbon, fluorine forms the strongest bond, seen from the carbon-fluorine bond energy of 456-486 kJ per mole compared to the carbon-chlorine bond of 350 kJ per mole, and the carbon-hydrogen bond which varies between 425-435 kJ per mole. At the same time it is important to note that the bond lengths associated with the bond dissociation energies differ with the respect to the different halogens bonded to carbon. The much shorter fluorine-carbon bond, 1.31 Å, substantiates the difference in bond dissociation energies if it is compared to the other carbon-halogen bond lengths, eg. carbon-chlorine bond length of 1.78 Å.

Another interesting phenomenon is the relative difference in the bond lengths of perfluoro methanes (Table 2.1) if compared to the carbon-hydrogen bond. The decrease of bond lengths are again accompanied by the increase in dissociation energies.

Table 2.1 Bond lengths (Å) and bond dissociation energies (kJ/mol) of halogenated methanes (Source:Banks, 1998).

Halogen	CH_3X	CH_2X_2	CHX_3	CX_4
F	1.385 Å 456 kJ/mol	1.358 Å 510 kJ/mol	1.332 Å 535 kJ/mol	1.317 Å 543 kJ/mol
Cl	1.782 Å 350 kJ/mol	1.772 Å 339 kJ/mol	1.767 Å 325 kJ/mol	1.766 Å 301 kJ/mol
Br	1.939 Å 289 kJ/mol	1.934 Å 267 kJ/mol	1.930 Å 259 kJ/mol	1.942 Å 295 kJ/mol

Smart, 1983, rationalized this decrease in bond lengths as the result of an increase in π character, i.e. that any p-carbon electrons would be shared by the fluorine atom and that this will lead to an increase in s-character of the carbon-fluorine bond and subsequent decrease in bond lengths. Furthermore, the carbon-fluorine bond in fluoromethane would be more p-electron rich and longer in bond length than fluoroform. Friesen and Hedberg, 1980, reported on the pronounced differences in the gauche effect, manifested in the molecular conformation, of vicinal electronegative substituents. This phenomenon is shown in the presence of 1,2- difluoroethane to exist in the synclinical conformation as opposed to the antiperiplanar position. This feature is a common occurrence of halogenated compounds but is amplified most in the fluorine substituted compounds.

Finally the electronegativity of fluorine can also effect distribution within a molecule substituted by other groups and thereby effecting the polarity (dipolar moment), reactivity and stability of the entire molecule, and the acid characteristics of adjacent groups.

2.3 Influence of fluorine on the physical and chemical reactivity of molecules.

The difference in the physical, chemical and biological activity of fluorinated and non-fluorinated compounds can be directly attributed to the difference in electronegativity of fluorine and hydrogen, the greater carbon-fluorine bond strength

relative to the carbon-hydrogen bond strength, the ability of fluorine to donate a free electron pair and form intermolecular hydrogen bonds.

The high electronegativity associated with fluorine has pronounced electron withdrawing effects *via* (a) the creation of an induced dipole along a chain of bonded atoms known as a sigma withdrawing effect, Iσ, or (b) through space electrostatic interaction known as the field effect. These two effects are well illustrated in the example of the relative difference in acidity of trifluoroacetic acid and acetic acid. In an aqueous medium trifluoroacetic acid (pKa 0.3) exists as a stronger acid than acetic acid (pKa 2.24). But in the gas phase this tendency is not constant with trifluoroacetic acid being less acidic than trichloroacetic acid. This irregularity can be ascribed to the smaller polarisability of carbon-fluorine bond (0.53 Å) relative to that of the carbon-chlorine bond (2.61 Å). This decrease in polarizability renders a carbon-fluorine more comparable to that of an carbon-hydrogen bond (Welch, 1990, and references cited).

The influence of the π system on the electronic effects of fluorine directly opposes the charge induced dipole effect described above by the back donation of non-bonded electrons from the fluorine atom to the π system in an I_π repulsive interaction. These interactions are manifested in the reactivity of α-fluorinated anions toward radicals and the addition reactions of nucleophiles to fluorinated alkenes. α-Fluorinated cations on the other hand are stabilized by the presence of fluorine by the interactions of the empty p-orbital of the carbon atom and the filled orbitals of the fluorine atom.

Another chemical reactivity that prevails from the properties of fluorine is the capability of fluorinated carbon-carbon bonds in determining the stereochemistry of a specific reaction (Lin *et al.*, 1988; Cheung *et al.*, 1986; O'Hagan and Rzepa, 1997). The carbon-carbon bond with no fluorine substituents and subsequently the higher electron density has the ability to donate electrons to bond forming orbitals and thus control the stereochemistry in such a manner.

2.4 The influence of fluorine on the biological reactivity of molecules.

Selective fluorination of organic compounds has been an extremely effective synthetic tool for altering biological activity and probing structural activity relationships. The suitability of fluorine as substituent for hydrogen in biologically potent compounds is twofold.

Firstly, as explained in the foregoing discussion, the pronounced difference in the electronic effects brought upon by fluorination. Secondly the inability of this effect to be neutralise by a sterically hindered substituent, i.e. fluorine is comparable in size to hydrogen, the Van der Waal's radii being 1.35 Å and 1.20 Å respectively. From a potential substitution point of view the carbon-fluorine bond length of 1.39 Å is comparable to the carbon-oxygen bond length of 1.43 Å which leads to the assumption that these substitution could be energetically feasible.

Fried and Sabo, 1954, explored the reactivity of organofluorine compounds by the introduction of fluorine into biologically active metabolites, which led to the blocking of reactivity. Fluorine hydrogen-substitution synthetic methods has been used extensively to elucidate the role that intra- and inter-molecular bonding plays in rendering compounds biologically active. Fluorine has the ability to mimic the role of the electron pair accepting hydrogen by its ability to donate non-bonded electrons to a species with a vacant orbital.

Fluorine can act in the capacity of a leaving group in enzyme inhibition and thus contribute in clarifying the susceptibility of the compound to enzymatic action. The step-wise fluorine substitution of hydroxyl groups reveals the effect that hydroxylation has in a molecular system as was done in the successful study of vitamin D_3 analogs. To many of the metabolic transformations the strongly carbon bonded fluorine is rendered resistant. But at the same time fluorine may also be employed in addition-elimination reactions where its greater leaving group capabilities relative to that of hydrogen is exploited. The ability of fluorine to increase lipophilicity and hence the increase in the bioavailability of the compound underlines its pharmacological application (Welch, 1990, and references cited).

Finally the natural stable isotope of fluorine, ^{19}F, a spin quantum number of one-half and chemical shift range of about 300 ppm, provides a very sensitive analytical tool in nuclear magnetic resonance studies. The pharmokinetic profiles of certain compounds can be defined by the selective introduction of the fluorine probe into the desired compound. The synthetic short-half life isotope, ^{18}F, allows for the *in vitro* study of living tissue by its positron emission. The technique, positron emission tomography (PET), provides a non-invasive diagnostic tool which complements existing techniques such as X-ray analysis, and supercedes such techniques with respect to time (PET allows for real time assays) and the health aspects concerned.

The success of PET relies on the half-life of the administered isotope. ^{18}F has a half life of 110 minutes which makes it suitable for the brain imaging of patients suffering from Parkinsons disease, compared to 20 minutes of ^{11}C, 10 minutes of ^{13}N and 2 minutes of ^{15}O. ^{18}F labeled fluorodopa PET studies have revealed an considerable amount of information concerning the chemistry and metabolism present in the brain tissue. This has also led to a further application in the diagnosis of breast tumours in the form of ^{18}F-labeled estrogen probe (Reivich and Alavi, 1985).

2.5 Applications of organofluorine compounds in bio-organic chemistry.

2.5.1 Amino acids and amines.

The importance of enzymatic decarboxylation of amino acids in biosynthetic pathways may be obtained by the use of a specific fluorinated analogue as inhibitors of the decarboxylation enzymes in studying these pathways. Examples of physiologically important amines formed by decarboxylation are dopamine, 5-hydroxytryptamine, histamine and gamma-aminobutyric acid (GABA) (Figure 2.3). Catecholamines are essential in the regulation of central and peripheral blood pressure (Versteeg *et al.*, 1977). Douglass, 1975, observed elevated histamine levels in diseases such as allergies, hypersensitivity, gastric ulcers and inflammation. Elevated putrescine levels are known to be associated with rapid cell proliferation, including tumour growth (Dunzendofer and Russell, 1978).

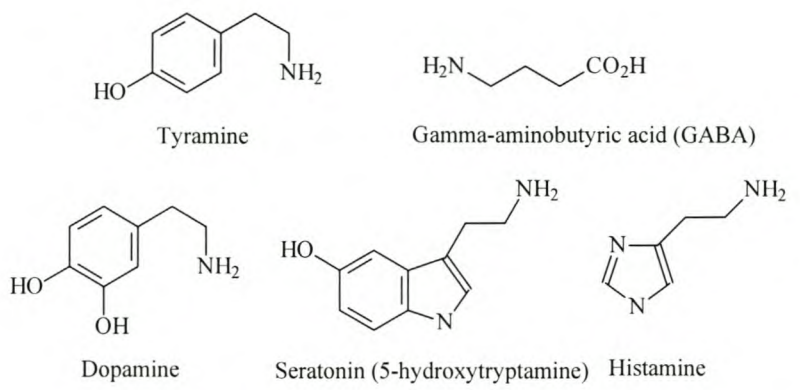


Figure 2.3 Physiologically important amines formed by decarboxylation of the respective amino acid.

Fluoromethylated amino acids have been recognized as potent suicide inhibitors of enzymatic decarboxylation reactions (Kollonitsch *et al*, 1978) as seen in Table 2.2. The enzymatic inactivation is thought to be dependant on the loss of fluoride from the methyl group, which leads to the formation of a reactive Michael type acceptor. The acceptor covalently bonds to the decarboxylase enzyme, thus inhibiting any further binding to substrates.

Table 2.2 Fluoromethylamino enzyme inhibitors (Source: Welch, 1987).

Amino acid analogue	Inhibited enzyme
Fluoromethylglutamic acid	Glutamate decarboxylase
Fluoromethylornithine	Ornithine decarboxylase
Fluoromethyldopamine	Dopa decarboxylase
Fluoromethyltyrosine	Dopa decarboxylase
Fluoromethylhistidine	Histidine decarboxylase (mammalian)
Fluoromethylhistamine	Histamine decarboxylase

Kingsbury *et al*., 1983, reported on the incorporation of *m*-fluorophenylalanine in the synthesis of di- and tri-peptides. The resultant fluoro-peptides have the ability to easily penetrate fungal cell walls, which leads to the release of the toxic *m*-fluorophenylalanine into the cell plasma. In contrast *m*-fluorophenylalanine is inherently non-toxic to *Candida Albicans* as can be seen from Table 2.3.

Table 2.3 *m*-Fluorophenylalanine-containing peptide as agents against *Candida Albicans* (Source: Kingsbury *et al.*, 1983).

m-Fluorophenylalanine containing peptides	**MIC[a] against *C. albicans* strain**	
	B311	**759**
L-mFPhe	>250	>250
L-mFPhe-L-Ala	2	4
L-mFPhe-L-Ala-L-Ala	0.5	2
L-mFPhe-L-Met-L-Met	8	63
L-mFPhe-L-Ala-L-mFPhe	2	63

[a] Minimum inhibitory concentration in μg/ml.

In contrast to the above, α-monofluoromethyl- and difluoromethyl- amino acids have been defined as stringent enzyme-activated irreversible inhibitors of the parent α-amino acid decarboxylase. Versteeg *et al.*, 1977; reported on the importance of catecholamines in the regulation of central and peripheral blood pressure. Fluorine substitution on the aromatic ring of catecholamines has a striking effect on there selectivities for α- and β-subtypes of adrenergic receptors. This led to the progressive testing of new analogues in the quest to determine the submolecular mechanisms at work at the cathecholamine-adrenergic receptor interface. The results obtained indicated that the direct effect of the carbon-fluorine bond on antagonist-receptor interaction may be more important than the indirect effect of the carbon-fluorine bond on neighbouring side chains.

Walborsky *et al.*, 1955, discovered that amino acids substituted with trifluoromethyl groups can act as possible antimetabolites. The synthesis of amino acid analogues, i.e. the substitution of a methyl group for a trifluoromethyl groups, is essential for probing enzyme activity. The resultant high electron density that exists on the trifluoromethyl groups may initiate the association with enzymes and the subsequent formation of strong hydrogen bonds that could render the enzyme inactive due to the blocking of functional groups responsible for metabolic responses.

Initially the fluorination of amines was found to be problematic due to the non-selective fluorination of the compounds by the then available fluorination reagents. The careful optimisation of the reaction conditions with respect to the solvents used in the synthesis, i.e. the use of hydrogen fluoride employed to protect the amine functionality, has led to the successful synthesis of β-fluorinated amines which are

important substrates in the design of the antimetabolites and drugs because of the optimal change in electron distribution and minimal effect on the structural design of the molecule.

2.5.2 Steroids

As mentioned, the fluorination of steroids manifested in the work of Fried and Sabo, 1954, was indicative of the effect that fluorine substitution can have on biologically active molecules. The synthesis of fluorinated analogues of vitamin D_3 has been employed to prevent the metabolic hydroxylation (fluorination at C1, C3 or C25, see Figure 2.4) and thus the subsequent activation of the vitamin D_3 analogues of the active steroid hormone, **I**. The selective fluorination of the hydroxylation positions allows for the elucidation of the role of each hydroxyl group in the reactivity of the molecule.

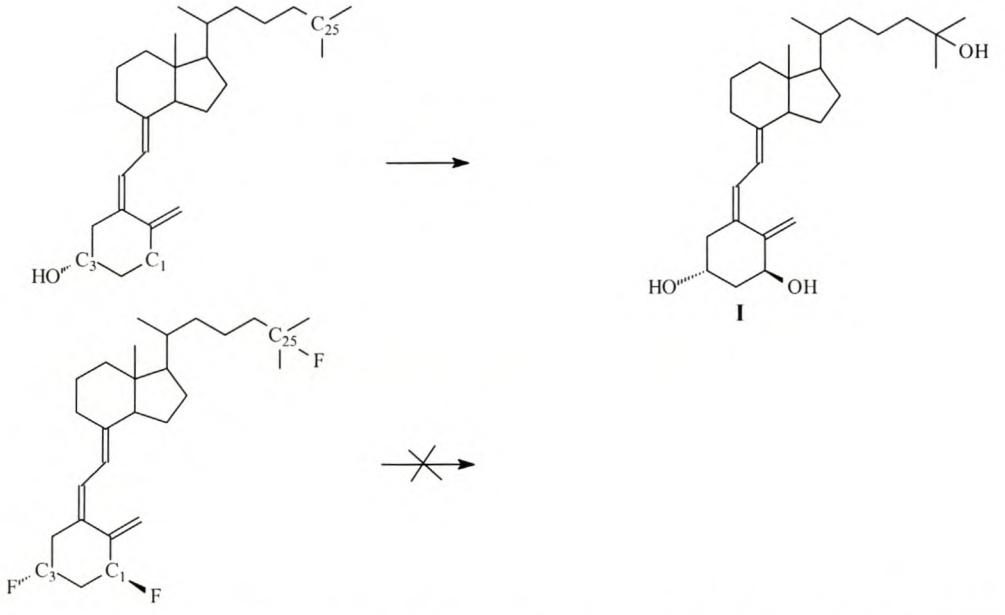


Figure 2.4 The activation of Vitamin D_3 by hydroxylation. Hydroxylation is inhibited in fluoro analogues.

2.5.3 Carbohydrates

The selective fluorination of carbohydrates has found wide application in the probing of biochemical mechanisms. These resultant fluoro-glycosides have many

applications in biochemistry, medicinal chemistry and pharmacology (Bessel *et al.*, 1973).

In addition ^{18}F-labelled sugars have found utility as *in vivo* imaging agents for carbohydrate metabolism. 2-Deoxy-2-fluoro-glucose-(^{18}F) has been established as a useful radiopharmaceutical for studying glucose metabolism in both healthy and diseased tissue (Schelvert *et al.*, 1982). Radio labeled 2-deoxy-2-fluoromannose was shown to be effective in the application of positron emission tomography for the imaging of subcutaneous AH109 A tumor in rabbits (Fukuda *et al.*, 1982, see Figure 2.5).

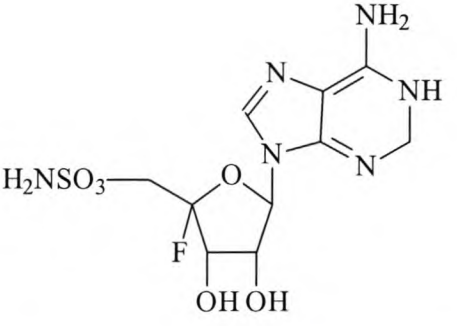


Figure 2.5 The naturally occurring fluorosugar constituent of nucleocidin.

The importance of fluorine substitution of hydroxyl groups in carbohydrates is manifested in the profound difference obtained by this substitution in the electronic effects experienced by neighbouring groups in their constant immediate steric environment. These substitutions are accomplished without the loss of the potential hydrogen bonding. At the same time this contributes to difficult synthesis and the total synthesis is virtually impossible because of the stereochemical control needed in adjacent asymmetric centers of the fluorinated carbohydrates (Chmielewski *et al.*, 1982). Von der Osten *et al.*, 1990, reported on the role that aldolase can play in a convergent enzymatic synthesis of fluorosugars *via* easily fluorinated building blocks.

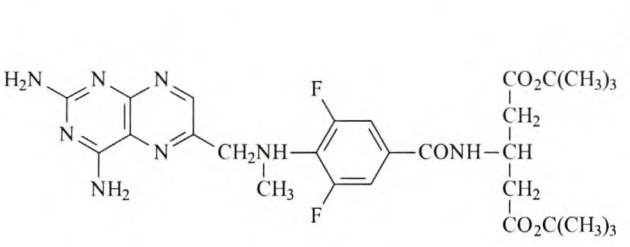
2.5.4 Nucleic acids

Fluorinated nucleic acid analogues have been found to exhibit a wide spectrum of biological activity. Bergstrom *et al.*, 1984, observed antiviral activity of some of the fluorine substituted naturally occurring nucleic acids, whereas Shimizu *et al.*, 1984,

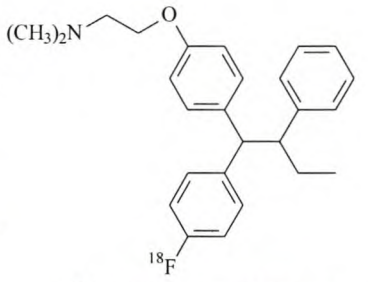
Yeatts *et al.*, 2000; Mehta *et al.*, 2000, reported on antitumor and antifungal activity of 5-fluoruracil. The electronic effect brought about by fluorine substitution is again responsible for the marked difference in reactivity. Fluorine acts as replacement for both hydroxyl and hydrogen groups, whereas difluoromethylene probes the oxygen activity.

2.5.5 Aromatic compounds

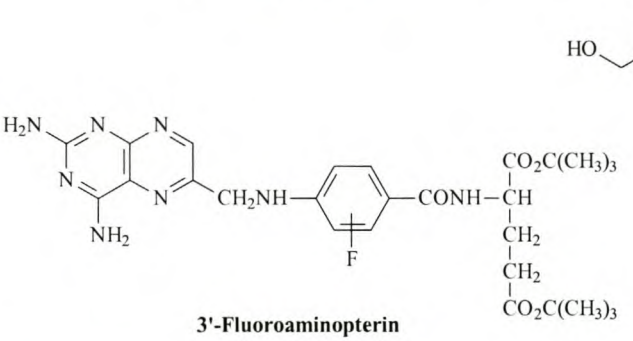
Fluorinated aromatics have found wide application as antibiotics, sedatives, essential agrochemicals and radioactive imaging agents. Henkin and Washtien, 1983 reported on the improved antitumor activity of 2′, 3′-fluoroaminopterin (Figure 2.6) on L1210 cell or human cancer HuTu80 cells as compared to methotrexate. 3′,5′-Difluoromethotrexate (Figure 2.6) has been used successfully to study the binding of materials to dihydrofolate reductase. When bound to the enzyme, the fluorine substituents are in different chemical environments and it is possible to observe the effect of added NADH or NADPH to the bound methotrexate analogue (Welch, 1987). Radio labeled fluorotamoxifen (Figure 2.6) was found to be a good estrogen receptor binding agent, allowing for the study of estrogen distribution patterns.



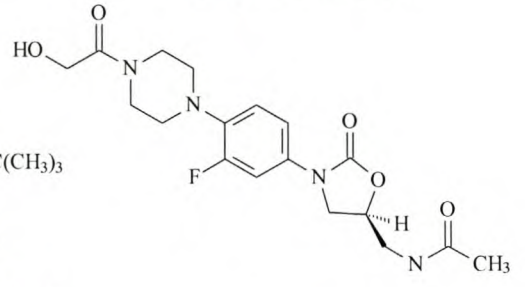
3',5'-Difluoromethotrexate



^{18}F-labelled Fluorotamoxifen



3'-Fluoroaminopterin



Eperezolid

Figure 2.6 Biologically active aromatic fluorides.

The remarkable impact of a new found antibiotic class, the oxazolidinones, on the treatment of multiple resistant strains of *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pneumoniae* and certain *Enterococus* species, has been reported (Zurenko *et al.*, 2001; Dailey *et al.*, 2001 and Oramus-Shirley *et al.*, 2001). The lead agents, linezolid (Figure 2.1) and eperezolid (Figure 2.6) were found to be highly effective against methicillin-resistant *S. aureus* strains. Methicillin together with vancomycin and ticoplanin are seen as the last line of defence against life threathening infections including pneumonia, surgical infections and other hospital acquired opportunistic infections. The reports on intermediate resistance to vancomycin resistance, emphasize the need for a new class of antibiotics such as the oxazolidinones. Structure activity relationship studies revealed that the aromatic fluorine improved the antibacterial activity and efficacy.

2.6 Synthesis of organofluorine compounds

The numerous fields of application of selective fluorination, as a tool to probe biological activity, testify to the importance of a good understanding into the chemistry of the fluoro substrates and more importantly the fluorinating reagents and methodologies employed. The optimization of these methodologies will lead to an even greater elucidation of certain structure activity relationships contained in the biologically active molecules.

Two distinct strategies are available when planning the synthesis of a fluorinated molecule: (1) incorporate a starting material already containing the fluoro group needed in a *de novo* synthesis of the desired compound (the "building-block" approach); or (2) insert the fluoro group required at a convenient stage using a fluorinating agent. Depending on the target molecule, either or both approaches may be meaningful. In our research effort emphasis was placed on the latter of the two strategies, since in principle, it is always more convenient, if possible, to incorporate fluorine in an existing natural product than building the desired molecule from simpler constituents.

It remains a challenge, however, to selectively incorporate fluorine into a multifunctional compound. This section reviews modern methods available for

selective aromatic fluorination, with specific reference to the synthetic approaches employed in our attempts to fluorinate ochratoxin.

2.6.1 Halogen replacement

From fluorine's chemical nature it can be appreciated that most of its application in synthetic chemistry revolves around the fluoride species (F^-). In contrast to the relative ease of chlorination and bromination of aromatic hydrocarbons, in which an aromatic hydrogen is electrophilically substituted and therefore lost as a proton, the introduction of fluorine is normally achieved *via* nucleophilic attack by an F^- species under more severe conditions. The halogen-exchange methodology involves the displacement of a larger halogen from the aromatic carbon by a fluorine substituent in the form of an F^- species *via* an addition-elimination mechanism. As mentioned before the reaction proceeds at an acceptable rate only if the leaving halogen group is activated by an *ortho*- or *para* electron-withdrawing substituent (Figure 2.7).

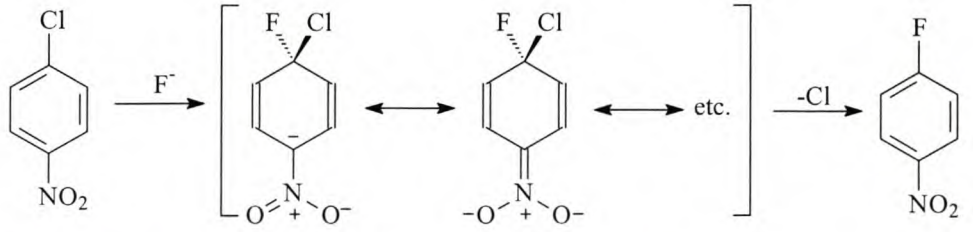
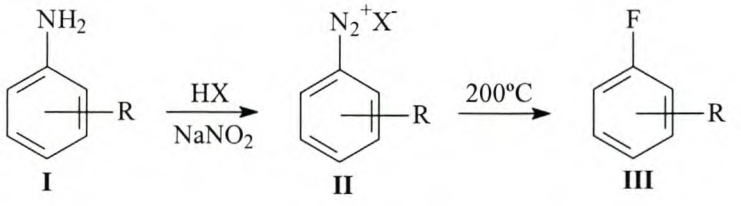


Figure 2.7. Fluorination by the halogen-exchange route.

The most effective activating groups (NO_2, CN, COF, CHO, SO_2F) withdraw electron density from the aromatic ring (specifically the *ortho* and *para* positions) *via* both the inductive (-I) and the mesomeric (-M) effects (March, 1992). Since the C-X bond is not broken in the initial rate determining step, the order of ease of nucleophilic displacement of halogen from activated aromatic systems (NO_2-Ar-X) is X= F > Cl > Br > I. This allows for the synthesis of fluoroaromatics from the corresponding inexpensive chloro-compound using a large excess of alkali-metal fluoride sources *viz.* CsF, RbF, KF, NaF and LiF (Moilet, 1994). Although the fluoride is a better leaving group the dynamics of the equilibrium allows for washing out of the chloride by the sheer excess of the fluoride.Halogen replacement *via* arylmetallic intermediates are discussed in section 2.6.4.

2.6.2 Nitrogen replacement -The Balz-Schiemann Reaction

This was the first widely used method for the introduction of a fluorine atom onto an aromatic ring (Balz and Schiemann, 1927). It involves a three stage process starting with the diazotization of an aromatic amine **I** to produce a diazonium fluoride or tetrafluoroborate **II**, which is then decomposed thermally (the fluorodediazotization step) to afford the fluoro aromatic **III**, nitrogen and boron trifluoride in the case of tetrafluoroborate (Figure 2.8).



$X^- = F^-, BF_4^-$
R = H or one or more substituents.

Figure 2.8 Fluorination by the diazoroute.

The key to the method is the relative ease in which the highly insoluble arenediazonium tetrafluoroborate can be isolated in an aqueous medium. The yields of the isolated arene diazonium salts are generally high, often exceeding 90%. However, it has been shown that the presence of hydroxyl or carboxylic acid ring substituents could adversely affect yields by increasing the aqueous solubility of the salts (Moillet, 1994). The lower product yields in the dediazotization step can be ascribed to numerous factors. Arenediazonium tetrafluoroborates containing electron-donating groups *ortho* or *para* to the diazonium substituent are less stable and decompose at lower temperatures than those containing *meta* substituted equivalents and electron withdrawing groups. The presence of boron trifluoride as a strong Lewis acid as well as the role that the diazonium group plays as a powerful activator for the displacement of nucleophillic groups in *ortho* and *para* positions is evident in the resultant byproducts observed.

As an extension of the Balz-Schiemann reaction, F^- has been tried as an alternative to the tetrafluoroborate. In this approach an aromatic amine is diazotized with nitrosyl

fluoride, generated *in situ* from hydrogen fluoride and sodium nitrite. This method avoids the isolation of the fluoride salt prior to decomposition, and no recycling of gaseous products as in the case with tetrafluoroborate.

It is obvious from the reaction conditions employed in the Balz-Schiemann reaction that this approach is limited to the fluorination of relatively simple aromatic compounds. It could thus only feature in the early stages of a *de novo* synthesis of a highly functionalised molecule.

2.6.3 Hydrogen replacement

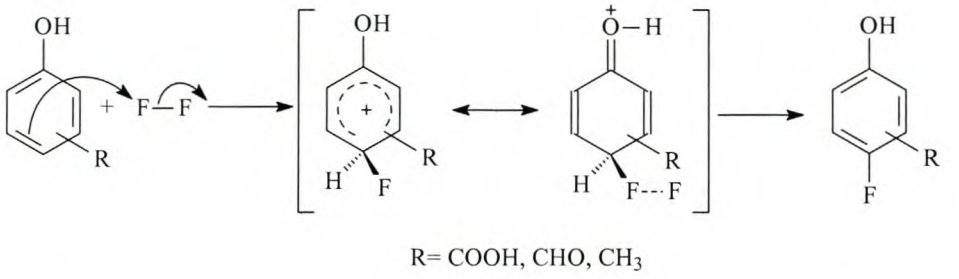
2.6.3.1 Direct elemental fluorination of aromatic systems.

From its discovery in 1886 by Henri Moissan until the 1960's elemental fluorine had been considered too reactive and dangerous to be practical for the fluorination of organic molecules. Fluorine is such a strong oxidizing agent that it reacts with almost any organic substrate, usually exothermically, and often with explosive results (Grakauskas and Baum, 1970). In addition the poor solubility of fluorine in organic solvents leads to reactions taking place at the liquid-gas interface which subsequently afford several unwanted side reactions (Cacace *et al.*, 1980). These effects have been minimized by the use of dilute solutions of fluorine in inert gases such as nitrogen or argon, which allows for greater control and selectivity.

The applications of fluorine in organic synthesis have flourished with the advent of high quality fluorine and fluorine-nitrogen mixtures being commercially available as well the development of moderating fluorine carriers (section 2.6.3.2). The great expense of user-friendly fluorination agents such as acyl hypofluorites, caesium fluoroxysulfate and xenon difluoride as well as the fact that elemental fluorine is used in the production of these reagents, has prompted renewed interest in the use of elemental fluorine in the direct fluorination of especially aromatic compounds (Chambers *et al.* 1999; Moillet, 2001).

The first attempts to substitute an aromatic hydrogen for a fluorine group was met with limited success. Cacace *et al.*, 1980; employing molecular fluorine (<0.76% F_2 in N_2) in $CFCl_3$ at low temperatures, substituted a variety of aromatic rings (benzene,

toluene, anisole and nitrobenzene) at low conversions (0.01%). Grakauskas, 1970, reported that the substitution reactions followed first-order kinetics, in which the conversion is only determined by the amount of aromatic substrate present. The mechanism proposed involves the attack on the benzene ring by a polarized fluorine molecule leading to an arenium ion (See figure 2.9).



R= COOH, CHO, CH_3

Figure 2.9 S_N2-type reaction mechanism for the fluorination of substituted phenols as proposed by Cacace *et al.*, 1980.

In addition to the proposed mechanism by Cacace and coworkers, Chambers *et al.*, 1997, suggested that fluorination occurs by both one (See figure 2.10) and two electron transfer processes. This explains to a great extent the multitude of secondary reaction products obtained in the fluorination of aromatic compounds by early researchers.

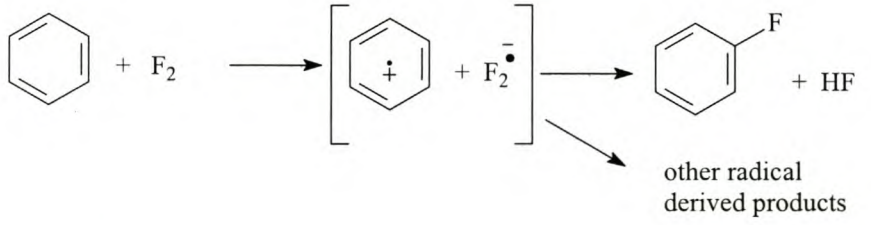


Figure 2.10 Aromatic fluorination *via* 1-electron transfer (Hutchison and Sanford, 1997).

Grakauskas, 1970, reported on the fluorination of methyl benzoate in acetonitrile at -20°C. The reported yields (74% *p*-, *o*- and *m*-fluorobenzoates) were the first to be of a synthetically useful scale and the isomer ratios (1:3:5, respectively) suggest that the fluorination occurs *via* electrophilic aromatic substitution analogous to the ionic

reaction of other halogens. Sams *et al.*, 1978; employed molecular sieves in an attempt to minimize polymer formation, a secondary reaction that was prone to occur at high conversions. At optimized reaction conditions (-78°C, no solvent), Sams obtained almost 20% *o*- and *p*-difluorobenzenes from fluorobenzenes.

Misaki, 1981 and 1982, reported on the monofluorination of oxygenated aromatic substrates in high yields using molecular fluorine (11% in nitrogen) at various temperatures. For the fluorination of a 10% solution of phenol at -20°C, a 10 % conversion to the monofluorinated product afforded an *ortho* to *para* ratio of 22:1, whereas during higher conversions the substitution of the *para* position was favoured. This was ascribed to the fact that the *ortho* isomer underwent secondary reactions to an experimentally observed byproduct. Misaki, 1982, further emphasized that temperature also played an important role in the isomeric ratios. For the fluorination of phenol at lower temperatures increased conversion and fewer side reactions altering the isomeric ratios occurred.

Misaki, 1982, also reported on the fluorination of methyl substituted phenols. For *o*-cresols the *ortho*/*para* substitution was approximately 1:1 irrespective of solvent used or temperature employed. The fluorination of *m*-cresol furnished a mixture of *o*-substituted products, the 2-fluoroderivative being the major product. The fluorination of *p*-cresol **I**, were accompanied by the formation of the *o*-derivative **II** but also of fluorocyclohexadienyl ketone **III** as a major byproduct (Figure 2.11).

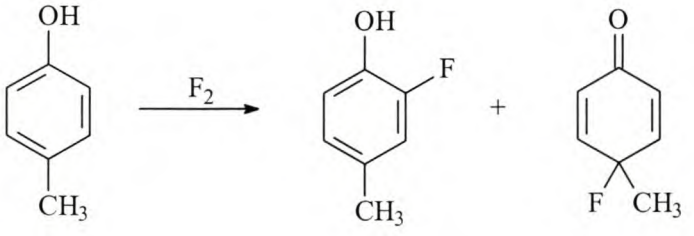


Figure 2.11 The fluorination of *p*-cresol by F_2 (Misaki, 1982).

Misaki, 1982, extended his studies to the fluorination of substituted phenols such as, salicylic acid, salicylaldehyde and phenyl salicylate with addition of hydrogen

fluoride. 5-Fluorosalicylic acid was isolated in good yield (73%) from salicylic acid in hydrogen fluoride, while phenyl salicylate fluorination afforded a mixture of phenyl 3- and 5-fluoro salicylate in an even greater yield (89 %) under similar conditions. Of interest was the fact that no oxidation of the aldehyde group occurred in the fluorination of salicylaldehyde as opposed to the oxidation of salicyl alcohol to the fluoro salicylaldehyde during fluorination in acetonitrile (Figure 2.12)

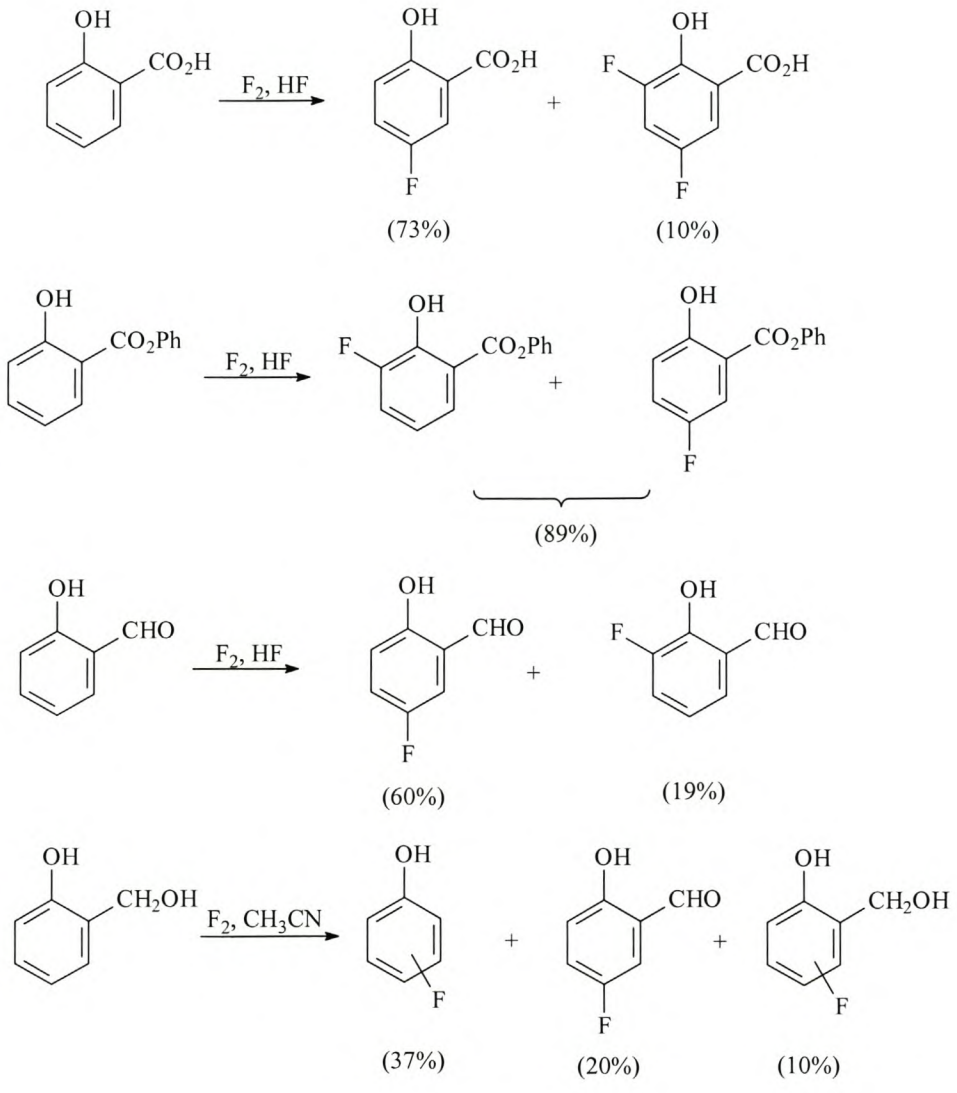


Figure 2.12 The direct fluorination of aryl oxygen compounds using F_2 (Misaki, 1982).

With a view to promoting the two electron process, i.e. encouraging the apparent electrophilicity of fluorine, Chambers *et al.*, 1996, investigated the effect of strong acids as solvent on fluorination of aromatics. It was found that for formic and sulfuric acid, both strong acids of high dielectric constants, fluorine was polarised to such an extent, that the electronegative end of the molecule is protonated by the acid and the electropositive end is free to react with the nucleophilic aromatic substrate. The preferred solvent is therefore one which assists electrophilic reactions while suppressing radical reactions. Although formic acid is not inert to fluorine, the product of the reaction i.e. hydrogen fluoride and carbon dioxide are easily removed from the reaction chamber. Ring sulfonation, competing with the fluorination reactions involving sulfuric acid, proved to be of no advantage yet marked success has been achieved, particularly with aromatic fluorination reactions involving a relatively electron deficient ring (Chambers *et al.*, 1996). Side reactions of sulfonation, due to greater nucleophilicity, are prone to react with electrophilic fluorine.

Chambers *et al.*, 1999, reported an increase in both yields and selectivity when replacing acetonitrile with sulfuric, or formic acid in the fluorination of the compounds employed by Misaki, 1981 and 1982. The selectivity is particularly high when both groups in a disubstituted aromatic compound direct the attacking electrophile to the same ring carbon atom. For example 3,4-difluorobenzoic acid can be obtained in good yields (80%) from 4-fluorobenzoic acid in the presence of high concentrations of sulfuric acid (Figure 2.13).

In addition direct fluorination of 4-chloronitrobenzene to yield 4-chloro-3-fluoronitrobenzene in good yield (68%), which in turn is an isomer of 3-chloro-4-fluoronitrobenzene (produced with relative ease by the Halex process, section 2.6.1), illustrates the use of this methodology to produce different isomers that would otherwise have been synthesized with great difficulty (Chambers *et al.*, 1999)(Figure 2.13).

Chambers *et al*, 1999, reported the fluorination of a benzaldehyde in which the ring is substituted with an electron donating group, e.g. 4-methoxybenzaldehyde, could be fluorinated in a satisfactory yield (60%) by the addition of sulfuric acid. In contrast in the fluorination of 4-fluoro-benzaldehyde, the major reaction was the oxidation to 4-

fluorobenzoic acids followed by the fluorination in the 3-position. Furthermore, the presence of methyl groups as ring substituents lowers the yield of aromatic ring fluorinated products due to the reaction of fluorine with benzylic hydrogens and the subsequent increase in tar formation.

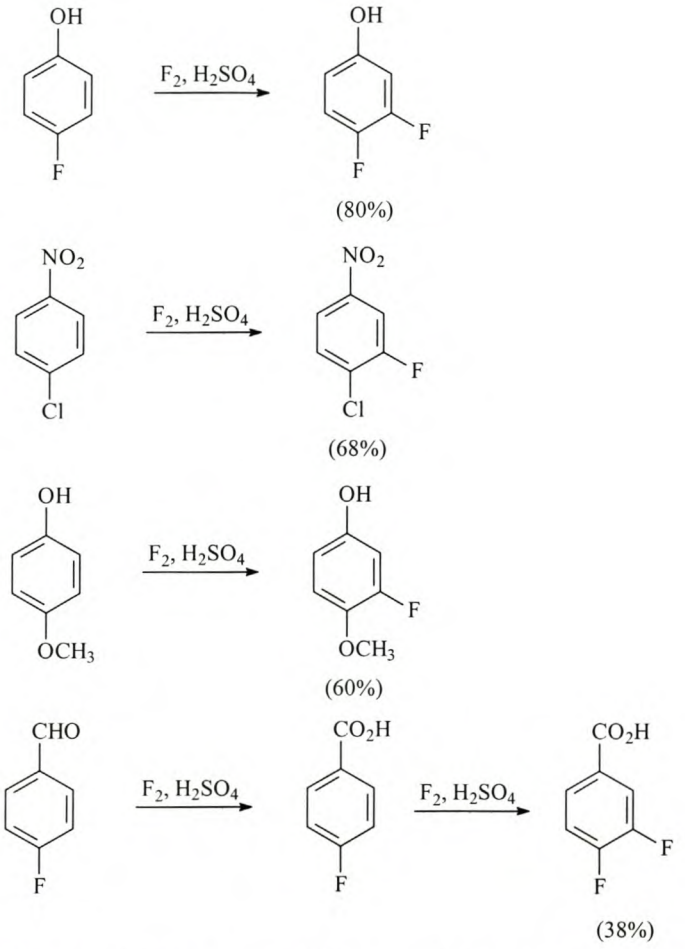


Figure 2.13 Direct aromatic fluorination by F_2 in the presence of H_2SO_4 (Chambers *et al*, 1996).

The synthesis of 2-fluoro-vanilin (4-hydroxy-3-methoxybenzaldehyde) is an example of the high selectivity obtained in employing fluorine together with sulfuric acid for direct fluorination of multifunctional aromatic compounds (Moilliet, 2001). From the directing effects of the substituents it would normally be expected that the 5-fluoro isomer would be produced in highest yield. It was established that aromatic compounds containing a carbonyl group in the 1-position and an alkoxy or hydroxy group in the 3-position preferred the formation of the 2-fluoro isomer. This was illustrated in the synthesis of 2-fluoro-3-methoxybenzaldehyde from 3-

methoxybenzaldehyde in almost quantitative yield (>90%). Moillet *et al.*, 2001, rationalised this phenomenon by the protonation of the carbonyl group leading to a positive charge on the carbonyl carbon together with an association with the alkoxy- or hydroxyl group at the 3-position. This leads to the polarized fluorine molecule being kept close to the 2-position, which allows for subsequent fluorination of the 2-position (60%).

Elemental fluorine is therefore of use as a selective fluorinating agent for aromatic molecules where the electrophilic directional effects of the substituents on the ring are complimentary. This gives a selection of products which is unusual in the sense that the orientation is different from those made by nucleophilic fluorination.

2.6.3.2 Electrophilic fluorination with fluorine carriers

The difficulties associated with direct fluorination using elemental fluorine, with regard to the highly specialized apparatus and operational expertise needed in its application as well as other factors discussed above, stimulated the development of alternate sources of electrophilic fluorine that can easily and safely be handled in organic synthesis. Perchloro fluoride, fluoroxytrifluoromethane, acyl- and perfluoroacyl hypofluorites and xenon difluoride were among the first fluorine carrier reagents available.

Apart from increased selectivity of electrophilic fluorination (as compared to fluorine) displayed by these reagents, a number of limitations have precluded their widespread use. Virtually all of these reagents are strong oxidizing agents and are either very expensive or require the use of fluorine for their *in situ* generation. In recent years, a number of N-F fluorinating agents have emerged as generally safer, cost effective and easier to handle, sources of selective fluoronium (F^+) ions.

2.6.3.2.1 N-F reagents

Banks and Williamson, 1964, first reported on the potential of compounds containing N-F bonds to act as electrophilic fluorination agents (see Figure 2.14). This was

illustrated by conversion of the sodium salt of 2-nitropropane to 2-fluoro-2-nitropropane using perfluoro-*N*-fluoropiperidine (**1**).

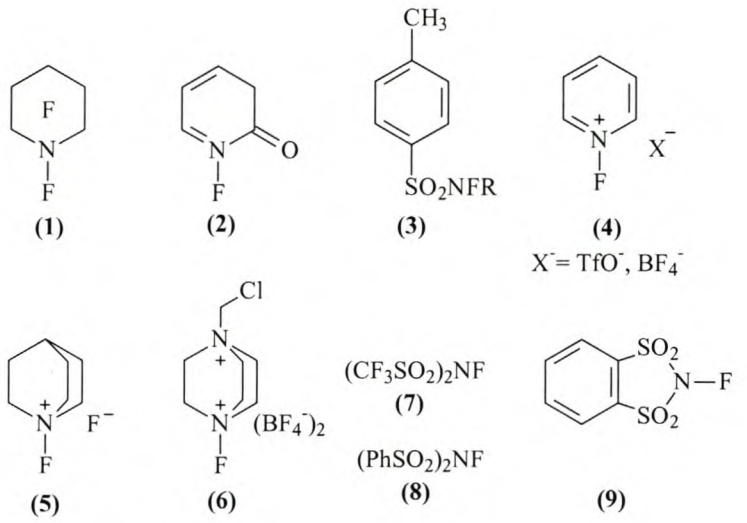


Figure 2.14 Various electrophilic N-F fluorinating agents

Other applications with N-F reagents, structurally altered to suit certain synthetic needs, were soon to follow. Purrington and Jones, 1983, reported on the use of *N*-fluoropyridin-2(1H)-one (**2**); and Barnette, 1984, on *N*-fluoro-*N*-alkylsulfonamides (**3**). This was superceded in reactivity by both *N*-fluoropyridinium salts, **4** (Umemoto *et al.*, 1986) and *N*-fluoroquinclidinium fluoride (**5**) (Banks *et al.*, 1986). Finally, in 1987 Demarteau reported that *N*-fluorotrifluoromethylsulfonimide (**7**) was the most powerful F^+ delivery agent of the N-F class (Lal *et al.*, 1996 and references cited).

There are clearly two types of N-F fluorinating reagents available for electrophilic fluorination: a) neutral, R_2NF compounds and b) quaternary ammonium R_3N^+F A^- salts where A^- is a non-nucleophilic anion. In both cases the R_2N^-- and R_3N are designed to be good leaving groups, thus facilitating the reactivity of the bound fluorine with the nucleophiles.

2.6.3.2.1.1 1-Chloromethyl-4-fluoro-1,4-diazoniabicyclo [2.2.2] octane *bis*(tetrafluoroborate), Selectfluor™ (F-TEDA-BF_4) 6.

Banks and coworkers developed the Selectfluor™ class of compounds in 1992, as a

more potent electrophilic fluorinating agent relative to the previously developed *N*-fluoroquinuclidinium fluoride (Banks *et al.*, 1988). The commercially available compound, F-TEDA-BF_4 **6,** is a white, free-flowing crystalline solid which can be handled safely in ambient atmosphere. It incorporates a chloromethyl group, two quaternary ammonium groups of which one carries the electrophilic fluorine atom and two tetrafluoroborate counter anions, rendering the compound neutral. The combination of these properties allows for a balance between the F-electrophilicity (F^+-strength) and overall thermal stability of the compound.

Compared to other N-F reagents, it is second only to the well-known DesMarteau compound [$(CF_3SO_2)_2NF$ (**7**)] in terms of its fluorinating capacity and thermal stability (up to about 195°C) (Banks *et al.*, 1996). Furthermore, the electrophilicity of the F-group can be modulated by simply substituting the methyl chloride group with a stronger or weaker electron withdrawing group. Within the F-TEDA class the order of reactivity follows the relative electron withdrawing power of the alkylating organic group (e.g. CF_3CH_2> $ClCH_2$> Me etc.). In addition, a variety of anion combinations can be selected which allows for different solubility characteristics.

For the past decade Selectfluor™ has become the electrophilic fluorination agent of choice for a wide variety of applications (Figure 2.15). The diversity in applications is illustrated by the array of functionalities incorporated in compounds fluorinated. These include alkenyl boronic acids and trifluoroborates (Petasis *et al.*, 1997), steroids (Godard, 1994) and pyrimidine bases (Brunavs *et al.*, 1994; Lal *et al.*, 1995), the synthesis of fluoro-carbohydrates and glycosides (Vincent *et al.*, 1999) and important aromatic substrates (Banks *et al.*, 1996; Zupan *et al.*, 1995 and Lal, 1995, see figure 2.15).

Electrophilic fluorination of aromatic compounds is enhanced by the presence of electron releasing ring substituents (+*I*, +*M*). This is evident in the unsuccessful fluorination (under reasonable conditions) of benzene compared to that of toluene and xylene (see figure 2.15).

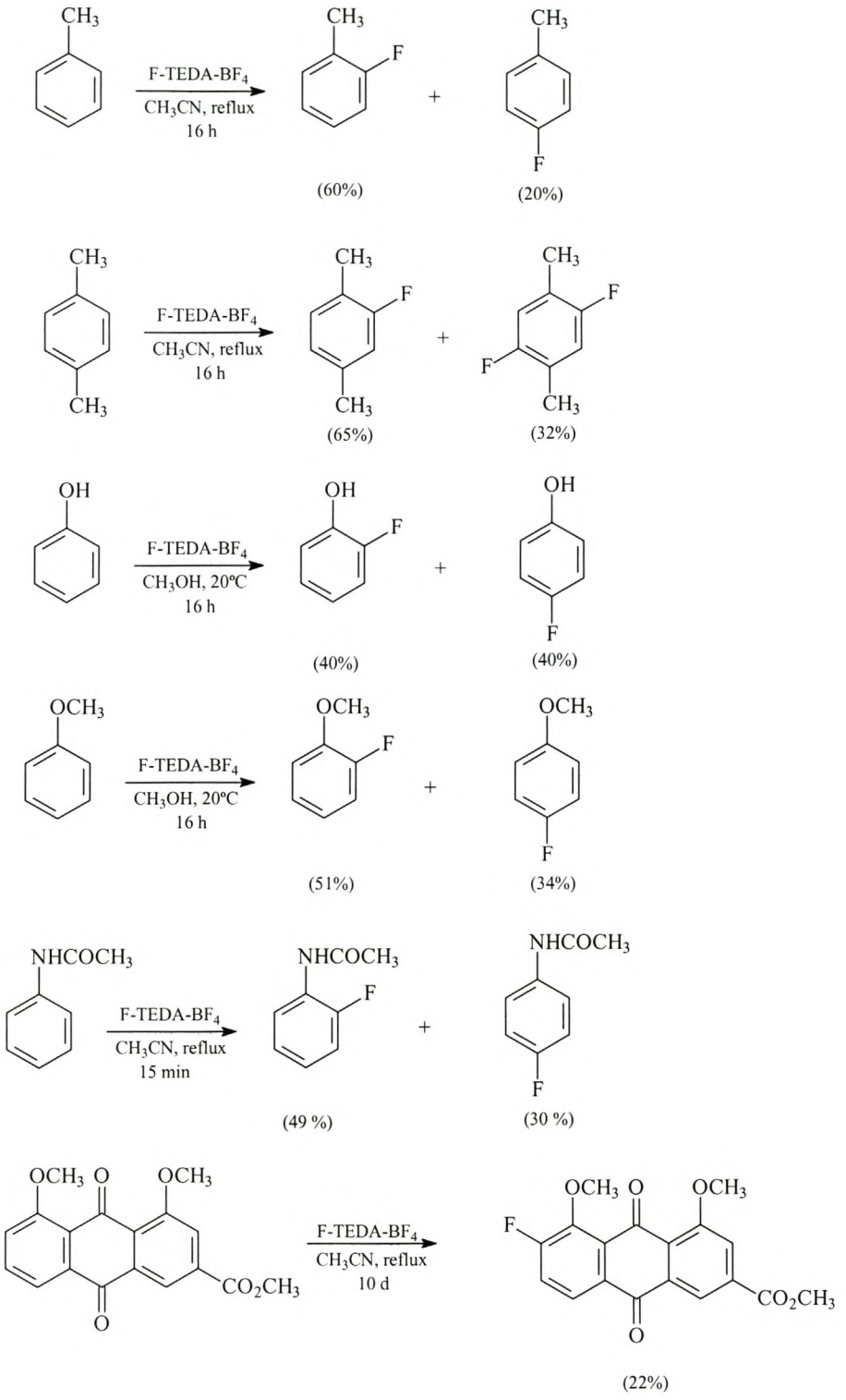


Figure 2.15 Selective aromatic fluorination using Selectfluor™.

2.6.3.2.1.2 *N*-fluorobenzenesulfonimide

N-fluorosulfonimides became of increased importance as electrophilic fluorination reagents by the development of the most reactive N-F reagent, *N*-fluoro(trifluoromethylsulfon)imide (see Figure 2.14, **7**), by Desmarteau and coworkers in 1987. Examples of selective fluorination with $(CF_3SO_2)_2NF$ at room temperature includes benzene (50%), toluene (80%, *o*:*p* = 3.5:1), anisole (100%, *o*:*p* = 3:1) phenol (high, *o*:*p* = 3:2) as well as other aromatic compounds. Unfortunately this compound, which is a low-boiling liquid (b.p. 90-91°C), is not commercially available and its five-step synthesis requires the use of liquid fluorine in a highly specialised apparatus (Singh and Desmarteau, 1987).

To circumvent these limitations *N*-fluorobenzenesulfonimide (NFBS), **8** (Davis *et al.*, 1995) and its closely related analogue, *N*-fluoro-*o*-benzenedisulfonimide (NFOBS), **9** (Davis and Han, 1991) were developed. These compounds are stable, easy to handle and find application as site-selective, electrophilic monofluorinating agents of enolates and silyl enol ethers, alkyl phosphonate carbanions (Differding *et al.*, 1991) as well as aromatic compounds (Davis *et al.*, 1995) (Figure 2.16).

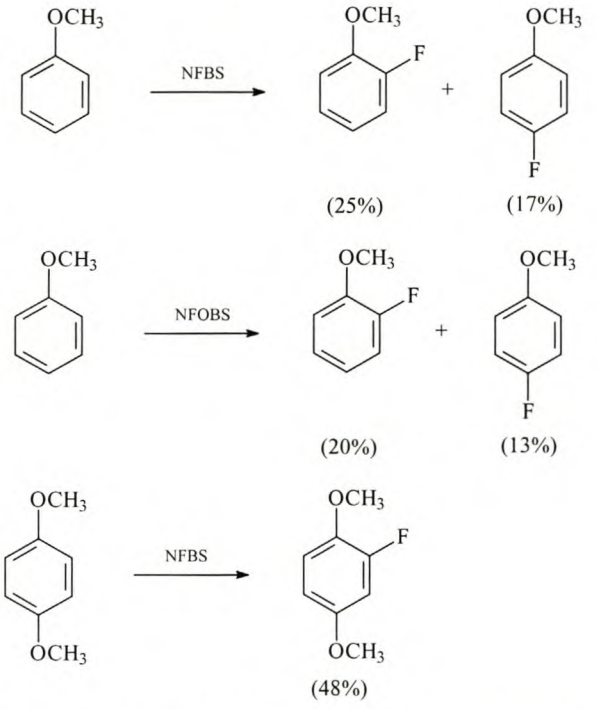


Figure 2.16 Fluorination of aromatic compounds by NFBS **8** and NFOBS **9**.(Davis and Han, 1991)

The fluorination of anisole by NFOBS yielded 42% of a 3:2 mixture of *o*- and *p*-fluoroanisole. Lower yields (33%, *o*:*p* = 3:2) were reported for NFBS under similar reaction conditions. With 1,4-dimethoxybenzene, NFOBS afforded 2-fluoro-1,4-dimethoxybenzene in 48% yield.

Alternatively, the regioselective synthesis of fluorinated aromatics can be accomplished by fluorination of *ortho*-lithiated aromatic substrates (Sniekus, 1994). These substrates, generated from the reaction of directive metallation group aromatics and alkyllithiums, when reacted with NFOBS or NFBS furnishes the corresponding *ortho*-fluorinated compounds in good yields (see Figure 2.17). In this case NFBS proves to be a more effective, affording higher yields of the fluorinated compound (Sniekus *et al.*, 1994).

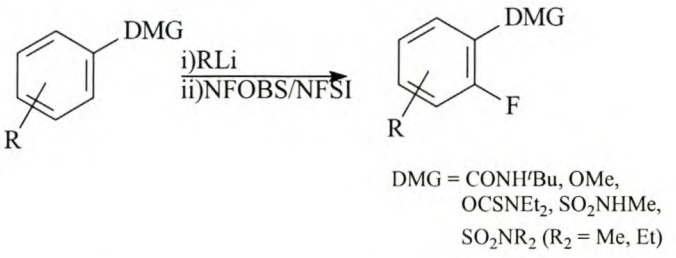


Figure 2.17 Directed *ortho*-metallation-mediated fluorination.

Sniekus *et al*, 1994, reported that fluorination of *ortho*-lithiated diethylbenzamide by NFOBS gave a 10% yield of the *N*,*N*-diethyl-*o*-fluorobenzamide. Furthermore the fluorination of *N*-*tert*-butyl-2-lithio-3-methoxybenzamide with NFBS afforded 2-fluoro-3-methoxy benzamide in 56 % yield.

2.6.3.2.2 O-F reagents

Perchloryl fluoride ($FClO_3$) was the first of the class of O-F reagents to emerge as a source of fluorine to electron-rich sites. The severe toxicity and health risks involved in its handling hampered its further applications and led to the development of fluoroxytrifluoromethane, CF_3OF (Barton *et al.*, 1968) and other reagents possessing the O-F moiety such as trifluoroacetyl hypofluorite, CF_3COOF and acetyl hypofluorite CH_3COOF (Rozen *et al.*, 1996).

Fifolt *et al.*, 1985, illustrated that aromatic derivatives, especially anilines, could be successfully fluorinated by using CF_3OF (produced *in situ*), at low temperatures for short periods of time. It was found that for reactions carried out in non-polar solvents, *ortho*-substitution was preferred almost exclusively. The proposed reaction mechanism includes a complex formation between the amino group and the CF_3OF (Figure 2.18). The reported yields of between 37-60% for the different fluorinated aniline derivatives were in agreement with the relative reactivity of the R-group towards hydrogen bonding.

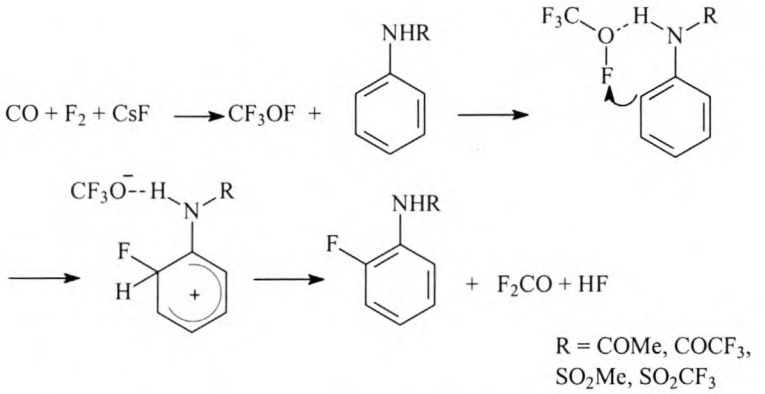


Figure 2.18 Proposed mechanism of fluorination of aniline derivatives by CF_3OF (Fifolt *et al.*, 1985).

Reactions involving toluenes, xylenes, anisole and cresols were less selective and were prone to low yields. However, Belanger *et al.*, 1988; reported on the formation of mono- and difluorinated 1,3-dimethoxybenzene in 60% overall yield from 1,3-dimethoxybenzene. Barton *et al*, 1972 illustrated by the fluorination of the antibiotic, griseofulvin (see Figure 2.19) that the use of CF_3OF as a selective electrophilic agent is not limited to simple mono or di-substituted aromatic rings only, but could also be applied to fairly complex molecules containing multiple sensitive functional groups.

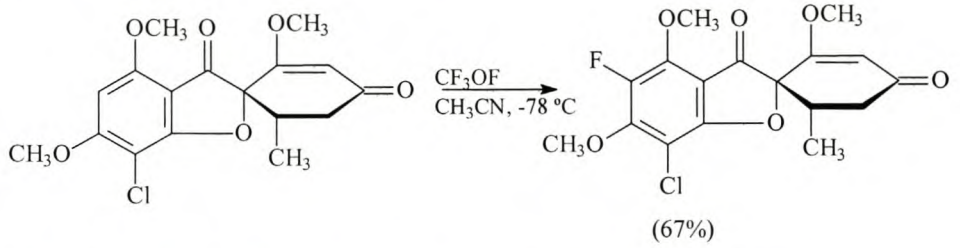
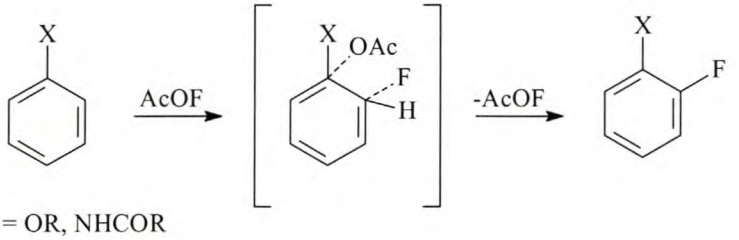


Figure 2.19 The fluorination of griseofulvin by Barton *et al.*, 1972.

Apart from the fluorination of aromatic compounds, fluoroxytrifluoromethane was also used for the highly selective fluorination of pyrimidines such as uracil, cytosine and thymine at temperatures from –78 to 25°C in very good yields, using aqueous trifluoroacetic acid as solvent (Barton *et al.*, 1972). The area where CF_3OF finds its most intense use, involves its reactions with various enols and silyl enol ethers, forming the α-fluorocarbonyl moiety through electrophilic fluorination (Middleton and Bingham, 1980).

Lerman *et al.*, 1984, observed that for the reaction of activated aromatic rings with acetyl hypofluorite the *ortho*-fluoro derivative was favoured with yields of up of 85%. The dominant *ortho*-substituent was a result of the addition of AcOF across the most electron-rich region of the aromatic ring (Figure 2.20). A subsequent spontaneous elimination of AcOH restored the aromaticity, but in the cases where this last step was not possible, the resulting cyclohexadiene reacted very rapidly with the reagent and tars were obtained. Examples of good conversions include 4-fluorosteroids and 3-fluoro tyrosine (Hebel *et al.*, 1986).



X = OR, NHCOR

Figure 2.20 The mechanism of aromatic fluorination by AcOF as proposed by Lerman *et al.*, 1984).

The fluorination of aromatic rings more activated than tyrosine, such as catechol derivatives, provide difficulty in fluorination owing to the fact that the AcOF adds across the most electron-rich area, in this case between the two adjacent hydroxyls. Nevertheless, in the fluorination of protected dopa derivatives with radiolabelled $AcO^{18}F$ two main fluorodopamine compounds were isolated and identified as 2- and 6-fluorodopamine (Rozen *et al.*, 1996).

Furthermore the reaction of phenol itself with acetyl hypofluorite is very vigorous and produces various oligomers, polymers, and carbonyl-containing compounds. The reaction can be controlled by introducing an electron-withdrawing group *ortho* to the phenolic hydroxyl group. This was illustrated by Lerman and Rozen, 1981, in the fluorination of methyl salicylate to afford the 5-fluorosalicylate in 20% yield. Barton *et al.*, 1968, reported on even higher yields (58%) for the synthesis of 5-fluoro salicylic acid directly from salicylic acid employing fluoroxytrifluoromethane as fluorinating agent.

By protecting the phenolic hydroxyl as an alkoxy group the fluorinated alkoxy derivative of methyl salicylate can be afforded in up to 40% yield (Lerman *et al.*, 1981). Belanger *et al.*, 1988, found that the fluorination of 2,6-dimethoxyacetophenone by fluoroxy trifluoromethane in Freon 11 at –78°C yielded 2,6-dimethoxy-3-fluoroacetophenone in almost quantitative yield.

The erratic yields obtained in the fluorination of electron rich aromatic compounds justified the search for other synthetic routes. Routes to consider include the reaction of acetyl hypofluorite with organometallic compounds such as aryl tin, silicon and mercury compounds resulting in the corresponding aryl fluorides. (2.6.4.2).

2.6.3.2.3 Xenon difluoride

$XePtF_6$ was the only reported stable compound derived from a noble gas before the synthesis of xenon tetrafluoride, XeF_4 and xenon difluoride, XeF_2 (Tius, 1995, and references cited). Comparable to elemental fluorine in its reactivity towards organic compounds, xenon difluoride by contrast is a colourless crystalline solid (mp 129°C) which provides an easy to handle source of fluorine. By taking special care in the design of the synthesis (i.e. in the choice of solvent, whether to use a catalyst or not, substrate and reagent concentration, the reaction vessel used) fluorination by xenon difluoride provides an easy route to fluorinated substrates (Dukat *et al.*, 1993; Ramsden and Smith, 1998). This is evident in the broad spectrum of applications, which include the fluorination of enol derivatives (Rozen and Filler, 1985), thioethers (Guilerm and Gâtel, 1994), sugars (Geilen *et al.*, 1992), aryl ketone and aldehydes

(Tius, 1995). Both homolytic and heterolytic Xe-F bond cleavage have been suggested in the elucidation of the mechanism of action in xenon difluoride reactions.

Several groups have reported fluorinations of aromatic rings using XeF_2. Shaw *et al.*, 1970, reported on the fluorination of several aromatic compounds in the liquid phase. Xenon difluoride was shown to react with benzene in CCl_4 to produce fluorobenzene in 68% yield. This was accompanied by the formation of biphenyl, fluorinated biphenyls and tars. Shaw and coworkers, further reported on the fluorination of toluene (32%, *o*:*p* = 1:3), anisole (65%, *o*:*p* = 1:2), nitrobenzene (81%, *o*:*m*:*p* = 1:3:1), trifluoromethylbenzene (71%, *meta* fluorinated) and aniline (51%, *o*:*p* = 4:1). Stavber and Zupan, 1983, emphasized the effects of catalysts on the fluorination of methyl substituted benzenes. It was found that in the presence of a catalytic amount of hydrogen fluoride, the fluorination of 1,2,4,5-tetramethylbenzene with an equimolar amount of xenon difluoride afforded the difluoro product. At higher concentrations of xenon difluoride (2 equiv.), the difluoroproduct was accompanied by the demethylated derivative, 1-fluoro-2,4,5-trimethylbenzene. Mackenzie and Fajer, 1970; employed xenon difluoride in the vapour phase successfully in their attempts to fluorinate aromatic compounds such as nitrobenzene and various fluorinated benzene derivatives.

The selective fluorination of phenols with xenon difluoride proved difficult, usually rendering mixtures of *o*-, *m*- and *p*-fluorophenols in low yields. This has led to alternate synthetic approaches being taken in the selective fluorination of phenols with XeF_2. Takemoto and Yamasaki, 1994, attempted to improve the selectivity by utilizing tertiary butyl protective substituents on the aromatic ring to avoid the formation of unwanted isomers (Figure 2.21). Regioselective direct fluorination was achieved by the reaction of 2,6-di-*tert*-butylphenol with xenon difluoride, affording 2,6-di-*tert*-butyl-4-fluorophenol in 49% yield. In contrast, under similar conditions reaction with elemental fluorine or acetyl hypofluorite with the protected phenol furnished biphenol as the major product. It was suggested that the size of fluorine or acetyl hypofluorite allows for interaction with the phenolic hydroxyl group, which leads to the formation of a phenoxy radical. Owing to the much larger size of xenon difluoride, similar interaction is not possible, which allows for para-substitution to be favoured. The subsequent removal of the protective groups did not prove difficult.

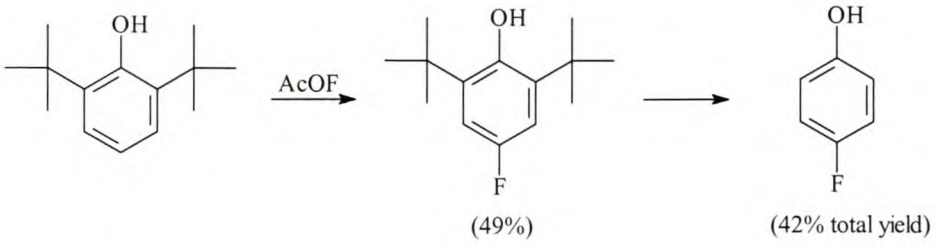


Figure 2.21 The synthesis of 4-fluorophenol *via* 2,6-di-*tert*-butyl-4-fluorophenol (Takemoto and Yamasaki, 1994).

Firnau, *et al.*, 1980, reported a simple and rapid synthetic route to L-6-fluorodopa. Direct fluorination of L-dopa with xenon difluoride was not possible, owing to the fact that the catechol constituent is susceptible to oxidation and will convert to *o*-quinone. Starting from the protected L-3-methoxy-4-hydroxyphenylalanine ethyl ester, selective fluorination of the 6-position by xenon difluoride in methylene chloride was achieved. The subsequent hydrolysis of the reaction mixture afforded L-6-fluorodopa in 25% overall yield.

Xenon difluoride reactivity towards aryltrimethylsilanes to yield the corresponding aryl mono fluorides *via* fluorodesilylation provides an excellent route for the specific introduction of fluorine into an aromatic ring. These reactions and other aspects of organometallic fluorination will be dealt with in the following section.

2.6.4 Fluorodemetalation reactions.

Although aromatic halodemetalation reactions have been known for a long time, these reactions do not feature prominently in organic synthesis. In recent years interest in the application of organometallic aryl compounds for the labeling of aromatic compounds, with especially radiobromine and radioiodine, has been prompted (Coenen and Moerlein, 1987).

These demetallation reactions allow for the site-selective halogen substitution of the labile metal functionality with high yield, which make them ideally suited for the mild and effective halogenation of short lived radionucleotides. For these purposes, however, positron-emitting fluorine-18 is an even more ideal candidate with respect to

radionucleotide half-life (110 min) and subsequent application as a radiopharmaceutical for positron emmision tomography. This has led to numerous applications of fluorodemetallation, for both radiofluorination as well as stable isotope aromatic fluorination, utilizing aromatic compounds containing tin, silicon, germanium, lead, and mercury substituents as precursors (Adam *et al.*, 1981, 1983 and 1984; Speranza *et al.*, 1984).

The rationale of this process is based on the lower stability of the metal-carbon bond as well as an increase in bond polarity relative to the corresponding carbon-hydrogen bond. Accompanied by the halophilicity of certain alkylmetal species, the electrophilic attack on the aromatic carbon is much more pronounced, giving higher yields and regioselectivity and is readily achieved even on deactivated aromatic rings. In the synthetic design consideration needs to be taken for the type of alkylmetal species used, its synthesis, the stability towards fluorodemetalation and the effect that the fluorination reagent can have on ring substituents.

2.6.4.1 Synthesis of aryl tin and aryl silicon compounds.

Group IV metals share common characteristics which allow for their application in fluorodemetallation reactions. Nucleophilic substitution at especially silicon is more facile than the corresponding nucleophilic substitution at carbon. This enables an electrophilic attack of the specific aryl carbon by a fluorine species. More importantly though is the extreme halophilicity of group IV elements, especially silicon towards fluorine. Furthermore, single bonds from these metals to the electronegative elements oxygen, chlorine and fluorine are very strong and thus allows for the complete substitution of the alkylmetal species on treatment with an electronegative fluorine specie. The steric effects of a trialkylmetal specie, e.g. Me_3Si are often not very large, which facilitates substitution of the aryl halide in the preparation of the arylmetal compound.

The most preferred preparative routes to aryl-group IV metal derivatives are shown in Figure 2.22.

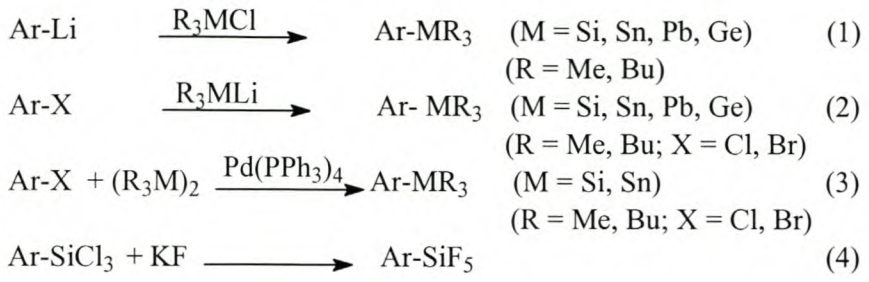
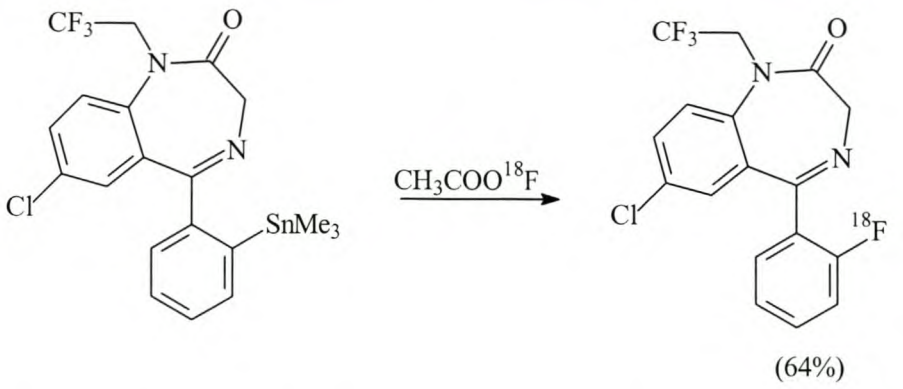


Figure 2.22 Preparation routes to aryl-group IV organometallics. (1) Sharp and Sniekus, 1985; (2) Shankarian *et al.*, 1985; (3) Azizian *et al.*, 1981; (4) Adam, 1986.

Of these methods the transmetallation reaction using either lithium or magnesium metallated aromatics are the most prevalent in organic synthesis. These compounds are normally synthesized from the corresponding halogenated (Br or I) aromatic derivative. Analogous to this method, the nucleophilic displacement of a halogen substituent by a tri-alkyl-metal species affords a route to prepare the arylmetal species with one less step. Both these methods have been limited to non-sensitive aromatic compounds, thus compounds containing no acid or base labile functional groups. Although shown to be successful in radio halogenation, the widespread use of pentafluorosilicates is hampered by their poor solubility in most solvents.

The palladium catalysed metallation of halogenated aromatic compounds using hexaalkyldistannanes or hexaalkyldisilanes provides a mild and efficient method for application to sensitive, multi-functional compounds. Duelfer *et al.*, 1991, illustrated this by the selective ^{18}F-labelling of 2-oxoquazepam *via* the fluorodemetalation of the easily prepared trimethylstannyl derivative (see figure 2.23). The organometallic compound was prepared under mild conditions by the catalytic stannylation of the bromo-oxoquazepam derivative in 64% yield.



(64%)

Figure 2.23 Selective ^{18}F-labelling of 2-oxoquazepam (Duelfer *et al.*, 1991)

Babin *et al.*, 1993, reported on the synthesis of various arylsilanes from aryl bromides using a catalytic amount of tetrakis(triphenylphospine)palladium(0) [$Pd(PPh_3)_4$] hexamethyldisilane, and hexamethyl phosporamide as solvent under stringent conditions (160°C for 96 hours). These include the following precursors: 2-trifluoromethyl bromobenzene (50% of the trimethylsilyl derivative), 3-trifluoromethyl bromobenzene (75%), 4-trifluoromethyl bromobenzene (80%), 2,4-difluoro bromobenzene (95%), 3-bromoacetophenone (40%) 1-bromo-3-cyanobenzene (50%) and for 1-bromo-4-cyanobenzene (95%). It is evident that electron withdrawing groups, situated *para* to the halogen substituent, increase the formation of the trimethylsilyl derivatives.

Goossen and Ferwanah, 2000, proposed a milder more efficient protocol for the catalytic silylation of both electron-rich and electron-poor aryl bromides (see Table 2.4). The catalytic system [tris-(dibenzylideneacetone)dipalladium(0), dba_3Pd_2] as catalyst, proposed included the use of two different sets of ligands (L) and bases tailored to suit the electronic nature of the different aryl bromides. For electron-rich substrates, diphenyl-2′-pyridylphospine (PPh_2Py) in combination with K_2CO_3 gave the best results, whereas for electron-poor substrates, 2-(di-*t*-butylphosphino)biphenyl (P[*t*-Bu]$_2$biPh)/KF was preferred. The reactions were carried out in either DMF or 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyridinone (DMPU), as solvent at 100°C for 2 hours.

Table 2.4 Pd-catalysed silylation of acetophenone under different conditions (Source: Goossen and Ferwanah, 2000).

Entry	R	Ligand	Base	Conversion (%)	Arylsilanes (%)	Reduction (%)	Biaryl (%)
1	$C(O)CH_3$	PPh_3	K_2CO_3	95	-	27	-
2	$C(O)CH_3$	PPh_3	KF	100	7	93	-
3	$C(O)CH_3$	PPh_2Py	KF	69	10	91	-
4	$C(O)CH_3$	$P(t\text{-}Bu)_2biPh$	KF	98	86	10	-
5	$C(O)CH_3$	$P(t\text{-}Bu)_2biPh$	K_2CO_3	98	3	7	-

Condition: 3 mol % Pd, 9 mol % ligand, 5 equiv. base, 2 equiv. H_2O, 100°, DMPU.

It was proposed that the aryl halide would oxidatively add to the palladium-phosphine complex, followed by activation of hexamethyldisilane by the resultant Pd(II) species, PdL_nArBr. It was further suggested that the electronic nature of the aryl group strongly affected the reactivity of this species towards hexamethyldisilane, which if not corrected could lead to unwanted side reactions. By employing different phosphines in the catalytic systems, the electronic nature of the palladium species could be balanced, and improved selectivity could be achieved. For 4-bromoacetophenone, the trimethylsilyl derivative was obtained in 86% yield, for 4-bromotoluene (87%), 4-bromoanisole (74%), 1-bromo-4-cyanobenzene (82%) and for ethyl 4-bromo-benzoate (84%).

2.6.4.2 The cleavage of aryl-metal bonds by fluorination.

Aromatic fluorodemetalation of trialkylaryltin and –silicon compounds has been achieved by various electrophilic fluorination reagents, including elemental fluorine (Adam *et al.*, 1983), acetylhypofluorite (Duelfer *et al.*, 1991) and xenon difluoride (Lothian and Ramsden, 1993). Yields of the fluorinated aryl compounds are affected by various factors. These include, the stability of the metal-aryl bond, electronic effects of other ring substituents and the formation of side products.

Coenen and Moerlein, 1987; evaluated the influence of the displaced metal and aromatic substituents on the fluorodemetallation of various group IV arylmetallic compounds using elemental fluorine and acetyl hypofluorite. They observed that for a given substituent, all fluorodemetallation yields decreased in the order Sn > Ge > Si.

The decrease in the fluorination yield by a factor of approximately three when going from stannylated to the silylated substrates corresponds with the increase in carbon-metal bond energies (Sn-C, 257 kJ/mol; Ge-C, 308 kJ/mol; Si-C, 352 kJ/mol) (Pilcher *et al.*, 1982) and the decrease in carbon-metal bond lengths (Sn-C, 1.54 Å; Ge-C, 1.36 Å; Si-C, 1.31 Å) (Dean, 1979).

For a given trimethylmetal substituent, the reactivity towards defluorometallation increased as electron withdrawing groups (NO_2, CF_3, Br) were replaced by electron donating groups (H, F). In contrast, no further increase in yield was obtained in the replacement with methyl and methoxy groups. In fact a decrease in the overall yield of the fluoroaromatic compound was observed. It was suggested that due to the relative low reactivity of silylated aryl compounds, certain side reactions are manifested. In the case of anisole, direct fluorination of the aromatic ring occurs in the ortho position (16% of 2-fluoroanisole). The reactivity of benzylic hydrogen towards fluorine radicals are evident in the small amounts of α-fluoro-toluene formed in the reactions of toluyl derivates.

2.7 Conclusion

From the foregoing discussion it is evident that a wide range of fluorinating reagents and protocols are available for synthesis of organofluorine compounds. In our research effort the choice of fluorinating reagent was based on not only the commercial availability of the reagent but also the extent to which the sensitive functional groups of OB would be tolerated by the reagent and reaction conditions.

As mentioned before, the use of elemental fluorine in synthesis requires specialised equipment and expertise not available to us. Furthermore, it has not yet been illustrated that the conditions employed in the use of F_2/N_2 mixture are mild enough to preserve sensitive functional groups such as found in OB. The fluorination of sensitive organic substrates has been achieved using CF_3OF and AcOF, however, these reagents are either not commercially available or require F_2 for their *in situ* preparation.

Elemental fluorine carriers such as N-F reagents, Selectfluor™ and *N*-fluorobenzenesulfonimide are widely available and provide for an easy to handle source of fluorine. These compounds have been used extensively in the electrophilic fluorination of aromatic compounds and show a moderate reactivity to functional groups. The use of other fluorine carriers, such as xenon difluoride, in the synthesis of aromatic fluorides provide for high reactivity (comparable to F_2) and requires relatively simple laboratory techniques. The wide range of aromatic compounds fluorinated using XeF_2 is testimony to its efficiency as fluorinating reagent.

Finally, the palladium catalysed metallation of halogenated aromatic compounds using hexamethyl- and hexabutyldistannanes provides a mild and efficient fluorination method for application on sensitive, multi-functional compounds such as OA and BrOB. The demetallation reaction allows for efficient halogen substitution of the labile metal functionality, which suggests a viable route for the synthesis of fluoro-OB.

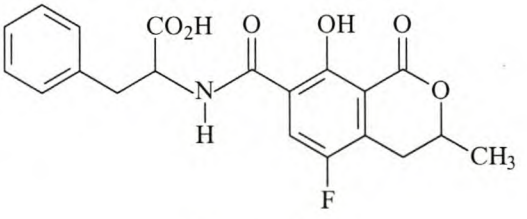
SECTION B: STUDY OBJECTIVES

2.8 Structure activity relationship studies of Ochratoxin A analogues.

Natural analogues of OA, i.e. OB, OC, Oα and 4-OH-OA provide an insight into not only the nature of OA toxicity but also the biological processes by which they are derived. In order to elucidate the role that a certain functional group plays in the toxicity of OA, numerous synthetic analogues are synthesised. The design and the synthesis of these analogues are directed to remove or to block a certain functional group in an attempt to ascertain the role the substituent plays in rendering the molecule toxic.

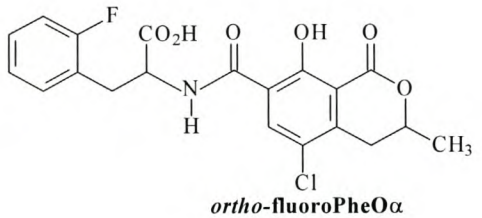
In our research effort we attempt to probe the role of the halogen and the amino acid groups in the biological activity of OA. Firstly, from the preceding discussion it is evident that the halogen group is important in the observed toxicity of OA. This is clearly illustrated in the substantial difference in toxicity of OA compared to OB. The synthesis of BrOB and IOB provided much to further rationalise the role that the halogen plays in the reactivity of OA. To complete the spectrum of halogen substituents, fluorine is still outstanding. Furthermore, from this chapter it is clear that fluorine plays an important biological role in its own right. Apart from the high electronegativity manifested by a fluorine substituent to an aromatic ring and the subsequent electronic effect on neighbouring substituents, the importance of steric bulk of the halogen group will be further clarified.

The regioselective introduction of fluorine into an aromatic compound thus becomes a salient factor in this study. Comparitive toxicological studies employing previously synthesized bromo-OB and iodo-OB, complemented by fluoro-OB should culminate the study of the role of the chloro group in OA toxicity.

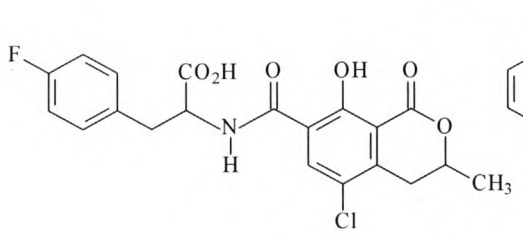


Fluoro-OB

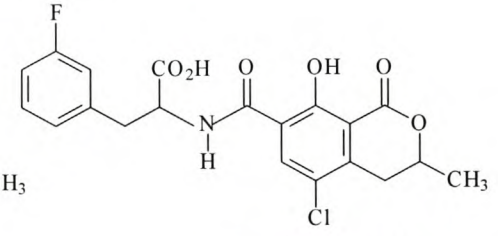
Secondly, the coupling of *o*-, *m*- and *p*- fluorosubstituted phenylalanine to Oα, will introduce another aspect in probing the reactivity of the hydrophobic side chain. The fluorosubstitution of the phenylalanine ring will alter the chemical reactivity of the relatively unreactive aromatic ring without significant change to its steric structure and elucidate its role in the inhibition of protein synthesis.



***ortho*-fluoroPheOα**

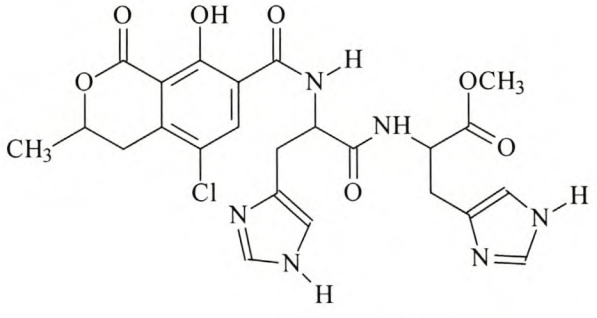


***para*-fluoroPheOα**



***meta*-fluoroPheOα**

Furthermore, the coupling of Oα to novel dipeptides, such as histidylhistidine (available in our laboratory), could provide invaluable information with respect to several aspects which include a comparison of histidine as such as a replacement for phenylalanine, as well as the implications of a dipeptide as side chain, heretofore not yet investigated.



Histidylhistidine-Oα

CHAPTER 3

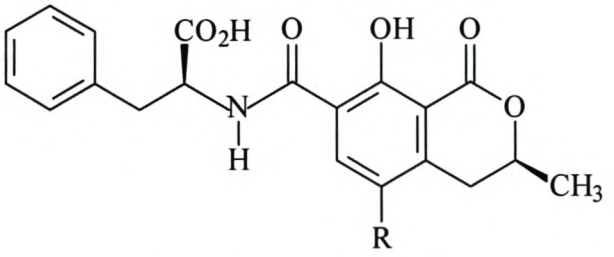
METHODOLOGY, RESULTS AND DISCUSSION

3.1 Introduction - Section Overview

The different methodologies used in our attempts to synthesise fluoro-ochratoxin B and other analogues are discussed in this chapter. It is important to note that both OA (**10**) and OB (**11**) are extremely expensive and that most reactions were done on milligram scale. All methods that were to be employed on these compounds were first studied and optimised on model compounds. An important part of our research effort was consequently focussed on the synthesis and modification of functionalised aromatic compounds.

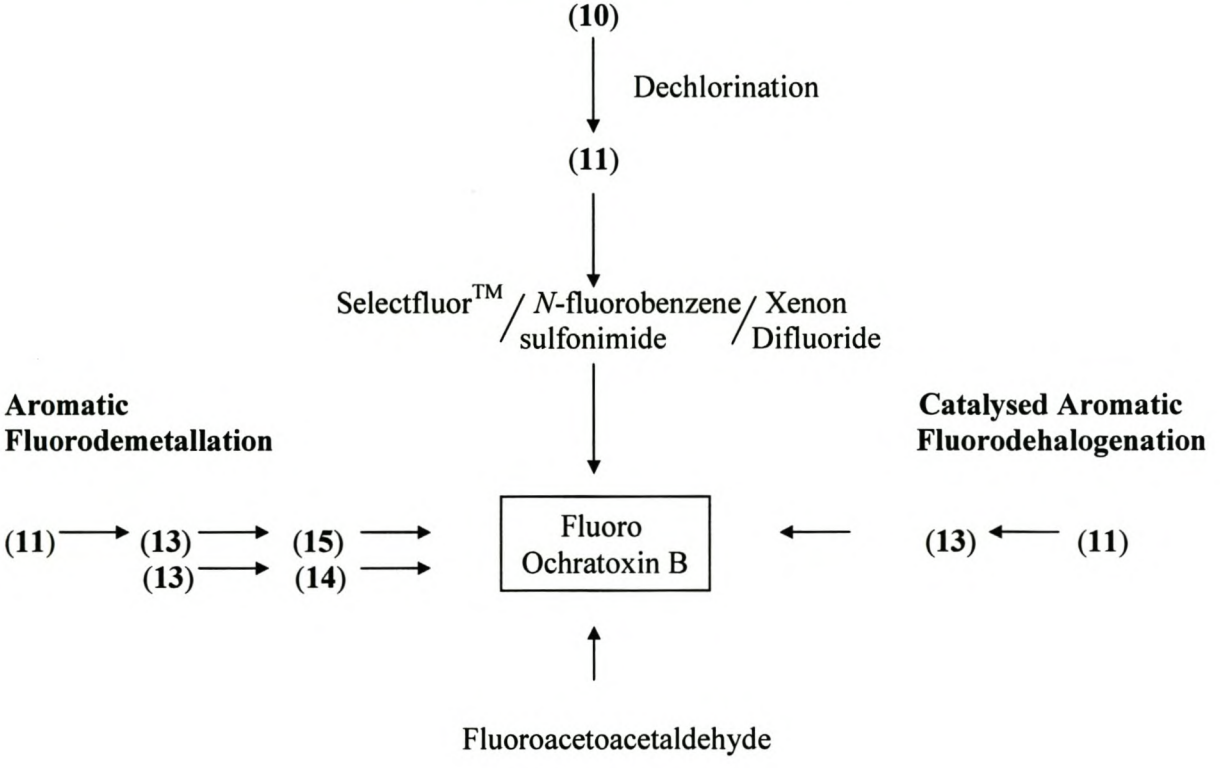
Our attempts included the direct electrophilic fluorination of OB (**11**) and different analogous aromatic model compounds by various fluorinating agents (section 3.2). They further involve a study to an alternative route to fluoro aromatic compounds from bromo and chloro analogues *via* the replacement of a trimethyl- and tributyl- stannyl (section 3.3.1) and trimethylsilyl (section 3.3.3) groups with fluorine by means of xenon difluoride (section 3.3.2). Efforts towards the direct catalytic fluorosubstitution of aryl halides are proposed (section 3.4). The synthesis of a key intermediate, fluoroacetoacetaldehyde, in a *de nova* synthetic route to fluoro-ochratoxin B is discussed (section 3.5). Figures 3.1 and 3.2 show a scheme of the different synthetic routes studied, including the route starting from the most important precursors, for both OA (**10**) and model substrates.

The synthesis of novel OA analogues with respect to the replacement of the L-phenylalanine moiety is discussed (section 3.6). This includes the conversion of OA (**10**) to Oα, by acid hydrolysis (section 3.6.1), followed by the coupling of *ortho*-, *meta*- and *para*- substituted DL-fluorophenylalanine to the lactone acid (section 3.6.2). This is followed by the synthesis of histidylhistidine methyl ester (section 3.6.3) and attempted coupling to ochratoxin α (section 3.6.4). The coupling of halosalicylic acids and salicylic



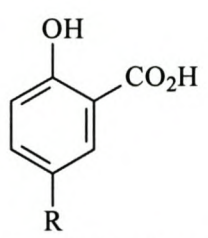
(10) R = Cl Ochratoxin A
(11) R = H Ochratoxin B
(12) R = F Fluoro Ochratoxin B
(13) R = Br Bromo Ochratoxin B
(14) R = Bu_3Sn
(15) R = Me_3Sn
(16) R = Me_3Si

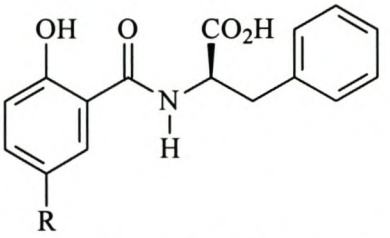
Direct Electrophilic Aromatic Fluorination



***De nova* Synthesis**

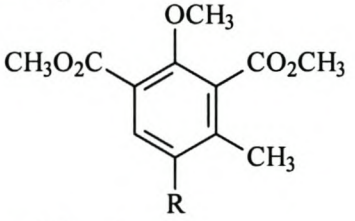
Figure 3.1 Synthetic routes employed in the attempted synthesis of fluoro-OB.

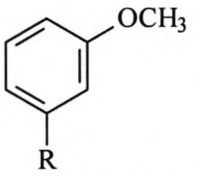




(17) R = H Salicylic acid
(18) R = Cl Chlorosalicylic acid
(19) R = F Fluorosalicylic acid
(20) R = Br Bromosalicylic acid
(21) R = Me_3Si

(22) R = H SAPhe
(23) R = Cl ClSAPhe
(24) R = F FSAPhe
(25) R = Br BrSAPhe
(26) R = Bu_3Sn

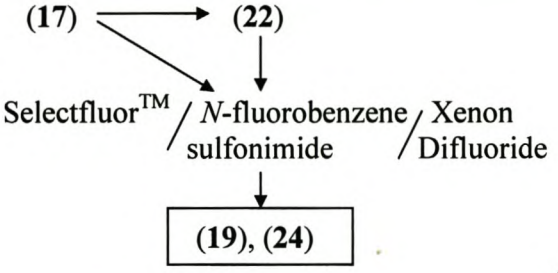




(27) R = Br
(28) R = Bu_3Sn
(29) R = F
(30) R = H

(31) R = Br
(32) R = F
(33) R= H

Direct Electrophilic Aromatic Fluorination



Aromatic Fluorodemetallation

Catalysed Aromatic Fluorodehalogenation

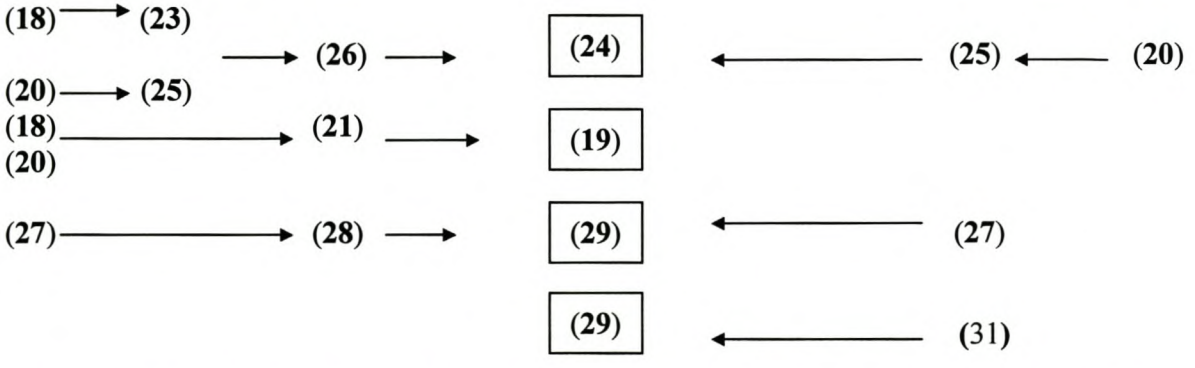


Figure 3.2 Synthetic routes employed in the attempted synthesis of fluoro aromatic compounds.

acid to L-Phe, for use as model aromatic substrates for fluorination is discussed (section 3.6.5). Peptide coupling by DCC carboxyl activation, including the protection of the phenolic hydroxyl group in 5ClSA for application on Oα (34) concludes the chapter (section 3.6.6).

3.2 Selective Electrophilic Fluorination

3.2.1 Selectfluor™ (6)

The methods described by Banks *et al*, 1996 for the fluorination of aromatic compounds were utilised. Fluorination was attempted on methoxybenzene, salicylic acid (**17**) and its phenylalanine coupled analogue, SAPhe (**22**). Although limited success was obtained by this method, it was further applied to small amounts of OB (**11**) and OB methyl ester. Most reactions were conducted at ambient temperature and in dry acetonitrile. Reactions were further studied at higher temperatures, increased reaction times, large excesses of reagents and in different solvents (e.g. water, methanol and 3-5% trifluoroacetic acid in water). The reactions were monitored by HPLC and UV spectroscopy, by comparing retention times and UV absorbance profiles with that of standards: salicylic acid and 5-fluorosalicylic acid.

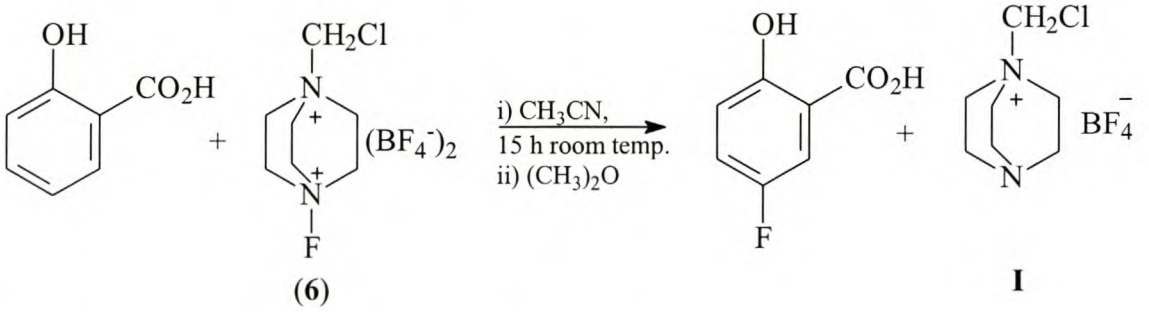


Figure 3.3 Fluorination of salicylic acid by Selectfluor™.

In all of the experiments conducted in this study with Selectfluor™ (**6**) the byproduct of the reaction, 1-chlomethyl-4-1-azoniabicyclo[2.2.2]octane tetrafluoroborate(**I**), was precipitated from the reaction mixture with the addition of diethyl ether and isolated by

filtration. ES-MS data corresponded with literature data (Banks *et al.*, 1996), molecular ion (M^+) peak *m/z* 160/162 ($C_7H_{15}N_2Cl$ requires 162.58).

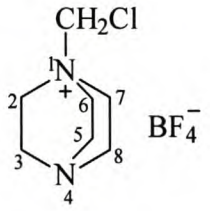


Figure 3.4. 1-Chloromethyl-4, 1-azoniabicyclo[2.2.2]octane tetrafluoroborate.

^{1}H-NMR of the salt indicated H-2, H-6 and H-7 to resonate as triplets at δ_H 3.60, H-3, H-5 and H-8 as triplets at δ_H 4.30 and the methylchloride group as as singlet at δ_H 5.20. ^{13}C revealed C-2, C-6 and C-7 resonance at δ_C 51.10, C-3, C5 and C-8 at δ_C 44.90 and the chloro bearing carbon at δ_C 69.80. These spectra are in perfect agreement with reported spectra by Banks *et al.*, 1996.

The attempted fluorination of methoxybenzene yielded an unidentified product (72% yield). From ^{1}H-NMR data it is suggested that fluorination was limited to the methoxy group accompanied by possible dimerisation at the *ortho*-position of the aromatic ring. The *ortho*-substitution was established by the resonance of the four aromatic protons as doublets (2-H) and triplets (2-H) at δ_H 6.7, 6.4, 6.3 and 6.2. Furthermore the resonance of the methoxy protons, which appears as a characteristic singlet at δ 3.8 and 3.9 for methoxybenzene and 2-fluoromethoxybenzene respectively, was absent. EI-MS data supported the presence of a dimeric analogue with *m/z* peaks at 260 and 204 (calculated for monofluoro dimer $C_{14}H_{12}O_2F_2$ = 250).

An attempt to fluorinate salicylic acid by the same method was met with limited success. Salicylic acid (**17**) was reacted over a prolonged time (72 h), at higher concentrations of the fluorinating reagent (reagent:substrate - 3:1) and at elevated temperatures (90°C). The reaction was monitored by HPLC with a diode array detector by periodically injecting samples of the reaction mixture and comparing the observed retention times with that of

the internal standards (salicylic acid **17** and 5-fluorosalicylic acid **19**). Figure 3.5 shows a chromatogram of the reaction mixture after 72 hours. The compounds were identified on the basis of their retention times.

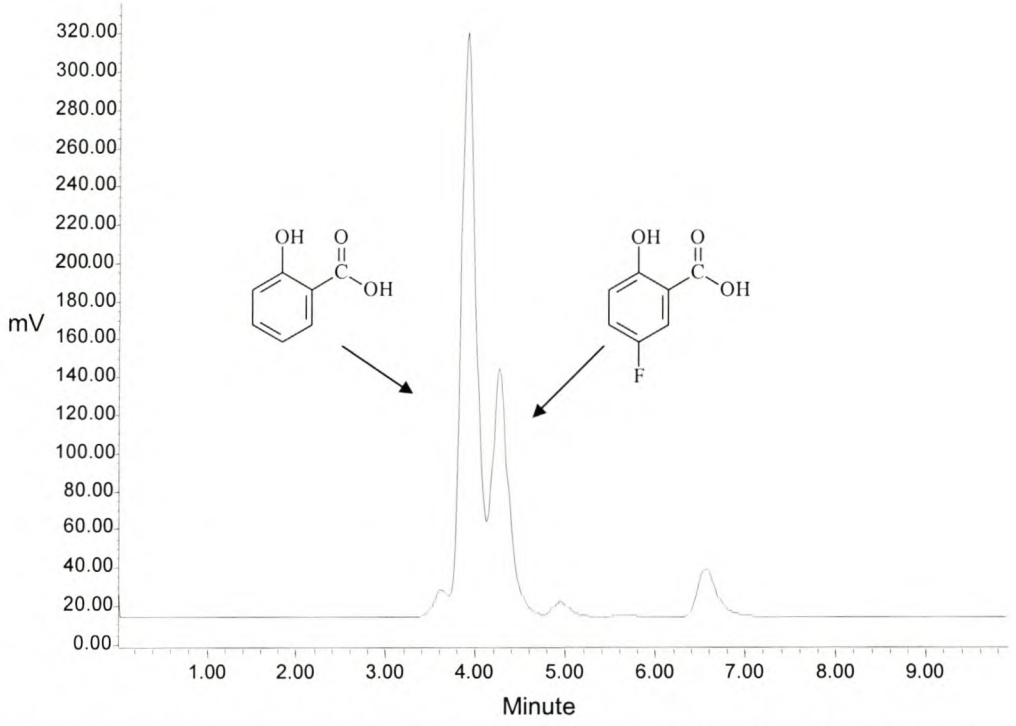


Figure 3.5 HPLC chromatogram of the fluorination of salicylic acid by Selectfluor™ after 72 h.

For salicylic acid at r_t = 3.91 min, and fluoro salicylic acid at r_t = 4.27 min. No attempt was made to further isolate the fluorinated product.

The method was further extended to SAPhe and small amounts of OB. In both cases the reactions were monitored by HPLC with a UV detector over a prolonged period of time (20 days). There was no indication that fluorination took place in either of the cases. These findings were confirmed by ^{1}H-NMR spectroscopy of the recovered starting materials.

3.2.2 *N*-Fluorobenzenesulfonimide

The method described by Davis, 1995, for the fluorination of anisole was used in the attempt to fluorinate salicylic acid. The method was further modified in the

concentrations of reagent used (reagent:substrate, 3:1) and the duration (72 h), in an attempt to drive the reaction to completion. HPLC monitoring followed by ^{1}H- and ^{13}C-NMR analysis of the reaction mixture indicated that no reaction had taken place. The attempt to fluorinate SAPhe and small amounts (2 mg) of OB over a prolonged reaction period (20 days), with an excess of reagent (reagent:substrate, 3:1) and under elevated temperatures (<70°C), was unsuccessful on the basis of HPLC monitoring. In both cases ^{1}H-NMR analysis confirmed that the substrates recovered were unreacted SAPhe and OB.

3.2.3 Xenon Difluoride

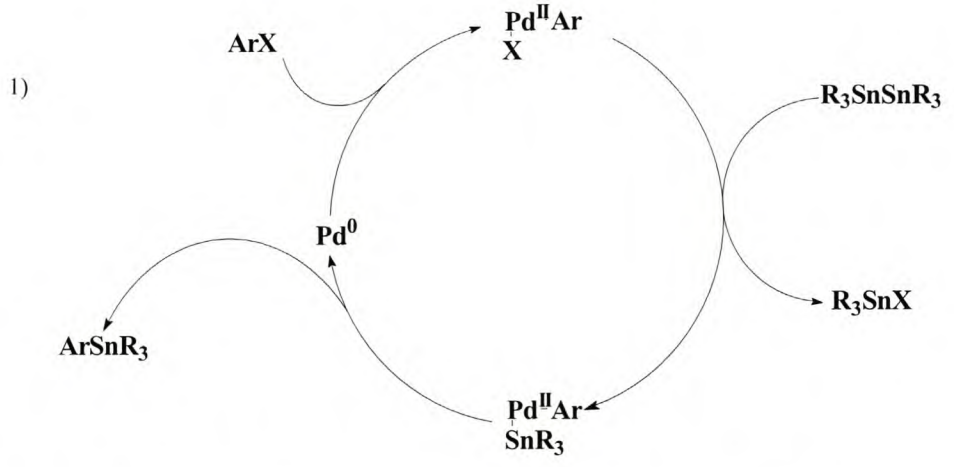
The method described by Ramsden and Smith, 1998, was utilised in the attempt to fluorinate model substrate, salicylic acid. The reaction conducted at low temperatures (-60 — 0°C) in a glass vessel in the absence of a catalyst (e.g. HF) was monitored by HPLC using a diode array detector. Extention of the reaction at higher temperatures (<50°C), and increased reagent concentrations did not drive the reaction to completion. ^{1}H- and ^{13}C-NMR analysis of the recovered product indicated a large proportion of substrate decomposition and small amounts of unreacted product.

The attempted fluorination of SAPhe (**22**) and small amounts of OB (**11**) (21 days reaction time), under similar rigorous condition was unsuccessful. In both cases the recovered products were identified by ^{1}H-NMR analysis as predominantly unreacted substrate.

In an attempt to ascertain the role that the free carboxylic acid has to play in blocking the reactivity of xenon difluoride, attempted fluorination was repeated on OB methyl ester (21 days reaction time). In this experiment α,α,α trifluorotoluene was used as solvent, in an attempt to suppress any radical process that accompanies the use of XeF_2. The reaction was monitored and the residue analysed in the same manner as described previously and yielded predominantly unreacted OB methyl ester as determined by ^{1}H-NMR.

3.3 Fluorodehalogenation of aryl halides *via* a palladium catalysed metal substitution followed by xenon difluoride fluorosubstitution.

The palladium-catalysed metallation of halogenated aromatic compounds using hexamethyl- and hexabutyldistannanes provides a mild and efficient fluorination method for application on sensitive, multi-functional compounds. The first intermediate in this synthetic route is the trialkyltin aromatic species. The synthesis of analogous silyl aromatic intermediates was attempted as alternative to the stannyl compounds. Bromo- and chloro-aromatic compounds provide the starting material for this synthetic route. These include halogenated salicylic acids (5ClSA, **18**; 5BrSA, **20**), and their respective phenylalanine analogs (CSAPhe, **23**; BrSAPhe, **25**) and 5-bromo-2-methoxy-4-methyl-isophthalic methyl ester (**27**). The catalyst employed was tetrakis(triphenylphospine) palladium (0). A further catalytic system, tris(dibenzylideneacetone)dipalladium(0), was used in the attempt to trimethylsilylate BrOB (**13**). Fluorodestannylation was attempted using xenon difluoride on the tributylstannyl specie of bromoisophtalic ester. The rationale of the synthetic approach, including the important intermediates, is shown in Figure 3.6.



2) $ArSnR_3 + XeF_2 \longrightarrow ArF + Xe + R_3SnF$

Ar	R	X
OB	Me, Bu	Br
N-(2-hydroxyphenyl)carbonyl-L-Phenylalanine	Bu	Cl, Br
2-methoxy-4-methyl 5-bromo isophthalic ethyl ester	Bu	Br

Figure 3.6 XeF_2 fluorodestannylation of aryltrialkylstannanes formed by palladium catalysed stannylation of aryl halides.

3.3.1 Catalytic Stannylation of Aryl Halides.

The substrates for this synthetic route are chloro or bromo aryl compounds. Although chlorides are not as readily replaced as the analogous bromo substituents during palladium catalysed trialkylstannylation, reactions were conducted on both substrates. This is exemplified in the attempt to replace the chloro group in OA (**10**), which will allow for a shorter route to the trialkylstannyl species than to follow the route *via* BrOB. Preparation of BrOB entails the dechlorination of OA to form OB, and then the bromination of OB.

3.3.1.1 Dehalogenation of OA.

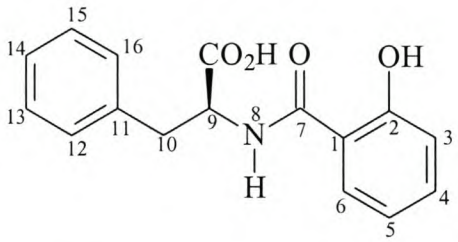
The method of Bredenkamp *et al*, 1989, was used in the dehalogenation of OA (**10**) to OB (**11**). Modifications, concerning reaction periods (over night), to this method suggested by Payne, 1999, were also adhered to. The method entails the treatment of the aromatic halide with ammonium formate in methanol, in the presence of 10% Pd/C. Furthermore, chloroform was replaced by ethyl acetate as solvent in the work up of larger amounts of OB (250 mg) (As desribed by Payne, 1999).

The successful dechlorination is clearly observed in the ^{1}H-NMR where the aromatic signal of OA (**10**) at δ_H 8.43 is replaced by two doublets at δ_H 6.82 and δ_H 8.33 showing mutual *o*-coupling of 7.98 Hz. Similarly in the ^{13}C spectra the Cl-bearing *ipso* singlet signal in OA at δ_C 123.2 is replaced by a singlet at δ_C 118.5 in OB. These spectra are in perfect agreement with the reported spectra of OA (Stander, 2000) and OB (de Jesus *et al.*, 1982).

Quantitative catalytic dechlorination was further applied to CSAPhe (**23**) in preparation of the dechloro analogue, SAPhe (**22**). This provided an alternative route for the preparation of SAPhe (**22**) since the coupling of phenylalanine to salicylic acid gave problems not encountered with the analogous 5-chlorosalicylic acid.

The successful dechlorination of CSAPhe (**23**) is clearly observed in the ^{1}H-NMR where the resonance at δ_H 6.80, assigned to H-5 appeared as a td owing to the *ortho* coupling to H-4 and H-6 (J = 8.9 Hz) and *meta* coupling to H-3 (J = 2.6 Hz). Furthermore the H-4 of CSAPhe (**23**) which appeared as a dd at δ_H 7.48 is replaced by a td at δ_H 6.97 in the dechlorinated product and shows mutual *o*-coupling to H-5 (J = 8.9 Hz). Similarly in the ^{13}C spectra the Cl-bearing *ipso* singlet signal in ClSAPhe (**23**) at δ_C 123.7 is replaced by a doublet at δ_C 119.0. These spectra are in agreement with the reported spectra of CSAPhe and SAPhe (Payne, 1998)(Table 3.1).

Table 3.1 ^{1}H- and ^{13}C-NMR data ($CDCl_3$) for SAPhe (**22**).



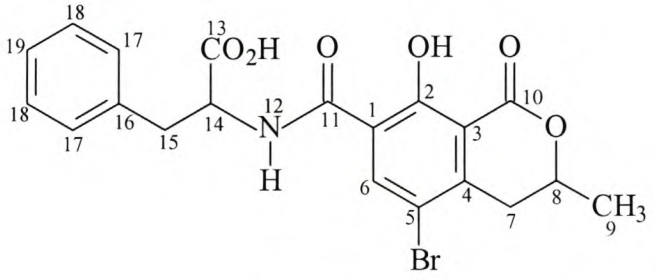
	δ_H/ppm	δ_C/ppm
1	-	118.7
2	-	161.6
3	7.18	113.8
4	6.97	134.8
5	6.80	119.0
6	7.18	125.7
8	5.00	169.8
9b	3.28	53.0
9a	3.10	37.3
10	-	135.2
11/15	7.29	12.9.4
12/14	7.29	128.9
13	7.38	127.6
16	-	175.7
NH	9.20	-

3.3.1.2 Bromination of OB, Steyn and Payne, 1999.

Pyridinium hydrobromide perbromide (PPB), was prepared freshly in adequate yield (67%), by the procedures reported by Fieser and Fieser, 1967. The process entails the rapid treatment of a cooled mixture of pyridine and 48% hydrobromic acid with bromine. The product was washed with an acetic acid:water (1:1) solution and recrystalised from

the same solution to yield a red crystalline product, that provided a stable, easy to handle source of bromine. OB (**11**) was subsequently treated with PPB in glacial acetic acid at 50°C for 6 h. The reaction was monitored by TLC (toluene:acetic acid 5:1) and proceeded smoothly and efficiently (98% yield). The successful bromination of OB (**11**) is clearly observed in the ^{1}H-NMR where it reveals a close similarity to OA (**10**)(Table 3.2). The most important feature is the diagnostic singlet signal at δ_H 7.20 for the aromatic proton (H-8). Similarly in the ^{13}C spectra the Br-bearing *ipso*-singlet signal at δ_C 112.3 for BrOB (**25**) revealed a downfield shift compared to OA (**10**) (δ_C123.2). These spectra are in perfect agreement with the reported spectra of BrOB (Steyn and Payne, 1999).

Table 3.2 ^{1}H- and ^{13}C-NMR data ($CDCl_3$) for BrOB (**13**).



	δ_H/ppm	δ_C/ppm
OH	12.7	-
NH	8.5	-
1	-	120.7
2	-	159.8
3	-	110.5
4	-	142.9
5	-	112.4
6	7.2	142.2
7a	2.8	34.9
7b	3.3	34.9
8	4.7	75.8
9 (3)	1.6	20.5
10	-	169.9
11	-	163.9
13	-	177.2
14	5.1	54.2
15	3.2	37.3
16	-	135.8
17	7.2	129.4
18	7.2	128.8
19	7.2	127.4

3.3.1.3 Attempted synthesis of trialkylstannyl aromatic intermediates.

The method employed by Duelfer *et al.*, 1991 for the selective ^{18}F-labelling of 2-oxoquazepam *via* the fluorodemetallation of the corresponding trimethylstannyl derivative was used for stannylation. The tributylstannylation was first attempted on 5ClSAPhe (**23**) and 5BrSAPhe (**25**). The limited success obtained on these compounds prompted further application on an aromatic nucleus, electronically more closely related to OA (**10**). A bromo substituted aromatic ester, 5-bromo 2-methoxy 4-methyl isopthalic methyl ester (**27**), was available to us and it proved to be a succesfull canditate for metallation. The method was further applied to BrOB (**13**).

For the stannylation of BrSAPhe (**25**), the reaction entailed treatment of the aryl bromide with hexabutylditin in xylene in the presence of tetrakis(triphenylphospine)palladium (0) as catalyst. The reaction mixture was heated under reflux in an argon atmosphere and monitored by TLC (toluene: acetic acid 5:1). Multiple products with increased polarity (rel. BrSAPhe, **25**) formed after 17 hours and upon purification of the crude product by column chromatography (silica / toluene: acetic acid 7:1) the fractions were isolated and characterised by ^{1}H-NMR and ES-MS.

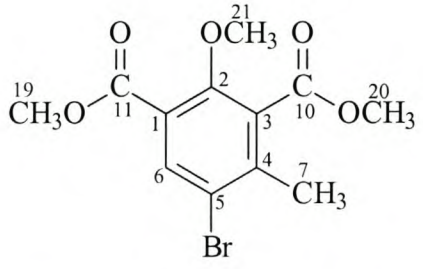
Apart from the unreacted substrate recovered (BrSAPhe, **25**), a byproduct of the reaction was isolated at Rf: 0.75, and was identified as tributylstannylbromide. The isotope profile at *m/z* 452 (^{122}Sn), 449 (^{119}Sn), 448 (^{118}Sn), 447 (^{117}Sn) and 446 (^{116}Sn) agrees for a $SnBr_2$ compound, $C_{12}H_{27}SnBr_2$ requires 448.38. The product at Rf: 0.30 was identified as the debrominated compound, SAPhe (**22**). ES-MS of this product indicated *m/z* molecular ion peak at 285. ^{1}H-NMR-data were in agreement with reported results (see section 3.3.1.1).

The third product (R_f 0.20) obtained was not identified. ^{1}H-NMR data supported a possible biaryl structure: the duplication of the phenylalanine protons (H12-16) and salicylic acid ring protons (H-3, H-4, H-6) resonance signals and the absence of a H-5 resonance peak (see figure 3.8). ES-MS supported the presence of a biaryl structure *m/z* peaks at 540, $C_{32}H_{28}N_2O_8$ = 540.56). In the case where ClSAPhe (**23**) was used as substrate, no reaction occurred under similar condition. ^{1}H- and ^{13}C-NMR data obtained

from the reaction mixture confirmed that the product isolated was predominantly the unreacted substrate. In contrast to BrSAPhe no biaryl formation was observed.

The increased reactivity of the bromo substrate to the chloro analogue was in agreement with literature findings. This prompted the use of a bromo aromatic compound exclusively, 5-bromo-2-methoxy-4-methylisophtalic methyl ester (**27**) as model substrate. Apart from the relatively inert phenylalanine ring, this compound represents the aromatic nucleus of OA (**10**) more closely than the halogenated salicylphenylalanine substrates. Using ^{1}H and ^{13}C-NMR data from BrOB (**13**), and other analogous compounds the chemical shifts were empirically assigned to the nuclei as shown in Table 3.3.

Table 3.3 ^{1}H- and ^{13}C-NMR data ($CDCl_3$) for 5-bromo-2-methoxy-4-methyl- isophthalic methyl ester, **27**.



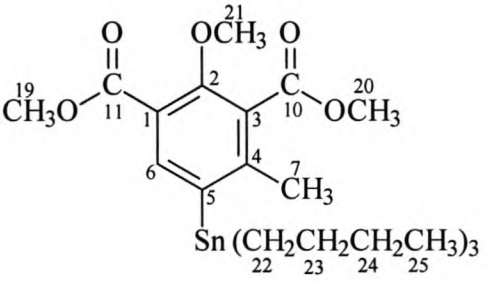
	δ_C/ppm	δ_H/ppm
1	133.9	-
2	156.2	-
3	119.7	-
4	141.8	-
5	124.8	-
6	136.2	8.05 (s)
7	20.3	2.35 (s)
10	165.1	-
11	167.5	-
19	51.9	3.95 (s)
20	51.9	3.93 (s)
21	63.5	3.92 (s)

The methyl ester (**27**) (0.289 mmol) was treated with hexabutylditin (1.44 mmol) in xylene in the presence of tetrakis(triphenylphosphine)palladium(0) (37.0 μmol) as catalyst. The reaction was protected from light and heated under argon at 115°C. The

reaction was monitored by TLC (petroleum ether: ethyl acetate 10:1) and after 19 h the reaction was complete. The solvent of the filtrate was evaporated under reduced pressure and purified by column chromatography (silica gel/ petroleum ether: ethyl acetate 10:1) to yield a colourless oil in 51% yield.

Chemical shifts were empirically assigned to the nuclei using ^{1}H and ^{13}C-NMR data from the bromoaryl methyl ester **27**, and analogous stannylated compounds (Table 3.4). A certain amount of the debrominated isophthalic methyl ester (<40%) was also identified in the fraction. It was represented by H-6 resonating as a doublet at δ_H 7.86 ($J_{H6,H5}$ = 8.9 Hz) and H-5 resonating as a doublet at δ_H 7.05 ($J_{H5,H6}$ = 9.0 Hz).

Table 3.4 ^{1}H NMR data ($CDCl_3$) for 2-methoxy-4-methyl-5-tributylstannyl isophthalic methyl ester



Proton	δ_H/ppm
6	8.95(s)
7	2.20(s)
19,20	3.95(s)
21	3.87(s)
22	1.10(m)
23	1.30(m)
24	1.51(m)
25	0.90(m)

The method employed for the methyl isophthalate, was applied to BrOB (**13**) and proved to be unsuccessful. The reaction, monitored by TLC (toluene: acetic acid 5:1), showed that multiple products had formed after 17 h. Upon purification by column chromatography (silica/ toluene: acetic acid 12:1) of the crude product, the fractions were isolated and characterised by ^{1}H-NMR and ES-MS.

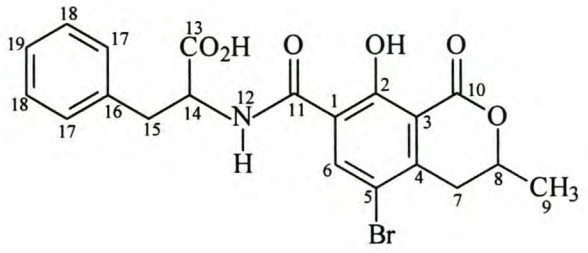
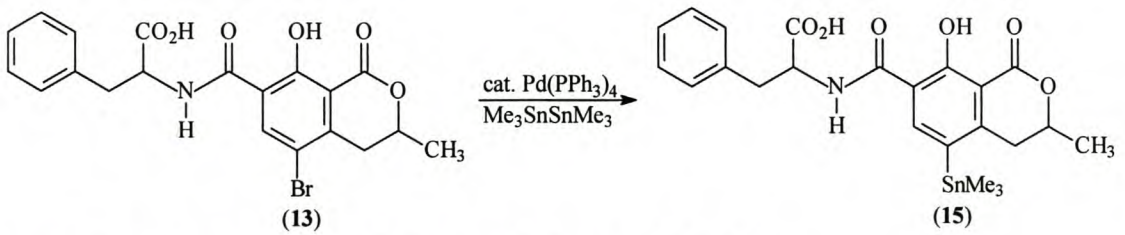


Figure 3.7 BrOB.

Tributylstannylbromide and unreacted BrOB (**13**) (Figure 3.7) was first separated from the mixture, followed by the isolation of debrominated product, OB (**11**). ^{1}H-NMR-data of the latter was in agreement with data obtained for OB. The H-6 resonates as a singlet at δ_H 7.2 for BrOB and as a doublet at δ 8.33 ($J_{H6,\ H5}$ = 8.00 Hz) for OB (**11**), H-5 resonates as a doublet at δ_H 6.82 ($J_{H5,\ H6}$ 8.0 Hz). ES-MS of odourless viscous oil that was isolated indicated that it was a tin bearing compound, by isotope profiles at *m/z* 657 (^{122}Sn), 654 (^{119}Sn), 653 (^{118}Sn), 652 (^{117}Sn) and 651 (^{116}Sn); tributylstannyl OB (**14**), $C_{32}H_{45}NO_6Sn$ requires 658.43). ^{1}H-NMR analysis was not in agreement with a possible stannylated OB compound showing none of the characteristic tin resonance peaks at high field. This either indicates that the desired product was identified by ES-MS but in insignificant quantities, or otherwise the peaks at *m/z* 657-653 belong to another compound not containing Bu_3Sn.

The moderate yields in the tributylstannylation of bromo isopthalic methyl ester (**27**), which is a very good electronic model of BrOB (**13**) where no desired product was detected under similar reaction conditions, suggested that steric considerations could be ascribed to this difference in reactivity. We therefore, decided to replace the hexabutylditin with hexamethylditin in a separate experiment (Figure 3.8).



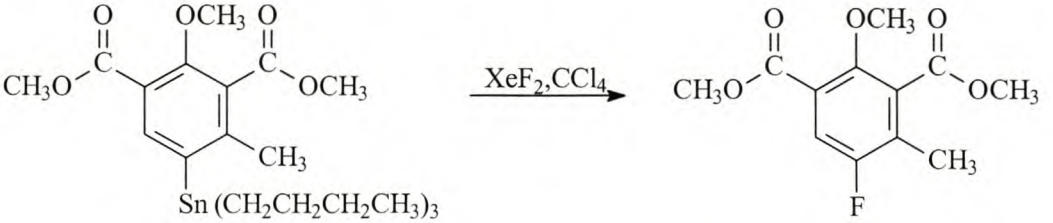
(**13**) (**15**)

Figure 3.8 The attempted catalytic stannylation of BrOB (**13**).

Two fractions were isolated by column chromatography following the reaction, and analysed by ^{1}H-NMR. Apart from the dehalogenated product, OB (**11**), the further fraction contained unreacted BrOB (**13**) and an unidentified product. This product shared the dihydroisocoumarin structure of BrOB (**13**) (H-7 appeared as two doublets at δ_H 2.77 (J = 12.0 Hz) and 2.84 (J = 12.2 Hz) but did not contain the phenylalanine side chain, i.e. the H-15 protons which resonate as a multiplet at δ_H 3.2, together with phenylalanine peaks (H-17—H-19) at δ_H 7.26 were absent. A small amount of the fraction (<10%) contained a trimethylstannyl substituted species. The characteristic tin singlet resonance at δ_H 0.5 Hz was observed. The absence of the phenylalanine proton resonance peaks suggested that the hexamethylditin could have reacted with the labile phenylalanine benzylic (C-15) protons. The use of BrOβ (the hydrolysis product of BrOB, **13**) as subtrate could circumvent this problem. This approach was not explored any further.

3.3.2 Fluorodestannylation by Xenon difluoride.

This procedure was conducted on the tributylstannylisophthalic ester intermediate (**28**) (Figure 3.9). Treatment of this compound with xenon difluoride in carbon tetrachloride, is a modification of the method used by Duelfer *et al.*, 1991, which described the use of $^{18}F_2$ gas, trapped at 0°C in acetonitrile containing a trimethylstannyl precursor, as the fluorine source. The limited availability of not only F_2 gas but also the highly specialised equipment, and expertise in its usage, prompted us to apply xenon difluoride as our source of fluorine. This approach has been implemented with great success by Lothian and Ramsden, 1993, in the rapid fluorodesilylation of aryltrimethylsilanes in fluorinated solvents.



(**28**) (**29**)

Figure 3.9 Attempted fluorodestannylation of the tributylstannylisophthalic ester intermediate by XeF_2.

Treatment of 2-methoxy-4-methyl-5-tributylstannylisophthalic methyl ester (**28**) with xenon difluoride to fluoro-substitute the halophilic tributylstannyl group, met with limited success. The rapid reaction of the fluorine source with the tin substrate afforded predominantly the protonated analogue, 2-methoxy-4-methylisophthalic methyl ester. It was characterised by H-6 and H-5 resonance peaks, featuring as doublets at δ_H 7.86 ($J_{H6, H5}$ = 8.9 Hz) and 7.05 ($J_{H5, H6}$ = 9.0 Hz). For successful fluorination at C-5, the ^{13}C-NMR, should have indicated the characteristic splitting patterns for the C-5 fluorine-bearing carbon atom ($J_{C, F} \approx$ 240 Hz) as well as the marked downfield shift associated with fluoro compounds. These features were not observed.

3.3.3 Attempted synthesis of trimethylsilyl aromatic intermediates.

The ability of tin to undergo undesired radical reactions, together with the prevalance of the benzylic hydrogen atoms that are prone to take part in radical processes during the replacement of the trialkyltin substituent with a fluorine substituent led to the investigation of the trialkylsilanes which is less apt to radical procesess. Furthermore, the fluoro substitution of aryltrimethylsilanes on aromatic species is rapid and can be achieved under farely mild conditions using xenon difluoride (Lothian and Ramsden, 1993). Our attempts were limited to the reaction of model compounds, 5ClSA (**18**) and 5BrSA (**20**) with hexamethyldisilane in the presence of tetrakis(triphenylphospine) palladium (0) as catalyst, as described in the method by Babin *et al.*, 1993.

The chloro substrate showed no reactivity towards catalytic silylation and the unreacted product was isolated in all the attempts incorporating varying temperatures, prolonged reaction times, elevated catalyst and hexamethyldisilane concentrations.

Although large amounts (up to 51%) of the unreacted acid was isolated, the 5-bromo salicylic acid was more reactive but non- selective for trimethylsilyl substitution. The product isolated was predominantly the debrominated compound (**17**) product, and small amounts of a biaryl compound was also obtained (Figure 3.10).

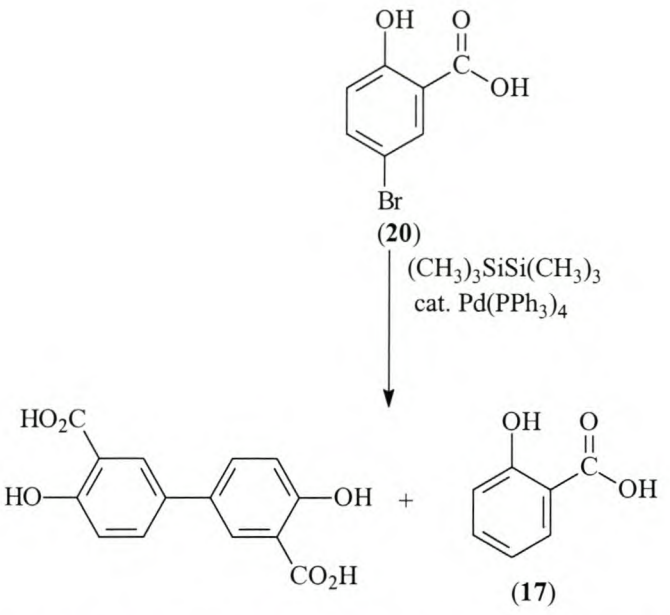


Figure 3.10 Non-selective side products in the attempted catalytic silylation of BrSA.

The limited success obtained, prompted the application of the method described by Kimes *et al.*, 1992; which entails much more harsh conditions. The method entails the treatment of arylbromides with hexamethyldisilane in the presence of tetrakis (triphenylphosphine)palladium(0) as catalyst at high temperatures 150-170°C in sealed reaction vessels for prolonged periods of time (2 days). These reaction conditions did not prove successful and afforded similar results as previously obtained under milder conditions. Furthermore, the application of this method on subtrates containing sensitive functional groups, such as BrOB (**13**) was not feasible.

An alternative of this method was found in a newly proposed protocol for the catalytic silylation of aryl bromides as desribed by Gooβen and Ferwanah, 2000. This method, which employs a catalytic system consisting of tris(dibenzylideneacetone)dipalladium(0) and different phosphines, has been proven to be mild and extremely efficient in affording arylsilanes from arylbromides.

The method is based on the rationale that arylbromides oxidatively adds to the palladium phosphine substrates, creating a Pd(II) species that will subsequently react with the hexamethyldisilane. In the case of a substituted aryl component, the Pd(II) species will be

activated in varying degrees, owing to the spectrum of substituents on the aromatic ring. BrOB (**13**) is a relatively electron rich aromatic ring, thus the use of an electron poor phosphine, such as diphenyl-2′-pyridylphosphine (PPh_2Py), will balance the electronic nature of the Pd (II) reactive intermediate.

However, the method did not prove successful in the silylation of BrOB (**13**), the product spectrum revealed that no biaryl specie had formed by furnishing predominantly the unreacted BrOB and trace amounts of the debrominated product, OB (**11**).

3.4 Direct catalytic fluorosubstitution of aryl bromides.

The low selectivity which accompanied the subtitution of the aryl bromide substrates for the trialkylstannyl- and -silyl groups, as well as the difficulty in isolating these intermediates prompted the investigation into a direct fluoro halogen subtitution method. The method involves the substitution of an aryl bromine atom for a nucleophilic fluoride (F^-, from tetrabutylammonium fluoride and cesium fluoride) in the presence of tetrakis(triphenylphosphine)palladium(0). The arylbromide substrates used were 5BrSAPhe (**25**), 3-bromoanisole (**30**) and 5-bromo-2-methoxy 4-methylisopthalic methyl ester (**27**) (Figure 3.11).

The first reaction entailed treatment of a mixture of 3-bromoanisole (**30**) in mesitylene with tetrabutylammonium fluoride in the presence of tetrakis(triphenylphosphine) palladium (0). After a prolonged reaction time (20 h), the crude product was analysed by ES-MS. The characteristic isotope peaks (*m/z* m and m+2) confirmed that no bromo substitution had taken place.

The reaction was repeated on BrSAPhe (**25**), using toluene as solvent. ES-MS analysis of the crude product revealed the base peak at *m/z* 215/217 which supports the bromosalicylic acid (**20**) structure (M-1; $C_7H_5O_3Br$ requires 216). Furthermore the peak at *m/z* 155, supports the analogous fluoro compound, fluorosalicylic acid (M-1; $C_7H_5O_3F$ requires 156). The fluorination could not be confirmed by ^{1}H-NMR, which implies that the fluoroproduct (**19**) was insignificant or that the peak at *m/z* 156 does not belong to **19**.

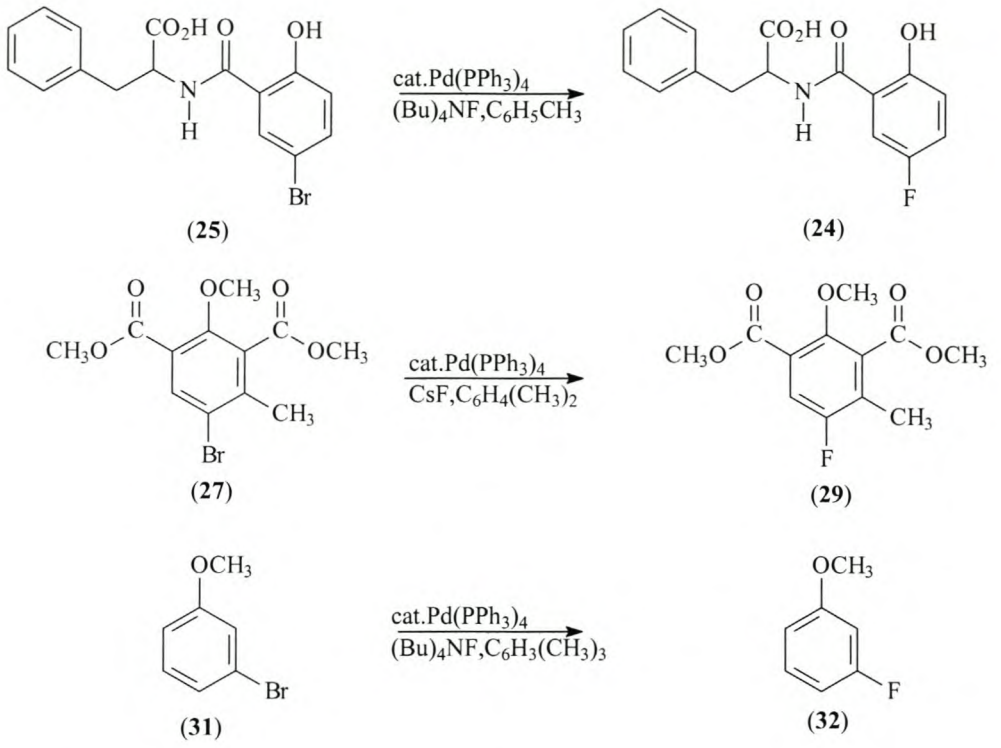


Figure 3.11 The attempted direct catalytic fluorosubstitution of aryl bromides.

In a final attempt, a mixture of 5-bromo-2-methoxy-4-methylisophtalic methyl ester (**27**) in xylene was reacted with cesium fluoride in the presence of tetrakis(triphenylphosphine) palladium (0) under reflux in a argon atmosphere for 21 h. ES-MS analysis of the crude mixture did not support the desired structure. The only product supported was the unreacted bromoisophtalic methyl ester (**27**) (*m/z* 316/318) and its debrominated analogue (**30**) (*m/z* 239).

3.5 Attempted synthesis of 2-fluoroacetoacetaldehyde.

It is clear from the discussion of a proposed method for the synthesis of fluoro-ochratoxin B, based on the diester approach of Kraus, 1981, that 2-fluoroacetoacetaldehyde is a key intermediate in this *de nova* synthesis route. The first step would include the synthesis of sodium acetoacetaldehyde followed by fluorination using Selectfluor™. Deprotonation by a mild base should render the corresponding sodium salt of fluoroacetoacetaldehyde.

The method used, was based on the synthesis of bromoacetoacetaldehyde from sodium acetoacetaldehyde by Lipinsky *et al.*, 1984.

3.5.1 Synthesis of sodium acetoacetaldehyde.

Sodium acetoacetaldehyde was prepared as described by Lipinsky *et al.*, 1984. The product was obtained in 64% yield. ^{1}H-NMR analysis revealed that a major impurity of this reaction proved to be sodium formate, the proton resonates as a singlet at δ_H 8.33 (Figure 3.12). The structure of sodium acetoacetaldehyde, is supported by the resonance of H-1 as a singlet at δ_H 8.75, H-2 a doublet at 5.13 and H-3 a singlet at 2.12. The mole fraction of the sodium acetoacetaldehyde to sodium formate was determined by the ratio of the single proton absorbance at δ_H 8.33 and 8.75.

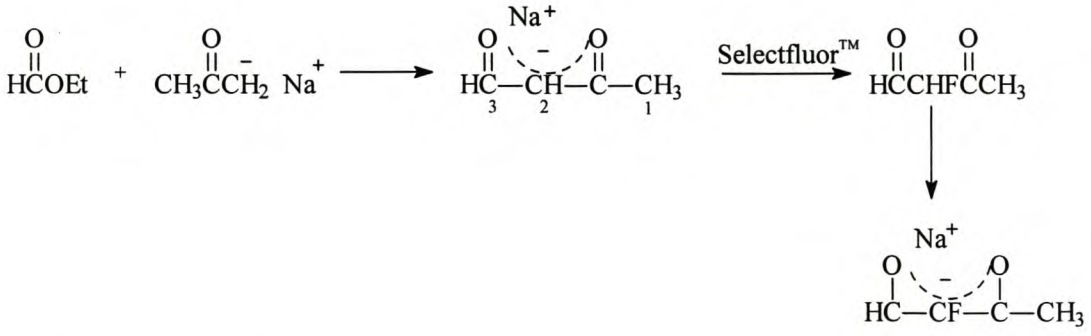


Figure 3.12 The synthesis of the sodium salt of fluoroacetoacetaldehyde.

3.5.2 Attempted fluorination of sodium acetoacetaldehyde.

Freshly prepared sodium acetoacetaldehyde (containing trace amounts of sodium formate) in acetonitrile was treated with Selectfluor™ at ambient temperatures for 72 h. The sodium salt had a poor solubility in acetonitrile, but upon addition of the fluorinating reagent it afforded a clear solution. However, ^{1}H-NMR analysis of the crude product revealed that no fluorination had taken place and that a large amount of product decompostion had occurred.

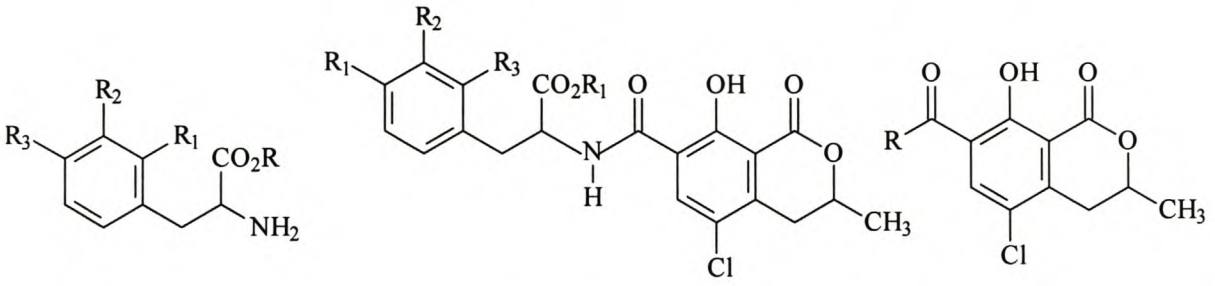
Product decomposition was ascribed to the presence of sodium formate in the starting substrate. The presence of excess Selectfluor™ in the reaction mixture could lead to the formation of hydrogen fluoride due to the oxidation of sodium formate, which could facilitate side reactions. No further attempts were made in the preparation of fluoro acetoacetaldehyde due to time constraints.

3.6 Peptide synthesis

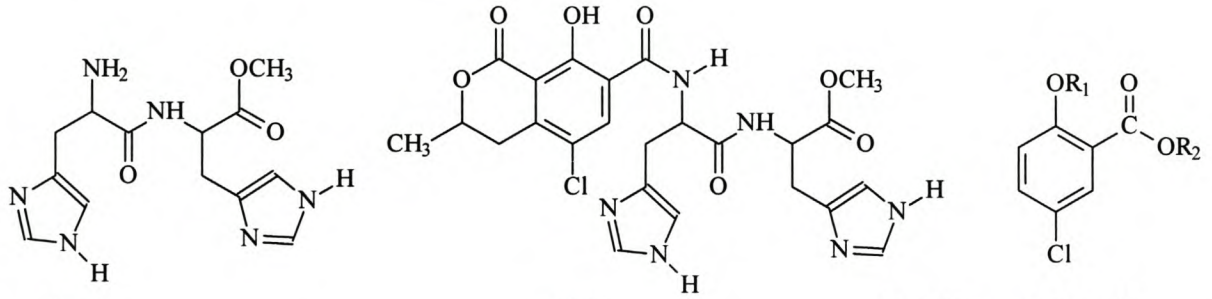
Peptide synthesis methodology forms an integral part in our structure activity relationship study of OA analogues. The areas of application includes (1) the coupling of fluoro substituted DL-phenylalanine to Oα, (**34**) (2) the synthesis of dipeptide histidylhistidine, and the attempted coupling to Oα, (**34**) (3) the synthesis of phenylalanine analogues, SAPhe (**22**), ClSAPhe (**23**) and BrSAPhe (**25**) for application in fluorination methodologies and (4) the coupling of F-Oβ to L-phenylalanine, as a route to fluoro-ochtaoxin B (**12**). Figure 3.13 illustrates the different synthetic routes and intermediates.

3.6.1 Acid hydrolysis of OA.

Oα (**34**), derived from the facile acid hydrolysis of OA, provides the access route to the synthesis of OA analogues by the modification to the L-phe side chain. The method by Xiao *et al.*, 1995, was utilised for the hydrolysis of OA (**10**) to afford Oα (34) which was used in the various coupling procedures. It entails treatment of the lactone acid with 6N HCl over a 72 h period under reflux. The crystals of Oα could be harvested directly from the reaction mixture and 12 h at ambient temperature was allowed for their formation. Repeated washing of the crystals with distilled water removed any traces of L-Phe. Residual Oα from the acid-water fraction was separated from Phe by partitioning into chloroform. Pure Oα crystals could be obtained by recrystallisation from methanol and water (1:1).



(36)	R_1=F	R_2=H	R_3=H	R_4=H	**(42)**	R_1=F	R_2=H	R_3 = H	R_4=H	**(34)**	R=OH
(37)	R_1=H	R_2=F	R_3=H	R_4=H	**(43)**	R_1=H	R_2=F	R_3 = H	R_4=H	**(35)**	R=Cl
(38)	R_1=H	R_2=H	R_3=F	R_4=H	**(44)**	R_1=H	R_2=H	R_3 = F	R_4=H		
(39)	R_1=F	R_2=H	R_3=H	R_4=CH_3	**(45)**	R_1=F	R_2=H	R_3 = H	R_4=CH_3		
(40)	R_1=H	R_2=F	R_3=H	R_4=CH_3	**(46)**	R_1=H	R_2=F	R_3 = H	R_4=CH_3		
(41)	R_1=H	R_2=H	R_3=F	R_4=CH_3	**(47)**	R_1=H	R_2=H	R_3 = F	R_4=CH_3		



(48) **(49)** **(50)** R_1= acyl R_2 = H
(51) R_1=R_2=TBDMS

Coupling of 2F-, 3F- and 4F- DL-Phe to Oα.

(42) ⟶ **(45)**
(43) ⟶ **(46)**
(44) ⟶ **(47)**

(10) ⟶ **(34)** ⟶ **(35)** + **(39)** ⟶ **(45)** ⟶ **(42)**
(35) + **(40)** ⟶ **(46)** ⟶ **(43)**
(35) + **(41)** ⟶ **(47)** ⟶ **(44)**

Coupling of dihistidine to Oα

(48) + **(34)** ⟶ **(49)**

Figure 3. 13 Peptide synthesis routes employed in synthesis of OA analogues.

3.6.2 Coupling of *ortho-*, *meta-* and *para-* substituted DL-fluorophenylalanine methyl ester to Oα-Cl (35).

The method by Steyn and Holzapfel., 1965, for the coupling of L-Phe to Oα was used extensively for the coupling of *o-*, *m-* and *p*-substituted DL-fluoro-phenylalanine to Oα. The first step in this method entails the derivatisation of the substituted DL-fluorophenylalanine to its methyl ester hydrochloride, followed by deprotonation to yield the methyl esters.

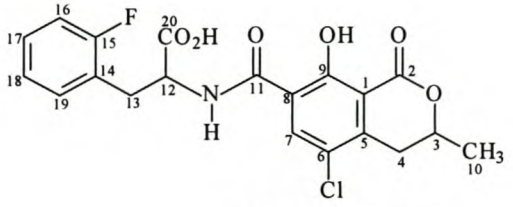
The coupling substrate, Oα-Cl (**35**) was obtained by the treatment of Oα (**34**) with freshly distilled thionyl chloride under reflux for 2 h in an argon atmosphere. In order to confirm the presence of Oα-Cl (**35**), and thus monitor the reaction by TLC, a drop of absolute ethanol was added to a fraction of the reaction mixture. The ethyl ester of Oα-Cl (**35**), the product of the reaction, migrates with the solvent. When hydrolysis was complete the thionyl chloride was evaporated under a dry stream of argon to afford the acyl chloride (**34**). This compound was immediately reacted in separate experiments with 2- (**39**), 3- (**40**) and 4- (**41**) F-DL-phenylalanine methyl esters. The reaction proved to be rapid and efficient and was complete after 2 hours. The isolated methyl esters were hydrolysed to produce the respective *ortho-* (**44**), *meta-* (**43**) and *para*-substituted (**42**) DL-fluorophenylalanine OA. The products were purified by crystalisation from methanol and diethyl ether. The structural assignments of the modified analogues are based on comparisons of their spectroscopic data with those of OA (**10**) and 2- (**36**), 3- (**37**) and 4- (**38**) F-DL-Phenylalanine (Table 3.5, 3.6 and 3.7).

Table 3.5 Infrared data (cm^{-1}) for *ortho*-substituted DL-fluorophenylalanine Oα and OA.

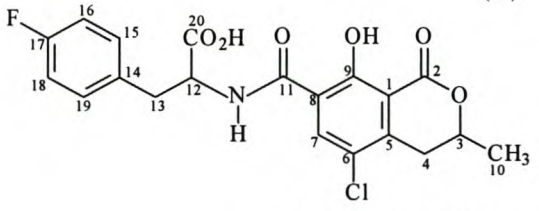
Compound	N–H vibration	HN–C=O vibration	C=O vibration	
			HOC=O	Amide/Lactone
OA	3398	1536	1737	1669
ortho-fluoroPhe Oα	3392	1530	1739	1671

The molecular ion (M^+) for the three analogues was obtained by EI-MS at *m/z* 435/437. The structure $C_{21}H_{19}O_6ClFN$ required m/z 435.8. Identical base peaks were observed for the three analogues at *m/z* 293.

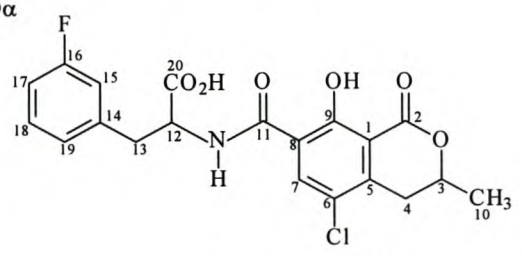
Table 3.6 ^{1}H- and ^{13}C-NMR data ($CDCl_3$) for *ortho*-, *meta*- and *para*- substituted DL-fluorophenylalanine Oα.



***ortho*-fluoroPheOα**
(44)



***para*-fluoroPheOα**
(42)



***meta*-fluoroPhe**
(43)

Carbon	*ortho*-fluoroPhe Oα			*meta*-fluoroPhe Oα			*para*-fluoroPhe Oα		
	δ_C/ppm	δ_H/ppm	$J_{H,H}$/Hz	δ_C/ppm	δ_H/ppm	$J_{H,H}$/Hz	δ_C/ppm	δ_H/ppm	$J_{H,H}$/Hz
1	110.05	-	-	111.36	-	-	111.72	-	-
2	169.66	-	-	168.00	-	-	168.55	-	-
3	75.85	4.76	16.8, 12.0	75.26	4.74	-	75.55	4.74	-
4	30.90	2.84 3.28	17.4, 3.8 17.4, 3.8	31.59	2.91 3.11	17.4, 12.0 8.4, 4.0	31.65	2.94 3.10	18.0, 12.1 18.0, 7.0
5	140.67	-	-	139.95	-	-	136.28	-	-
6	120.56	-	-	120.11	-	-	121.61	-	-
7	138.73	8.38		141.62	8.31	-	142.13	8.10	-
8	120.73	-		115.85	-	-	120.41	-	-
9	158.00	-		158.5	-	-	158.87	-	-
10	20.65	1.59	6.0	19.99	1.47	6.0	20.03	1.47	6.3
11	162.38	-	-	162.81	-	-	163.31	-	-
12	32.22	5.01	"complex"	53.60	4.84	"complex"	54.00	4.88	"complex"
13	31.06	3.43 3.21	13.8, 4.8 13.8, 6.0	36.09	3.10	8.4	35.68	3.12	8.4
14	128.72 128.67	-	-	121.00	3.21	3.5, 8.4 10.2	133.52	3..23	3.5,8.4, 10.2
15	160.27 162.57	7.26	-	135.80	7.07	-	115.41 115.13	7.23	-
16	131.72	7.04	-	162.70 161.09	-	-	131.33 131.44	7.14	-
17	124.06	7.20	-	135.80	7.04	-	159.96 163.18	-	-
18	131.72	6.98	-	130.02	7.32	-	131.33 131.44	7.14	-
19	124.02	-	-	125.28	7.10	-	115.40 115.13	7.23	-
20	172.70	-	-	172.06	-	-	172.70	-	-
N-H		8.56	-	-	8.69	-		-	-

Table 3.7 UV spectroscopy data for *ortho*-substituted DL-fluorophenylalanine Oα and OA.

Compound	λ_{max}/nm	ε/dm^3.mol^{-1}.cm^{-1}
OA	216	31500
	330	6050
ortho-fluoroPhe Oα	217	31450
	330	5900

3.6.3 The synthesis of histidylhistidine methyl ester.

Through a common interest in our research area, the methodology for the efficient synthesis of histidylhistidine was available to us. Furthermore, the aromatic nature of the constituent amino acid, L-histidines (52,53) resembles that of L-phe. The coupling of a dipeptide to Oα, a novel endeavor, could provide further insight into the role that the amide, if any, has to play in the toxicity of OA (**10**)

A general method by Rautenbach, 1999, for the synthesis of dipeptides using diisopropylcarbodiimide (DIPCDI) (**54**) as coupling reagent was utilised. The reaction mechanism is depicted in Figure 3.14.

The method entails the activation of the α-carboxyl group of t-butoxycarbonyl (tBOC)-histidine (tBOC-His) (**52**), by DIPCDI (**54**), to provide the activated carboxylic acid derivative (**56**). Treatment of the **56** with a solution of deprotonated (by triethylamine) histidine methyl ester dihydrochloride (His-OCH3) (**53**), in freshly distilled DMF, renders the dipeptide. The addition of hydroxybenzotriazole (HOBt) (**55**), a "trapping agent", to the reaction mixture prevents the racemisation of the amino acids during the coupling reaction (Lloyd-Williams, 1997).

A side product of the reaction, diisopropylurea (**57**), is highly insoluble in the DMF and easily removable by filtration. The use of t-BOC-His (**52**) allows for complete solubility in DMF and protection of the amino group to reactivity of the dehydrating agent. The t-BOC group can easily be removed by treatment of the dipeptide (**58**), with a 10%

aqueous solution of trifluoroacetic acid. The volatile side products of the reaction, isobutene and CO_2, can be removed by evaporation under reduced pressure.

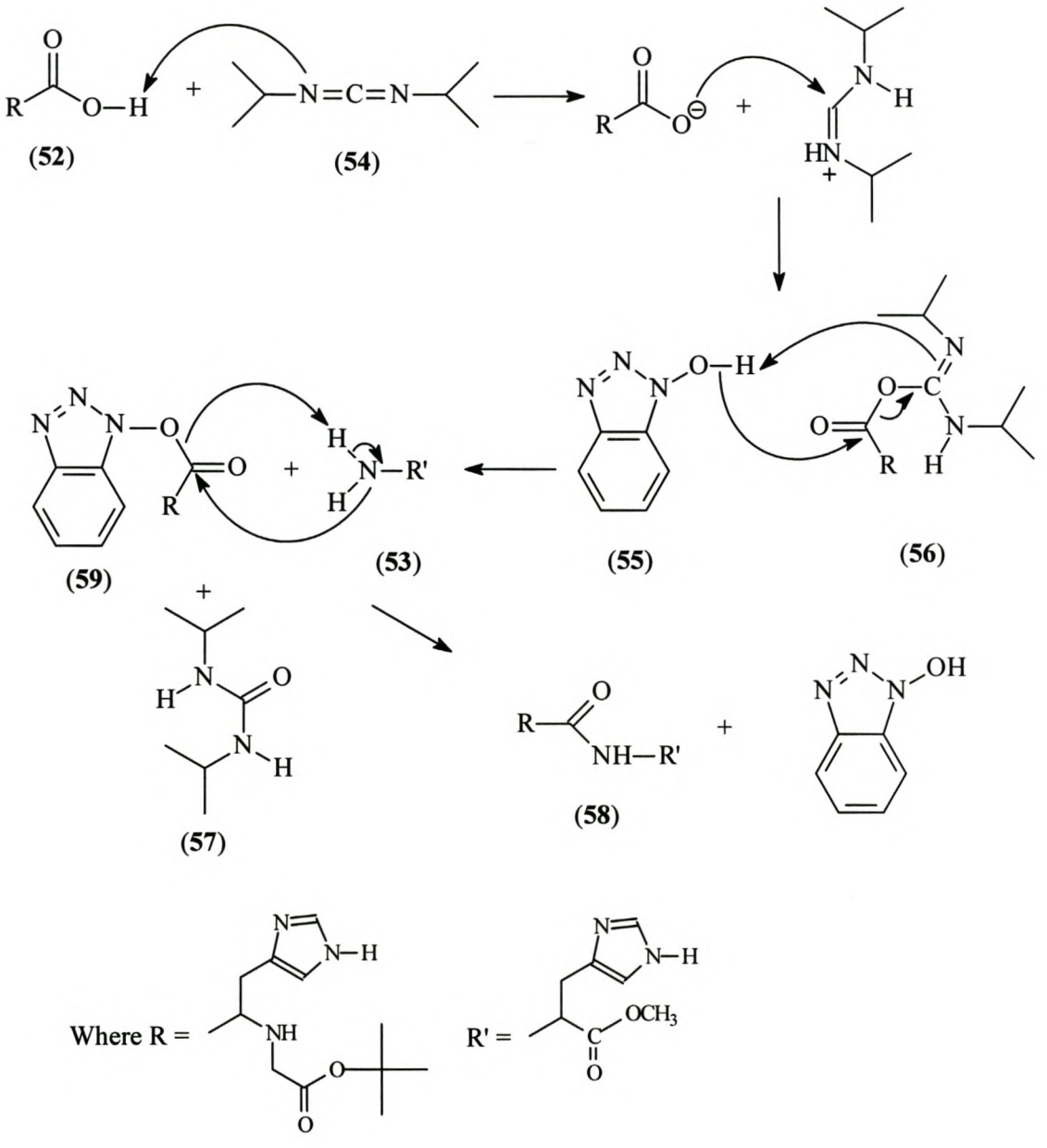


Figure 3.14 The mechanism of synthesis of histidylhistidine methyl ester by activation of DIPCDI (**54**) and HOBt (**55**).

The resultant white coloured residue was purified from the unreacted histidine amino acids (**52**, **53**) and trace amounts of diisopropylurea (**57**) by column chromatoghraphy (silca/ ethyl acetate:ethanol, 3:1).

Upon treatment of the concentrate containing the desired product, with a 10% aqueous TFA solution the methyl ester of histidylhistidine (**48**) was isolated in good yield (79%)(Figure 3.1.5). The white precipitate was recrystalised from a methanol: ether solution (with an excess diethylether). The histidyl-histidine methyl ester appeared to be highly hygroscopic, and was stored under argon.

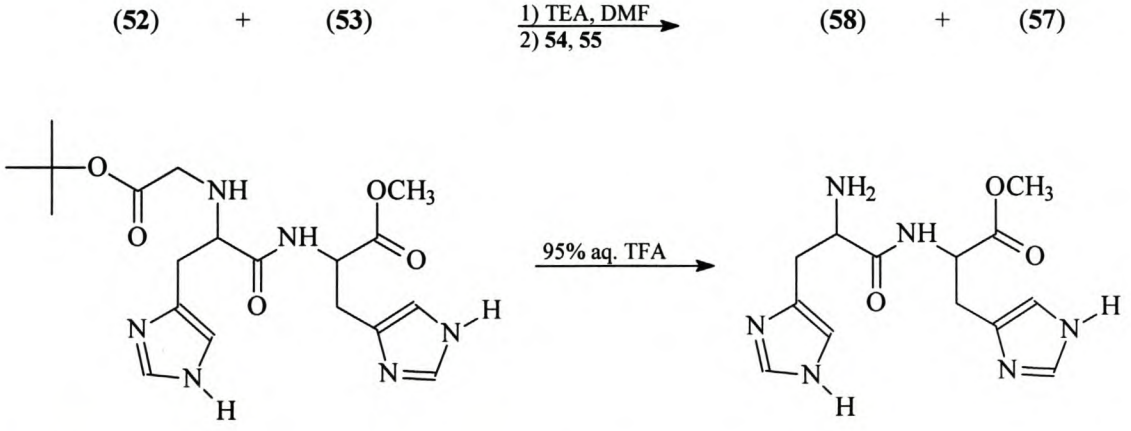
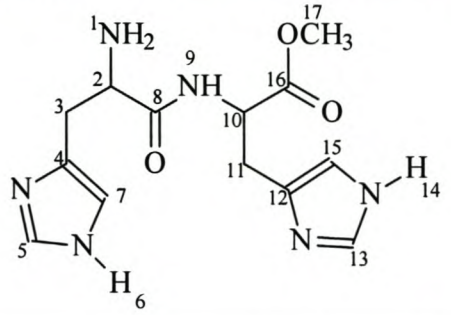


Figure 3.13 The synthesis of histidylhistidine methyl ester (**48**) by activation of DIPCDI (**54**) and HOBt (**55**).

Using the ^{1}H NMR data and the ^{13}C NMR data of analogous compounds and the precursors, the following assignments were made in Table 3.8.

The purified histidylhistidine methyl ester provided a dipeptide for the direct coupling to Oα-Cl.

Table 3.8 ^{1}H- and ^{13}C-NMR [$(CD_3)_2SO$] data for histidylhistidine methyl ester (**48**).



	δ_C/ppm	δ_H/ppm
2	4.56-4.59	52.10
3	3.03-3.07	26.41
4	-	130.06
5	8.15	134.08
7	7.14	116.08
8	-	167.89
9	9.23	-
10	4.15	52.19
11	3.12-3.17	27.62
12	-	130.46
13	8.51	134.69
15	7.28	118.07
16	-	170.40
17	3.64	51.68

3.6.4 Efforts to couple histidylhistidine methyl ester (48) to Oα-Cl (35).

The method used in the synthesis of Oα-Cl (**35**) and subsequent coupling to the fluoro substituted phenylalanine methyl esters (section 3.6.2), was utilised in the coupling of Oα-Cl (**35**) to histidylhistidine methyl ester (**48**).

The method utilised to couple the dipeptide methyl ester to Oα-Cl was unsatisfactory. This finding was confirmed by the analysis of the isolated product by ^{1}H- and ^{13}C-NMR and ES-MS, and by comparing these results with the spectroscopic data obtained from the building blocks, Oα (**34**) and dihystidine methyl ester. No further coupling techniques were explored in this regard.

3.6.5 The coupling of halosalicylic acids and salicylic acid to L-Phe.

The method used by Steyn and Holzapfel 1967, was used for the coupling of phenylalanine to salicylic acid and other halogenated salicylic acid derivatives. Salicylic acid, 5-chloro- and 5-bromosalicylic acid were respectively refluxed in thionyl chloride to afford the corresponding acyl chlorides. Treatment of the latter with sodium azide afforded the acyl azide. In each case the salicyl azide, was isolated and reacted without purification with the triethylammonium salt of the amino acid. Reactions were monitored using TLC (toluene:acetic acid, 5:1) and reached completion in 48 hours. Payne, 1999, modified the method used by Steyn and Holzapfel, 1967, by using ethyl acetate instead of DMF as solvent for the reaction. Not only can the reaction be monitored more effectively in this solvent, but ethyl acetate can also be removed more easily by evaporation and is less toxic than DMF.

Furthermore, ClSAPhe (**23**) and BrSAPhe (**25**) could easily be purified by crystallisation from chloroform. SAPhe (**22**), is a colourless oil, and was purified by column chromatography (toluene:acetic acid 5:1). SAPhe could also be obtained by the catalytic dehalogenation using Pd/C in methanol and ammonium formate. The coupled products were afforded in fairly low yields, i.e. for ClSAPhe (**23**) (49%), BrSAPhe (**25**) (38%) and SAPhe (**22**) (38% from the acid, quantitatively from ClSAPhe). ^{1}H-NMR and ^{13}C-NMR data for the coupled products were consistent with the data obtained by Payne, 1999.

These phenylalanine coupled salicylic acid derivatives provided, in the amino acid side chain and substituted aromatic substrate, the OA related functional groups for the application in fluorination techniques.

3.6.6 Peptide coupling by the DCC carboxyl activation.

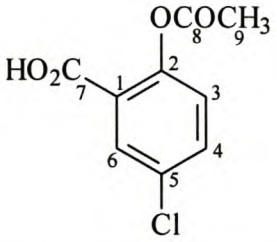
As discussed previously (section 2.3.1.2.3), the presence of the free hydroxyl group in OA (**10**) and halosalicylic acid allows for the intramolecular binding to the derivatised carbodiimide that leads to the formation of a undesired adduct. In an attempt to avoid the formation of this by-product, and increase the yield of the desired amide, the reactivity of

the hydroxyl group of 5ClSA (**18**) was blocked using different derivatives. In order to apply this method to Oα (**34**), the protective group employed should i) efficiently derivatise the hydroxyl group ii) be stable under coupling conditions and iii) easily be removed after coupling.

As part of this approach we derivatised the phenolic hydroxyl group of 5ClSA (**18**) by acetylation using acetyl chloride in pyridine, employing the method by Kendall *et al.*, 1979. Alternatively, protection of both hydroxyl and carboxyl group of 5ClSA (**18**) as the *tert*-butyldimethylsilyl (TBDMS) ether and ester respectively (**51**), can be achieved by treatment with *tert*-butyldimethylsilyl chloride, utilising the method by Ronald *et al.*, 1982.

5-Chlorosalicylic acid (**23**) was acetylated with acetyl chloride, to afford 2-acetoxy-5-chlorobenzoic acid in respectable yield (87%). The reaction was monitored with TLC (toluene:acetic acid, 5:1) where the product appeared as a dark spot under UV absorbance (Rf = 0.75). The structure of the product was confirmed by ^{1}H-NMR, ^{13}C-NMR. The assignments were made from data of 5-chlorosalicylic acid and other analogous compounds (Table 3.9).

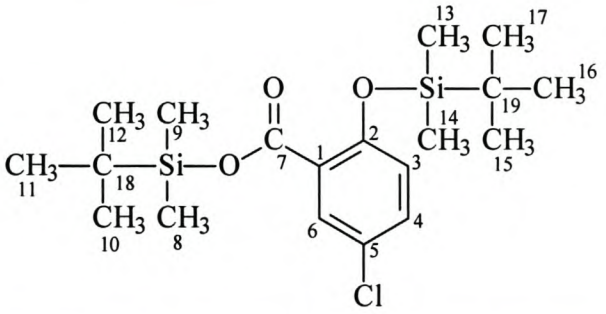
Tabel 3.9 ^{1}H- and ^{13}C- NMR data for 2-acetoxy 5-chlorosalicylic acid.



	δ_C/ppm	δ_H/ppm	$J_{H,H}$ /Hz
1	130.48	-	
2	169.50	-	
3	126.24	7.31	8.7
4	133.79	7.76	8.7, 2.7
5	149.28	-	
6	131.02	7.94	2.7
7	164.89		
8	156.12	-	
9	20.75	2.27	

tert-Butyldimethylsilyl 2-*tert*-butyldimethylsilyloxy-5-chlorobenzoate (**51**), was synthesised in good yield (84%). The structure of the product was confirmed by ^{1}H-NMR and ^{13}C-NMR. The assignments were made from data of 5-chlorosalicylic acid and other silylated compounds (Table 3.10).

Table 3.10 ^{1}H- and ^{13}C- NMR data for DiTBDMS-chlorosalicylate (**51**).



	δ_C/ppm	δ_H/ppm	$J_{H,H}$/Hz
1	125.36	-	
2	164.03	-	
3	122.93	6.80	8.4
4	132.63	7.27	9.0, 3.0
5	125.50	-	
6	131.10	7.65	3.0
7	154.50	-	
8,9	-4.70		
10-12	25.75	0.98	
13, 14	-4.01		
15-17	25.75	0.98	
18/19	25.71	0.34, 0.98	

The selective hydrolysis of the silyl ester will allow for the DCC activation and peptide coupling. Further application of this methodology on Oα could provide the protected intermediate needed for the coupling to amino acids by the use of DCC as coupling reagent.

CHAPTER 4

EXPERIMENTAL

4.1 General

4.1.1 The purification of organic solvents.

Acetonitrile: The solvent was primarily dried on 4A molecular sieves, followed by stirring with calcium hydride. The purified solvent was obtained, following fractional distillation immediately prior to use.

Diethyl ether and petroleum ether (60-80°C): The solvents were dried over sodium wire followed by fractional distillation immediately prior to use.

Carbon tetrachloride and Chloroform: The solvents were purified by elution through a column of activated alumina, followed by refluxing with potassium pentoxide and distilling immediately prior to use.

Ethyl acetate: The solvent was refluxed with a 10% (v/v) acetic anhydride solution in the presence of a catalytic amount of conc. sulphuric acid for 4 hours. The mixture was fractionally distilled and the distillate stirred with 2% (w/v) anhydrous potassium carbonate, filtered and redistilled.

Ethyl and methyl alcohol: The solvents were purified upon distillation from magnesium -ethoxide and –methoxide, respectively immediately prior to use.

Dimethyl formamide: The solvent was purified by stirring with 10% (w/v) potassium hydroxide, followed by filtration and fractionated vacuum distillation.

Pyridine: The solvent was purified upon stirring with calcium hydride and fractional distillation immediately prior to use.

Tetrahydrofuran: The solvent was purified by refluxing with a sodium-potassium liquid alloy (1:5) followed by fractional distillation immediately before use.

Toluene and Xylene: The solvents were purified by refluxing with potassium pentoxide followed by fractional distillation immediately before use.

Thionyl chloride: The reagent was purified by primarily refluxing with 5% (w/w) of dipentene (p-menta-1,8-diene). Following distillation of the mixture, the distillate was refluxed with 1-2% (w/w) of linseed oil and fractionally redistilled.

Reagents and chemicals: Reagents were purchased from Merck and Sigma-Aldrich, were of high purity and were used without further purification.

4.1.2 Chromatography

4.1.2.1 Thin layer chromatography (TLC): The method was used extensively to monitor the progress of reactions. The stationary phase used was precoated Kieselgel 60 F_{254} (Merck) plastic and glass-supported plates. The mobile phase used is specified where applicable in the experimental procedure. Apart from the visualization under UV light, the plates were treated with iodine vapour.

4.1.2.2 High performance liquid chromatography (HPLC): The method was used to monitor the progress of reactions, of which the samples were prepared in the following manner: Periodic samples (50 µl) were removed from the reaction mixture and the solvent evaporated under a stream of dry argon. The residue was suspended in chloroform (1 ml) and acidified with 1M HCl (200 µl). The aqueous phase was extracted three times with chloroform (3×0.5 ml) and the combined chloroform extracts were evaporated under a dry stream of argon. The residue was suspended in AR methanol (500 µl) and distilled water (500 µl) and assayed.

4.1.2.3 Preparative chromatography: The method described by Still *et al.*, 1966 for Flash chromatography was used in the separation and purification of reaction products. The stationary phase used was Kieselgel 60 (Merck, mesh size 230-400 nm) and the mobile phases are specified where applicable in the experimental procedure.

4.1.3 Instrumentation

4.3.1 Melting points: The melting points of compounds were determined using a Gallenkamp melting apparatus and are uncorrected.

4.1.3.2 Infrared spectroscopy: The IR spectra were recorded on a Perkin-Elmer 1720 FT-IR spectrometer using KBr pellets.

4.1.3.3 Mass spectrometry: EI mass spectra were recorded on a AMD 604 spectrometer at 70 eV. Electrospray mass spectra were recorded on a Micromass Quattro, triple quadropole mass spectrometer coupled to a HPLC system.

4.1.3.4 Ultraviolet-visible spectroscopy: UV spectra were recorded in methanol on GBC UV/VIS 920 spectrometer

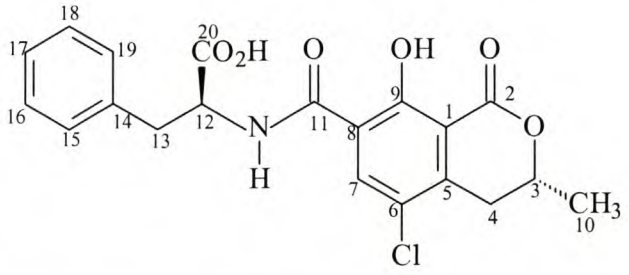
4.1.3.5 Nuclear magnetic resonance spectroscopy: ^{1}H and ^{13}C spectra were all recorded on a Varian VXR 300 MHz spectrometer (at 25°C) unless stated. ^{1}H and ^{13}C spectra were recorded at 300 MHz and 75 MHz, respectively. Spectra were further recorded on Varian Inova 600MHz (25°C) spectrometer. ^{1}H and ^{13}C-spectra were recorded at 600 MHz and 150 MHz respectively. All chemical shifts (ppm) are reported relative to tetramethylsilane (δ=0). The abbreviations used to describe ^{1}H-multiplicity are: s = singlet, d = doublet, t = triplet, q = quintet, m = multiplet, td = triplet of doublets etc. and for ^{13}C-multiplicity: S= singlet, D = doublet, T = triplet, Q = quintet.

4.1.3.6 High Performance Liquid Chromatography: Waters 2690 system fitted with a thermostatic column compartment, auto sampler, and Millennium software was used. Detection was achieved by fotodiode array (258 nm-330 nm) and fluorescence detectors (excitation wavelength was set at 331 nm and the emission wavelength at 460 nm). Separation was achieved on a C_{18} reversed phase column (4.6 mm × 150 mm, 5 µm, Discovery, Supelco, Bellefonte, PA, USA) at 30 °C, employing an isocratic mobile phase of methanol/water/acetic acid (60:40:2), an injection volume of 50 µL and a flow rate of 1 ml/min.

4.2Physicochemical data of relevant compounds.

4.2.1 Ochratoxin A (10)

Emperical formula: $C_{20}H_{18}ClNO_6$
Structural formula:



Melting point: 90°C (containing 1 mol of benzene)
171°C (from xylene) (Van der Merwe *et al.*, 1965)

Specific rotation: $[\alpha]_D^{21}$ (c 1.07, $CHCl_3$), -46.8° (Semenuik *et al.*, 1971)

ORD: (c 0.18, MeOH), $[\phi]_{600}$ -380°, $[\phi]_{589}$ -403°, $[\phi]_{342}$ -4600°, $[\phi]_{319}$ -0°, $[\phi]_{304}$ +1650°, $[\phi]_{269}$ -6270°(Steyn, 1984).

UV: (λ_{max} MeOH/0.0005 M H_2SO_4) 216, 330 nm (ε 31,500 and 6400, respectively); (λ_{max}^{MeOH}210 and 330 nm (ε 37 060 and 6050, respectively)(Steyn, 1984).

IR: (ν_{max} $CHCl_3$), 3380, 2988, 2928, 1723, 1674, 1612, 1528, 1425, 1381, 1304, 1260, 1170, 1140, 1107, 827 cm^{-1} (Steyn, 1984). The distinctive absorption bands are; carboxylic acid carbonyl stretch 1723 cm^{-1}(strong) and 1500 to 1700 cm^{-1} (broad), secondary amide 1665, 1535 (strong) and 3430 cm^{-1}(medium), lactone group 1678, 1132 cm^{-1} (strong) (Van der Merwe et al., 1965).

EI-MS: *m/z* M^+, 403 (13%), 359 (31%), 358 (18%), 357 (14%), 258 (52%), 257 (97%), 256 (100%), 255 (86%), 242 (49%), 241 (94%), 240 (98%), 239 (81%) and 238 (99%). (Steyn, 1984).

^{1}H NMR: ^{1}H NMR (500 MHz) of ochratoxin A in $CDCl_3$.(Stander, 2000)

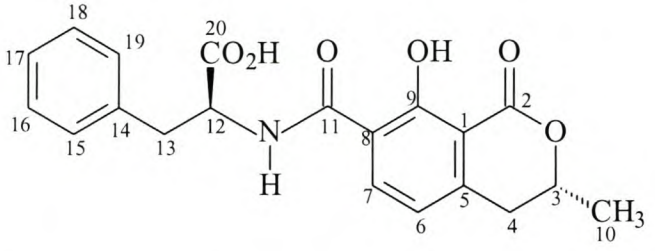
Proton	δ_H/ppm	*J*/Hz	Multiplicity	Coupling
OH	12.74		s	
NH	8.50	7.07	d	$J_{NH, 12}$
3	4.76	11.75, 6.05, 3.49	d, q, d	$J_{3, 4a}$, $J_{3, 10}$, $J_{3, 4b}$
4a	2.86	17.47, 11.75	d, d	$J_{4a, 4b}$, $J_{4a, 3}$
4b	3.29	17.47, 3.49	d, d	$J_{4b, 4a}$, $J_{4b, 3}$
7	8.43		s	
10(3)	1.60	6.05	d	$J_{10, 3}$
12	5.06	7.07, 7.07, 5.35	d, d, d	$J_{12, 13a}$, $J_{12, NH}$, $J_{12, 13b}$
13a	3.23	14.08, 7.07	d, d	$J_{13a, 13b}$, $J_{13a, 12}$
13b	3.36	14.08, 5.35	d, d	$J_{13b, 13a}$, $J_{13b, 12}$
15-19	7.27		m	

^{13}C NMR: ^{13}C chemical shifts and directly bonded [^1J(C,H)] coupling constants of OA (de Jesus et al., 1980).

Carbon	δ_C/ppm[a]	^1J(C,H)	Multiplicity
1	110.1		S
2	169.6		S
3	75.9	149.7	D
4	32.2	131.9	T
5	141.0		S
6	123.2		S
7	138.9	168.9	D
8	120.3		S
9	159.1		S
10	20.6	128.3	Q
11	163.0		S
12	54.3	144.5	D
13	37.5	130.3	T
14	135.9		S
15,19	129.4	158.9	D
16,18	128.6	159.5	D
17	127.2	160.8	D
20	174.9		S

4.2.2 Ochratoxin B (11)

Emperical formula: $C_{20}H_{19}NO_6$
Structural formula:



Melting point: 220-221°C (from MeOH) (Van der Merwe *et al.*, 1965).

Specific rotation: $[\alpha]_D^{21}$ (c 0.29, MeOH), -56 (Steyn, 1967).

ORD: (c 0.18, MeOH), $[\phi]_{600}$ -203°, $[\phi]_{589}$ -206°, $[\phi]_{325}$ -5200°, $[\phi]_{300}$ -0°, $[\phi]_{295}$ -1456°, $[\phi]_{284}$ -0°, $[\phi]_{266}$ -2860° (Steyn, 1967).

UV: (λ_{max}^{EtOH} 218 and 318 nm (ε 34300 and 6750, respectively)(Steyn, 1967)

IR: (ν_{max} Nujol), 3460 (N-H), 1728 (C=O acid), 1666 (C=O lactone), 1658 (C=O amide), 1532 (CO-N amide), and 1130 cm^{-1} (CO-O lactone)(Steyn, 1967)

EI-MS: *m/z* M^+, 369 (2%), 221 (57%), and 205 (100%) (Steyn, 1967)

^{1}***H-NMR:*** (500 MHz) of OB in $CDCl_3$ (Bredenkamp, 1989; this thesis).

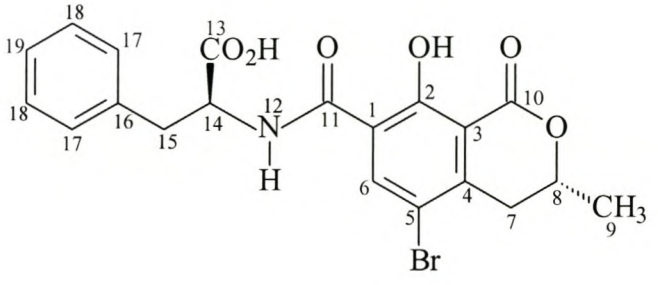
Proton	δ_H/ppm	*J*/Hz	Multiplicity	Coupling
NH	8.48	7.4	d	$J_{NH,\ 12}$
3	4.83	11.3, 6.3, 3.4	d, q, d	$J_{3,\ 4a}$, $J_{3,\ 10}$, $J_{3,\ 4b}$
4a	2.97	16.8, 11.3	d, d	$J_{4a,\ 4b}$, $J_{4}a,\ _{3}$
4b	3.08	17.2, 3.8	d, d	$J_{4b,\ 4a}$, $J_{4b,\ 3}$
6	6.95	8.0	d	$J_{6,\ 7}$
7	8.07	7.9	d	$J_{7,\ 6}$
10(3)	1.42	6.3	d	$J_{10,\ 3}$
12	4.72	4.9, 7.5, 7.5	td	$J_{12,\ 13}$a, $J_{12,\ NH}$, $J_{12,\ 13b}$
13a	3.08	13.5, 7.5	d,d	$J_{13a,\ 13b}$, $J_{13a,\ 12}$
13b	3.20	13.9, 5.0	dd	$J_{13b,\ 13a}$, $J_{13b,\ 12}$
15-19	7.18-7.28	-	m	-

^{13}C NMR: ^{13}C (126 MHz) chemical shifts of OB in $CDCl_3$ (Bredenkamp et al., 1989; this thesis).

Carbon	δ_C/ppm
1	109.3
2	169.1
3	76.1
4	33.6
5	144.7
6	118.5
7	136.9
8	118.3
9	159.4
10	20.1
11	163.6
12	53.8
13	36.7
14	137.0
15,19	129.2
16,18	128.2
17	126.6

4.2.3 Bromo-Ochratoxin B (13)

Emperical formula: $C_{20}H_{18}BrNO_6$
Structural formula:



Melting point: 140.6°C (from chloroform) Steyn and Payne, 1999.

UV: λ_{max} (MeOH)/nm 217.3 (ε/dm^{3}mol^{-1}cm^{-1}30500) and 332 (ε/dm^{3}mol^{-1}cm^{-1} 5150) (Steyn and Payne, 1999).

IR: ν_{max} (KBr), 3368 (N-H),1730 (C=O acid), 1671 (C=O lactone and amide) and 1526 (CO-N amide) cm^{-1}.(Steyn and Payne, 1999; this thesis)

ES-MS: *m/z*; 450 [M$^+$(^{81}Br), 100%], 448 [M$^+$(^{79}Br), 100%], 404 (33%), 402 (32%) and 322 (14%)(Steyn and Payne; this thesis)

^{1}H-NMR: (300 MHz) of Br-OB in $CDCl_3$ (Steyn and Payne, 1989; this thesis).

Proton	δ_H/ppm	*J*/Hz	Multiplicity	Coupling
OH	12.7	-	-	-
NH	8.5	7.1	d	$J_{NH,\ 14}$
6	7.2	-	S	-
7a	2.8	17.4, 11.5	d, d	$J_{7a,\ 7b}$, $J_{7a,\ 8}$
7b	3.3	-	m	"complex multiplet"
8	4.7	-	m	"complex multiplet"
9 (3)	1.6	6.3	d	$J_{9,\ 8}$
14	5.1	5.4, 7.1, 7.6	td	$J_{14,\ 15}$, $J_{14,\ NH}$, $J_{14,\ 15}$
15	3.2	-	m	"complex multiplet"
17-19	7.2		m	

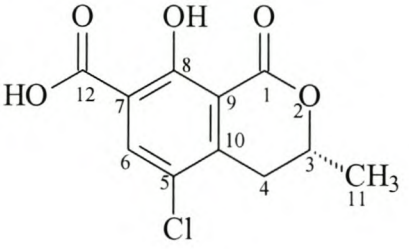
^{13}C NMR: ^{13}C chemical shifts of Br-OB in $CDCl_3$ (Steyn and Payne, 1989; this thesis).

Carbon	δ_C/ppm
1	120.7
2	159.8
3	110.5
4	142.9
5	112.4
6	142.2
7	34.9
8	75.8
9	20.5
10	169.9
11	163.9
13	177.2
14	54.2
15	37.3
16	135.8
17	129.4
18	128.8
19	127.4

4.2.4 Ochratoxin α (34)

Emperical formula: $C_{11}H_9ClO_5$

Structural formula:



Melting point: 245-246°C (from methanol) (Xiao *et al.*, 1995).

UV: λ_{max} (EtOH)/nm 218 (ε/dm^3 mol^{-1}cm^{-1}23 000) and 335 (ε/dm^3 mol^{-1}cm^{-1} 6200)(Xiao et al., 1995).

Fluorescence: 335 nm (excitation), 433 nm (emission)(Xiao *et al.*, 1995).

IR: ν_{max} (KBr), 3368 (N-H), 1730 (COOH), 1671 (lactone and amide CO absorption) and 1526 (amide II) cm^{-1}(Xiao *et al.*, 1995).

EI-MS: *m/z* 258 [M$^+$(^{37}Cl), 45%] 256 [M$^+$(^{35}Cl), 15%], 214 (35%), 212 (100%), 194 (70%)(Xiao *et al.*, 1995; this thesis).

^{1}H-NMR: (300 MHz) of Oα in $CDCl_3$ (Xiao et al. 1995; this thesis).

Proton	δ_H/ppm	*J*/Hz	Multiplicity	Coupling
OH	13.30	-	s	-
COOH	10.45		s	-
3	4.82	-	m	"complex"
4a	2.91	17.6, 11.6	d, d	$J_{4a,\,4b}$, $J_{4a,\,3}$
4b	3.30	17.6, 3.5	d, d	$J_{4b,\,4a}$, $J_{4b,\,3}$
6	8.41	-	s	-
11(3)	1.63	6.3	d	$J_{11,\,3}$

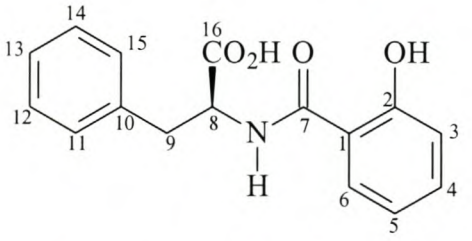
^{13}C NMR: ^{13}C chemical shifts of Oα in $(CD_3)_2SO$ (Kraus , 1981; this thesis)

Carbon	δ_C/ppm
1	167.03
3	74.23
4	32.08
5	120.44
6	135.88
7	117.73
8	160.31
9	112.31
10	143.08
11	20.00
12	165.24

4.2.5 *N*-(2-hydroxyphenylcarbonyl)-L-phenylalanine, SA Phe (22).

Emperical formula: $C_{16}H_{15}NO_4$

Structural formula:



UV: λ_{max} (MeOH)/nm 208 (ε/dm^{3}mol^{-1}cm^{-1} 35600), 235 (ε/dm^{3}mol^{-1}cm^{-1} 10050) 302 (ε/dm^3 mol^{-1}cm^{-1} 4500)(Payne, 1999).

IR: ν_{max} (Nujol) cm^{-1}; 3428 (N-H), 1722 (C=O acid), 1645 (C=O amide) 1534 cm^{-1} (CO-N amide)(Payne, 1999).

EI-MS: *m/z*; M^+, 285 (12%), 148 (13%), 137 (42%) 121 (53%) and 91 (16%) (Payne, 1999; this thesis).

^{1}H NMR: (300 MHz) of SAPhe in $CDCl_3$ (Payne, 1999; this thesis).

Proton	δ_H/ppm	*J*/Hz	Multiplicity	Coupling
3	7.18	7.6, 2.1	d, d	$J_{3,4}$, $J_{3,5}$
4	6.97	8.9, 2.6	d, d	$J_{4,3}$, $J_{4,5}$
5	6.80	8.9, 8.9, 2.6	td	$J_{5,4}$, $J_{5,6}$, $J_{5,7}$
6	7.18	7.6, 2.1	d, d	$J_{6,5}$, $J_{6,4}$
8	5.00	4.9, 7.1, 5.1	td	$J_{8,9a}$, $J_{8,NH}$, $J_{8,9b}$
9b	3.28	14.0, 5.1	d, d	$J_{9b,a}$, $J_{9b,8}$
9a	3.10	14.0, 5.0	d, d	$J_{9a,b}$, $J_{9a,8}$
11/15	7.29		m	-
12/14	7.29		m	-
13	7.38		m	-
NH	9.20	7.0	d	$J_{N,8}$

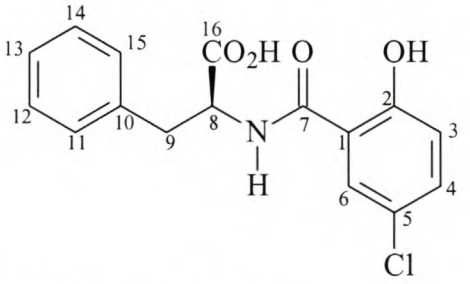
^{13}C NMR: ^{13}C chemical shifts of SAPhe in $CDCl_3$ (Payne, 1999; this thesis).

Carbon	δ_C/ppm
1	118.7
2	161.6
3	113.8
4	134.8
5	119.0
6	125.7
7	169.8
8	53.0
9	37.3
10	135.2
11/15	129.4
12/14	128.9
13	127.6
16	175.7

4.2.6 *N*-(5-chloro-2-hydroxyphenylcarbonyl)-L-phenylalanine, ClSAPhe (23).

Emperical formula: $C_{16}H_{14}ClNO_4$

Structural formula:



Melting point: 182°-183°C (Payne, 1999; this thesis)

UV: λ_{max} (MeOH)/nm 208 (ε/dm^{3}mol^{-1}cm^{-1} 38020) and 312 (ε/dm^{3}mol^{-1}cm^{-1} 3940).

IR: ν_{max} (KBr) cm^{-1}, 3368 (N-H), 1722 (C=O acid), 1632 (C=O amide) 1534 cm^{-1} (CO-N amide)(Payne, 1999; this thesis).

EI-MS: *m/z*; 321 [M$^+$(^{37}Cl), 13%], 319 [M$^+$(^{35}Cl), 39%], 184 (13%),182 (37%), 173 (23%), 171 (69%), 157 (32%), 155 (100%)(Payne, 1999; this thesis).

^{1}H NMR: (300 MHz) of ClSAPhe in $CDCl_3$ (Payne, 1999; this thesis).

Proton	δ_H/ppm	*J*/Hz	Multiplicity	Coupling
3	6.98	8.7	d, d	$J_{3,4}$
4	7.48	8.7, 2.8	d, d	$J_{4,3}$, $J_{4,6}$
6	7.96	3.0	d	$J_{6,4}$
8	4.72	7.0, 7.0, 5.2	td	$J_{8,9a}$, $J_{8,NH}$, $J_{8,9b}$
9b	3.29	14.0, 5.4	d, d	$J_{9b,a}$, $J_{9b,8}$
9a	3.10	14.0, 5.4	d, d	$J_{9a,b}$, $J_{9a,8}$
11/15	7.28		m	
12/14	7.28		m	
13	7.35		m	
NH	9.05	6.0	d	$J_{N,8}$

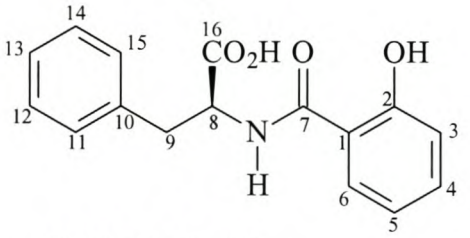
^{13}C NMR: ^{13}C chemical shifts of ClSAPhe in $CDCl_3$ (Payne, 1999; this thesis).

Carbon	δ_C/ppm
1	120.3
2	160.3
3	114.7
4	134.7
5	123.7
6	125.2
7	168.7
8	52.9
9	37.3
10	135.1
11/15	129.4
12/14	129.1
13	127.8
16	168.7

4.2.7 *N*-(5-bromo-7-hydroxyphenylcarbonyl)-L-phenylalanine, BrSAPhe (25).

Emperical formula: $C_{16}H_{14}BrNO_4$

Structural formula:



Melting point: 178°C (Payne, 1999; this thesis)

UV: λ_{max} (MeOH)/nm 210 (ε/dm^3 mol^{-1}cm^{-1} 68744) and 312 (ε/dm^3 mol^{-1}cm^{-1} 5616)

IR: ν_{max} (KBr) cm^{-1}; 3360 (N-H), 1747 (C=O acid), 1636 (C=O amide) 1526 cm^{-1} (CO-N amide)(Payne, 1999).

EI-MS: *m/z*; 365 [M$^+$(^{81}Br), 39%], 363 [M$^+$(^{79}Br), 39%], 226/228 (33%), 215/217 (73%) and 199/201 (100%)(Payne, 1999; this thesis).

^{1}H NMR:(300 MHz) of BrSAPhe in $CDCl_3$ (Payne, 1999; this thesis).

Proton	δ_H/ppm	*J*/Hz	Multiplicity	Coupling
3	6.87	8.7	d,d	$J_{3,4}$
4	7.20		m	
6	7.20		m	
8	4.76	7.1, 7.2, 5.3	td	$J_{8,9a}$, $J_{8,NH}$, $J_{8,9b}$
9b	3.24	12.5, 6.4	d,d	$J_{9b,a}$, $J_{9b,8}$
9a	3.10	12.6, 6.4	d,d	$J_{9a,b}$, $J_{9a,8}$
11/15	3.10			
12/14	7.30		m	
13	7.35		m	
NH	9.03	6.0	d	$J_{N,8}$

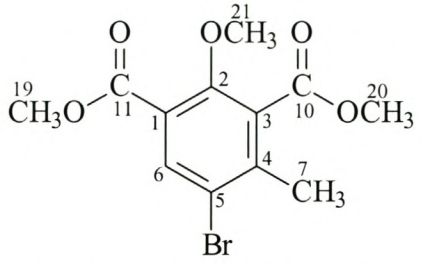
^{13}C NMR: ^{13}C chemical shifts of BrSAPhe in $CDCl_3$ (Payne, 1999; this thesis).

Carbon	δ_C/ppm
1	120.5
2	160.6
3	116.9
4	137.2
5	110.4
6	130.2
7	169.1
8	54.3
9	37.2
10	137.9
11/15	128.7
12/14	128.9
13	127.2
16	172.2

4.2.8 5-Bromo-2-methoxy-4-methylisophthalic methyl ester (27)

Emperical formula: $C_{12}H_{13}BrO_5$

Structural formula:



EI-MS: *m/z*; 318 [M^+(^{81}Br), 59%], 316 [M^+(^{79}Br), 54%], 288 (100%), 286 (100%).

^{1}H NMR:(300 MHz) of **27** in $CDCl_3$.

Proton	δ_H/ppm	Multiplicity
6	8.05	s
7	2.35	s
19	3.95	s
20	3.93	s
21	3.92	s

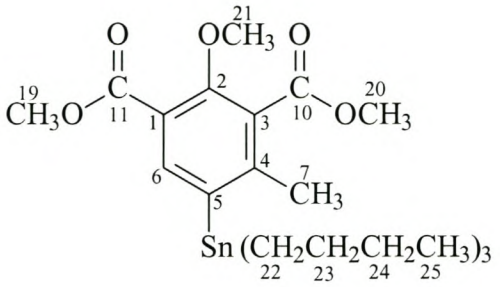
^{13}C NMR: ^{13}C chemical shifts of **27** in $CDCl_3$.

Carbon	δ_C/ppm
1	133.9
2	156.2
3	119.7
4	141.8
5	124.8
6	136.2
7	20.3
10	165.1
11	167.5
19	51.9
20	51.9
21	63.5

4.2.9 2-Methoxy-4-methyl-5-tributylisophthalic methyl ester (28) (Payne, 1999)

Emperical formula: $C_{24}H_{40}O_5Sn$

Structural formula:



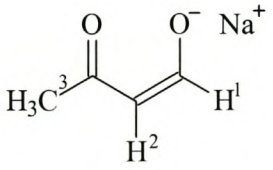
^{1}H NMR: ^{13}C chemical shifts (300 MHz) of **28** in $CDCl_3$.

Proton	δ_H/ppm	Multiplicity
6	8.95	S
7	2.20	S
19	3.95	S
20	3.95	S
21	3.87	S
22	1.10	M
23	1.30	M
24	1.51	M
25	0.90	M

4.2.10 Sodium acetoacetaldehyde

Emperical formula:$C_4H_5O_2Na$

Structural formula:



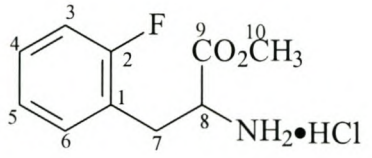
^{1}H NMR:(60 MHz) of sodium acetoacetaldehyde in $CDCl_3$

Proton	δ_H/ppm[a]	Multiplicity
1	8.75	S
2	5.13	D
3	2.12	S

4.2.11 DL-2-Fluorophenylalanine methyl ester hydrochloride (39)

Emperical formula: $C_{10}H_{13}ClFNO_2$

Structural formula:



EI-MS: *m/z*; M^+ 198 (4%), 177 (5%), 138 (92%) and 88 (100 %)

^{1}H NMR: (300 MHz) of **39** in $CDCl_3$.

Proton	δ_H/ppm[a]	*J*/Hz	Multiplicity	Coupling
3	7.12	12.0, 7.0, 3.0	td	$J_{3,4}$, $J_{3,5}$ $J_{3,F}$
4	7.28	-	m	"complex"
5	7.04	12.0, 11.7, 5.0	td	$J_{5,4}$, $J_{5,6}$ $J_{5,3}$
6	7.44	11.5, 7.5,	d, d	$J_{6,5}$, $J_{6,4}$
7a	3.36	12.0, 6.0	d, d	$J_{7a,7b}$, $J_{7a,8}$
7b	3.48	9.0, 15.0	d, d	$J_{7b,7a}$, $J_{7b,8}$
8	4.24	6.0, 9.0, 9.0	td	$J_{8,7a}$, $J_{8,NH}$, $J_{8,7b}$
10	3.70	-	s	-
NH	9.05	-	broaden	-

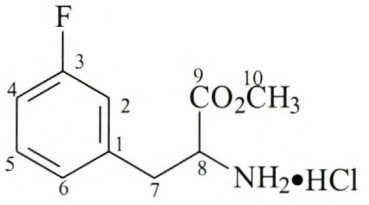
^{13}C NMR: ^{13}C chemical shifts of **39** in $CDCl_3$.

Carbon	δ_C/ppm[a]	*J*/Hz	Multiplicity
1	121.24	15.4	D
	121.04		
2	162.74	250.2	D
	159.50		
3	115.05	27.0	D
	114.70		
4	132.00	4.7	D
	131.94		
5	124.17	3.9	D
	124.12		
6	129.28	8.5	D
	129.17		
7	29.62	-	S
8	30.26	-	S
9	169.04	-	S
10	52.28	-	S

4.2.12 DL-3-Fluorophenylalanine methyl ester hydrochloride (40)

Emperical formula: $C_{10}H_{13}ClFNO_2$

Structural formula:



EI-MS: *m/z*; M^+ 198 (2%), 38 (84%) and 88 (100%)

^{1}H NMR: (300 MHz) of **40** in $CDCl_3$.

Proton	δ_H/ppm[a]	*J*/Hz	Multiplicity	Coupling
2	7.09		m	"complex"
4	6.79	12.2, 7.0, 3.5	td	$J_{4,5}$, $J_{4,6}$, $J_{6,F}$
5	7.29		m	"complex"
6	7.09		m	"complex"
7a	3.36	12.0, 6.0	d, d	$J_{7a,7b}$, $J_{7a,8}$
7b	3.48	9.0, 15.0	d, d	$J_{7b,7a}$, $J_{7b,8}$
8	4.24		m	
10	3.75			
NH	9.04		broaden	

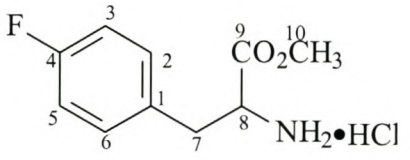
^{13}C NMR: Chemical shifts of **40** in $CDCl_3$.

Carbon	δ_C/ppm[a]	*J(C-F)*/Hz	Multiplicity
1	137.07	7.7	D
	136.97		
2	114.59	21.6	D
	114.31		
3	164.41	251.7	D
	161.15		
4	116.81	22.4	D
	116.52		
5	130.43	9.3	D
	130.31		
6	125.47	3.1	D
	125.43		
7	30.89		S
8	35.80		S
9	169.20		S
10	52.81		S

4.2.13 DL-4-Fluorophenylalanine methyl ester hydrochloride (41)

Emperical formula: $C_{10}H_{13}O_2NClF$

Structural formula:



EI-MS: *m/z*; M^+, 197 (3%), 138 (86%) and 88 (100 %)

^{1}H NMR: (300 MHz) of **41** in $CDCl_3$.

Proton	δ_H/ppm[a]	J/Hz	Multiplicity	Coupling
2, 6	7.05		m	"Complex"
3, 5	7.33		m	"Complex"
7a	3.29	12.1, 6.0	d, d	$J_{7a, 7b}$, $J_{7a, 8}$
7b	3.41	8.7, 15.0	d, d	$J_{7b, 7a}$, $J_{7b, 8}$
8	4.18		m	
10	3.76		s	
NH	9.27		broaden	

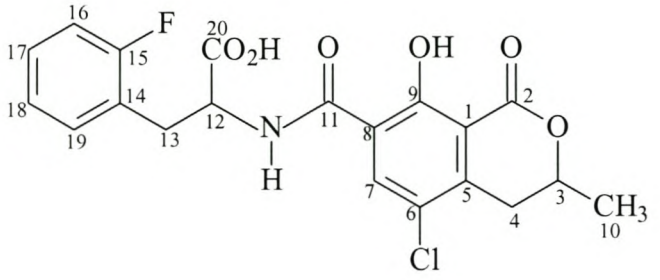
^{13}C NMR: Chemical shifts of **41** in $CDCl_3$.

Carbon	δ_C/ppm[a]	*J(C-F)*/Hz	Multiplicity
1	130.40	3.1	D
	130.36		
2, 6	131.43	8.5	D
	131.32		
3, 5	115.63	22.4	D
	115.34		
4	163.69	251.7	D
	160.43		
7	35.25		S
8	53.931		S
9	169.26		S
10	52.678		S

4.2.14 *ortho*-fluorophenylalanine Oα (44)

Emperical formula: $C_{20}H_{17}ClFNO_6$

Structural formula:



EI-MS: *m/z* ,437 [$M^+(^{37}Cl)$, 2%], 435 [$M^+(^{35}Cl)$, 6%],378 (5%), 376 (16%), 241 (35%) 239 (100%).

UV: λ_{max} (MeOH)/nm 217 (ε/$dm^3mol^{-1}cm^{-1}$ 31450), 330 (ε/$dm^3mol^{-1}cm^{-1}$ 5900).

IR: ν_{max} (Nujol) cm^{-1}; 3392 (N-H), 1739 (C=O acid), 1671 (C=O amide) 1530 cm^{-1} (CO-N amide).

^{1}H NMR: (300 MHz) of **44** in $CDCl_3$.

Proton	δ_H/ppm[a]	*J*/Hz	Multiplicity	Coupling
3	4.76		m	"complex"
4a	2.84	17.4, 3.8	d, d	$J_{4a,4b}$, $J_{4a,3}$
4b	3.28	17.4, 3.8	d, d	$J_{4b,4a}$, $J_{4b,3}$
7	8.38		s	
10	1.59	6.0	d	$J_{10,3}$
12	5.01		m	"complex"
13a	3.43,	13.8, 4.8	d, d	$J_{13a,13b}$, $J_{13a,12}$
13b	3.21	13.8, 6.8	d, d	$J_{13b,13a}$, $J_{13b,12}$
16	7.04		m	
17	7.20		m	
18	6.98		m	
19	7.26		m	
N-H	8.56		broaden	

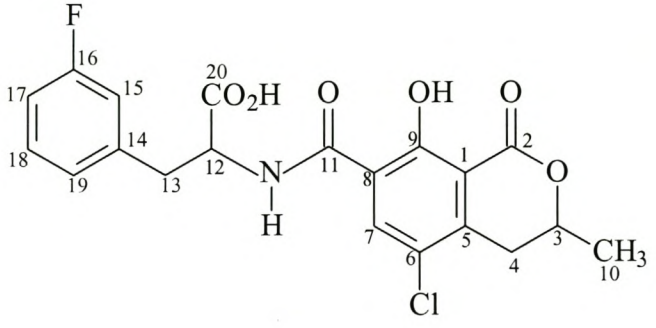
^{13}C NMR: ^{13}C chemical shifts of **44** in $CDCl_3$.

Carbon	δ_C/ppm[a]	*J(C-F)*/Hz
1	110.05	
2	169.99	
3	75.85	
4	30.90	
5	140.67	
6	120.56	
7	138.73	
8	120.73	
9	158.00	
10	20.65	
11	162.38	
12	32.22	
13	31.06	
14	128.72, 128.67	3.9
15	160.20, 162.57	184.0
16	131.72	
17	124.06	
18	131.72	
19	124.02	
20	172.70	

4.2.15 *meta*-fluorophenylalanine Oα (43)

Emperical formula: $C_{20}H_{17}ClFNO_6$

Structural formula:



EI-MS: *m/z*; 437 [M^+(^{37}Cl), 2%], 435 [M^+(^{35}Cl), 6%],378 (5%), 376 (16%), 241 (35%) 239 (100%).

^{1}H NMR: (300 MHz) of **43** in $CDCl_3$.

Proton	δ_H/ppm[a]	*J*/Hz	Multiplicity	Coupling
3	4.74	-	m	"complex"
4a	2.91	17.4, 12.0	d, d	$J_{4a,4b}$, $J_{4a,3}$
4b	3.11	8.4 , 4.0	d, d	$J_{4b,4a}$, $J_{4b,3}$
7	8.31		s	
10	1.47	6.0	d	$J_{10,3}$
12	4.84		m	"complex"
13a	3.10	13.8, 4.8	d, d	$J_{13a,13b}$, $J_{13a,12}$
13b	3.21	13.5, 8.4, 10.2	d, d	$J_{13b,13a}$, $J_{13b,12}$
15	7.07		m	
17	7.04		m	
18	7.32		m	
19	7.10		m	
N-H	8.56		broaden	

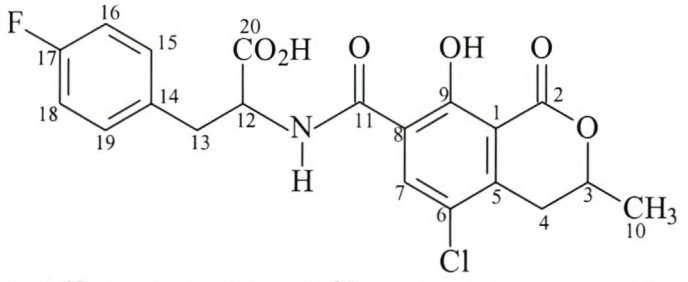
^{13}C NMR: ^{13}C chemical shifts of **43** in $CDCl_3$

Carbon	δ_C/ppm[a]	*J(C-F)*/Hz
1	111.36	
2	168.00	
3	75.26	
4	31.56	
5	139.95	
6	120.11	
7	141.62	
8	115.85	
9	158.50	
10	19.99	
11	162.81	
12	53.60	
13	36.09	
14	121.00	
15	135.80	
16	162.70, 161.09	124.1
17	135.80	
18	130.02	
19	125.28	
20	172.06	

4.2.16 *para*-fluorophenylalanine Oα (42)

Emperical formula: $C_{20}H_{17}ClFNO_6$

Structural formula:



EI-MS: *m/z*; 437 [$M^+(^{37}Cl)$, 2%], 435 [$M^+(^{35}Cl)$, 6%], 378 (5%), 376 (16%), 241 (35%) 239 (100%).

^{1}H NMR: (300 MHz) of **42** in $CDCl_3$.

Proton	δ_H/ppm[a]	*J*/Hz	Multiplicity	Coupling
3	4.74	-	m	"complex"
4a	2.94	17.4, 12.0	d, d	$J_{4a,4b}$, $J_{4a,3}$
4b	3.10	8.4 , 4.0	d, d	$J_{4b,4a}$, $J_{4b,3}$
7	8.10		s	
10	1.47	6.0	d	$J_{10,3}$
12	4.88		m	"complex"
13a	3.12	13.8, 4.8	d, d	$J_{13a,13b}$, $J_{13a,12}$
13b	3.23	13.5, 8.4, 10.2	d, d	$J_{13b,13a}$, $J_{13b,12}$
15,19	7.23		m	
16,18	7.14		m	

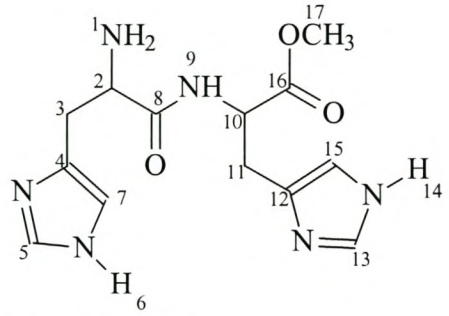
^{13}C NMR: ^{13}C chemical shifts of **43** in $CDCl_3$

Carbon	δ_C/ppm[a]	*J(C-F)*/Hz
1	111.72	
2	168.55	
3	75.55	
4	31.65	
5	136.28	
6	121.61	
7	142.13	
8	120.41	
9	158.87	
10	20.03	
11	163.31	
12	54.00	
13	35.68	
14	133.52	
15	115.41, 115.13	21.6
16	131.33, 131.44	8.5
17	159.96, 163.18	248.6
18	131.33, 131.44	8.5
19	115.40, 115.13	20.9
20	172.70	

4.2.17 Histidylhistidine methyl ester (48).

Emperical formula: $C_{13}H_{13}N_6O_3$

Structural formula:



ES-MS: *m/z*; M^+, 306 (72%), 291 (20%), 135 (100%).

^{1}H NMR: (300 MHz) of **48** in $CDCl_3$

Proton	δ_H/ppm[a]	Multiplicity
2	4.56-4.59	m
3	3.03-3.07	m
5	8.15	s
7	7.14	s
9	9.23	s
10	4.15	m
11	3.12-3.17	m
13	8.51	s
15	7.28	s
17(3H)	3.64	s

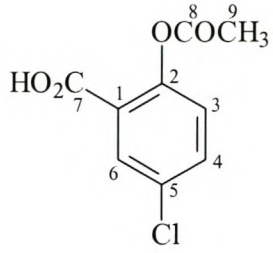
^{13}C NMR: ^{13}C chemical shifts of **43** in $CDCl_3$

Carbon	δ_C/ppm[a]
2	52.10
3	26.41
4	130.06
5	134.08
7	116.08
8	167.89
10	52.19
11	27.62
12	130.46
13	134.69
15	118.07
16	170.40
17	51.68

4.2.18 2-acetoxy-5-chlorobenzoic acid

Emperical formula: $C_9H_7ClO_4$

Structural formula:



Melting point: 130°C (Steyn, 1967)

UV: λ_{max} (MeOH)/nm 222 (ε/dm^3 mol^{-1}cm^{-1} 9400) and 288 (ε/dm^3 mol^{-1}cm^{-1} 1350) (Steyn, 1967).

IR: CO (1767, 1741 and 1704 cm^{-1}) (Steyn, 1967).

EI-MS: *m/z*; 216 [M$^+$(^{37}Cl), 2%], 214 [M$^+$(^{35}Cl), 6%], 174 (80%), 172 (26%), 154 (100%) and 156 (36%).

^{1}H NMR: (300 MHz) of **48** in $CDCl_3$

Proton	δ_H/ppm[a]	J/Hz	Multiplicity	Coupling
3	7.31	8.7	d	$H_{3\text{-}4}$,
4	7.76	8.7, 2.7	d,d	$H_{4\text{-}3}$, $H_{4\text{-}6}$
6	7.94	2.7	d	$H_{6\text{-}4}$
9	2.27	-	-	-

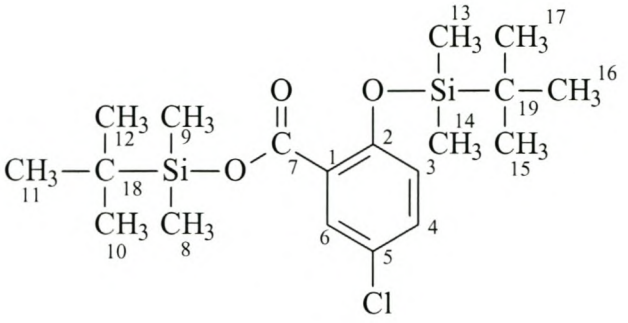
^{13}C NMR: ^{13}C chemical shifts of **43** in $CDCl_3$

Carbon	δ_C/ppm[a]
1	130.48
2	169.50
3	126.24
4	133.79
5	149.28
6	131.02
7	164.89
8	156.12
9	20.75

4.2.19 *tert*-Butyldimethylsilyl 2-*tert*-butyldimethylsilyloxy-5-chlorobenzoate (51)

Emperical formula: $C_{19}H_{33}O_3NClSi_2$

Structural formula:



EI-MS: *m/z*; 345 [$M^+(^{37}Cl)$, 1%], 343 [$M^+(^{35}Cl)$, 3%], 231 (39%) and 229 (100%).

^{1}H NMR: (300 MHz) of **51** in $CDCl_3$

Proton	δ_H/ppm[a]	J/Hz	Multiplicity	Coupling
3	6.80	8.4	d	$J_{3,4}$
4	7.27	9.0, 3.0	d,d	$J_{4,3}$, $J_{4,6}$
6	7.65	3.0	d	$J_{6,4}$
8-9	0.34			
10-12	0.98			
13/14	0.18			
15-17	0.98			

^{13}C NMR: Chemical shifts of **43** in $CDCl_3$

Carbon	δ_C/ppm[a]
1	125.36
2	164.03
3	122.93
4	132.63
5	125.50
6	131.10
7	154.50
8-9	-4.70
10-12	25.75
13/14	-4.01
15-17	25.75
18/19	25.71

4.3 Experimental Procedures

4.3.1 Selective electrophilic aromatic fluorination:

4.3.1.1 Using 1-(Chloromethyl)-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane bis(tetrafluoroborate), Selectfluor™ (6).

4.3.1.1.1 Fluorination of methoxybenzene.

A magnetically stirred degassed solution of Selectfluor™ (**6**) (556 mg, 1.57 mmol) and methoxybenzene (115 mg, 1.06 mmol) in dry acetonitrile (10 ml) was heated at 90°C for 15 h under an argon atmosphere. The reaction was monitored by TLC (hexane:ethyl acetate 10:1) and after the completion of the reaction the mixture was cooled to room temperature. The clear solution was diluted with dry diethyl ether (30 ml) and a white precipitate was isolated by filtration to yield a white solid, 1-(chloromethyl)-1,4-diazoniabicyclo[2.2.2]octane bis(tetrafluoroborate), 458 mg (1.36 mmol). The solvent of the filtrate was removed under reduced pressure, which afforded an unidentified dark coloured oil, 98 mg. The oil was not identified, but ^{1}H-,^{13}C-NMR and ES-MS results suggest that no fluorination had taken place and revealed the presence of predominantly an *o*-substituted dimer.

4.3.1.1.2 Attempts to fluorinate other compounds using Selectfluor™ (6).

This method was further employed on salicylic acid (**17**) (223 mg, 1.61 mmol), SAPhe () (65 mg, 0.240 mmol), OB (**11**)(2 mg, 5.4 μmol) and OB methyl ester (18 mg, 47 μmol). The reactions was conducted at elevated temperatures (70-90°C) and the reaction period prolonged (20 d) during which the reaction was monitored by HPLC (see section 4.1.) using a UV detector (328 nm). In the case of salicylic acid (**6**) as substrate small amounts of fluorosalicylic acid was identified by HPLC and UV-detection (332 nm). The predominant product of the reaction was unreacted salicylic acid (**17**). Similarly in the cases of SAPhe (**22**), OB (**11**) and OB methyl ester as substrates the unreacted products were quantitatively recovered and identified by ^{1}H-NMR and ES-MS (in the case of OB methyl ester).

4.3.1.2 Using *N*-Fluorobenzenesulfonimide (8)

4.3.1.2.1 Fluorination of salicylic acid (17)

Salicylic acid (**17**) (148 mg, 1.07 mmol) was dissolved in dry THF (10 ml), cooled in an ice bath to 0°C and treated with *N*-fluorobenzenesulfonimide (**8**) (111 mg, 0.352 mmol). The reaction was stirred under an argon atmosphere and the reaction temperature was kept between 0 and 8°C. The reaction was monitored by TLC (toluene:acetic acid 5:1). After 12 h a further aliquote of sulfonimide (111 mg, 0.352 mmol) was added and the temperature was gradually increased to 70°C and stirred for another 12 h. Monitoring indicated that no reaction had occurred. The reaction mixture was allowed to cool, the solvent removed *in vacuo* and the residue suspended in chloroform (5 ml). The solution was acidified with 1M HCl (1 ml) and the aqueous phase extracted three times with chloroform (3× 5 ml) after separation. The combined extracts were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to yield a white residue, 115 mg, identified as unreacted salicylic acid by ^{1}H- and ^{13}C-NMR.

4.2.1.2.2 Attempts to fluorinate other compounds using N-Fluorobenzenesulfonimide (8).

This method was further employed on SAPhe (**22**) (67 mg, 0.247 mmol) and OB (**11**) (2 mg, 5.4 μmol). The reactions were followed by HPLC using a UV-detector (258-332 nm) over an extended period (20 d) (see section 4.1.). In both cases the unreacted product were recovered quantitatively and identified by ^{1}H-NMR.

4.3.1.3 Using Xenon difluoride.

4.3.1.3.1 Fluorination of salicylic acid (17).

To a degassed solution of salicylic acid (**17**) (82 mg, 0.594 mmol) in dry dichloromethane (3 ml) was added xenon difluoride (101 mg, 0.597 mmol) in a glass vessel. The reaction mixture was stirred under an argon atmosphere and the reaction temperature maintained between –60 and 0°C. The reaction was monitored by TLC (toluene:acetic acid 5:1) and after 18 h indicated that no reaction was occurring.

The mixture was subsequently diluted with dichloromethane (10 ml) and washed with a 1M $NaHCO_3$ solution. The aqueous phase was acidified with 1M HCl (2 ml) and the

extracted with chloroform (3×10 ml). The combined organic extracts were dried over anhydrous Na_2SO_4, filtered and the solvent evaporated under reduced pressure to yield an unidentified black coloured oil. ^{1}H- and ^{13}C-NMR analysis of the recovered product indicated a large proportion of substrate decomposition and small amounts of the unreacted product, salicylic acid (**17**).

4.3.1.3.2 Attempts to fluorinate other compounds using xenon difluoride.

This method was further employed on SAPhe (**22**) (59 mg, 0.218 mmol) and OB (**11**) (2mg, 5.4 μmol). In the case of OB (**11**) (20 mg, 54 μmol) the reaction were conducted in α,α,α- trifluorotoluene (5 ml) as solvent. The reactions were monitored by HPLC using a UV detector (332 nm) over an extended period of time (21 d) (see section 4.1.). The recovered products were identified by ^{1}H-, ^{13}C-NMR and ES-MS analysis as the unreacted substrates SAPhe (**22**) and OB (**11**) in each case.

4.3.2 Fluorodehalogenation of aryl halides, via a palladium catalysed metal substitution, followed by xenon difluoride fluorosubstitution.

4.3.2.1 Dehalogenation of OA (10) (Bredenkamp *et al.*, 1989)

Ochratoxin A (250 mg, 0.619 mmol) was added to a suspension of 10% Pd/C (158 mg) and ammonium formate (230 mg, 3.75 mmol) in AR methanol (10 ml). The reaction mixture was stirred at room temperature in a closed vessel. The reaction was monitored by TLC (toluene:acetic acid, 5:1). Upon completion of the reaction (overnight) the mixture was filtered, and the solvent evaporated under reduced pressure.

The residue was dissolved in ethyl acetate (10 ml) and treated with 1M HCl (10 ml). The aqueous phase was extracted three times with ethyl acetate (3×10 ml) and the combined organic extracts evaporated under reduced pressure. The residue was recrystallised from methanol to yield OB (**11**) in quantitative yield, 228 mg (0.618mmol) (Characterised by ^{1}H-, ^{13}C-NMR and EI-MS see section 4.2.2).

4.3.2.2 Dehalogenation of CSAPhe (23)

CSAPhe (**23**) (200 mg, 0.625 mmol) was added to a suspension of 10% Pd/C (189 mg) and ammonium formate (270 mg, 3.98 mmol) in AR methanol (10 ml). The reaction mixture was stirred at room temperature in a closed vessel. The reaction was monitored by TLC (toluene:acetic acid, 5:1). Upon completion of the reaction (overnight) the mixture was filtered, and the solvent evaporated under reduced pressure.

The residue was dissolved in ethyl acetate (10 ml) and treated with 1M HCl (10 ml). The aqueous phase was extracted three times with ethyl acetate (3×10 ml) and the combined organic extracts evaporated under reduced pressure. The resultant oil was purified by column chromatography (silica/ toluene:acetic acid – 7:1) to yield a colourless oil, SAPhe (**17**), 149 mg (0.525 mmol, 84%)(Characterised by ^{1}H-, ^{13}C-NMR and EI-MS see section 4.2.5).

4.3.2.3 Synthesis of BrOB

4.3.2.3.1 Preparation of Pyridinium hydrobromide perbromide.

The experimental procedures reported by Fieser and Fieser, 1967, were employed. A mixture of pyridine (15 ml, 0.18 mol) and 48% hydrobromic acid (30 ml, 170.0 mmol) was cooled on ice and treated with bromine (27.00 g, 0.169 mmol) whilst the reaction mixture was stirred. Upon completion of the addition of bromine the reaction mixture was filtered and the crystalline material washed with acetic acid:water (1:1). The product was recrystallised from the fresh acetic acid:water (1:1) solution to yield an orange coloured crystalline product, pyridinium hydrobromide perbromide, 31.00 g (94.20 mmol, 55%).

***4.3.2.3.2 Bromination of OB* (11) (Steyn and Payne, 1999)**

A mixture of ochratoxin B (11) (200 mg, 0.544 mmol) in glacial acetic acid (20 ml) was treated with pyridinium hydrobromide perbromide (261 mg, 0.816 mmol) and stirred at 50°C. The reaction was monitored by TLC (toluene:acetic acid, 5:1) and the reaction was complete in 6 h. The reaction mixture was poured into water (20 ml) and extracted three times with chloroform (3×20 ml). The combined organic extracts were washed twice with

water to remove the excess acetic acid, dried over anhydrous Na_2SO_4, filtered and the filtrate concentrated under reduced pressure to yield, Br-OB (**13**), 220 mg (0.49 mmol, 88%). Rf values for OB and BrOB were 0.48 and 0.85, respectively.(Characterised by ^{1}H-, ^{13}C-NMR and ES-MS see section 4.2.3).

4.3.2.4 Attempted catalytic stannylation of aryl halides, followed by XeF_2 fluorosubstitution.

4.3.2.4.1 Catalytic Stannylation of 5-bromo-2-methoxy- 4-methylisophthalic methyl ester (27) using hexabutylditin in the presence of tetrakis(triphenylphosphine) palladium(0).

A 10 ml round bottom flask equipped with a reflux condenser and a magnetic stirring bar was charged with 5-bromo-2-methoxy-4-methylisopthtalic methyl ester (**27**) (100 mg, 289 μmol), tetrakis(triphenylphosphine)palladium(0) (43 mg, 37.0 μmol) and xylene (3 ml). Hexabutylditin (830 mg, 1.44 mmol) was introduced. The reaction was protected from light and heated under argon at 115°C. The reaction was monitored by TLC (petroleum ether (60-80°C): ethyl acetate - 10:1) and the reaction was complete after 19 h. The reaction mixture was cooled to room temperature and filtered. The filter cake was washed with xylene (9 ml in three portions) and benzene (2 ml). The solvent of the filtrate was evaporated under reduced pressure and purified by column chromatography (silica gel, petroleum ether (60-80°C): ethyl acetate, 10:1) to yield a colourless oil, 2-methoxy-4-methyl 5-tributylstannylisophthalic methyl ester (**28**), 82 mg (155 μmol, 53%) (Characterised by ^{1}H-NMR see section 4.2.8).

4.3.2.4.2 Attempts to catalytically metallate other aryl halides.

The reaction was further employed on BrSAPhe (**20**) (100 mg, 0.275 mmol), CSAPhe (**18**) (100 mg, 0.313 mmol) and BrOB (**13**) (78 mg, 0.174 mmol). In the case of the latter, both hexabutyl- and hexamethyl distannane was used as nucleophilic agent. ^{1}H-, ^{13}C-NMR and ES-MS data of the recovered products, in the case of CSAPhe (**18**) as substrate, revealed that no reaction occurred. Multiple products were observed by analysis of the ^{1}H-, ^{13}C-NMR and ES-MS data of the recovered products in the case of BrSAPhe as substrate. This included predominantly unreacted BrSAPhe (**25**), SAPhe (**22**) and small amounts of an unidentified biaryl compound. The tributylstannylation of OB

was unsuccessful and the recovered products were identified by ^{1}H-, ^{13}C-NMR and ES-MS as predominantly Br-OB (**13**), OB (**11**) and trace amounts of an unidentified stannylated product. Where hexamethylditin was used as nucleophilic agent, unreacted Br-OB (**13**), OB (**11**) and trace amounts of an unidentified stannylated compound were identified by ^{1}H-, ^{13}C-NMR and ES-MS.

4.3.2.4.3 Attempted fluorodestannylation of 2-methoxy-4-methyl-5-tributylstannyl-isopthalic methyl ester (28) by XeF_2

To a degassed solution of the diester (**28**) (52 mg, 99 μmol) in dry carbon tetrachloride (5 ml) was added xenon difluoride (31 mg, 183 μmol) in a glass vessel. The reaction mixture was stirred at room temperature under an argon atmosphere and monitored by TLC (petroleum ether (60-80°C):ethyl acetate, 10:1). After the completion of the reaction, the yellow solution was diluted with carbon tetrachloride (10 ml) and washed with a 1M $NaHCO_3$ solution. The aqueous phase was acidified and extracted with chloroform (3×10 ml). The combined organic extracts were dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, petroleum ether:ethyl acetate, 10:1) to yield predominantly the reduced product (**30**), 23 mg (63%)(^{1}H-, ^{13}C-NMR discussed in section 3.3.2).

4.3.2.5 Attempted catalytic silylation of aryl halides

4.3.2.5.1 Catalytic silylation of 5-bromosalicylic acid (20) catalysed by tetrakis (triphenylphosphine)palladium(0)

A 50 ml round bottom flask equipped with a reflux condenser and a magnetic stirring bar was charged with 5-bromosalicylic acid (**20**) (300 mg, 1.38 mmol) and tetrakis(triphenylphosphine)palladium(0) (19 mg, 16.8 μmol). The reaction flask was purged with argon and degassed xylene (7 ml) and hexamethyldisilane (853 mg, 5.83 mmol) was added via an airtight syringe. The resulting yellow mixture was stirred at 140°C for 48 h in which time the reaction was monitored by TLC (toluene:acetic acid 5:1). After 24 h a black precipitate, decomplexed palladium catalyst, had formed and another aliquot of the catalyst was added (12 mg, 10.4 μmol) and the reaction stirred for a further 24 h at 140°C. The reaction mixture was cooled, petroleum ether (30-60°C) (30

ml) was added, and the suspension filtered. The filtrate was evaporated under reduced pressure to yield a dark red oil which was purified by column chromatography (silica gel/ toluene:acetic acid, 7:1). The major products isolated were a) 5-bromosalicylic acid (**20**), 175 mg (58% unreacted) and b) salicylic acid (**17**) (45 mg, 23% reduced) as characterized by ^{1}H-, ^{13}C-NMR and ES-MS.

4.3.2.5.2. Catalytic silylation of bromo-ochratoxin B using hexamethyldisilane in the presence of tris-(dibenzylideneacetone)dipalladium (0).

A 50 ml round bottom flask equipped with a reflux condenser and a magnetic stirring bar was charged with tris(dibenzylideneacetone)dipalladium(0) (2 mg, 2.20 μmol), diphenyl-2'-pyridylphospine (25.09 mg, 9.53 μmol), bromo-OB (**13**) (47 mg, 105 μmol) and K_2CO_3 (70 mg, 509 μmol). The reaction vessel was purged with argon and degassed DMPU (5 ml) was added *via* syringe. The resulting orange slurry was stirred for 10 min at room temperature. Subsequently, water (4 mg, 214 μmol) and hexamethyldisilane (18 mg, 118 μmol) were added *via* syringe and the reaction mixture was stirred at 100°C for 22 h. After this time a palladium mirror had formed indicating completion of the reaction. To the reaction mixture was added NH_4OH (to pH of 11) and extracted three times with diethyl ether (10 ml). The alkaline aqueous phase was acidified with 1M HCl (to pH 3) and extracted three times with chloroform (3×10 ml). The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by column chromatography, (silica gel/ toluene:acetic acid, 12:1) to yield, unreacted bromo-OB (**13**) 12 mg (26.7 μmol, 25%) and OB 9 mg (24.4, 23%)(Characterised by ^{1}H-, ^{13}C-NMR and EI-, ES-MS see section 4.2.3 and 4.2.2, respectively).

4.3.3 Direct catalytic fluorosubstitution of aryl bromides.

4.3.3.1. Attempts to fluorinate *N*-(5-bromo-2-hydroxyphenylcarbonyl)-L-phenylalanine (25)(5-BrSAPhe) using tetrabutylammonium fluoride in the presence of tetrakis(triphenylphosphine)palladium(0).

A 50 ml round bottom flask equipped with a reflux condenser and a magnetic stirring bar was charged with 5-BrSAPhe (100 mg, 275 μmol), 1M tetrabutylammonium fluoride (TBAF) in THF (0.7 ml) and toluene (10 ml). The reaction vessel was purged with argon

and the THF removed in vacuo. Tetrakis(triphenylphospine)palladium(0) (4 mg, 3.6 μmol) was added to the degassed solution and the mixture heated under reflux in an argon atmosphere. The reaction was monitored with TLC (toluene: acetic acid - 5:1) and after 21 h the reaction mixture was allowed to cool to room temperature, filtered and the solvent of the filtrate evaporated under reduced pressure. The residue was suspended in chloroform (10 ml) and shaken up with 1M HCl (10 ml). The aqueous phase was extracted three times with chloroform (3×5 ml). The combined chloroform extracts were evaporated under reduced pressure to afford predominantly unreacted BrSAPhe (**25**) (Characterised by ^{1}H-, ^{13}C-NMR and ES-MS see section 4.2.7).

4.3.3.2 Attempts to fluorinate other compounds.

The reaction was further employed to 3-bromoanisole (**31**) (1.00 g, 5.34 mmol) using TBAF (1M in THF) (0.4 ml, 0.4 mmol) as well as 5-bromo-2-methoxy-4-methylisophthalic methyl ester (**27**) (100 mg, 0.315 mmol) using cesium fluoride (205 mg, 0.350 mmol). In the case of **31** as substrate, ES-MS analysis of the crude reaction mixture indicated that no reaction had taken place. In the case of **27**, ES-MS analysis of the crude reaction product indicated predominantly unreacted substrate, **27** and small amounts of the debrominated product, **30**.

4.3.4 Attempted synthesis of 2-fluoroacetoacetaldehyde.

4.3.4.1 Synthesis of sodium acetoacetaldehyde

To a slurry of freshly prepared sodium ethoxide (27.00 g, 500 mmol) in dry diethyl ether (226.8 g) under nitrogen was added drop-wise a solution of acetone (29.05 g, 500 mmol) and ethyl formate (37.05 g, 500 mmol). The reaction was stirred during the 30-minute addition period under nitrogen, and the internal reaction temperature was maintained at 25-30°C in an ice bath. After the addition was complete, the stirring was continued for 15 minutes, and the resulting tan solid was collected by filtration, washed with ether, and then dried under an argon to prevent water absorption. After further drying under reduced pressure a pale tan powder, sodium acetoacetaldehyde was isolated, 38.0 g (0.33 mol, 64%). The purity (from sodium formate) was established by rapid ^{1}H-NMR (60 MHz)

analysis of the product. The mole fraction of the sodium acetoacetaldehyde to sodium formate was determined by the ratio of the single proton absorbance at δ_H 8.33 and 8.75 (Characterised by ^{1}H-NMR see section 4.2.10).

4.3.4.2 Fluorination of sodium acetoacetaldehyde

Selectfluor™ (324 mg, 926 μmol) was added to a solution of sodium acetoacetaldehyde (100 mg, 926 μmol) in dry acetonitrile (10 ml) and stirred for 72 h at ambient temperature. The solvent was removed under reduced pressure, the residue acidified with a 1M HCl solution (10 ml) and extracted with dichloromethane (15 ml). The organic extracts were dried over anhydrous Na_2SO_4, filtered and the solvent removed under reduced pressure to yield a white coloured solid. ^{1}H-NMR of the product suggested that decomposition had occurred.

4.3.5 Peptide synthesis

4.3.5.1 Synthesis of *ortho*-, *meta*- and *para*-substituted DL-fluorophenylalanine Oα (44, 43 and 42, respectively).

4.3.5.1.1 Acid hydrolysis of OA (10)

OA (250 mg, 0.62 mmol) was heated with 6M HCl (100 ml) and refluxed for 72 h. The hydrolysed Oα (34) crystallized from the acidic solution at room temperature over 12 h without purification. The crystals of Oα were isolated by filtration and were washed repeatedly with distilled water to remove L-Phe. Pure Oα crystals were obtained following recrystalisation from a mixture of methanol and water (1:1). Residual Oα from the acid-water fraction was separated from L-Phe by partitioning into chloroform (250 ml). The chloroform extract was concentrated under reduced pressure. The residue (50 mg) was reconstituted from a mixture of methanol and diethyl ether (3:1). The total yield of Oα (34) from OA (**10**) was 95 mg, (0.37 mmol, 60%) (Characterised by ^{1}H-, ^{13}C-NMR and EI-MS see section 4.2.4).

4.3.5.1.2 Synthesis of the acyl chloride of Oα (Oα-Cl)(35)

A 50 ml round bottom flask equipped with a reflux condenser and a magnetic stirring bar were charged with Oα (50 mg, 0.195 mmol) and freshly distilled thionyl chloride (10 ml, 137 mmol) and heated under reflux in an argon atmosphere for 3 hours. The solution was allowed to cool to room temperature, and the excess thionyl chloride evaporated under a dry stream of argon. The acyl chloride (**35**) was further dried under vacuum pressure at room temperature for 12 h to remove any residual SO_2 or HCl. The product (**35**) was protected from any moisture and stored under argon. The presence of the product was established by treatment with ethyl alcohol and monitoring the ethyl ester of Oα, the product of the reaction, by TLC (neutral $CHCl_3$).

4.3.5.1.3 Preparation of DL-2-fluorophenylalanine methyl ester (39).

DL-2-fluorophenylalanine (36) (250 mg, 1.36 mmol) was added to a cold solution (-10°C) of AR methanol (3 ml) and thionyl chloride (400 µl, 5.52 mmol) and heated under reflux for 2 h. The reaction mixture was allowed to cool and the solvent evaporated under reduced pressure. The white precipitate was filtered off and recrystallised from a mixture of methanol and ether (1:1) and afforded, DL-2-fluoro-phenylalanine methyl ester hydrochloride 302 mg (1.29 mmol, 95%). (Characterised by ^{1}H-, ^{13}C-NMR and EI-MS see section 4.2.11).

The methyl ester hydrochloride salt (200 mg, 0.855 mmol) was dissolved in ether (5 ml) and cooled on ice. 0.5M Potassium carbonate (2.5 ml) was added to the solution and stirred for 1 h. The organic layer was separated and dried over anhydrous Na_2SO_4, filtered and the solvent removed from the filtrate under reduced pressure to yield 157 mg of a colourless oil, DL-2-fluoro-phenylalanine methyl ester (**39**) (0.79 mmol, 92%).

4.3.5.1.4 Preparation of DL-3- and -4-phenylalanine methyl esters (40, 41 respectively).

The isomeric fluorophenylalanine methyl esters were prepared using the identical procedures used in section 4.6.1.3 yielding 280 mg (1.20 mmol, 88%) of 3-fluoro- and 300 mg (1.28 mmol, 94%) of 4-fluoro-phenylalanine methyl ester hydrochloride

Both hydrochloride salts were converted, as described in section 4.6.1.3, to the respective methyl esters (both colourless oils) to yield 141 mg of DL-3-fluoro-phenylalanine methyl ester (**40**) (0.71 mmol, 83%) and 148 mg of 4-fluoro-phenylalanine methyl ester (**41**) (0.74 mmol, 87 %) (Characterised by ^{1}H-, ^{13}C-NMR and EI-MS see section 4.2.12 and 4.2.13).

4.3.5.1.5 Coupling of Oα (34) to DL-2-fluorophenylalanine methyl ester (39).

The Oα-Cl (**35**) (50 mg, 18 μmol) was dissolved in dry pyridine (3.5 ml) and cooled on ice. A solution of DL-2-flurophenylalanine methyl ester (**39**) (46 mg, 230 μmol) and triethylamine (23 mg, 230 μmol) in ethyl acetate (3.5 ml) was added drop-wise to the cold acyl chloride solution. The reaction was stirred for 7 h at room temperature, and thereafter washed with 0.1M HCl, 0.1M Na_2CO_3 and water, respectively. The organic layer was dried over anhydrous Na_2SO_4, filtered and the solvent of the filtrate evaporated under reduced pressure. The dried residue was treated with 0.5M NaOH at room temperature for 12 h. The mixture was then acidified with 6M HCl to pH < 1.0 and allowed to stand for 12 h at room temperature followed by extraction with chloroform (3×5 ml). The combined extracts were dried over anhydrous Na_2SO_4, filtered and the solvent of the filtrate removed under reduced pressure to yield, 68 mg (0.16 mmol, 88%) *ortho*-fluorophenylalanine Oα (**44**) (Characterised by ^{1}H-, ^{13}C-NMR and EI-MS see section 4.2.14).

4.3.5.1.6 Coupling of Oα (34) to DL-3- and -4- fluorophenylalanine methyl ester (40, 41 respectively).

The coupling of these isomeric fluorophenylalanine methyl esters was done using an identical procedure as in 4.6.1.5 yielding 45 mg *meta*-(0.11 mmol, 61%) and 62 mg *para*-(0.15 mmol, 83%) fluorophenylalanine Oα (**43**, **42** respectively) (Characterised by ^{1}H-, ^{13}C-NMR and EI-MS see section 4.2.15 and 4.2.16)..

4.3.5.2 Attempted synthesis of histidylhistidine Oα (49)

4.3.5.2.1 Synthesis of histidylhistidine methyl ester (48)

L-Histidine methyl ester dihydrochloride (0.284 mg, 1.175 mmol) and triethylamine (237 mg, 2.35 mmol) was dissolved in freshly distilled *N,N*-dimethylformamide (15 ml), and treated with a solution of *N*α-(*tert*-butoxycarbonyl)-L-histidine (300 mg, 1.175 mmol) in dimethylformamide (15 ml). Diisopropylcarbodiimide (177 mg, 1.41 mmol) and 1-hydroxybenzotriazole hydrate (190 mg, 1.41 mmol) was added to the above solution and stirred at room temperature in a closed vessel for 15 h.

After the completion of the reaction the solvent was concentrated *in vacuo* and the white precipitate, diisopropylurea (**57**), separated by filtration. The rest of the solvent was removed from the filtrate under reduced pressure and the residue purified by column chromatography (silica gel/ ethylacetate:ethanol - 3:1). The fractions containing the product were combined and the solvent evaporated under reduced pressure. The residue was treated with 95% trifluoroacetic acid for 1 h and concentrated under high vacuum. The residue was recrystalised from methanol:ether (excess ether) to yield a white solid, histidylhistidine methyl ester (**48**), 284 mg (930 μmol, 79%)(Characterised by ^{1}H-, ^{13}C-NMR and ES-MS see section 4.2.17).

4.3.5.2.2 Attempted coupling of Oa-Cl (35) to histidylhistidine methyl ester (48)

The procedure as described in 4.6.1.5 was employed for the attempted coupling of the Oα-Cl (**35**) to the histidylhistidine methyl ester (**48**). The reaction was monitored by TLC (toluene:acetic acid, 5:1) and after 7 h indicated that a reaction had occurred. ^{1}H-, ^{13}C-NMR and EI-MS data from the resultant product indicated that no coupling took place.

4.3.5.3 Synthesis of model peptides.

4.3.5.3.1 Peptide coupling of L-Phe to salicylic acid (17)

A round bottom flask (100 ml) equipped with a reflux condenser and a magnetic stirring bar was charged with salicylic acid (**17**) (1 g, 7.25 mmol) and freshly distilled thionyl chloride (10 ml, 137 mmol) and heated under reflux in an argon atmosphere for 3 hours.

The solution was allowed to cool to room temperature, and the thionyl chloride evaporated under a stream of dry argon.

The colourless oil residue, salicyl chloride, was dissolved in ethyl acetate (10 ml) and cooled on ice. A cold solution of sodium azide (1.4 g, 23.1 mmol) in water (10 ml) was added drop-wise to the salicyl chloride solution and stirred for 25 min on ice. The ethyl acetate was separated from the aqueous phase and extracted three times with ethyl acetate (3×10 ml). The combined extracts were dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure, which yielded a white solid, salicyl azide 745 mg (4.572 mmol, 63%). The presence of the azide was established by the increased in Rf value when separated by TLC (toluene:acetic acid – 5:1). The azide appears as a dark spot compared to the blue absorbance of salicylic acid when viewed under UV-light.

The salicyl azide (745 mg, 4.572 mmol) was redissolved in ethyl acetate (10 ml) and treated with a solution of L-Phenylalanine (1.131 g, 6.858 mmol) and triethylamine (0.692 g, 6.858 mmol) in water (10 ml). The reaction was stirred at room temperature for 56 h, in which time the reaction was monitored by TLC (toluene: acetic acid - 5:1). After completion of the reaction, the organic layer was separated, 1M Na_2CO_3 (10 ml) was added to the aqueous phase which in turn was extracted three times (3 ×10 ml) with ethyl acetate.

The aqueous phase was acidified with 1M HCl to pH 2 and extracted three times (3 ×10 ml) with chloroform. The combined organic extracts were dried over anhydrous Na_2SO_4, filtered and the filtrate evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, toluene:acetic acid, 5:1) to yield a colourless oil, SAPhe, 791mg (2.785 mmol, 38%) (Characterised by ^{1}H-, ^{13}C-NMR and ES-MS see section 4.2.5).

4.3.5.3.2 Peptide coupling of L-Phe to 5-chlorosalicylic acid (18) and 5-bromosalicylic acid (20)

The method used in 4.6.3.1 was further applied in the preparation of ClSAPhe (**23**) and BrSAPhe (**25**). These products were purified by crystallization from chloroform in a total yield of 49% and 38%, respectively (Characterised by ^{1}H-, ^{13}C-NMR and ES-MS see section 4.2.6 and 4.2.7).

4.3.5.4 Phenolic hydroxyl derivatisation of 5-chlorosalicylic acid (18).

4.3.5.4.1 By acetylation, as 2-acetoxy-5-chlorobenzoic acid (50).

A solution 5-chlorosalicylic acid (**18**) (500 mg, 2.90 mmol) and pyridine (0.52 g, 6.12 mmol) in dichloromethane (20 ml) was cooled on ice and treated with acetyl chloride (424 mg, 5.4 mmol). The reaction was stirred at room temperature for 10 h in a closed vessel. The reaction mixture was poured onto ice and acidified with 1M HCl (5 ml) and extracted with chloroform (3 ×10ml). The combined chloroform extracts were dried over anhydrous sodium sulfate, filtered and the solvent evaporated under reduced pressure. The residue was recrystalised from chloroform to yield a white solid **50**, 540 mg (2.53 mmol, 87%)(Characterised by ^{1}H-, ^{13}C-NMR and EI-MS see section 4.2.18).

4.3.5.4.2 By* tert-*butyldimethylsilylation, as* tert-*butyldimethylsilyl 2-*tert-*butyldimethyl silyloxy-5-chlorobenzoate (51).

A solution of 5-chlorosalicylic acid (**18**) (500 mg, 2.897 mmol), *tert*-butyldimethylsilyl chloride (1.091g, 7.238 mmol) and imidazole (0.789 mg, 11.59 mmol) in DMF (15 ml) was stirred at 50°C under an argon atmosphere for 15 h. The reaction mixture was poured into water and extracted three times (3×10 ml) with petroleum ether (60-80°C). The combined extracts were washed with a brine solution, dried over anhydrous Na_2SO_4, filtered and the solvent evaporated under reduced pressure. The residue was purified by column chromatography (silica, petroleum ether (60-80°C):ether, 10:1) to yield a colourless oil **51**, 980 mg (2.44 mmol, 84%)(Characterised by ^{1}H-, ^{13}C-NMR and EI-MS see section 4.2.19).

CONCLUSION

The synthesis of fluoro-Ochratoxin B.

From our investigation into the synthesis of fluoro-OB, have emerged a number of possible synthetic routes. These include:

a) The use of *ortho*-directed metallation methodology described by Sibi *et al.*, 1985, on fluorinated starting materials, such as 5-fluorosalicylic acid or the corresponding benzamide analogue. This synthetic sequence will circumvent the problems encountered in the selective aromatic fluorination of the multifunctional aromatic ring.

b) The successful fluorination of sodium acetoacetaldehyde will allow for the incorporation of this key intermediate into the synthetic procedure employed by Kraus, 1981 for the preparation of OA.

c) The palladium catalysed trimethylstannylation of BrOB revealed that metallation was successful at the halogen position and could possibly be enhanced in the absence of labile phenylalanine benzylic protons. The use of BrOβ, the hydrolysis product of BrOB, as subtrate could avoid this problem.

The coupling of other amino acids and dipeptides to Oα

d) The coupling of L-Histidine, which shares a commonality with L-Phe in the aromatic nature of their side chains, to Oα will not only probe the amino acid moiety but also allow for further coupling of histidylhistidine. The effect that the presence of a dipeptide coupled to Oα will have on the toxicity of OA could further be explored in the coupling of glycylphenylalanine to Oα.

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