SYNTHESIS OF NOVEL THYROID HORMONE ANALOGUES

A Thesis submitted to the

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Master of Science

by

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The Languages, especially the dead,
The sciences, and most of all the abstruse,
The arts, at least all such as could be said
To the most remote from common use.

Byron

SUMMARY

A series of twenty two 2-phenoxybenzoic acids were prepared in various yields using two different methods. It was found that the method involving a modified Ullmann coupling of a phenol with 2-chlorobenzoic acid gave the best yields. Reaction between diphenyliodo-2-carboxylate and a phenoxide gave lower yields and xanthone as byproduct. Using the modified Ullmann reaction 3-phenoxybenzoic acid and two thiophenoxybenzoic acids were also prepared in good yield.

Transthyretin (TTR) was isolated from human plasma by column chromatography and the prepared phenoxybenzoic acids were tested for binding to the isolated protein. The most potent compounds were found to be 3-phenoxybenzoic acid (**26**), 2-(2,4-dimethylphenoxy)benzoic acid (**10**), 2-(2,3-dimethylphenoxy)benzoic acid (**9**) and 2-(3-iodophenoxy)benzoic acid (**25**). All of the compounds tested were up to two orders of magnitude less active than the corresponding nitrogen bridged anthranilic acids, indicating that replacing the nitrogen atom with either an oxygen or sulfur lowers activity. Interestingly, 3-phenoxybenzoic acid binds more strongly to TTR than 2-phenoxybenzoic acid and is almost as active as 2-phenoxyanthranilic acid. This indicates that the preparation of analogues with the carboxylic acid in alternative positions on the ring may lead to even more potent anti-thyroid drugs.

Declaration

The work described in this thesis has not been previously submitted for any other degree at this or any other University. The studies presented in this thesis represent my own original work except where due acknowledgment has been made. This thesis is less than 100,000 words in length, exclusive of tables and bibliographies

M. Radovanovic

CONTENTS

Acknowledgments

Firstly and foremostly, I would like to acknowledge that I have great friends who have encouraged and supported me throughout this ordeal. They were my inspiration, my motivation and my excitation. Secondly, I like to recognise other individuals who over the years indirectly contributed towards the completion of this thesis.

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Dedication

Убек и сеставу

To a gentle man who always believed in me.

Тапи

To a sweet woman, whose childlike temerity was always so endearing.

Баби.

List of Abbreviations

T_4	Thyroxine
T_3	3,5-Triiodothyronine
Tg	Thyroglobulin
TBG	Thyroxine-Binding Globulin
TTR	Transthyretin
ALB	Albumin
HSA	Human Serum Albumin
rT_3	reverse 3,3',5'-triiodothyronine
TSH	Thyroid Stimulating Hormone
TRH	Thyrotropin-releasing Hormone
PTU	Propylthiouracil
Triac	acetic acid analogue of T_3
NSAID	Non-steroidal Anti-inflammatory Drug
DPIC	Diphenyliodonium-2-carboxylate
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
RBP	Retinol binding Protein
SDS	Sodium dodecyl sulphate
KDa	kilodaltons, 1000 atomic mass units

List of Figures and Tables

Chapter 1

Literature Review

We can't all be heroes because someone
has to sit on the curb and clap as they go by.

Will Rodgers

1.1 INTRODUCTION

This thesis is concerned with the preparation of thyroid hormone analogues that target thyroid hormone transport. The work will provide data regarding the development of analogues of this type as potential anti-thyroid drugs. This chapter gives an overview of the thyroid system, and the diseases associated with malfunction of the thyroid system. It also describes the thyroid hormones and some anti-thyroid compounds that compete with the thyroid hormones for their binding sites. The general aim of the study is to understand the interaction of several non-thyroidal compounds with the plasma transport protein, TTR, providing a basis for the experimental results presented in the following chapters.

1.2 THE THYROID SYSTEM

The thyroid gland is part of the endocrine system which is involved in many biological processes throughout the body by regulating protein synthesis in the target tissue. The mechanism of the action of iodothyronines on cell metabolism and on organ development are not well understood. The pituitary gland, an endocrine gland, controls the thyroid hormone system, which is in turn under the control of the hypothalamus. The whole system is of enormous complexity, with many factors controlling thyroid hormone levels. The thyroid gland develops in the embryo from tissue that grows out of the floor of the pharynx, becomes detached, and eventually completes its differentiation in the neck near the upper end of the trachea.

In humans, the thyroid gland consists of two lobes of endocrine tissue positioned on either side of the trachea (Fig 1.1).[1] The lobes are connected by a small bridge of thyroid tissue running across the front of the trachea known as the isthmus. These lobes consist of numerous tiny hollow sacs called follicles. The shell of each follicle consists of closely packed cells and is wrapped in a thin membrane covered with a

dense mesh of blood capillaries which ensure a steady and abundant supply of fresh plasma to the cells. Apart from the blood vessels, there are lymph and nerve vessels that also surround the follicle.

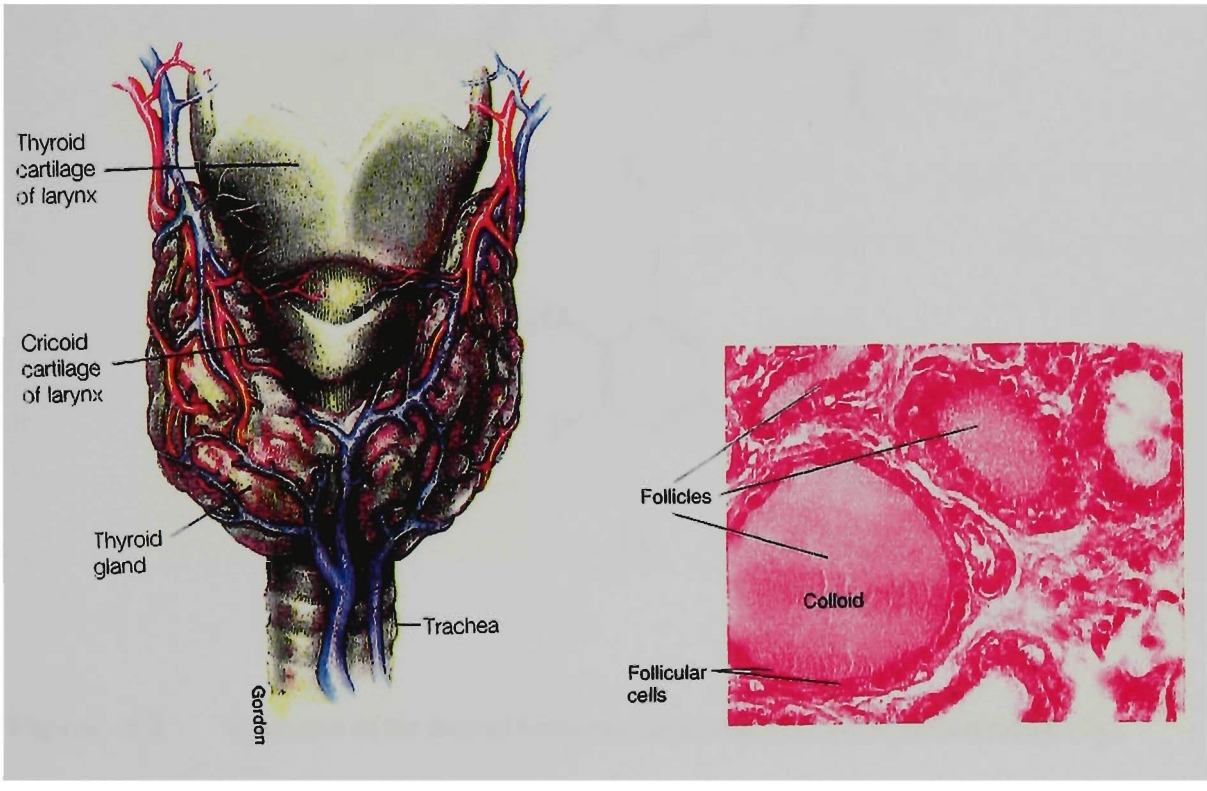
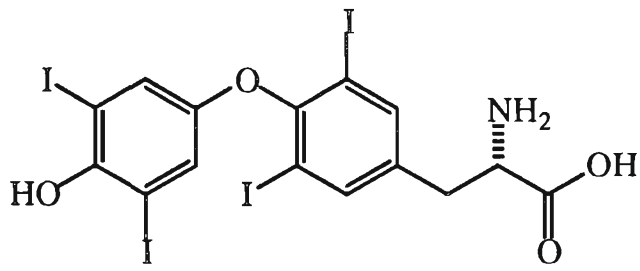
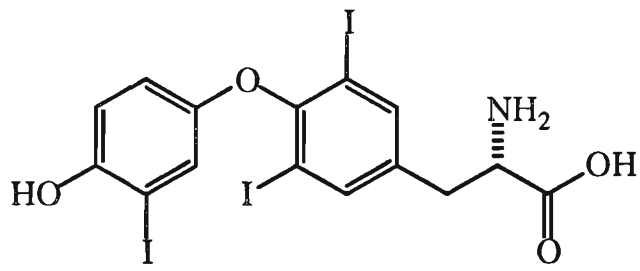


Figure 1.1 (a) A schematic diagram of the thyroid gland. (b) A photomicrograph of a thyroid gland showing numerous thyroid follicles. Each follicle consists of follicular cells surrounding the fluid known as colloid, which contains thyroglobulin. Adapted from Fox.[1]

Every follicle of the thyroid gland makes thyroid hormone, stores it, and secretes it into the bloodstream. Thyroid hormones generally refer to the two principle hormones synthesised within the thyroid gland. These substances, L-tetraiodothyronine (thyroxine, T_4) and L-triiodothyronine (T_3) (Fig 1.2) play an extremely important role in growth, development and differentiation of cells and also in the regulation of a wide range of metabolic processes in both humans and animals.[2] Under normal conditions, the most abundant hormone is thyroxine.



T_4



T_3

Figure 1.2 Structures of the thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3).

1.3 HISTORY OF THYROID GLANDS

The thyroid gland was known as early as the seventeenth century,[3] however, at that time its secretory role was not fully understood. It was during the nineteenth century that the relationship between the thyroid and various body functions was studied. Clinical symptoms such as heart failure,[4] palpitations and protrusion of the eyeballs were associated with the swelling of the thyroid gland (goitre),[5] and so the concept of an internal secretory function of the gland was formulated in 1836.[6] In 1883, Reverdin and Reverdin,[7] and Kocher[8] described the clinical features of thyroid deficiency following a surgical thyroidectomy, and found the symptoms to parallel the condition myxoedema (most severe form of hypothyroidism). Two years later, Horsley[9] recognised that the loss of the thyroid caused both the spontaneous and

operative disorders; until then the prevailing view had been that asphyxia or injury to the central nervous system was the fundamental cause of thyroid deficiency.

Work was then initiated to search for the active constituent of the gland. In 1895, Dr. Eugene Baumann[10] treated sheep thyroid with sulphuric acid and isolated in high concentrations an organo-iodine compound which he named 'thyroidin'. Later, in 1899, Oswald[11] purified the material further and found that the iodinated compound was a globulin, and the name was changed to thyroglobulin. This protein is responsible for thyroid hormone iodination.

In an effort to determine the active component of the thyroid gland, Kendall[12] isolated another substance as pure crystals from bovine thyroid glands by treating them with sodium hydroxide. He estimated the molecular weight of the substance and assigned the structure as 4,5,6-trihydro-4,5,6-triiodo-2-oxy-ß-indole propionic acid, which he named thyroxin (Fig. 1.3). He claimed that this substance, rather than thyroglobulin, was the active component of the gland.[13]

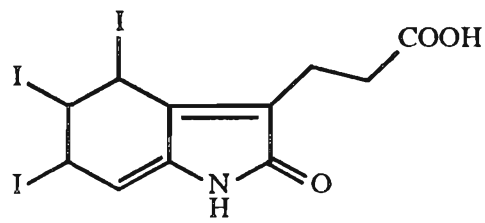
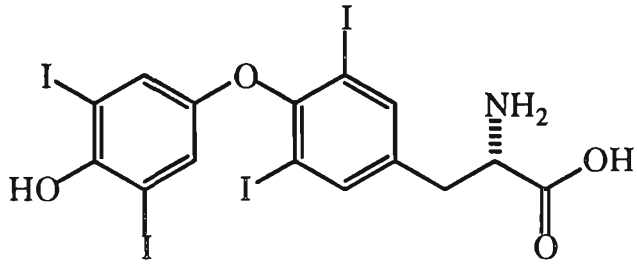


Figure 1.3 Structure of thyroxin proposed by Kendall in 1915.[12]

Harrington[14] and later Harrington and Barger,[15] improved the isolation procedure of thyroxin by extracting it with barium hydroxide to obtain an insoluble barium salt from which thyroxin could be generated. Through the characterisation of the structure isolated, they identified amino acid and phenolic components of the molecule. Harrington established the empirical formula of the material to be $C_{15}H_{11}I_4NO_4$ and

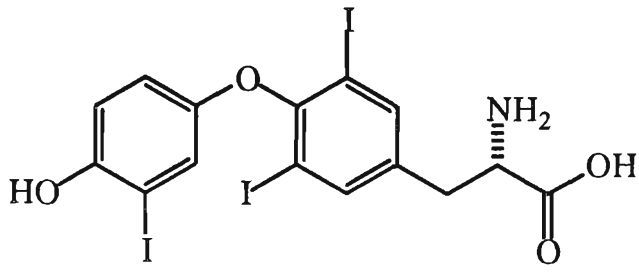
proposed the structure for thyroxin, later called thyroxine or T_4 (Fig 1.4). This structure was confirmed as correct when synthesised thyroxine displayed identical chemical and physical properties to the extracted material.



T_4

Figure 1.4 Structure of thyroxine T_4, confirmed by Harington.

Subsequently, a thyroxine like compound was discovered but with only three iodine atoms.[16] This compound was called T_3 and the assigned structure (Fig 1.5) was confirmed by Roche and co-workers,[17] and it was shown to be more physiologically potent than thyroxine. Using techniques involving radioactive isotopes of iodine and paper chromatography, it was possible to separate and identify small amounts of other iodinated compounds in thyroid hydrolysates and blood plasma.[18] Since the 1950's a number of iodinated compounds with structures similar to T_3 and T_4 have been isolated. These iodinated compounds are collectively known as iodothyronines. Iodothyronines have been found to be the only hormones known to be amino acids and are the only naturally occurring hormones containing a diphenyl ether linkage.[19]



T_3

Figure 1.5 Structure of triiodothyronine T_3 established by Roche and coworkers.

The use of radioactive ^{131}I and synthetic thyroxine labelled with ^{131}I, enabled detailed information on the transport mechanism of thyroxine in blood to be generated. This work also resulted in the discovery of human serum albumin (HSA), thyroxine-binding globulin (TBG), and a third thyroid hormone carrying protein, transthyretin (TTR). The thyroid hormones T_4 and T_3 are transported in the blood by these carrier proteins, which serve to facilitate their uniform delivery to different tissues as well as to store the hormones in blood, in a non-diffusable form, maintaining the concentration of free hormone at a constant level.[20, 21]

1.4 BIOSYNTHESIS AND METABOLISM OF T_4 AND T_3

Thyroid hormone biosynthesis begins with the accumulation of inorganic iodide, circulating in the bloodstream, in the follicular cells of the thyroid gland. The inorganic iodide is oxidised to iodine by the enzyme iodide peroxidase. The active iodine is used for the iodination of tyrosine residues on a glycoprotein called thyroglobulin. Thyroglobulin (Tg), with a molecular weight of more than 600 kDa, is stored within the gland in follicles as the main component of a substance called the thyroid colloid. This arrangement, which provides a reserve of thyroid hormones, perhaps reflects the frequent scarcity of environmental iodine, particularily on land and in freshwater. The oxidative coupling of two diiodotyrosine residues then forms

thyroxine (T_4). During this process a smaller quantity of triiodothyronine (T_3) is also formed. Both hormones are released into the blood stream following complete proteolysis of the thyroglobulin molecule.[22]

Iodotyrosines are also formed as a result of the hydrolysis of thryroglobulin and have no biologic activity. They are considered to be the precursors of the biologically active hormones T_4 and T_3. The iodine is removed from iodotyrosine by an enzyme known as deiodinase. Iodine, from metabolised thyroid hormone, is removed largely in the liver and in the kidneys, and most of it returns to the thyroid gland, an economy that emphasizes the need for conservation; some iodine, however, is lost in the alimentary tract. The half-life of thyroxine is comparatively long, six to seven days, whereas, the half-life of T_3 is much shorter.[23]

1.4.1 Conversion of T_4 to T_3

Thyroxine is the major circulating thyroid hormone, whereas T_3 is more physiologically active at the nuclear receptor. It is worth noting that the relative binding affinities of T_4 and T_3 are reversed at the nuclear receptor and TTR sites, i.e. T_3 is the more potent hormone at the nuclear receptor site while T_4 is more potent at the TTR site.[19] Upon release from carrier proteins in peripheral tissues, T_4 is deiodinated to T_3 by the enzyme 5'-monodeiodinase, which is present in tissues throughout the body but is most active in the liver and kidney, where about 60% of the conversion takes place. This enzyme, which is associated with microsomal or cellular membranes and functions in most tissues of the body, is also responsible for the selective deiodination of T_4 to a range of other naturally occurring hormones of similar structure which differ in the degree of iodination (Fig 1.6). All of these hormones are present *in vivo* in at least trace amounts. As yet the physiological function of these metabolites is not known. Virtually all rT_3 (reverse 3,3',5'-triiodothyronine) is produced by the monodeiodination of the inner ring of thyroxine, but it is not physiologically active. It

has been suggested that the selective deiodination of T_4 may represent an important peripheral regulatory mechanism for modulating the quantity of biologically active hormone at the tissue level.[24, 25]

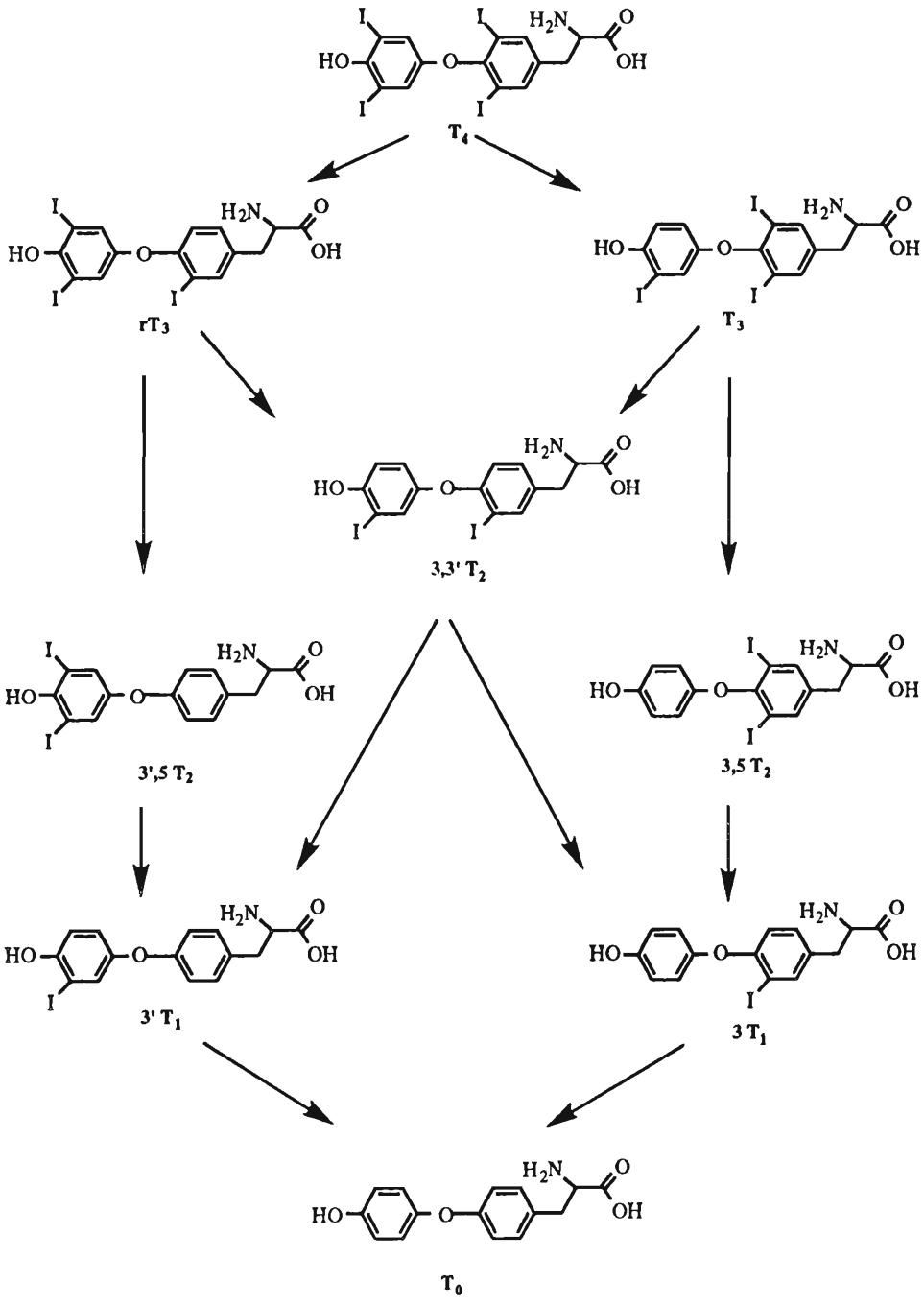


Figure 1.6 Deiodination cascade of the nine naturally occurring thyroid hormones.

1.4.2 Regulation of Thyroid Hormones

Thyroid hormone synthesis and secretion are regulated by extrathyroidal (thyrotropin) and intrathyroidal (autoregulatory) mechanisms.[26] Although thyrotropin is the major modulator of thyroid activity, autoregulatory mechanisms are also important and significant.

The level of thyroid hormone circulating in the blood is controlled by a glycoprotein hormone called thyroid stimulating hormone (TSH) which is synthesised in the pituitary gland. TSH is a glycoprotein with a molecular weight of 28 kDa, consisting of two polypeptide chains, designated α and β, that are linked by non-covalent bonds. TSH in plasma has a half life of about an hour. This hormone mediates the rate at which iodine is extracted from the blood and taken into the follicular cells of the thyroid. In turn, this controls the rate at which T_3 and T_4 are hydrolysed from thyroglobulin and secreted into the bloodstream.

The synthesis of TSH is regulated by several compounds produced in the hypothalamus: thyrotropin-releasing hormone (TRH), somastostatin and dopamine. TRH is present throughout the brain but it is at its highest concentration in the hypothalamus. Hypothalamic production and release of TRH are controlled by poorly understood neural pathways in the brain. TRH is transported *via* the hypothalamic-hypophyseal portal system to the anterior pituitary where it stimulates the function of TSH. The output of TRH is regulated by levels of T_4 and T_3 in the blood stream.

TRH enhances TSH production while somatostatin and dopamine inhibit its release. A stable level of circulating thyroid hormone is maintained by a negative feedback mechanism (Fig 1.7). High levels of thyroid hormone inhibit TRH and TSH production by the hypothalamus and pituitary respectively. [27]

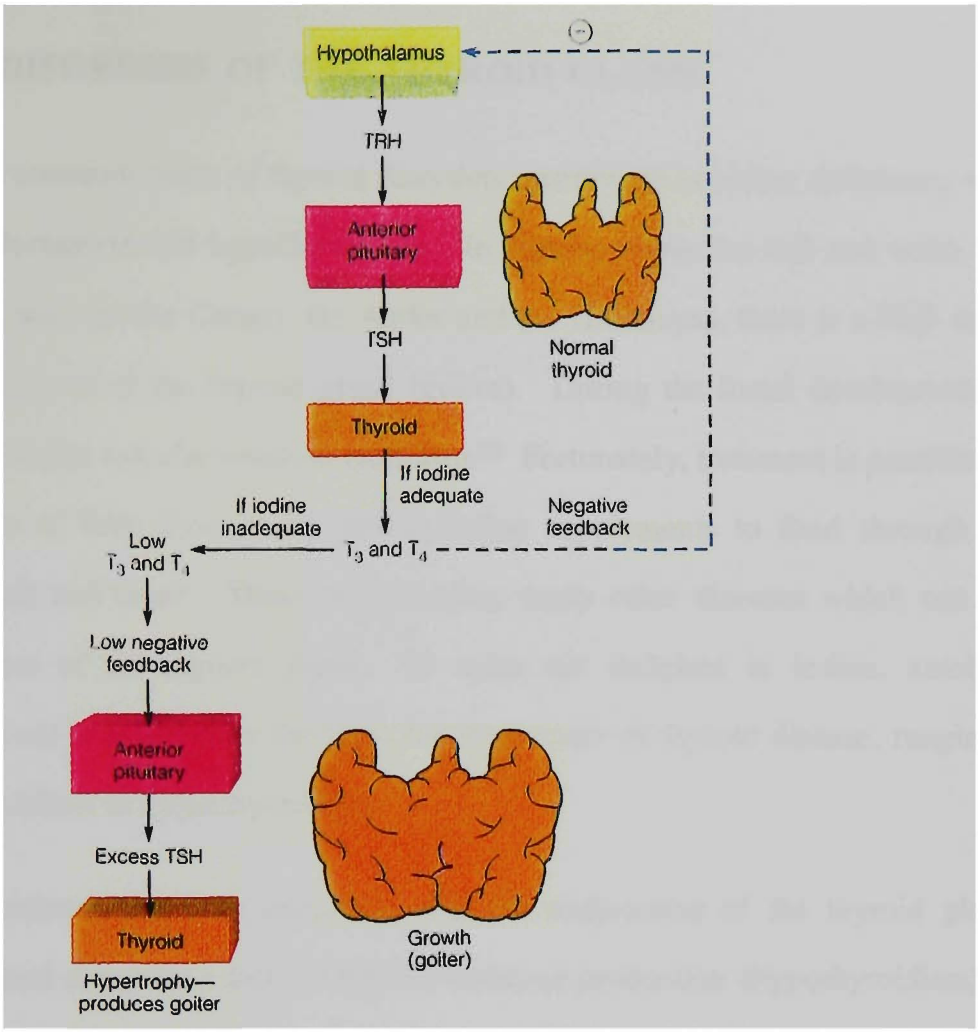


Figure 1.7 Pathways in the control, synthesis and secretion of thyroid hormones.[1]

In addition to the major control of thyroid function by the hypothalamic-pituitary axis, the thyroid is able to regulate uptake of iodide and thyroid hormone synthesis by intrathyroidal mechanisms independant of TSH. Increasing amounts of iodide inhibit iodide organification which occurs at a critical level of intrathyroidal inorganic iodide. Thyroglobulin iodination and thyroid hormone synthesis subsequently decrease. This is known as Wolff-Chaikoff block.[27] The normal thyroid gland escapes from the Wolff-Chaikoff block and hypothyroidism does not ensue because of intrathyroidal feedback inhibition of the iodide transport mechanism by an organic iodine intermediate.

1.5 DISORDERS OF THE THYROID GLAND

The most common cause of thyroid disorders worldwide is iodine deficiency resulting in goitre formation and hypothyroidism. In regions where the soil and water are low in iodine, such as the Congo, the Andes and the Himalayas, there is a high incidence of enlargement of the thyroid gland (goitre). During the foetal development stage, deficient iodine can also result in cretinism.[28] Fortunately, treatment is possible for the prevention of both diseases by adding iodine supplements to food through use of iodised salt and flour. There are however, many other diseases which can lead to malfunction of the thyroid gland. In areas not deficient in iodine, autoimmune processes are believed to be the basis for most cases of thyroid disease, ranging from hypothyroidism to hyperthyroidism.

Two disorders commonly associated with the malfunction of the thyroid gland are characterised either by a lack of thyroid hormone production (hypothyroidism) or by an overproduction of thyroid hormone (hyperthyroidism, thyrotoxicosis, and toxic goitre). Both conditions are more common in females than in males.

1.5.1 Hypothyroidism

Thyroid underfunction, or hypothyroidism, resulting from a deficiency of T_4 and T_3, may originate in the thyroid itself or in the pituitary or hypothalamus, which control the thyroid. A rough dry, and yellow skin, coarse scalp hair, and hoarse voice suggest that thyroid hormones may be deficient, but none of these signs need be present. Intellectual functions may become impaired, and personality changes or severe emotional illnesses may appear. Lowered basal metabolic rate and physical signs such as slurring of speech, enlargement of the tongue, and weight gain can occur.[29] Once diagnosed the condition is easily treated with synthetic Thyroxine.

1.5.2 Hyperthyroidism

Autoimmune diseases and cancer are major causes of dysfunction of the thyroid gland. Overactivity of the thyroid gland with diffuse enlargement of the gland and excess production of thyroid hormones, often accompanied by eye protrusion (exophthalmos, or Graves' disease), is an auto-immune disorder. Toxic nodular goitre, or Plummer's disease, results from hyperfunction of thyroid adenomata, or tumors. It usually occurs without eye signs, but the eyes often appear larger because of an unusual stare. Hyperthyroidism or thyrotoxicosis may result from an ovarian struma (a goitre-like, iodine containing tumor) that secretes thyroid hormones, from a testicular, placental, or other tumor that is secreting TSH-like material that stimulates the thyroid gland, or from ingestion of huge amounts of thyroid hormone.[28,30] Hyperactive behaviour, thyroid enlargement, smooth and hot skin, excessive sweating, tremors, rapid pulse, and eye signs are the most frequent features of thyrotoxicosis, although virtually any organ or tissue may be affected. With respect to the central nervous system, emotional and personality disturbances, abnormal brain waves, and nerve-muscle transmission, or convulsions may occasionally develop in thyrotoxicosis. The circulatory changes in thyrotoxicosis involve increased heat production and heat loss, dilatation of blood vessels, decreased arterial tone, increased movement of blood, greater output of blood by the heart, and a rise in blood pressure.[28,29]

Endocrine glands other than the thyroid may be affected when excesses of thyroid hormones are present. Occasionally, urine volumes and thirst are excessive, which may indicate interference with the action of vasopressin, one of the hormones that regulate water excretion by the kidney. Growth hormone and blood sugar levels may be diminished, skin pigmentation increases in some individuals, and loss of menstruation is common when thyroid overactivity is severe, although pregnancy can occur.[29] Diagnosis of thyroid overactivity is supported by increased oxygen

consumption or elevated basal metabolic rate. Acute hyperthyroidism is known as thyroid storm and can be fatal. Treatment usually involves the use of anti-thyroid drugs or surgical removal of part of the thyroid gland, followed by hormone replacement therapy.

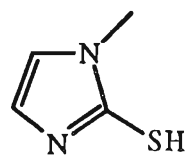
1.5.3 Anti-thyroid Drugs

The selection of a particular treatment for an overactive thyroid is determined by a number of variables including the age and sex of the person, any associated disorders, and the size and shape of the gland. A variety of compounds are known to inhibit thyroid hormone synthesis *in vivo* at different stages. These compounds can block biosynthesis at various stages, including iodine uptake/concentration and iodination of the tyrosine residues, at doses that do not have major adverse effects on other organ systems. However, other compounds do affect metabolic processes in diverse tissues as well as affecting one or more steps in thyroidal iodine metabolism.[31]

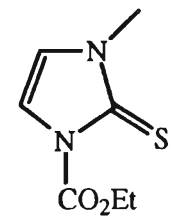
Thyroid blocking agents, also known as goitrogenic substances either interfere with the "pump" that concentrates iodide in the thyroid cells or prevent the binding of iodine within the thyroid. These thyroid blocking agents include, ClO_4^-, TcO_4^-, SCN^- ReO_4^- and BF_4^-. These complex anions, which cannot be metabolised by the thyroid gland, accumulate in the gland and as a result, competitively inhibit the uptake of iodide. The perchlorate anion is effective in the treatment of hyperthyroidism but has not been widely used because of serious toxic reactions like aplastic anaemia and nephrosis.

Another class of compounds, currently used as anti-thyroid drugs are the thioamide drugs: Carbimazole, Methimazole and Propylthiouracil (PTU)[31] (Fig 1.8). These drugs target the biosynthesis of thyroid hormone by affecting the coupling of iodotyrosyl residues to form iodothyronines. This coupling is catalysed by thyroid peroxidase and it is believed that these agents interfere with the peroxidatic reaction.[21] The adverse reaction experienced by some patients with the use of these drugs is called

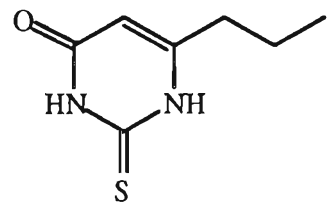
agranulocytosis, a disorder in which there is a decreased number of circulating granulocytes which are a type of leucocyte.[32]



Methimazole



Carbimazole



Propylthiouracil

Figure 1.8 Anti-thyroid drugs: Carbimazole, Methimazole and Propylthiouracil.

A class of anti-thyroid drugs known as thioamide drugs, are effective in treating hyperthyroidism. They are slow acting drugs and do not affect the level of thyroid hormones already circulating in the body. Treatment is designed to provide temporary relief of hyperthyroidism until spontaneous remission occurs. More effective drugs would have to have a much faster response time. One way of improving response time would be to target the transport of thyroid hormones within the body. In this regard, several drugs which include barbital, 2,4-dinitrophenol, and drugs like salicylates and penicillin have been shown to interact with the thyroid transport system and exhibit some binding to TTR.[33,34]

1.6 MECHANISM OF THYROID TRANSPORT

In the blood stream, the majority of thyroid hormones are bound to thyroxine-binding globulin (TBG). The rest is distributed between TTR and albumin (ALB) with a small fraction transported by a variety of other proteins. T_4 is transported to peripheral tissues and converted to the more physiologically active hormone T_3. Here the unbound hormone passes into the cell and exerts its action on at least one nuclear receptor site. The whole system is controlled by a negative feedback mechanism.

It had been presumed that thyroid hormones are bound by proteins in the blood stream to increase serum concentration of the hormones, however, Schreiber and Richardson[35] found that higher hormone levels could be obtained without the presence of the binding proteins, since normal hormone levels are three orders of magnitude less than the solubility limit. Mendel[20] has shown that in the absence of thyroxine-binding proteins, thyroxine will preferentially partition into the first cell membranes encountered. It would therefore seem that the function of these proteins is to ensure even distribution of the hormones throughout the cells.

1.6.1 Transport Proteins

In euthyroid (normal) patients, the concentration of T_4 in the blood is 100nM and 1-2nM T_3. The thyroid hormones are almost exclusively bound to their respective carrier proteins during transport and in this form the thyroid hormones are biologically inactive. Only the free hormone regulates the pituitary feedback mechanism. A dynamic equilibrium exists between the plasma and intracellular free hormone pools. Protein bound thyroid hormones act as a "buffer" responsible for keeping a large store of hormones in the extrathyroidal space in a nondiffusable form, and may act as donors of hormones from the circulation to the cell.

The serum proteins differ by several orders of magnitude in their binding capacity and affinity for thyroxine. TBG is the most efficient of the three carrier proteins in binding T_4 and T_3, it has the highest affinity, and carries approximately 75% of the thyroid hormone. TTR and albumin have lower affinities but higher concentrations in serum and carry approximately 15 and 10% respectively of the total thyroid hormone load.[36]

1.6.2 Thyroxine-Binding Globulin

Thyroxine-binding globulin (TBG), an α-globulin with a molecular weight of approximately 54 kDa, has the highest affinity for the thyroid hormone of all the proteins and the lowest serum concentration.[25] TBG is an acidic glycoprotein consisting of 395 amino acids with half of the peptide groups equally distributed between α-helical and β-sheet structure.[37] TBG has one binding site for thyroid hormone which has a high affinity for T_4 ($K_a = \sim 10^{10}\ M^{-1}$) and a lower affinity for T_3 ($K_a = \sim 10^{9}\ M^{-1}$).[21] The details of the binding site are still unclear and hence the molecular aspects are poorly understood. The amino acid sequence of TBG shows 42% homology with α_1-antitrypsin, identifying TBG as a member of the serpin family of proteins.[25]

TBG, synthesised by the liver parenchymal cells, is relatively unstable and can be readily denatured to multiple electrophoretic forms making it difficult to purify and crystallise the protein.[38] The three dimensional representation of TBG shown in Figure 1.9 was built up by aligning "conserved" protein sequences with the known X-ray crystal structure of α_1-antitrypsin.[39] Models of TBG have been built using different sequence alignments[40] which allowed the identification of a hydrophobic cleft within the protein as the T_4 binding site.[41] It has been suggested that the thyroid hormones are bound in the centre of a β-barrel structure where the iodo-tyrosine sits deep into the binding cavity and the alanine sidechain interacts with charged residues.

The high affinity of TBG for the thyroid hormones is attributed to the large number of aromatic residues surrounding the proposed binding site.

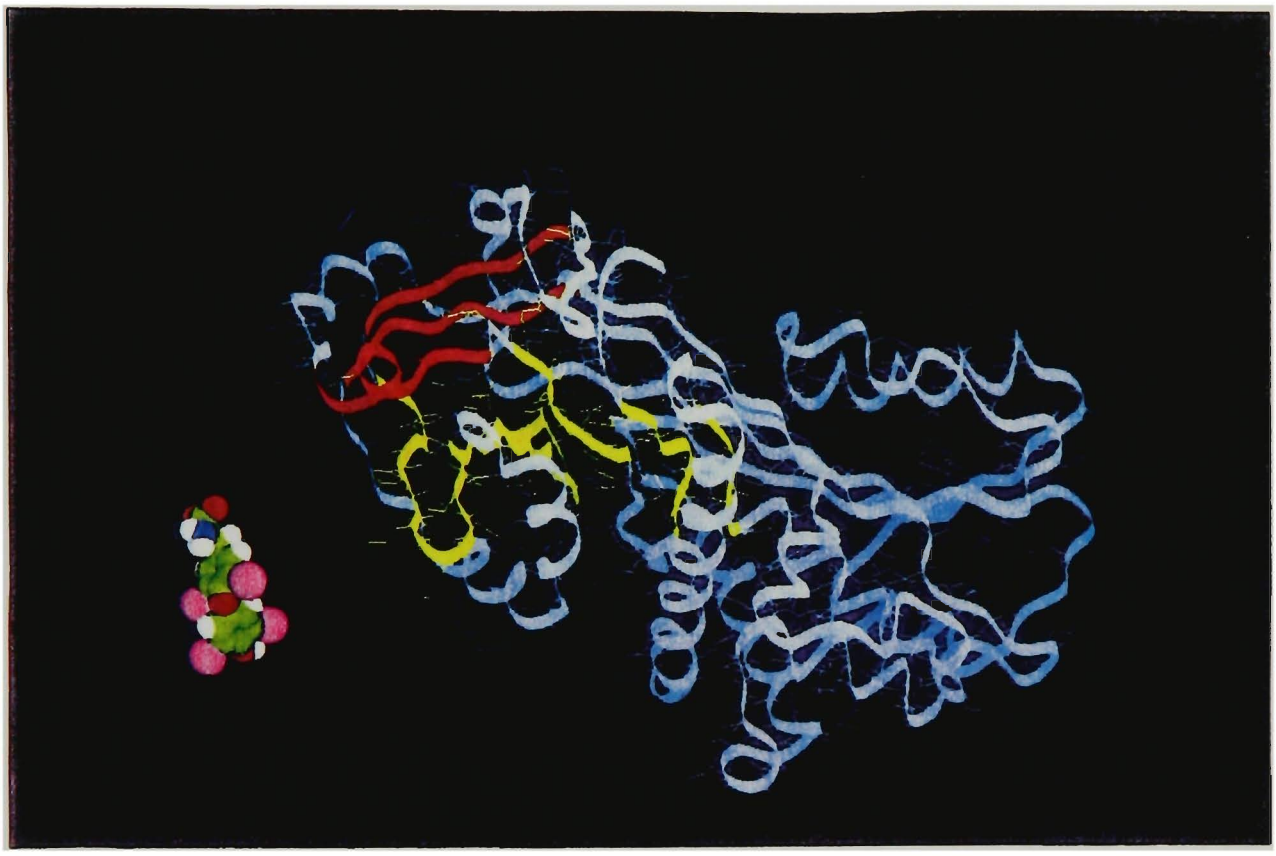


Figure 1.9 Thyroxine binding globulin model showing the β barrel cleft, the binding domain of thyroxine. Thyroxine is shown adjacent to the binding site to illustrate their relative dimensions. (Taken from J. Jarvis)[41]

1.6.3 Transthyretin

Transthyretin (TTR) is a tryptophan rich protein with a molecular weight of approximately 55kDa. It is principally synthesised in the liver. Unlike TBG, TTR is not glycosylated. It is found in high concentrations (250-300 mg/l) in blood serum, and is responsible for much of the immediate delivery of T_4 and T_3 to cells, although only 15- 20% of T_4 in plasma is bound to TTR.[27] This is because the affinity of the hormones for TTR is lower than for TBG therefore they dissociate from TTR more rapidly.

Isolation and purification of stable TTR is possible using standard chromatographic techniques. The stability of the molecule is attributed to its unusually high content of β-structure. The three dimensional structure of TTR has been extensively studied using high resolution X-ray crystallographic techniques.[42,43] These studies have shown that TTR is a tetramer made up of four identical polypeptide chains, each composed of 127 amino acids, which are held together by non-covalent interactions. The subunits are approximately 50% β-sheet, with only 5% of the amino acid residues found in a single short α-helix. The TTR protein is formed by an edge-to-edge interaction of the concave β-sheets of two of the monomers. The two dimers then form a structure called a β-barrel, which is a cylindrical channel about 55Å long and 8Å wide. The β-barrel occurs as a result of the concave faces of the β–sheets directing inwards[22] (Fig 1.10).

Crystallographic data gathered by Blake and co-workers[43] shows that at the center of the TTR-T_4 complex hydrophilic residues Ser117 and Thr119 project into the channel resulting in two binding sites. The side chain carboxyl and amino groups of T_4 are adjacent to Lys15 and Glu54 respectively near the channel entrance. The phenolic hydroxyl group is in a hydrophilic area near the centre of the molecule. The iodines are positioned in a hydrophobic pocket surrounded by Leu17, Leu110, Thr106, Ala108 and Val121.

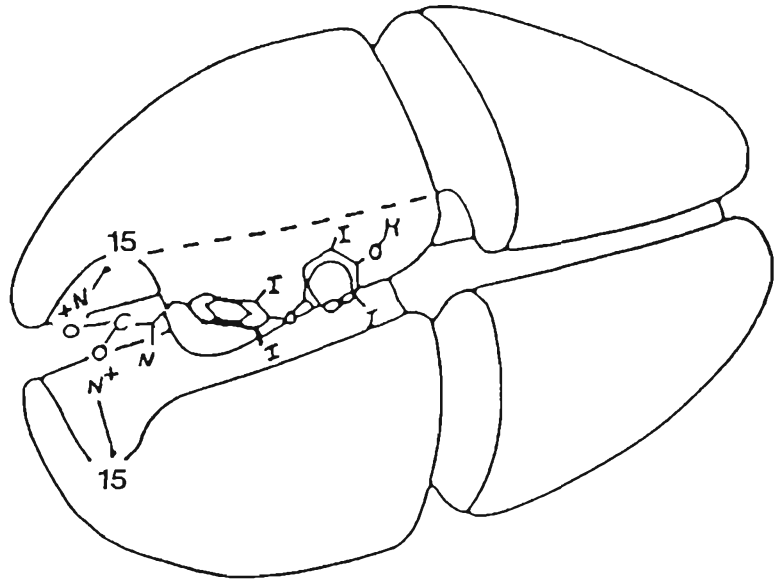


Figure 1.10 Schematic diagram of transthyretin showing the four subunits and the binding orientation of T_4. The side chain carboxylate ion is shown forming ion-paired associations with two lysine-15 side chains of TTR. (Taken from Blake)[44]

Even though there are two binding sites within TTR, only one molecule of T_4 binds to TTR with high affinity. The secondary binding site shows a much lower affinity for thyroid hormones. This is explained by a strong negative cooperativity in the binding of T_4 and T_3.[25] The exact mechanism of this cooperativity is not clearly understood. Under physiological conditions only one ligand is carried per TTR molecule.

1.6.4 Albumin

Human serum albumin (HSA) is the most abundant protein in the blood, and is important in maintaining the colloid osmotic blood pressure. Albumin has the lowest affinity of the carrier proteins for T_4 and T_3 but because of it high serum concentratrion it binds significant amounts of thyroid hormone (approximately 10% of T_3 and T_4). HSA is also involved in transporting a multitude of small molecules in blood, including calcium and sodium ions, fatty acids, hormones and aqueous media. Thyroid hormones and tryptophan are the only amino acids that bind to the same site in albumin.

HSA is a single polypeptide chain of 65 kDa consisting of 585 amino acids. Recently, the three dimensional structure of HSA was determined by X-ray crystallography, which shows it to consist of 28 α-helices held in place by 17 disulfide bridges.[45] The protein is composed of three domains each consisting of two smaller subdomains. Crystallographic binding studies have shown the presence of one high affinity T_4 site and numerous sites of lower affinity located in the two subdomains.[46,47] HSA is the only thyroxine-binding protein in which the binding site is constructed from α-helices, rather than β-sheets.[25]

1.6.5 Other Thyroid Hormone Binding Serum Proteins.

There are a small number of other serum proteins that bind thyroid hormones. One of these is a protein known as 27K Protein, a dimer with a molecular weight of approximately 66 kDa. This protein, which may carry about 3% of the total T_4 and 6% of the total T_3 in serum, was shown to have two binding sites for T_4 with an affinity slightly less than TTR. Lipoproteins, like the mitochondrial proteins, cytoplasmic and endoplasmic reticulum proteins, have not normally been classified as thyroid transport proteins, but have been found to bind L-T_4, D-T_4, rT_3 and triac, the corresponding analogue of T_3, with affinities higher than that of TTR. The T_4 binding sites of the lipoproteins have been predicted to have β-sheet structure.[48]

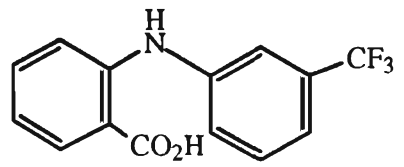
1.7 INTERACTIONS OF NON-THYROIDAL COMPOUNDS

There have been many thyroidal and non-thyroidal compounds that have been found to interact with the T_4-TTR site. These include thyroid hormones and their analogues,[49] dioxins,[50] flavonoids,[43,51] (a group of natural and semi-synthetic compounds which possess a wide range of pharmacodynamic properties) and clinical drugs such as the salicylate derivatives.[33] Members of each class of these compounds were studied and assessed for their ability to displace T_4 from TTR. Another class of compounds that has aroused interest in the last decade are the non-steroidal anti-inflammatory drugs

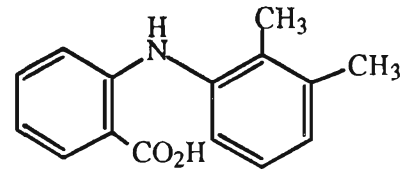
(NSAID).[19] These compounds have been studied extensively by various groups and will be discussed below in more detail.

1.7.1 Non-thyroidal Drugs

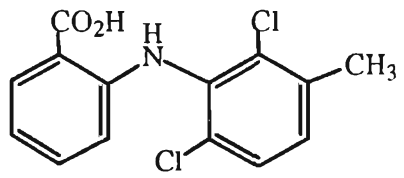
A range of non-thyroidal drugs, commonly prescribed as non-steroidal anti-inflammatory agents and diuretics, are known to displace thyroid hormones, in particular T_4, from transport proteins. An investigation of the clinical implications of TTR displacement has been carried out[52,53] and has led to the design of a new class of thyroid hormone analogues based on modifications of the anti-inflammatory agent flufenamic acid (Fig 1.11). Compounds of the anthranilic acid class of NSAID; flufenamic acid, meclofenamic acid and mefenamic acid were potent displacers of T_4 from TTR (Table 1.1). Within this series, the trifluoromethyl functional group of flufenamic acid appears to be a much better binding moiety than the methyl group of both mefanamic acid and meclofenamic acid. Flufenamic acid binds to TTR with twice the affinity of T_4, although it binds to TBG with four times less affinity then T_4. When comparing substitution patterns, it would appear that substitution at the 3' position is more important than at the 2' position. It is assumed that substitution at the 2' and 6' positions would not cause any significant conformational barriers to rotation around the inter-ring bonds in these molecules.[19] As well as these compounds, sulindac, furosemide, fenclofenac and milrinone (Fig. 1.11) have also been shown to displace labelled T_4 from isolated TTR.[54,55] Milrinone, a positive cardiac intropic agent, has an affinity for TTR 50,000 times greater than for TBG. A crystal structure[56] of the milrinone-TTR complex shows that it binds to the two sites on TTR. The ligand binds 3.5Å closer to the centre of TTR than T_4, with the hydroxyl group situated innermost, the two rings sitting perpendicular while one of the nitrogens interacts with the Lys15 residue at the channel entrance.



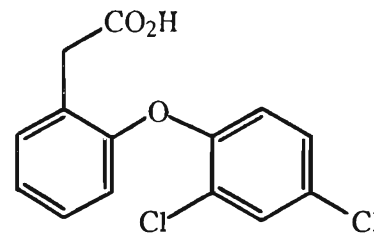
Flufenamic acid



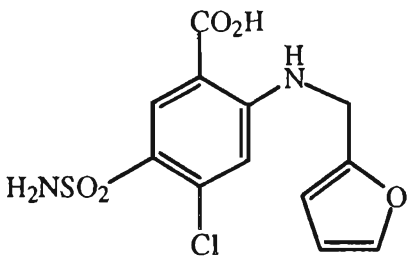
Mefenamic acid



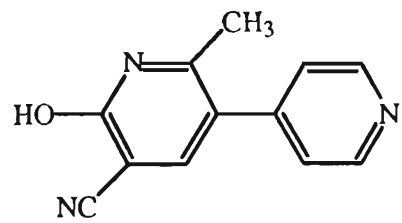
Meclofenamic acid



Fenclofenac



Furosemide



Milrinone

Figure 1.11 Non-thyroidal compounds which displace T_4 from the thyroid hormone transport protein, TTR.

These compounds belong to several structural classes and have different drug actions. From observation these compounds exhibit some broad structural similarities; most of the drugs are acidic, they contain aromatic moieties connected by single bonds or by a bridging atom and the linkage between the two aromatic rings allows for free rotation around the bond.

Table 1.1 Relative affinities of selected drugs for TTR as determined by Munro *et al* [141]

Compound	% Affinity for TTR
T_4	100
Flufenamic acid	174.6
Mefenamic acid	26.5
Meclofenamic acid	20.4
Milrinone	4.8
Sulindac	1.8
Fenclofenac	0.8
Furosemide	<0.024
VCP6	523

Chalmers[53] has synthesised and tested a series of phenylanthranilic acids with various substitution patterns. Structure activity studies conducted on these compounds showed that the carboxylic acid group is essential for binding and that 3' substitution increases affinity. In addition the more lipophilic the substituent at C3' the better the cell uptake of the compound.

Some of these analogues exhibited a high affininity for the T_4 binding sites of TTR. In particular (3-chlorophenyl)anthranilic acid, VCP6 (Fig. 1.12), was shown to have an affinity for TTR five times that of T_4 (Table 1.1).

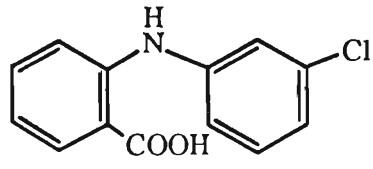


Figure 1.12 Potent anthranilic acid analogue 2-(3-chlorophenyl)anthranilic acid (VCP6).

The crystal structure[57] of TTR-VCP6 shows the carboxyl group hydrogen bonds with Lys15 positioned near the binding site and the 3'-chlorine occupies the site where the iodine of thyroxine normally binds. These results clearly indicate the potential of compounds of this type to act as anti-thyroid drugs. Whilst these studies have delineated the importance of the substituents on the aromatic rings no information is available regarding the nature of the bridging atom.

1.8 SCOPE OF THE WORK

The aim of this work was to prepare a series of aryloxybenzoic acids and arylthiobenzoic acids, and to conduct binding studies in order to provide information regarding the binding of these analogues to TTR. The proposed research will be directed towards the synthesis of drugs capable of binding to thyroid hormone binding proteins with significantly higher affinities than T_4.

Chapter 2

Structure-Activity Relationships

Anyone who isn't confused doesn't really understand the situation.

Edward R. Murrow

2.1 INTRODUCTION

The design of novel thyroid hormone analogues requires an understanding of the structure as well as a knowledge of the molecular interactions of T_4 and T_3, both *in vitro* as well as *in vivo* if the aim of an enhanced bioactive compound is to be attained. To achieve this understanding the following aspects of thyroid hormones are discussed in this chapter: molecular conformations and structure-activity relationships.

2.2 STEREOCHEMISTRY AND CONFORMATIONAL ANALYSIS

Conformational analysis is the study of those parameters that describe the three dimensional arrangement of the atoms in a molecule. The conformational analysis of thyroid hormones is important in understanding their structure activity relationships as it provides information as to how these compounds are able to interact at a receptor site. The hormonal response is initiated by the binding of T_3 to a nuclear receptor but the molecular details of this interaction are unknown. It has been proposed that the structural and stereochemical features of the thyroid hormones are responsible for hormone action.[58] A variety of techniques have been applied to the study of the conformation of thyroid hormones, including NMR, X-ray crystallographic analysis[37] and theoretical methods. The comparison of these methods of conformational analysis has been reviewed by Andrews *et al.*[42]

The possible conformations of the thyronine skeleton are described by the angles; ϕ, ϕ', τ_1, τ_2, and τ_3 (Fig. 2.1). The symbols ϕ and ϕ' represent the angles the planes of the inner and outer rings make with the ether bridge, whilst τ_1, τ_2, and τ_3 describes rotation about the bonds in the alanine side chain.

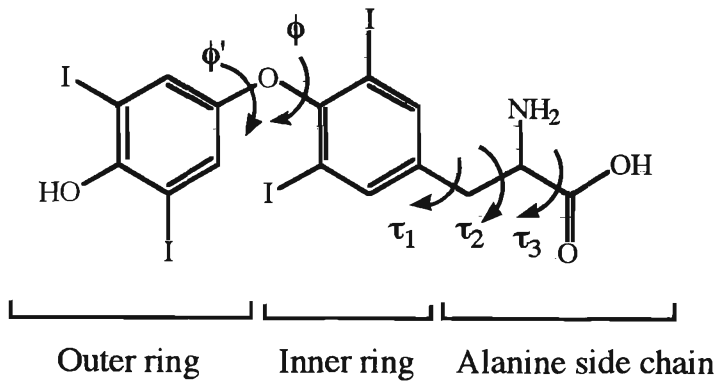


Figure 2.1 Basic structure of T_4 depicting the outer ring, inner ring, alanine side chain and the torsion angles ϕ, ϕ', τ_1, τ_2, and τ_3.

Most of the thyroid hormones and thyroid hormone analogues that have been crystallised have been shown to adopt similar conformations. X-ray crystallographic studies[59] on thyroid hormone analogues reveal that the two phenyl rings are linked by a C-O-C angle of 120°, and that the two phenyl rings are perpendicular to one another (ϕ = 90°, ϕ' = 0°) (Fig. 2.2). In these so called 'skewed' conformations steric interactions between the 3,5-iodo groups and the 2',6' hydrogens are minimised.[60] Thyroid hormones can also adopt 'twist skewed' forms, where $\phi > 90°$ and the inner ring twists so that $\phi' < 0°$.

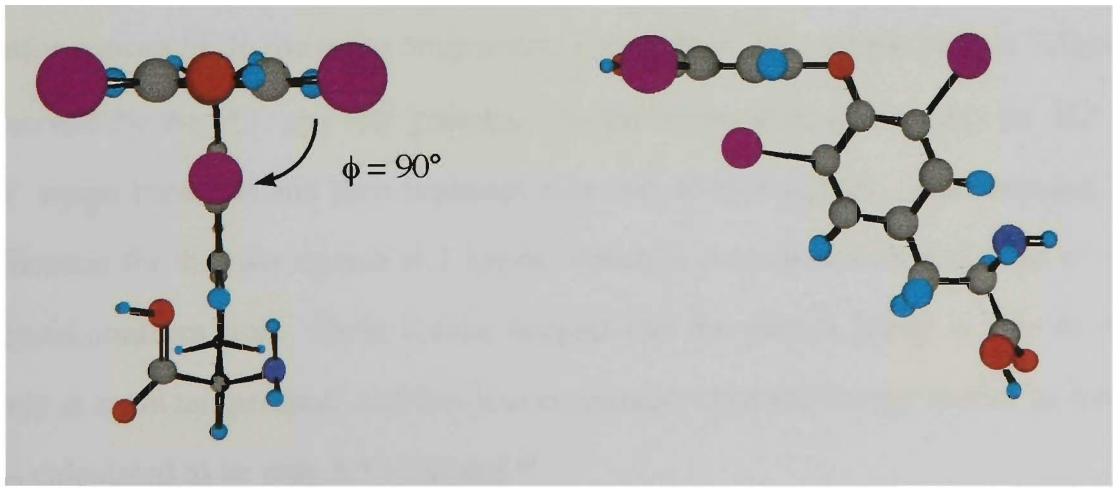


Figure 2.2 The skewed conformation of T_4 where two phenyl rings are perpendicular to one another ϕ=90°.

The skewed conformations leave the 3' and 5' positions non-equivalent relative to the inner ring. For T_3 and other analogues with only one iodine atom on the outer ring, this results in either a distal (away) or proximal (near) conformation of the outer ring substituent (Fig. 2.3).[61] The distal conformation is more active and appears to be the conformation in which the hormones bind to the nuclear receptor.[62, 63]

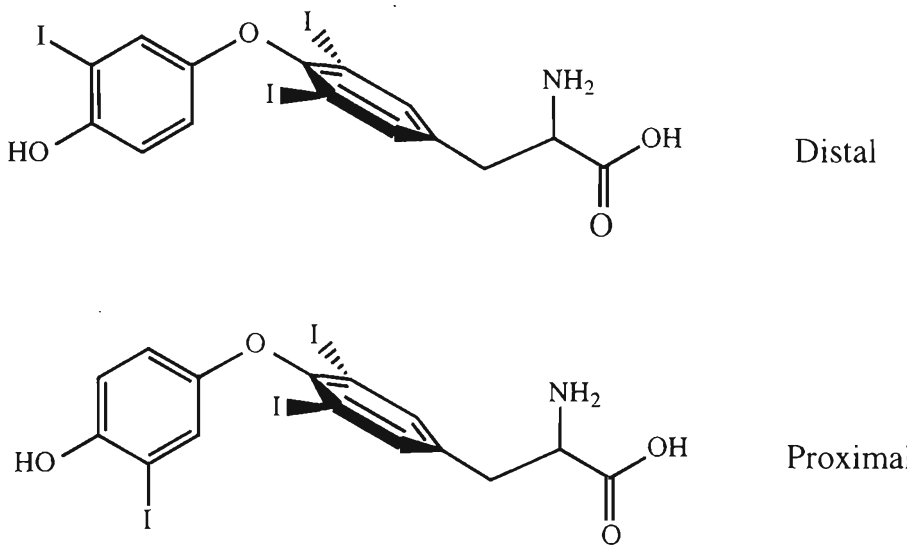


Figure 2.3 Distal and proximal conformations of T_3.

While X-ray crystallographic studies[42] have established the solid state conformations of thyroid hormone analogues, NMR is the main tool for elucidating solution conformations.[64] In the room temperature spectrum of T_4 a single peak at 7.1ppm is observed for the H2' and H6' protons. As the temperature is lowered, the H2' and H6' signal broadens and then separates into two distinct signals. The chemical shift difference for the two signals is 1.1ppm, which is consistent with a skewed or twist skewed conformation. These results suggest that the phenyl group is able to rotate freely at room temperature, and this was confirmed when the energy barrier to rotation was calculated to be only 8.5 kcal/mol.[61]

Two mechanisms have been suggested to explain this rotation about the ether bridge. The first relies solely upon rotation about ϕ', and the second involves co-operative rotation about ϕ and ϕ'. As yet, quantum mechanical calculations have been unable to indicate which is the preferred pathway.[42]

Work by Cody[65,37] has shown that the amino and carboxyl groups of the alanine sidechain prefer to be out of the plane of the ring, i.e. $\tau_1 = 90°$, avoiding any steric clashes with the inner ring.

The three energy minima observed for τ_2 describe relative orientations of the amino group. When $\tau_2 = -60°$ the carboxylic acid group is extended away from the inner ring, and when $\tau_2 = 180°$ the amino group is away from the inner ring. Both functionalities are folded back towards the inner ring when $\tau_2 = 60°$ (Fig. 2.4). Analysis of the proton NMR coupling constants indicates that all three conformers are present in solution.[42] Examples of all three low energy rotamers have been observed in crystal structures of thyroid hormone analogues, which suggests that there are minimal energy differences between the three staggered forms.[37,42]

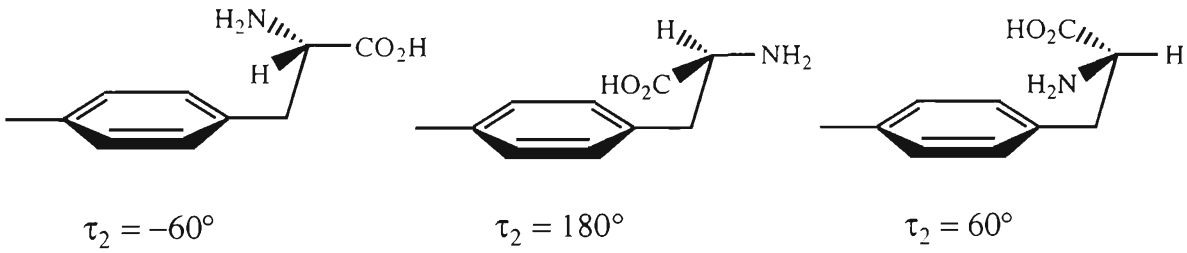


Figure 2.4 The amino acid conformations τ_2 when is -60°, 180° and 60°.

2.3 STRUCTURE-ACTIVITY RELATIONSHIPS

The thyroid hormones cause a multitude of physiological effects, and as result, a variety of assay systems have been developed to measure the relative potencies of the

hormones and their analogues. The rat antigoitre[42], rat oxygen consumption[62,65-67] and tadpole metamorphosis[68] have been the most widely used assays to determine the relative activities of thyroid analogues *in vivo*. By analysing the structure-activity relationships for a large number of compounds, quantitative structure-activity relationships have been developed which can accurately predict the effect of substitution on activity[62,66,68,69,70].

There has been a lot of data gathered on structure activity relationships for thyroid hormones and their analogues.[62,65,67] It has been found that for a compound to exhibit thyromimetic activity, it must be of the general form phenyl-x-phenyl. Single ring compounds such as 3,5-diiodotyrosine show no thyromimetic activity.[71]

The T_4 skeleton can be divided into five sections; the alanine side chain, the inner ring, the bridging atom, the outer ring and the phenolic hydroxyl group (Fig. 2.5). Each of these will be discussed separately in the following sections.

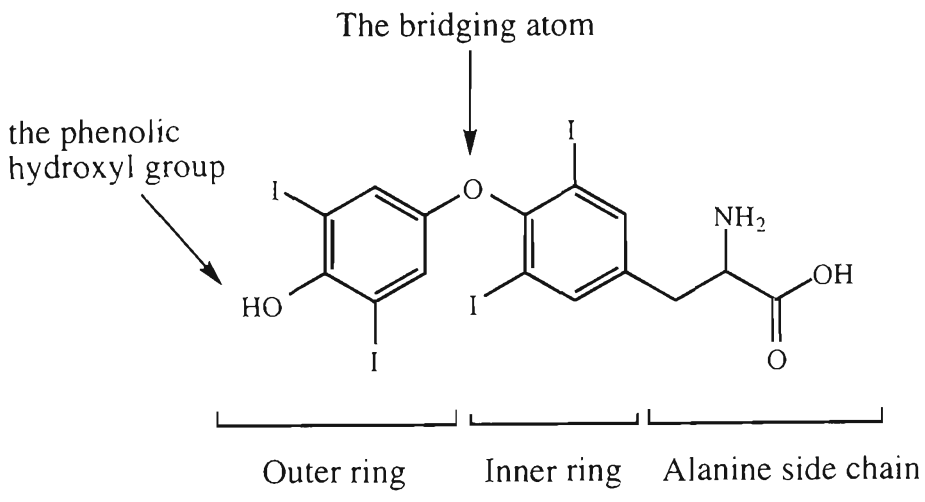


Figure 2.5 The five sections of T_4: the phenolic hydroxyl group, the outer ring, the bridging atom, the inner ring and the alanine side chain.

2.3.1 Alanine Sidechain

The aliphatic sidechain is an important part of the thyroid hormone, it is situated *para* to the bridging group; analogues with the sidechain at other positions show no activity. The length of the sidechain has also been found to be important. When the alanine side chain was replaced by a variety of carboxylic acid derivatives, activity rose from the weakly active formic acid analogues (Fig. 2.6, n=0) to maximal activity for the acetic acid group (n=1) and decreases for the longer propionic (n=2) and butyric acid (n=3) derivatives. Longer chain carboxylic acid derivatives showed negligible activity. No significant biological activity is observed if the carboxylic acid functionality is removed to form an ethylamine side chain.

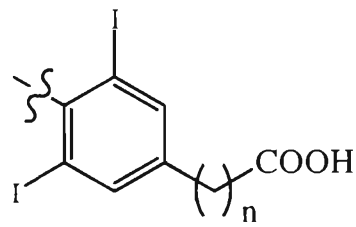


Figure 2.6 Derivatives of T_4 containing different length carboxylic acid chains.

Analogues without the amino group have a higher affinity for the nuclear receptor, however, the thyromimetic activity is less than that of derivatives containing the alanine sidechain. Similarly, analogues with a D-alanine sidechain showed much less activity for the nuclear receptor, resulting in an 87% decrease in activity for T_4 and a 93% reduction for T_3.[62]

2.3.2 Inner Ring Substituents

The rat antigoitre assay has been used to study the effect on activity of replacing the ring iodine atoms with a variety of substituents.[62,68] The replacement in T_3 and T_4 of one or both of the inner ring iodine atoms leads to loss of activity *in vivo*. The reason

for this is the iodine atoms maintain the T_4 skeleton in a conformation suitable for binding, by restricting the motion of the two rings about the bridging atom.[72-75] Analogues with less bulky atoms (eg: Br) in the 3 and 5 positions do not bind as well and are less active. Substituents larger than iodine render the molecule inactive.

Moderate levels of activity have been observed for analogues containing methyl groups in the 3 and 5 positions. 3,5-Dimethyl-3'-isopropylthyronine (Fig. 2.7) crosses the placental barrier in animals and is hormonally active in the fetus after administering it to the mother.[76-78] These types of analogues could be useful in treating foetal thyroid hormone deficiencies since T_4 and T_3 do not cross the placenta.

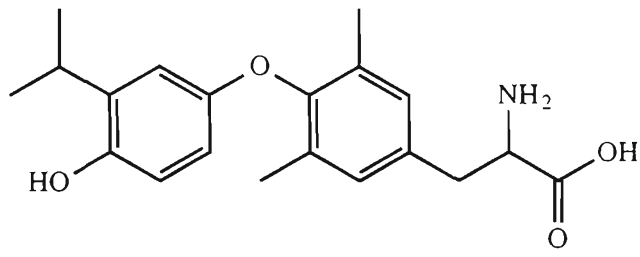


Figure 2.7 3,5-Dimethyl-3'-isopropylthyronine

Alkyl substitution at the 3 and 5 positions with groups bulkier and less symmetrical than methyl groups (eg. isopropyl and *sec*-butyl) results in inactive compounds. Polar group substitution at the 3 and 5 position (eg. nitro[79], amino, carboxyl, cyano[80]) also produced inactive compounds. Similar results have been reported for bulky lipophilic groups such as thioethyl and thiophenyl.[81] The reason why iodine atoms increase activity much more than alkyl groups is believed to be due to the formation of hydrogen bonds and charge transfer complexes between protein binding sites and the iodine atoms.[82]

2.3.3 The Bridging Atom

Rat antigoitre and tadpole metamorphosis assay studies have been used to study the importance of the bridging oxygen atom.[83] Replacing the bridging oxygen with sulfur,[84] or with a methylene group, produces highly active compounds. The activities are 1.5 times higher for the sulfur analogue and 3 times higher for the methylene bridged compound compared to T_4. The ring orientations in these compounds show negligible change from the T_4 and T_3 structures. The sulfur and methylene analogues however, do not participate in the quinoid oxidation and electron transfer reactions which has been proposed as a possible mode of hormone action.[85] This seems to indicate that the three dimensional structure and the receptor fit of the hormone are more important than any role played by intermediate reactions.[83]

When the aromatic rings are attached directly as biphenyl analogues, activity is essentially lost. The inter-ring angle is changed from 120° of the diaryl ether to a 180° orientation in a biphenyl, and the length of the molecule is greatly shortened (Fig. 2.8).

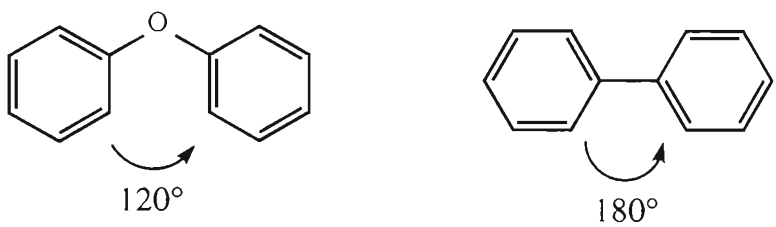


Figure 2.8 Comparison of the inter-ring angle for a diaryl ether and a biphenyl.

2.3.4 Outer Ring Substituents

Rat antigoitre assay studies[62,66,68,69,70] were carried out to show the major effects on biological activity produced by variation of the substituents in the 3' and/or 5' positions on the outer ring. As the size of the halogen in the 3´ position is changed,

activity decreases with decreasing atomic weight (ie. I < Br < Cl < F). This decrease in activity has been attributed to an increase in ionization of the phenolic hydroxy group and a resulting increase in binding to the transport protein. This increase in binding results in a decrease in the release of the hormone from the protein.

The unsubstituted structure 3,5-T_2, which contains no iodine atoms on the outer ring (Fig 2.9) shows low activity. The addition of a methyl group in the 3' position increases the activity by sixteen times compared to 3,5-T_2. Substituting an ethyl or isopropyl group in the 3' position further enhances the activity. If the alkyl group is in the compact form, -$C(CH_3)_x$, the activity is higher than the linear chain analogue. The isopropyl analogue is four times more active than the *n*-propyl substituted compound and is the most active alkyl analogue, being 1.4 times more potent than T_3. The greater activity of the alkyl substituted compounds is attributed to the lack of an iodine atom on the outer ring, thus preventing its metabolism by 5'-deiodinases.[62,82]

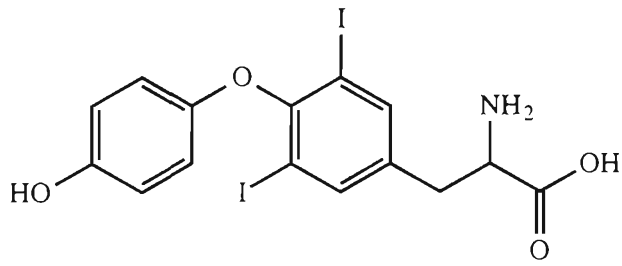


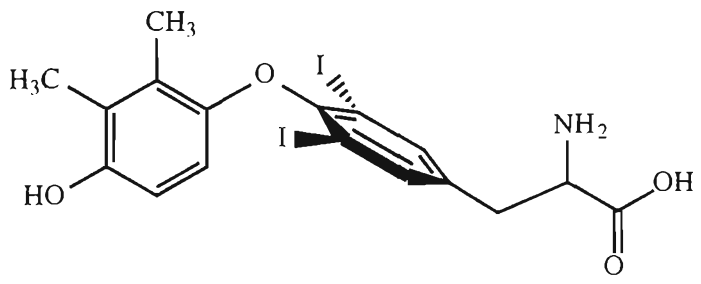
Figure 2.9 The structure of 3,5-T_2.

Polar substituents (eg. nitro, hydroxyl) reduce activity to below that of 3,5-T_2. Analogues substituted at both the 3' and 5' position show lower activity *in vivo* than the corresponding mono-substituted analogue, thus the disubstituted hormone T_4 is less active than the mono-substituted T_3.

Hormonal activity is dependent on the ability of 3,5-diiodo-3'-substituted thyronines to bind to a transport protein or a receptor site which is affected by their ability to

spatially orientate themselves in either the distal or proximal form. The hormone analogue, 3,5-diiodo-2',3-dimethylthyronine, is forced into the distal position due to steric hindrance between the inner ring and the 2'-methyl group (Fig. 2.10a) . The activity of this compound is one half of T_4. The 2',5' substituted analogue (Fig 2.10b) is locked into the proximal spatial position, the less favoured orientation with respect to biological activity, and shows an activity of one hundredth of T_4.

a



b

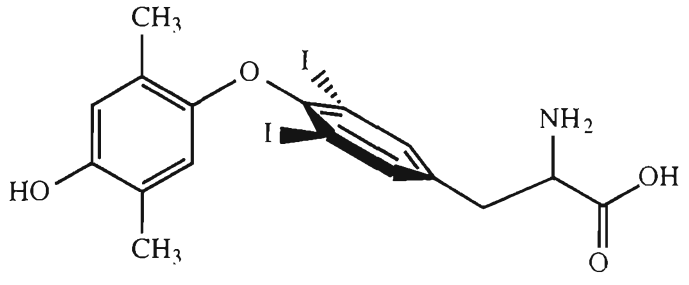


Figure 2.10 a) 3,5-diiodo-2',3'-dimethyl thyronine in the distal form; **b)** 3,5-diiodo-2',5'-dimethyl thyronine in the proximal form

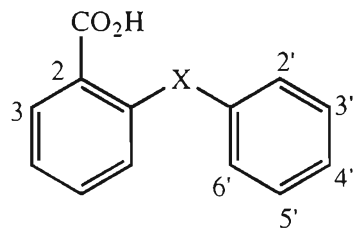
2.3.5 The 4'-Phenolic Hydroxyl Group

The effects of groups other than 4'-hydroxy of the outer ring has been examined.[60,61] 4'-Amino analogues exhibit weak activity compared to T_4, perhaps due to weak hydrogen-bonding of aromatic amino groups. The 4'-deoxy-3'-substituted-3,5 thyronines, (3'= CH_3, CF_3, I), have high activity values *in vivo* which almost parallels the activity values of the corresponding 4'-hydroxy analogues. It has been suggested that metabolic 4'-hydroxylation occurs as an activating step.[86] Addition of a methyl group on the 4'-position renders the compound inactive as metabolic 4'-

hydroxylation is prevented. Since an alkyl ether in the 4'-position is easily cleaved, the 4'-methyl ether analogues show high activity apparently due to their metabolic cleavage. Isomers of T_4 containing the hydroxy group in other positions on the outer ring generated analogues of weak activity.[87]

2.4 STRUCTURE-ACTIVITY OF 2-PHENOXYBENZOIC ACIDS

Now that the relationship between the structure and the activity of the thyroid hormones has been examined, we can use this information as a guide to design compounds that show thyromimetic activity. Chalmers,[53] has studied the structure-activity relationship of phenylanthranilic acids in order to develop compounds with high thyromimetic activity. The current study focuses on analogues of phenylanthranilic acid which contain either oxygen or sulfur bridging atoms (Fig 2.11).



X = O, S

Figure 2.11 General structure of the analogues prepared in this study showing the numbering sequence.

The analogues prepared were chosen to cover a wide range of structure-activity relationships. Analogues containing oxygen and sulfur linked aryl rings were prepared to examine the effect the bridging atom has on thyroid hormone activity. Compounds containing the carboxyl group in either the 2- or 3-position were studied to ascertain whether the position of the acid moiety contributes to thyromimetic

activity. Substituents were placed in the 2', 3', 4' and 5' positions, mono and disubstituted analogues were prepared and the type of substituent varied from the electron donating methyl and methoxy groups to highly electron withdrawing nitro and fluoro groups. The range of analogues chosen would provide general information regarding the structure activity relationships of compounds of this type.

2.5 CONCLUSION

A detailed knowledge of structural requirements for protein-hormone binding is essential when designing hormone analogues as potential therapeutic drugs. When thyroxine binds to a protein receptor, the shape and function of hormone and receptor changes. It is important to investigate the extent and nature of these changes in order to gain an insight into the mode of action of these hormones. Conformational and crystallographic techniques provide a picture of the mechanism by which hormones and their analogues interact at the molecular level. This background information contributes to the design and development of novel therapeutic drugs. The synthesis and binding assay studies of a selection of novel anti-thyroid compounds based on the 2-phenoxybenzoic acid skeleton is described in the following chapters.

Chapter 3

Preparation of Aryloxybenzoic Acids

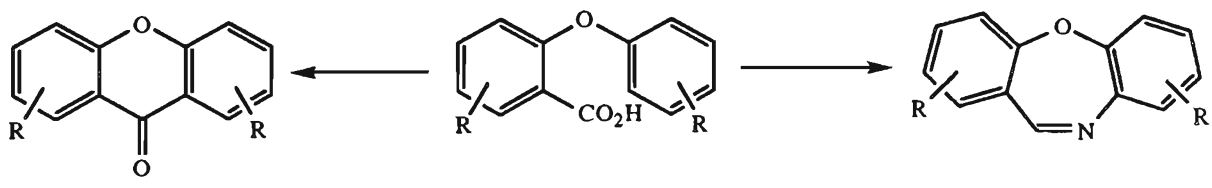
What we anticipate seldom occurs,
what we least expected generally happens.

Benjamin Disraeli

3.1 INTRODUCTION

Phenylanthranilic acid derivatives, which have been shown to bind to the thyroid hormone binding site in TTR, contain a diphenylamine linkage. The thyroid hormone T_4 is a diphenyl ether and therefore compounds containing the phenoxybenzoic acid skeleton will more closely mimic the bridging of the phenyl rings in T_4. Comparison of the binding affinities for TTR between the two classes of compounds will provide information regarding the importance of the bridging atom for thyromimetic activity.

2-Phenoxybenzoic acids (**1**) are useful intermediates in the synthesis of xanthones (**2**) and various tricyclic compounds such as dibenzoxazepines (**3**) (Scheme 1). A considerable number of 2-phenoxybenzoic acid derivatives have been prepared and used as non-steroidal anti-inflammatory drugs,[88] but antithyroid activity of these compounds have not been reported. Derivatives of diphenyl ethers, as well as, diphenyl methanes, diphenyl sulfides and diphenyl amines, have shown properties characteristic of potential antidepressants[89] and can also act as general cerebral activators.[90]



(2) (1) (3)

Scheme 1

The preparation of diaryl ethers has been extensively investigated and there are several methods available for their synthesis. 2-Phenoxybenzoic acids can be prepared by the proceedure of Beringer and co-workers,[91] from the reaction between sodium phenoxide and diphenyliodonium carboxylate (DPIC) in an appropriate solvent (Fig 3.1a). 2-Phenoxybenzoic acids can also be obtained by the reaction of an aryl halide

with a phenol as described by Ullmann[92,93] (Fig 3.1b) or from salicylic acid and an arylhalide as described by Goldberg and collegues (Fig 3.1c).[94]

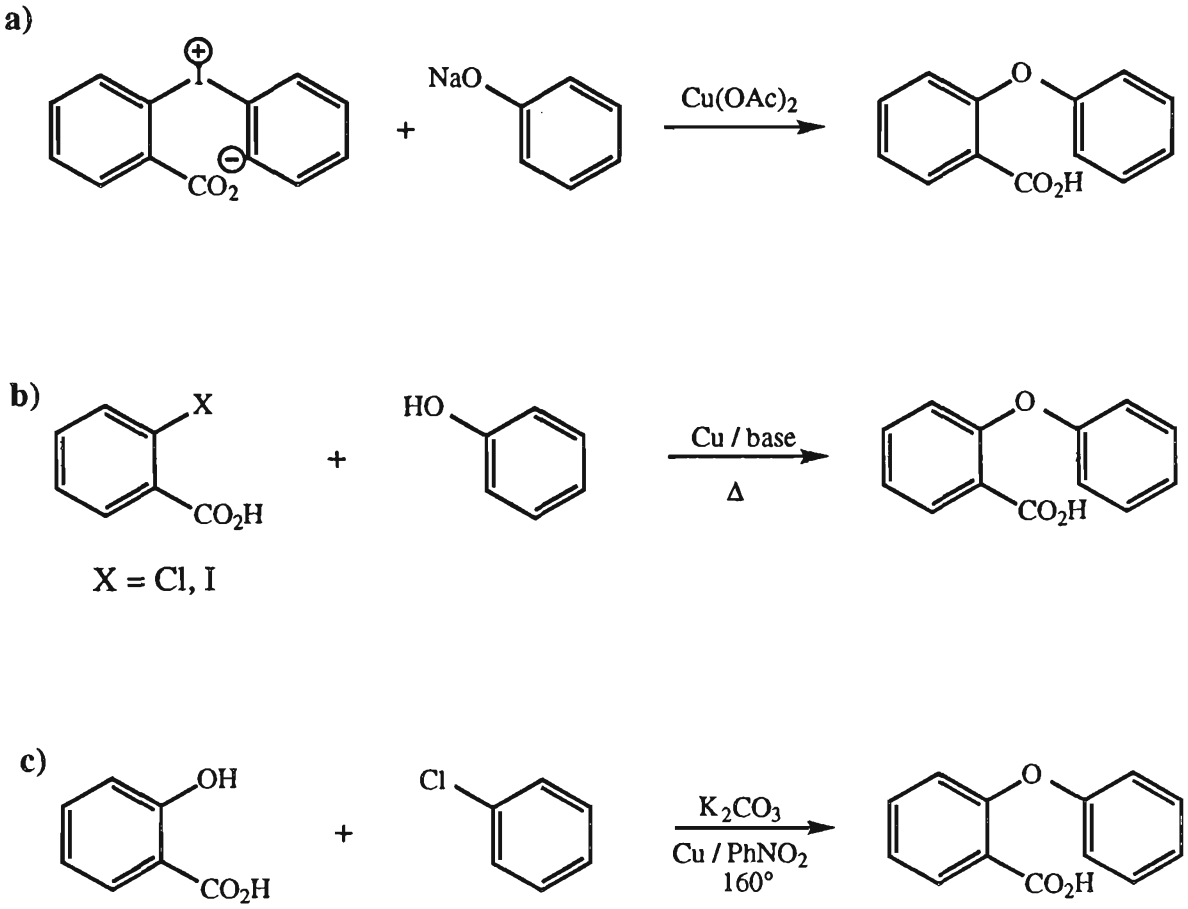


Figure 3.1 Three methods that are used for the synthesis of 2-Aryloxybenzoic acids.

3.1.1 Preparation of Diaryl Ethers Using DPIC

Diaryliodonium salts have been the subject of considerable research interest over the last hundred years due to their ability to arylate a wide variety of nucleophilic substrates.[95] DPIC is highly specific for nucleophilic attack on the carboxylate-bearing ring as the presence of 2-carboxyl group has a marked activating effect.[96] DPIC is a useful reagent for the preparation of 2-substituted benzoic acids under mild conditions which allows its use in the presence of functional groups that are known to be heat sensitive.

The reaction is catalysed by copper (II) salts, in contrast to other aryliodonium reactions which use Cu (I) species.[97,98] In the absence of copper, little or no reaction is observed. For reaction to occur it appears to be necessary for reduction of the copper (II) salt to copper (I) to take place within the reaction.[99-103]

The mechanism by which copper catalyses the reaction of iodonium salts is not well understood. From kinetic evidence,[98] aryl radicals and diaryliodine have been proposed as possible reaction intermediates although Beringer and co-workers[104] argued against the formation of aryl radicals in the reaction of diaryliodonium salts in certain solvents. Another possible mechanism involves the intermediacy of a copper phenoxide.[96] Copper phenoxides are known for their stablility,[105] and when pyridine is used as a solvent it would appear that pyridine complexes to the copper phenoxide.[106] This stabilises the intermediate against thermal decomposition and increases the reactivity of the copper phenoxides.[107] The formation of the copper-pyridine complex also greatly increases its solubility in pyridine.

DPIC is also known for its usefulness as a precursor to benzyne.[108-110] The aryl coupling reactions however, occur at temperatures of 80-100°C, well below those at which benzyne formation and other side reactions become important.

3.1.2 Preparation of Diaryl Ethers Using An Ullmann Condensation

The formation of 2-phenoxybenzoic acids by copper catalysed condensation of a 2-halobenzoic acid and a phenol is a widely used reaction and is traditionally known as the Ullmann coupling reaction. This reaction involves the substitution of the halogen atom of a halobenzene by an aromatic nucleophile to give a diaryl species. Ullmann discovered that the addition of a catalytic amount of powdered copper to the reaction mixture resulted in the facile substitution of nonactivated halogen atoms in good yield.[111,112]

An important factor in the Ullmann synthesis is the choice of solvent since the reaction mixture should remain homogeneous throughout. Immiscibility of the components and precipitation of product on the surface of undissolved reagents lead to low yields. A wide variety of solvents have been used, including pyridine,[113] DMF,[114] nitrobenzene,[115] DMSO,[116,117] *N,N*-dimethyl acetamide[118] and *n*-amyl alcohol.[119] Rewcastle and co-workers,[120] greatly improved yields by use of the phase-transfer catalyst, tris[2-(2-methoxyethoxy)ethyl]amine (TDA-1) in dioxane.

The reaction is catalysed by both Cu (I) and Cu (II) complexes, and reactions employing CuCl, CuBr, CuI, CuO and $Cu(OAc)_2$ have been reported. If non-activated phenols and halides are used more catalyst is required, but the quantity needed is still small. The mechanism by which copper catalyses the Ullmann reaction is still unknown, but one popular theory is that, in the case of the 2-halobenzoic acid, a copper chelate[121] is formed as the reactive species. The coordinate bond between the halogen and the copper atom enhances the polarization of the carbon-halogen bond and facilitates nucleophilic attack by the phenol at the ring carbon atom.

The reaction is usually carried out at high temperatures (>180°C) which can pose problems when working with heat sensitive functional groups. Pauson[122] described the reaction of phenoxide anion with (chlorobenzene)$Mn(CO)_3$ under mild conditions to give the required diaryl ether. This procedure is particularly useful in the presence of fragile substituents.

3.2 PREPARATION OF 2-ARYLOXYBENZOIC ACIDS

A series of 2-aryloxybenzoic acids were prepared and the yields obtained are shown in Table 3.1. Two methods were investigated for the preparation of these compounds. Method 1 involves a modified Ullmann-Goldberg proceedure,[92,123] which uses a copper catalyst for the condensation of a phenol with either 2-chloro or 2-iodobenzoic acid at temperatures greater than 180°C. At least one equivalent of base, usually

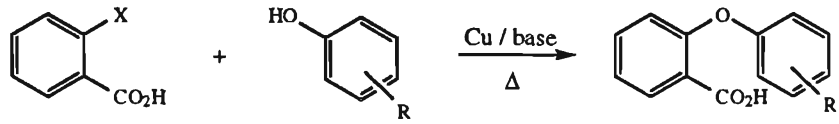
potassium carbonate,[124] is used in the reaction to neutralise the acid liberated, otherwise the reaction slows and decarboxylation tends to occur.[125] Method 2 utilised the condensation of DPIC and a sodium phenoxide in the presence of a catalytic amount of copper (II) acetate, in a mixture of pyridine and picoline.

All of the compounds were prepared in good to moderate yield with Method 1 giving significantly better yields. The poor yields or total lack of product obtained using Method 2 was due to the formation of xanthone (**2**). It was thought that this by-product was formed *via* intra-molecular cyclisation during the work-up procedure. In order to prevent this reaction, the crude reaction mixture was treated with acidic methanol and the corresponding methyl ester was isolated. Hydrolysis of the ester using potassium hydroxide gave the desired acid.

Where a sufficient amount of material for the binding assay was obtained using one method, the preparation of the compound using the alternative method was not attempted.

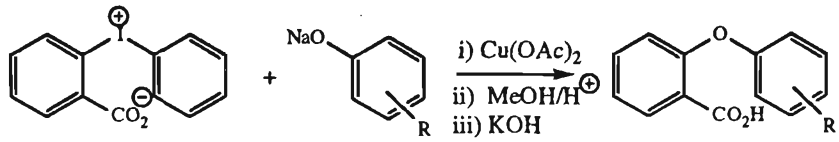
Table 3.1 The preparation of 2-aryloxybenzoic acids using the Ullmann (**1**) and DPIC (**2**) methods.

Method 1



X = Cl, I

Method 2



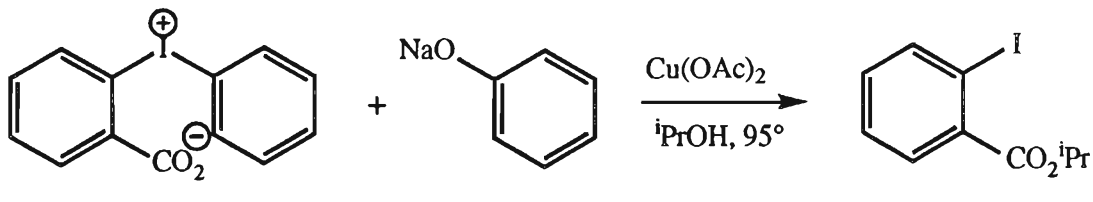
		Yield (%)	
Compound	R	Method 1	Method 2
4	H	*	26
5	3-CH_3	40	*
6	4-CH_3	43	*
7	2,5-CH_3	53	0
8	3,5-CH_3	*	13
9	2,3-CH_3	48	0
10	2,4-CH_3	57	*
11	3-Cl	*	17
12	4-Cl	60	12
13	2,3-Cl	*	24
14	3,4-Cl	31	5
15	3,5-Cl	32	18
16	4-F	49	*
17	3-F	39	*
18	2-F	42	*
19	3-CF_3	52	0
20	3,5-CF_3	37	0
21	3-NO_2	55	0
22	4-NO_2	61	0
23	4-CN	30	0
24	3-I	*	11
25	4-OCH_3	*	22

* : Compounds that were not attempted using the Method.

3.2.1 Reaction Conditions

A number of modifications were investigated in order to improve the yields obtained using Method 2. Condensation of a phenol with DPIC (Method 2) occurred at relatively mild temperatures (80-100°C). Temperatures below 70°C usually resulted in the recovery of the starting materials and increasing the temperature above 100°C produced a dark sticky mass resembling tar.

The solvent generally used for the preparation of phenylanthranilic acids involving the reaction of DPIC and substituted anilines is isopropanol.[53] The use of isopropanol for the reaction between DPIC and a phenol resulted in the almost exclusive formation of isopropyl 2-iodobenzoate (Scheme 2). The use of an aprotic solvent such as DMF was investigated but this resulted in very low yields of product from a mixture which contained both starting material and iodobenzoic acid. Pyridine was found to give better yields and fewer tarry by-products. The addition of 2-picoline as a co-solvent gave even better yields. It was not possible to use neat 2-picoline due to the insolubility of the DPIC, sufficient pyridine was therefore added to solubilise the reactants. Another advantage of using this solvent system was that the reaction time was reduced considerably from 12-16 hours in pyridine to only 3-4 hours in the pyridine/picoline mixture. Precipitation of the product after about 3 hours indicated that the reaction had stopped. No improvement in yield was observed if heating was continued after the precipitate had formed.



Scheme 2

3.2.2 Substituent Effects

Using Method 1 the products were formed in varying yields (30-61%) (Table 1). This variation in yield does not appear to be due solely to the electronic effects of the substituents attached to the phenol. It would be expected that phenols with electron donating substituents would have been more nucleophilic than those with electron withdrawing substituents and thus leading to higher yields of product. In fact phenols containing electron withdrawing groups generally gave higher yields than those containing electron donating groups. Thus, 2-(4-nitrophenoxy)benzoic acid (**22**) was formed in better yield (61%) than 2-(4-methylphenoxy)benzoic acid (**6**) (43%). This may be due to the ease with which the phenolate anion is formed in the reaction, since phenols with electron withdrawing substituents have a lower pK_a (4-nitrophenol: pK_a = 7.15) than phenols with electron donating groups (4-methylphenol: pK_a = 10.26).

As expected phenols containing two electron donating methyl groups (**7**, **9**, **10**) gave higher yields (48-57%) than phenols containing a single methyl group (**5**, **6**) (40-43%) due to the increase in nucleophilicity of the doubly substituted phenols. There does not appear to be any correlation between substituent effect (pK_a or nucleophilicity) and yield for the phenols containing electron withdrawing groups.

Steric effects generally did not influence the reactivity of the phenols, compounds containing a methyl group in the 2-position (**7, 9, 10**) gave similar yields to phenols with 3- and 4-methyl groups (**5, 6, 8**). The only steric effect observed was in the attempted preparation of the 2,6-dimethyl derivative, irrespective of the reaction conditions used, no product was obtained.

3.2.3 Spectral Features of 2-Aryloxylbenzoic Acids.

The compounds synthesised were analysed using ^{1}H and ^{13}C NMR, IR and mass spectroscopy. NMR was particularly useful for confirming the nature of the reaction products. The carboxylate bearing aromatic ring of aryloxybenzoic acids display a consistent and characteristic pattern of resonances in the ^{1}H NMR spectra. The H_6 proton is substantially deshielded by the carboxyl group and is evident as a doublet of doublets due to coupling with the protons in the 4- and 5-positions. H_4 and H_5 appear as triplets caused by coupling to their adjacent protons, at chemical shifts of approximately δ = 7.3 and 7.5ppm respectively. H_3 is the most upfield aromatic proton due to the electron donating effects of the oxygen group. In the spectrum of the unsubstituted phenylsalicylic acid, the equivalent protons $H_{2'}$ and $H_{6'}$ are situated the furthest upfield and appear as doublet of doublets. The carboxylic acid proton is not seen in $CDCl_3$. Figure 3.2 shows a typical ^{1}H NMR of a 2-aryloxybenzoic acid.

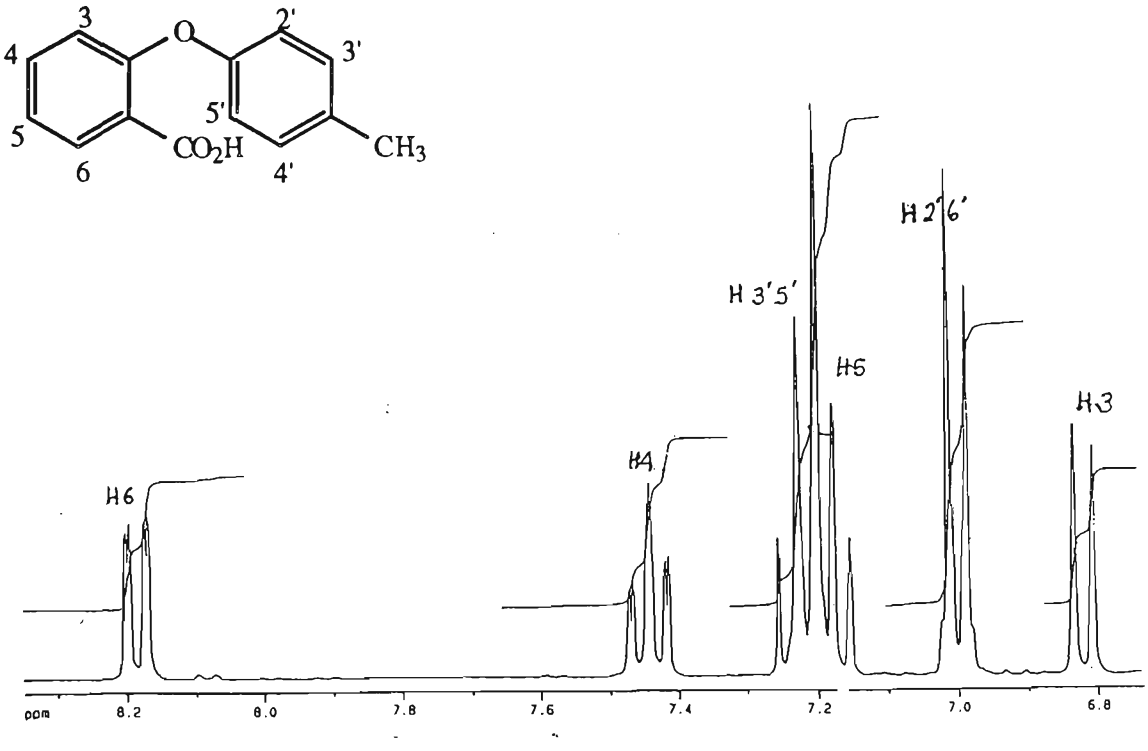
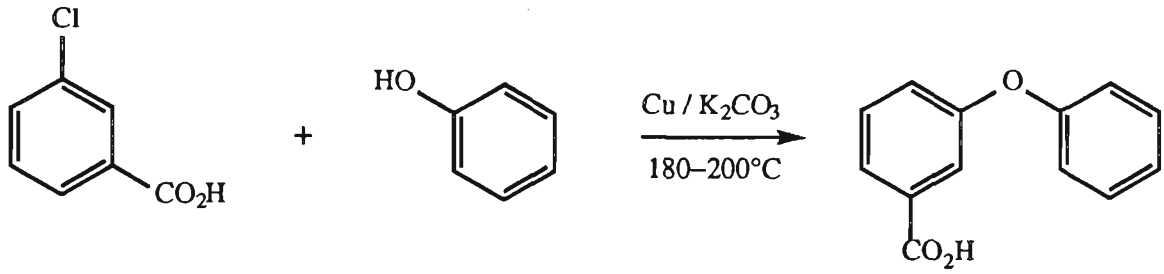


Figure 3.2 A typical ^{1}H NMR spectrum of 2-(4-methylphenoxy)benzoic acid (**6**) at 300 MHz.

3.3 PREPARATION OF 3-PHENOXYBENZOIC ACID (26)

The phenylanthranilic acid derivatives which have been tested for potential anti-thyroid activity all contain a carboxylic acid in the 2-position. In order to investigate the importance of the position of the carboxylic acid for thyromimetic actvity, 3-phenoxybenzoic acid (**26**) was synthesised.

3-Phenoxybenzoic acid (**26**) was prepared in reasonable yield (56%) using a modified version of the Ullmann-Goldberg reaction, in which sodium phenoxide was coupled with 3-chlorobenzoic acid in the presence of a copper catalyst (Scheme 3).



(**26**) 56% yield

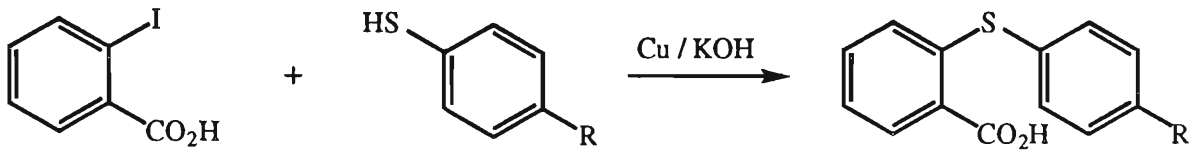
Scheme 3

3.4 PREPARATION OF ARYLTHIOBENZOIC ACIDS

Arylthiobenzoic acids, like aryloxybenzoic acids, have been extensively investigated and are important intermediates to thioxanthenes and many polycyclic systems which show anti-inflammatory activity.[126] Thioxanthene derivatives have also been used in the treatment of various forms of mental illness such as schizophrenia[127] and show potential anti-depressant activity.[128] Arylthiobenzoic acids have not been tested for thyromimetic activity.

The synthesis of arylthiobenzoic acids has been reported using a similar procedure to that used for the preparation of aryloxybenzoic acids, involving reaction between

iodobenzoic acid and a thiophenol, catalysed by copper in aqueous potassium hydroxide.[129,114] Using this procedure 2-(4-fluorothiophenoxy)benzoic acid (**27**) and 2-(4-methoxythiophenoxy)benzoic acid (**28**) were prepared in high yield (Scheme 4).



27 R = F 80%
28 R = OMe 65%

Scheme 4

In order to determine whether thiophenols reacted with DPIC in a similar fashion to phenols and arylamines to form arylthiobenzoic acids, DPIC was treated with 4-methoxythiophenol using similar conditions to that described earlier for the preparation of the aryloxybenzoic acids. A tarry residue was obtained from which no identifiable products could be isolated.

An alternative procedure involving palladium coupling similar to that described by Migita[130] for the preparation of diarylthioethers was also attempted. In this case starting material was recovered indicating that the presence of the carboxyl group has a deactivating effect.

3.4.1 Spectral Features of 2-Arylthiobenzoic Acids.

The carboxylic acid bearing aromatic ring of compounds (**27**) and (**28**) display consistent and characteristic resonances and compare well with the signals for the corresponding aryloxybenzoic acids. It is observed that the sulfur atom has a far greater deshielding effect on $H_{2'}$ and $H_{6'}$ than does an oxygen atom. This is evident when comparing compounds (**27**) and (**28**) with the corresponding oxygen

analogues, (**16**) and (**25**). The $H_{2'}$ and $H_{6'}$ signals appear further downfield from the $H_{3'}$ and $H_{5'}$ signals whereas for the corresponding aryloxybenzoic acids the $H_{3'}$ and $H_{5'}$ signals are downfield from the $H_{2'}$ and $H_{6'}$ signals (Fig 3.3). This effect can be readily observed in the NMR spectra of 4-fluorothiophenol and 4-fluorophenol.[131] The extra splitting observed for $H_{2'}$ and $H_{6'}$ in (**27**) is due to fluorine coupling [J_{HF} = 5.7 Hz (meta) and J_{HF} = 9 Hz (ortho)] and confirms the assignments.

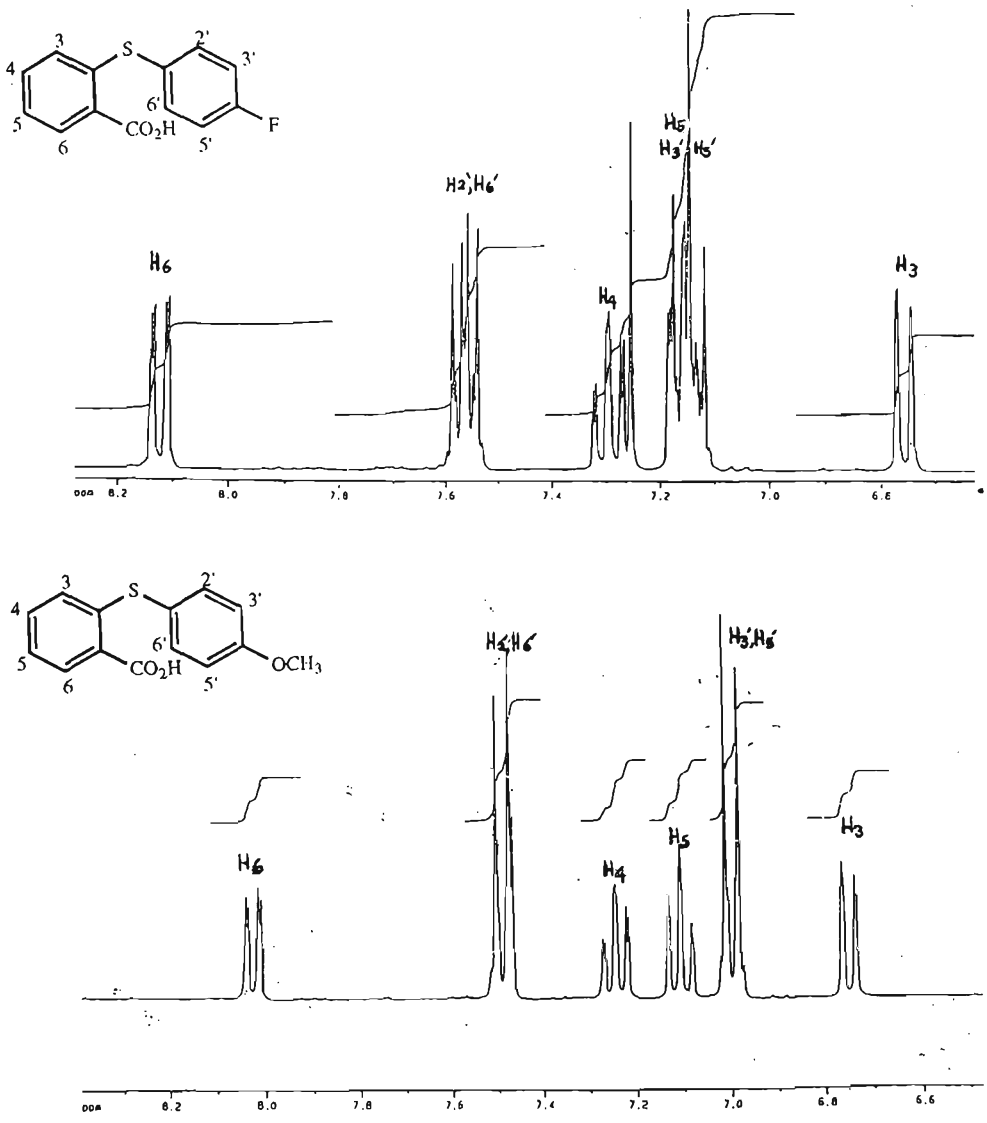


Figure 3.5 A typical ^1H NMR spectra of 2-(4-fluorothiophenoxy)benzoic acid **27** 2-(4-methoxythiophenoxy)benzoic acid **28** using a 300 MHz NMR spectrometer

3.5 CONCLUSION

This chapter describes the synthesis of 2-aryloxybenzoic acids using a modified Ullmann method and a procedure employing a diphenyliodocarboxylate salt. The former method gave the products in higher yields. Substitution on the phenol did not appear to have a marked effect upon the yield of the reaction. Using the modified Ullmann procedure it was also possible to prepare 3-phenoxybenzoic acid and substituted arylthiobenzoic acids in good yield.

Chapter 4

Binding of 2-Aryloxybenzoic Acids and Related Compounds to TTR

We dissect nature along lines laid down
by our native language.
Language is not simply a reporting device
for experience but a defining framework for it.

Benjamin Whorf

4.1 INTRODUCTION

Transthyretin (TTR) is a highly acidic protein comprising of four identical 127 residue subunits linked together to form a symmetrical structure with a channel running through the center, which contains the thyroxine binding site.[19] The three dimensional crystal structure has been well characterised and reveals multiple modes of binding which helps to explain the electron density observed in the binding site.[41,43]

Most thyroid binding proteins are difficult to isolate in an active form because of their low concentrations in blood plasma. Thyroxine binding globulin (TBG) has the lowest plasma concentration and is the least stable, thus isolation and purification is difficult. However, TTR is found in abundance and remains quite stable which makes it easier to isolate. The typical plasma level of TTR is 300 mgL^{-1}, therefore complete recovery from a 250 mL pack of plasma should provide 75 mg of TTR.

4.1.1 Isolation of TTR From Plasma

TTR was purified to homogeneity in 1965 by Oppenheimer[132] and since then there have been many publications on methods for the isolation of TTR including polyacrylamide gel electrophoresis, precipitation and chromatography.[133-136] The precipitation method involves removing polymerised fibrinogen from plasma followed by washing with sodium chloride and phenol to precipitate unwanted proteins. After centrifugation, washing with phosphate buffer and elution through a Sephadex G-75 column pure TTR is obtained.[137,138] Column chromatography has been one of the most popular techniques used for the isolation of TTR from plasma. The procedure involves the use of three successive chromatographic steps: cation exchange, affinity chromatography and gel filtration.[139] This proceedure has been modified and undertaken successfully in the labortatory by Munro,[19] Chalmers[53] and Duggan,[82] therefore it was the method adopted in this study to obtain pure TTR.

The first of the three columns employed an anion exchange resin which binds to TTR. The column most commonly used is one in which retinol binding protein (RBP) is attached to a solid support such as Sepharose-4B.[140] At low ionic strength the TTR-RBP complex binds to the Sepharose support, while most of the unwanted proteins are eluted from the column and discarded. Elution of the column with a sodium chloride gradient from 0.15 M to 0.50 M is usually used to increase the ionic strength and elute the TTR-RBP complex. In this study, an increase in ionic strength from 0.15 M to 0.30 M was used to elute the TTR complex. This stepwise increase was more rapid than a gradient and was found to give good resolution of the protein. The salt concentration was only taken up to 0.30 M instead of 0.50 M as this was found to be adequate to elute the TTR with good resolution. Because TTR contains a high number of acidic residues, it eluted after most of the bound protein. A fluorimeter was used to detect the fractions containing the fluorescent RBP-TTR complex. A final rinse using 0.15 M sodium chloride was used to remove any impurities and to regenerate the column.

The crude complex was then loaded onto a Blue Sepharose CL-6B dye affinity column which selectively binds the impurities HSA (Human Serum Albumin) and RBP whilst free TTR is eluted. The column results in the dissociation of the TTR-RBP complex[139] and the absence of fluoresence in the collected fractions indicates the cleavage of RBP from the complex. The RBP starts to elute immediately after the TTR and continues to do so until the column is cleaned with a high salt and thiocyanate buffer.

The final stage of the purification involved loading the collected protein onto a long gel filteration column which separates the TTR from any contaminants on the basis of size. The gel filteration media used in this study was Ultragel AcA-34. A buffer solution was used to elute the protein and SDS-PAGE was used to identify and check the purity of TTR. The pure TTR was lyophilised and stored at 4°C. This procedure afforded

23.0 mg of purified TTR from approximately 177 ml of plasma for use in binding studies.

4.2 TTR BINDING STUDIES

Studies have been performed on structurally diverse compounds to assess their ability to bind to TTR by displacing thyroxine. These studies have prompted research into the factors important for binding to TTR. There is an abundance of information about binding studies of thyroid hormone and their analogues to TTR,[37,49,62] however not much is known on the binding affinities of non-thyroidal hormone compounds. Munro,[141] Chalmers[53] and other workers[33,34] have investigated a class of non-thyroidal compounds and this work has resulted in both qualitative and quantitative models for the binding of phenylanthranilic acids to TTR. These compounds are effective in displacing T_4 from the binding site of TTR. In the current study the binding of a new series of compounds is investigated.

This chapter reports the structure-activity relationships for the binding of a series of aryloxybenzoic acids and thioaryloxybenzoic acids to TTR isolated from human serum. The assays were carried out by John Barlow in the Unit of Endocrinology at the Alfred Hospital.

4.2.1 Experimental Conditions

The TTR was isolated from human blood plasma using the method described by Dwulet and Benson. Binding affinities of the aryloxybenzoic acids and arylthiobenzoic acids were determined relative to $[^{125}I]T_4$. The protocol has been used to investigate the binding of a number of drugs and other compounds to TTR.[141] The affinity of each compound was measured by its ability to displace radiolabelled T_4 from the TTR binding site at a range of concentrations. The preparations bound T_4

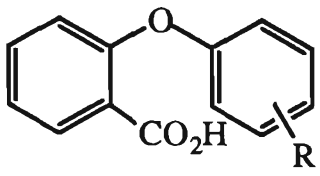
with an affinity of 64.9 +/- 9.9 nM (mean +/- SD, N = 4) at 0 - 4°C with approximately 1 mol binding sites per mol protein.

10 μg Purified TTR was incubated overnight at 4°C in 1 ml Tris buffer, pH 7.4 containing 20,000 cpm [^{125}I] - T_4 (DuPont-NEN, 135-165 μg) and competitor T_4 or drug. The incubation was terminated by the addition of 0.5 ml dextran:charcoal (0.1 : 1 %) followed by rapid centrifugation as described by Munro *et al.*[141] The activated charcoal binds to free [^{125}I]T_4 in solution separating it from the bound form. Residual unbound radioactivity was determined in the charcoal pellet and the fraction of [^{125}I]T_4 bound calculated by subtraction from the radioactivity found in identical incubates without any added competitor. The relative potency of each drug was determined using a five point competition curve generated with drug concentrations between 1-100 μM. The curve determined for each drug was compared with a similar curve for T_4 determined over the concentration range 0.01-1 μM.

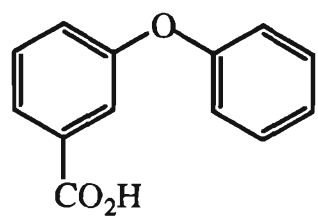
4.2.2 Results

Twenty-five compounds were tested for their ability to bind to TTR and the relative binding affinities of all compounds tested are summarised in Table 4.1. Binding affinity is reported as the negative logarithm of the concentration required to produce 50% inhibition of [^{125}I]T_4 binding (pIC_{50}). All compounds were initially assayed at a fixed concentration of 10^{-4} M to ascertain which compounds show relatively good binding to TTR. Full displacement curves were determined for those compounds that showed binding greater than 50% T_4. Those that showed binding at concentrations of $> 10^{-4}$ M were not trialed further.

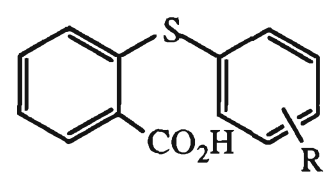
Table 4.1 TTR binding data for 2-aryloxybenzoic acids and 2-arylthiobenzoic acids. Full displacement curves were generated for those compounds that are shown in boxes.



(4)-(25)



(26)



(27) (28)

Compound	R	pIC_{50}
4	H	$>10^{-4}$M
5	3-CH_3	$>10^{-4}$M
6	4-CH_3	$>10^{-4}$M
7	2, 5- CH_3	4.22
8	3, 5- CH_3	4.52
9	2, 3 -CH_3	5.07
10	2, 4 -CH_3	5.22
11	3-Cl	$>10^{-4}$M
12	4-Cl	$>10^{-4}$M
13	2, 3 -Cl	$>10^{-4}$M
14	3, 4 -Cl	$>10^{-4}$M
15	3, 5- Cl	$>10^{-4}$M
16	4-F	$>10^{-4}$M
17	3-F	$>10^{-4}$M
18	2-F	$>10^{-4}$M
19	3-CF_3	$>10^{-4}$M
20	3, 5- CF_3	4.25
21	3-NO_2	$>10^{-4}$M
22	4-NO_2	$>10^{-4}$M
23	4-CN	4.39
24	3-I	5.09
25	4 -OCH_3	$>10^{-4}$M
26		5.04
27	4-F	4.97
28	4- OCH_3	4.97

The compounds can be divided into three classes, 2-aryloxybenzoic acids (**4**)-(**25**), 3-aryloxybenzoic acid (**26**) and 2-thioaryloxybenzoic acids (**27**)-(**28**). Each of these classes is discussed below.

4.2.3 2-Aryloxybenzoic Acids (4)-(25)

Twenty-two 2-aryloxybenzoic acids (**4-25**) were tested for their ability to bind to TTR. Seven of these compounds (**7-10, 20, 23-24**) were found to bind at greater than 50% T_4 when trialled at a fixed concentration of 10^{-4}M and full displacement curves for these compounds were determined and are shown in Figure 4.1. As can be seen all of the compounds were found to bind to TTR less strongly than the natural ligand T_4. In general these compounds are between 1.5 and 2 orders of magnitude less active than T_4.

The pIC_{50} of these eight compounds range from 4.22 for 2-(2,5-dimethylphenoxy)benzoic acid (**7**) to 5.22 for 2-(2,4-dimethylphenoxy)benzoic acid (**10**). Of these eight compounds five are disubstituted (**7-10, 20**) and five have substituents in either the 3- and/or 5-position (**7-9, 20, 24**). These compounds are therefore structurally similar to T_4 which has iodine atoms in the 3- and 5-position. These results are consistent with previous studies which have investigated analogues of T_4 (see Section 2.3.2). When the iodine atoms on the inner ring of T_4 are replaced with methyl groups the resultant compounds show anti-thyromimetic activity although they are less active than T_4. The 2-aryloxybenzoic acids with methyl groups in the 3- and/or 5-position (**7-9**) showed moderate activity. It has also been shown that replacing the iodine atoms in T_4 with halogens smaller than iodine results in inactive compounds. In our study, 2-aryloxybenzoic acids with either chlorine or fluorine atoms (**11-18**) attached to the ring showed no activity. These results support the earlier findings that not only is the position of substitution important but that steric and electronic properties also influence binding.

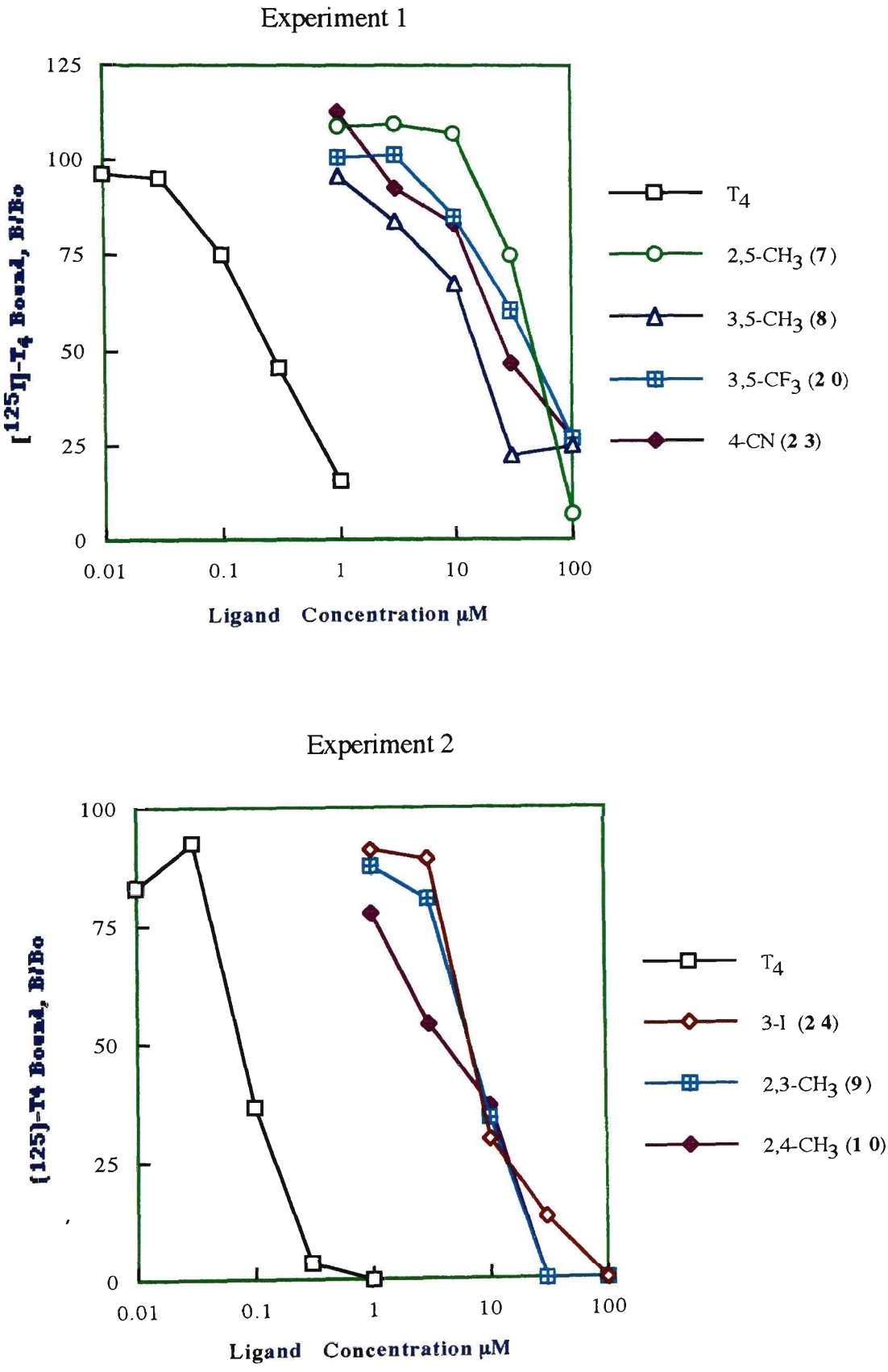
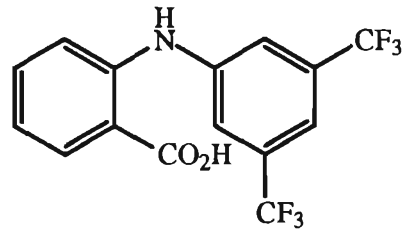


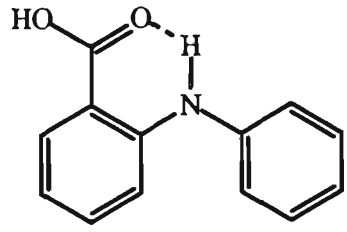
Figure 4.1 Full displacement curves for two experiments comparing the binding of T_4 to TTR with the 2-aryloxybenzoic acids (**7-10, 20, 23, 24**).

A series of phenylanthranilic acids, structurally similar to the 2-aryloxybenzoic acids in this study, have been previously tested for binding to TTR. In all cases the phenylanthranilic acids were significantly more active than the 2-aryloxybenzoic acids. For example, 2,5-ditrifluoromethyphenylanthranilic acid (**29**) (pIC_{50} = 7.09) was almost three orders of magnitude more active than the analogous 2-[3,5-bis(trifluoro-methyl)phenoxy]benzoic acid (**20**) (pIC_{50} = 4.25)



(**29**)

The lower activity of the oxygen bridged compounds clearly shows that the bridging atom between the two aromatic rings makes a contribution to binding interactions with TTR. A possible reason for this difference in activity is that the nitrogen atom can act as a hydrogen bond donor, whereas the oxygen atom can only act as a hydrogen bond acceptor.[19,53] Whether the N-H bond in the anthranilic acids form a hydrogen bond with TTR and contribute to increased binding or whether an intramolecular hydogen bond between the N-H and the carbonyl holds the molecule in a preferred conformation (**30**) for binding is not clear.



(**30**)

4.2.4 3-Phenoxybenzoic Acid

It has been shown that the presence of an carboxylic acid is crucial for significant binding since compounds without this acidic functionality or when the group is an methyl ester show no significant binding at the highest concentration tested.[19,53] Very little data however, is available concerning the effect the position of the acid group has on overall binding affinity. 3-Phenoxybenzoic acid (**26**) was found to be one of the more potent compounds tested (pIC_{50} = 5.04). This is significantly more potent than the corresponding 2-phenoxybenzoic acid (**4**) and it is almost as active as anthranilic acid (**31**) (pIC_{50} = 5.52). More compounds need to be prepared and tested to ascertain the importance of the position of the acid group for thyromimetic activity.

4.2.5 2-Thioaryloxybenzoic Acids

To further investigate the relationship between the ring junction atom and binding activity, compounds with a sulfur bridge were prepared and tested for TTR binding. 2-(4-Fluorophenylthio)benzoic acid (**27**) and 2-(4-methoxyphenylthio)benzoic acid (**28**) showed moderate binding activity (pIC_{50} = 4.97 for both) and their full displacement curves are shown in Figure 4.2. Both these species were found to be more potent than their corresponding oxygen analogues (**16** and **25**) which did not bind to a significant extent at 10^{-4} M. To clearly demonstrate a relationship between a sulfur linkage and increased TTR binding potential more analogues need to be tested.

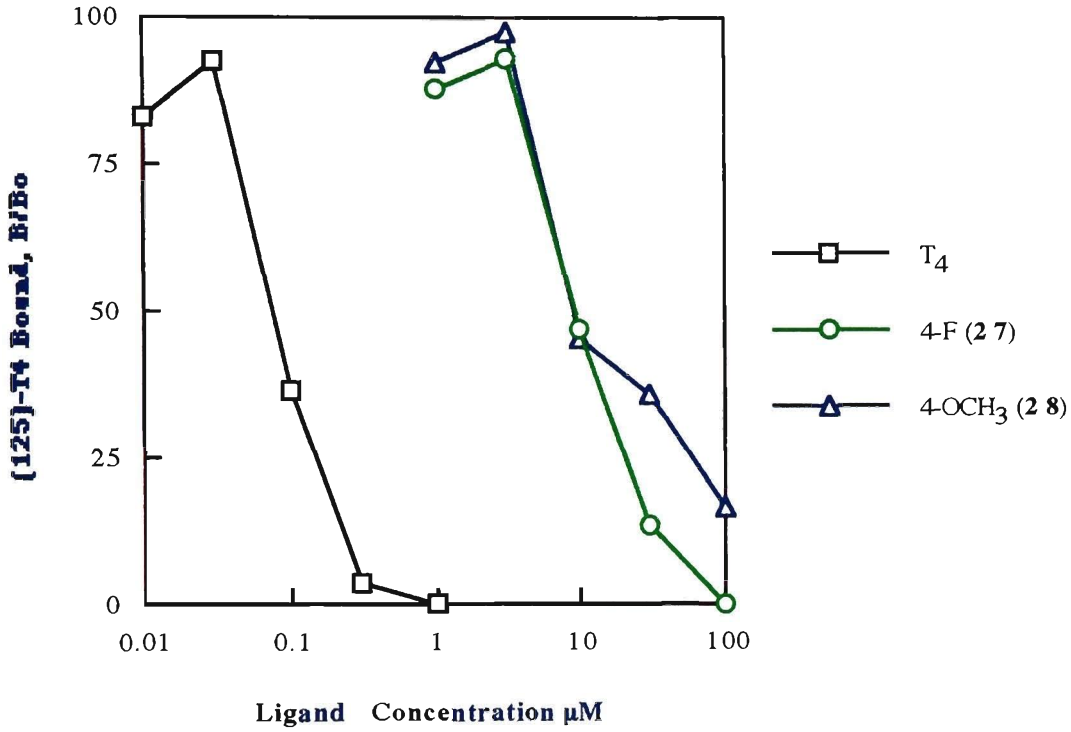


Figure 4.2 Full displacement curve comparing the binding of T_4 to TTR with 2-thioaryloxybenzoic acids (**27**, **28**).

4.3 CONCLUSION

This chapter describes the investigation of the binding of aryloxylbenzoic acids and other structurally related compounds to the plasma transport protein, TTR. The affinity of 25 aryloxybenzoic acids for TTR was determined with the most active being 2-(2,4-dimethylphenoxy)benzoic acid (**10**). All compounds examined were found to bind less strongly than the anthranilic acid derivatives. This indicates that replacement of the bridging oxygen atom with a nitrogen significantly reduces binding affinity. Compounds which contain a sulfur bridge are more active than the oxygen analogues but still less active than the compounds containing a nitrogen bridge. 3-Phenoxybenzoic acid was shown to bind more strongly than 2-phenoxybenzoic acid

which suggests that the position of the acid functionality in these compounds can also significantly alter binding ability.

This work has determined that 2-aryloxybenzoic acids are not suitable candidates for anti-thyroid drugs but more work is needed to delineate the potential of compounds containing the carboxyl group in alternative positions and sulfur bridged analogues.

Chapter 5

Experimental

Experience is the name everyone gives to mistakes.

Oscar Wilde

5.1 GENERAL

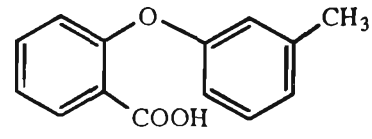
Melting points were determined on a Electrothermal melting point apparatus and are uncorrected. Infrared spectra were recorded as KBr disks on a Hitachi 270-30 grating spectrophotometer. Nuclear magnetic resonance spectra were recorded at room temperature on a Bruker AMX-WB300 NMR spectrometer at a ^{1}H frequency of 300 MHz and a ^{13}C frequency of 75.4 MHz. Unless stated otherwise, NMR spectra were recorded in deuteriochloroform ($CDCl_3$) with tetramethylsilane as an internal standard (δ 0.00). Mass spectra were recorded with a JEOL JMS-DX300 mass spectrometer in electron impact mode (70 eV). Fast Atom Bombardment (FAB) mass spectra were recorded using a JEOL FABMS source using xenon as the ionising particle. Microanalysis were carried out by Chemical and Micro Analytical Services Pty Ltd (CMAS).

"Work-up" means dilution with an organic solvent, washing the organic phase with water and acidification of the aqueous extract, isolation and purification of the compound. All solvents were purified by distillation and dried over appropriate drying sieves. All phenols were purified by distillation using a Buchi GKR-50 and kept dry by placing in a dessicator. Purification by chromatography was performed on columns (20-30 cm) of Merck silica gel 60 (230 mesh). Radial chromatography was carried out using a chromatotron model 7924T on glass plates coated with silica gell (2mm) (Merck, 60PF/UV254) with petroleum ether (bp. 40-60°C) and ethyl acetate mixture as eluent.

Thin layer chromatography was on Merck silica gel 60 F254 aluminium backed plates. Compounds were visualized on t.l.c. by UV light. Unless otherwise specified, all reactions were performed under an inert atmosphere of nitrogen, and purfication was achieved by recrystallisation in an appropriate solvent.

5.2 PREPARATION OF 2-ARYLOXYBENZOIC ACIDS

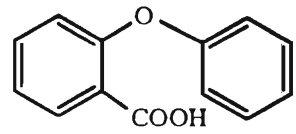
2-(3-methylphenoxy)benzoic acid (5)



Method 1

2-Chlorobenzoic acid (2 g, 12.7 mmol), 3-methylphenol (2.78 g, 25.7 mmol), anhydrous potassium carbonate (1.79 g, 12.9 mmol) and copper bronze (6 mg) were heated at 160-170°C in nitrobenzene (10 ml) for 6h. The dark solution was allowed to cool and the resultant mass was taken up in water (50 ml). Phenol was removed by extraction with ether (3 x 100 ml) and the aqueous layer acidified with concentrated hydrochloric acid. The coloured precipitate was collected and dissolved in aqueous K_2CO_3, the solution filtered (charcoal), and the acid reprecipitated. The solid was recrystallised from aqueous ethanol to give the desired product (1.16 g, 40%). mp 94-95.5°C (lit.[142] 91-93°C); ^{1}H NMR ($CDCl_3$) δ 8.21 (1 H, dd, J = 7.83, 1.8 Hz, C(6)H), 7.46 (1 H, td, J = 7.83, 1.8 Hz, C(4)H), 7.30 (1 H, t, J = 7.74 Hz, C(5)H), 7.20 (1 H, td, J = 7.59, 1.06 Hz, C(5')H), 7.07 (1 H, d, J = 6.97 Hz, C(4')H), 6.91 (2 H, m, C(2')H, C(6')H), 6.85 (1 H, dd, J = 8.4, 0.91 Hz, C(3)H), 2.37 (3 H, s, CH_3); ^{13}C NMR ($CDCl_3$) δ 167.4 (COOH), 157.3 (C2), 155.3 (C1'), 140.4 (C3'), 133.6 (C4), 129.8 (C6), 128.4 (C5'), 125.6 (C4'), 123.4 (C5), 120.4 (C2'), 120.2 (C1), 118.5 (C3), 116.7 (C6'), 18.2 (CH_3); IR (KBr) νmax 3150-2800 (COOH), 1710 (C=O), 1620 (Ar), 1500 cm^{-1}; MS (FAB) (m/e) 229 (100) (M+1), 228 (27), 211 (83), 121 (32).

2-phenoxybenzoic acid (4)

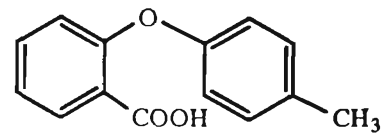


Method 2

Sodium (0.4 g, 17.5 mmol) was added to phenol (1.65 g, 17.5 mmol) in methanol (10 ml) stirred for 5 min and the methanol evaporated. The resultant mass was added to a solution of diphenyliodonium-2-carboxylate (DPIC) (3 g, 8.76 mmol) and copper (II) acetate (30 mg) in 2-picoline (20 ml). Pyridine (10 ml) was added to ensure total dissolution of the DPIC and the solution was heated at 90°C for 3h. The crude product was evaporated several times with toluene and the diaryl ether salt was refluxed for 2 hrs in methanol (20 ml) and H_2SO_4 (1 ml) to form the methyl ester. The reaction mixture was neutralised with $NaHCO_3$ and evaporated. Ether (50 ml) was added and the solution washed with water (3 x 10 ml), saturated sodium chloride (10 ml) and dried over $MgSO_4$. The solvent was evaporated and the resultant methyl ester was purified on a silica column to give a brown oil. The pure methyl ester was hydrolysed in refluxing 3M KOH solution (10 ml) and methanol (2 ml) for 1.5 - 3hrs. The mixture was neutralised with Dowex resin (H^+), filtered and the resin washed thoroughly with methanol. The solvent was evaporated and the resulting mixture was triturated between ethyl acetate and very little water several times. This was followed by drying of the organic layer with $MgSO_4$ and finally evaporation of the solvent which led to a white solid (0.48 g, 26 %); mp 109-111.2°C (lit.[143] 113 °C); ^1H NMR (d_6-DMSO) δ 7.78 (1 H, dd, J = 7.72, 1.72 Hz, C(6)H), 7.53 (1 H, td, J = 7.80, 1.74 Hz, C(4)H), 7.32 (2 H, t, J = 7.99 Hz, C(3')H, C(5')H), 7.25 (1 H, td, J = 7.51, 0.94 Hz, C(5)H), 7.07 (1 H, t, J = 7.38 Hz, C(4')H), 6.96 (1 H, d, J = 7.87 Hz, C(3)H), 6.86 (2 H, d, J = 8.4 Hz, C(2')H, C(6')H); ^{13}C NMR ($CDCl_3$)

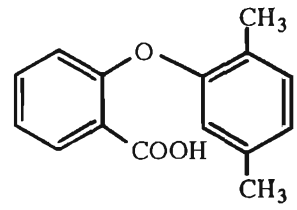
δ 168.1 (COOH), 156.8 (C2), 155.2 (C1'), 134.1 (C4), 132.5 (C6), 129.4 (C3', C5'), 123.9 (C4'), 122.8 (C5), 120.0 (C1), 119.0 (C2', C6'), 118.5 (C3); IR (KBr) νmax 3200-2400 (COOH), 1700 (C=O), 1600, 1500 cm^{-1}; MS (m/e) 215 (76) (M+1) , 214 (16), 197 (99), 121 (100).

2-(4-methylphenoxy)benzoic acid (6)



The title compound was prepared from 4-methylphenol (3.43 g, 31.7 mmol), 2-chlorobenzoic acid (2 g, 12.7 mmol), using Method 1. The glutinous material was purified using radial chromatography and the resultant solid was recrystallised from aqueous ethanol to give the title compound as a white solid (1.24 g, 43%); mp 118°C (lit.[92,89] 118.5°C, 130-132°C); ^1H NMR ($CDCl_3$) δ 8.18 (1 H, dd, J = 7.83, 1.65 Hz, C(6)H), 7.44 (1 H, td, J = 7.67, 1.83 Hz, C(4)H), 7.22 (3 H, m, C(5)H), C(3')H, C(5')H), 7.00 (2 H, d, J = 8.43 Hz, C(2')H, C(6')H), 6.82 (1 H, d, J = 8.37 Hz, C(3)H), 2.36 (3 H, s, CH_3); ^{13}C NMR ($CDCl_3$) δ 167.6 (COOH), 157.3 (C2), 153.9 (C1'), 133.9 (C4), 133.7 (C4'), 132.4 (C6), 130.3 (C3', C5'), 122.9 (C5), 121.4 (C1), 119.2 (C2', C6'), 118.8 (C3), 20.5 (CH_3); IR (KBr) νmax 3150-2800 (COOH), 1700 (C=O), 1620 (Ar), 1595, 1500 cm^{-1}; MS (FAB) (m/e) 229 (100) (M+1), 228 (30), 121 (100).

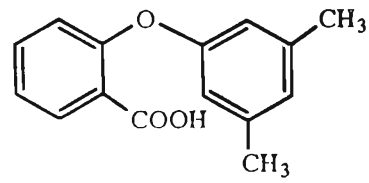
2-(2,5-dimethylphenoxy)benzoic acid (7)



The title compound was prepared from 2,5-dimethylphenol (2.95 g, 24.2 mmol), and 2-iodobenzoic acid (3 g, 12.1 mmol) using Method 1. After cooling the crude product

was powdered and extracted with dilute sodium hydroxide solution (50 ml), filtered and acidified with cold 2M HCl. The coloured solid was collected put through a short column of silica and recrystallised from aqueous ethanol to give a white solid (1.55 g, 53%). mp 131.4°C (lit.[144] 130°C); ^{1}H NMR ($CDCl_3$) δ 8.22 (1 H, dd, J = 7.86, 1.74 Hz, C(6)H), 7.43 (1 H, td, J = 7.84, 1.86 Hz, C(4)H), 7.16 (2 H, m, C(5)H, C(3')H), 7.03 (1 H, d, J = 7.68 Hz, C(4')H), 6.86 (1 H, s, C(6')H), 6.66 (1 H, d, J = 8.40 Hz, C(3)H), 2.32 (3 H, s, CH_3), 2.16 (3 H, s, CH_3); ^{13}C NMR ($CDCl_3$) δ 166.8 (COOH), 157.6 (C2), 151.8 (C1'), 137.8 (C3'), 134.8 (C5'), 133.5 (C4), 131.6 (C6), 127.1 (C2'), 126.7 (C5), 122.8 (C1), 121.4 (C4'), 118.5 (C6'), 115.9 (C3) 20.84 (CH_3), 15.58 (CH_3); IR (KBr) νmax 3150-2800 (COOH), 1710 (C=O), 1620, 1500 cm^{-1}; MS (FAB) (m/e) 243 (99) (M+1), 242 (33), 225 (100), 121 (39).

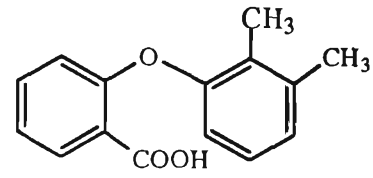
2-(3,5-dimethylphenoxy)benzoic acid (8)



The title compound was prepared from 3,5-dimethylphenol (3.72 g, 30.5 mmol) and diphenyliodonium-2-carboxylate (5.22 g, 15.3 mmol) using Method 2 to afford a white solid (0.46 g, 13%); mp 93.2-93.9°C; (Found M-H 241.0865 $C_{15}H_{13}O_3$ requires 241.0865) ^{1}H NMR ($CDCl_3$) δ 8.20 (1 H, dd, J = 7.87, 1.77 Hz, C(6)H), 7.49 (1 H, td, J = 7.83, 1.77 Hz, C(4)H), 7.21 (1 H, t, J = 7.58 Hz, C(5)H), 6.90 (1 H, s, C(4')H), 6.89 (1 H, d, J = 7.53 Hz, C(3)H), 6.75 (2 H, s, C(2')H, C(6')H), 2.34 (6 H, s, 2(CH_3)); ^{13}C NMR ($CDCl_3$) δ 166.6 (COOH), 156.9 (C2), 154.2 (C1'), 139.6 (C3', C5'), 134.1 (C4), 132.7 (C6), 126.3 (C5), 122.7 (C1), 117.5 (C3), 117.1 (C2', C6'), 20.7 (CH_3), 15.58 (CH_3); IR (KBr) νmax 3100-2800 (COOH), 1700 (C=O), 1610, 1500 cm^{-1}; MS (FAB) (m/e) 243 (57)

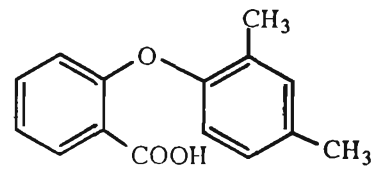
(M+1), 242 (28), 225 (100), 121 (38

2-(2,3-dimethylphenoxy)benzoic acid (9)



The title compound was prepared from 2,3-dimethylphenol (1.97 g, 16.1 mmol) and 2-iodobenzoic acid (2 g, 8.06 mmol) to give a white solid after column chromatography (0.93 g, 48%) mp 182.6°C (lit.[97] 178-180°C); ^{1}H NMR ($CDCl_3$) δ 8.23 (1 H, dd, J = 7.83, 1.8 Hz, C(6)H), 7.42 (1 H, td, J = 7.86, 1.8 Hz, C(4)H), 7.16 (3 H, m, C(5)H, C(4')H, C(5')H), 6.90 (1 H, dd, J = 8.19, 1.5 Hz, C(3)H), 6.64 (1 H, dd, J = 8.41, 0.41 Hz, C(6')H), 2.35 (3 H, s, CH_3), 2.14 (3 H, s, CH_3); ^{13}C NMR ($CDCl_3$) δ 166.2 (COOH), 157.9 (C2), 151.9 (C1'), 139.9 (C3'), 135.1 (C4), 133.9 (C6), 129.5 (C2'), 127.9 (C5'), 127.1 (C4'), 123.1 (C5), 118.9 (C6'), 118.6 (C1), 115.9 (C3), 20.3 (CH_3), 12.5 (CH_3); IR (KBr) νmax 3150-2800 (COOH), 1700 (C=O), 1620, 1600, 1510 (CH_3) cm^{-1}; MS (FAB) (m/e) 243 (100) (M+1), 225 (100), 121 (35).

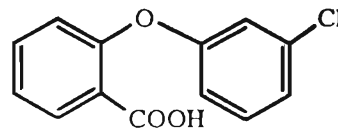
2-(2,4-dimethylphenoxy)benzoic acid (10)



The title compound was prepared from 2,4-dimethylphenol (1.97 g, 16.1 mmol) and 2-iodobenzoic acid (2 g, 8.06 mmol) using Method 1. The crude product was recrystallised from aqueous ethanol and further purified by column chromatography to give a white solid (1.12 g, 57%); mp 151.6°C (lit.[92] 152°C); ^{1}H NMR ($CDCl_3$) δ 8.23 (1 H, dd, J = 7.84, 1.77 Hz, C(6)H), 7.42 (1 H, td, J = 7.85, 1.77 Hz, C(4)H), 7.16 (1 H, t, J = 8.5 Hz, C(5)H), 7.13 (1 H, s, C(3')H), 7.07 (1 H, d, J = 8.97 Hz, C(6')H), 6.94 (1 H, d, J = 8.16 Hz, C(5')H), 6.65 (1 H, d, J = 8.11 Hz,

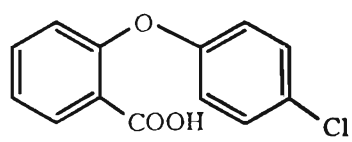
C(3)H), 2.36 (3 H, s, CH_3), 2.17 (3 H, s, CH_3); ^{13}C NMR ($CDCl_3$) δ 166.6 (COOH), 157.9 (C2), 149.8 (C1'), 136.1 (C4'), 135.0 (C4), 133.8 (C3'), 132.8 (C6), 130.4 (C2'), 128.5 (5'), 122.9 (C5), 121.2 (C6'), 118.5 (C1), 115.7 (C3), 20.9 (CH_3), 16.2 (CH_3); IR (KBr) νmax 3100-2800 (COOH), 1690 (C=O), 1610, 1500 (CH_3) cm^{-1}; MS (FAB) (m/e) 243 (100) (M+1), 225 (69), 185 (73), 121 (31).

2-(3-chlorophenoxy)benzoic acid (11)



The title compound was prepared from 3-chlorophenol (1.88 g, 14.6 mmol), diphenyliodonium-2-carboxylate (2.5 g, 7.3 mmol) in picoline (20 ml) and pyridine (10 ml) using Method 2 to give a white solid (0.31 g, 17%); mp 98.5°C (lit.[115] 98°C); ^1H NMR ($CDCl_3$) δ 8.15 (1 H, dd, J = 7.86, 1.71 Hz, C(6)H), 7.52 (1 H, td, J = 7.83, 1.83 Hz, C(4)H), 7.30 (1 H, t, J = 7.99 Hz, C(5')H), 7.25 (1 H, t, J = 7.49 Hz, C(5)H), 7.17 (1 H, dt, J = 8.01, 0.91 Hz, C(4')H), 7.06 (1 H, t, J = 2.1 Hz, C(2')H), 6.95 (2 H, m, C(3)H, C(6')H); ^{13}C NMR ($CDCl_3$) δ 168.1 (COOH), 156.9 (C2), 156.3 (C1'), 135.3 (C5'), 134.8 (C4), 133.2 (C3'), 130.7 (C6), 124.3 (C4'), 124.2 (C5), 121.0 (C1), 119.9 (C6'), 119.3 (C3), 117.1 (C2'); IR (KBr) νmax 3150-2800 (COOH), 1700 (C=O), 1600, 1495 cm^{-1}; MS (FAB) (m/e) 251 (56) (M+1), 249 (99) (M+1), 248 (22), 233 (47), 231 (100), 121 (56).

2-(4-chlorophenoxy)benzoic acid (5)

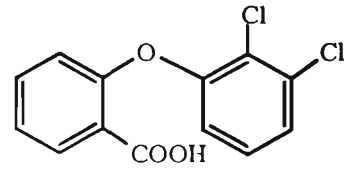


The title compound was prepared from 4-chlorophenol (4.08 g, 31.7 mmol) and 2-

chlorobenzoic acid (2 g, 12.7 mmol) using Method 1. The crude product was recrystallised from aqueous ethanol to give a white solid (1.89 g, 60%); mp 118.7°C (lit.[89,115] 119°C, 115 - 116°C); ^{1}H NMR ($CDCl_3$) δ 8.12 (1 H, dd, J = 7.81, 1.41 Hz, C(6)H), 7.53 (1 H, td, J = 7.65, 1.14 Hz, C(5)H), 7.37 (2 H, d, J = 6.93 Hz, C(3')H, C(5')H), 7.25 (1 H, t, J = 7.59 Hz, C(4)H), 7.01 (2 H, d, J = 6.93 Hz, C(2')H, C(6')H), 6.91 (1 H, d, J = 8.31 Hz, C(3)H); ^{13}C NMR ($CDCl_3$) δ 167.4 (COOH), 156.2 (C2), 154.0 (C1'), 134.2 (C4), 132.6 (C6), 129.4 (C3', C5'), 129.0 (C4'), 123.3 (C5), 120.2 (C1), 119.9 (C2', C6'), 118.6 (C3); IR (KBr) νmax 3150-2800 (COOH), 1700 (C=O), 1605, 1500 cm^{-1}; MS (FAB) (m/e) 251 (33), 249 (100) (M+1), 248 (25), 233 (36), 231 (98), 121 (77).

Preparation of the title compound using Method 2 gave an identical product in 12% yield.

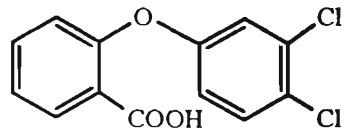
2-(2,3-dichlorophenoxy)benzoic acid (13)



The title compound was prepared from 2,3-dichlorophenol (2.38 g, 14.6 mmol) and diphenyliodonium-2-carboxylate (2 g, 5.85 mmol) using Method 2. The crude product was purified by column chromatography to give a white solid (0.39 g, 24%); mp 185.5°C; (Found C,54.85; H,2.75; O,16.72; $C_{13}H_8Cl_2O_3$ requires C,55.15; H,2.84; O,16.95%) ^{1}H NMR ($CDCl_3$) δ 8.19 (1 H, dd, J = 7.84, 1.74 Hz, C(6)H), 7.51 (1 H, td, J = 7.84, 1.8 Hz, C(4)H), 7.36 (1 H, dd, J = 8.10, 1.46 Hz, C(4')H), 7.26 (1 H, td, J = 7.60, 0.99 Hz, C(5)H), 7.23 (1 H, t, J = 8.19 Hz, C(5')H), 6.98 (1 H, dd, J = 8.15, 1.41 Hz, C(6')H), 6.78 (1 H, dd, J = 8.29, 0.78 Hz, C(3)H); ^{13}C NMR ($CDCl_3$) δ 166.0 (COOH), 156.0 (C2), 152.4 (C1'), 135 (C3'), 134.8 (C4), 133.6 (C6), 127.8 (C5'), 126.7 (C4'), 125.5 (C2'), 124.2

(C5), 121.0 (C1), 119.2 (C3), 117.7 (C6'); IR (KBr) νmax 3150-2800 (COOH), 1710 (C=O), 1620, 1500 cm^{-1}; MS (FAB) (m/e) 287 (10), 285 (38), 283 (64) (M+1), 185 (100), 110 (46).

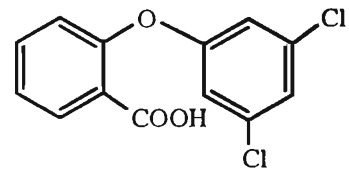
2-(3,4-dichlorophenoxy)benzoic acid (14)



The title compound was prepared using a modification of Method 1 as described by Desphande.[115] A solution of sodium ethoxide was prepared from sodium (0.88 g, 38.3 mmol) and ethanol (30 ml). To this was added 3,4-dichlorophenol (6.25 g, 38.3 mmol), 2-chlorobenzoic acid (3 g, 19.2 mmol) and copper bronze (0.15 g). The reaction mixture was heated at 190°C for 2h. Work-up was carried out according to Method 1. The product was purified by column chromatography (1.69 g, 31%); mp 126.8°C (lit.[115] 127°C); ^{1}H NMR ($CDCl_3$) δ 8.15 (1 H, dd, J = 7.84, 1.59 Hz, C(6)H), 7.57 (1 H, td, J = 7.79, 1.59 Hz, C(4)H), 7.43 (1 H, d, J = 8.82 Hz, C(5')H), 7.28 (1 H, t, J = 8.47 Hz, C(5)H), 7.14 (1 H, d, J = 2.76 Hz, C(2')H), 6.96 (1 H, d, J = 8.28 Hz, C(3)H), 6.91 (1 H, dd, J = 8.74, 2.79 Hz, C(6')H); ^{13}C NMR ($CDCl_3$) δ 168.9 (COOH), 156.1 (C1', C2), 135.2 (C4), 134.0 (C3'), 133.5 (C6), 131.4 (C5'), 128.0 (C4'), 124.9 (C5), 121 (C1), 120.8 (C2'), 120.6 (C3), 118.2 (C6'); IR (KBr) νmax 3150-2800 (COOH), 1710 (C=O), 1595, 1500 cm^{-1}; MS (FAB) (m/e) 287 (10), 285 (38), 283 (60) (M+1), 269 (12), 267 (65), 265 (100), 121 (84).

Preparation of the title compound using Method 2 gave a yield of 5%.

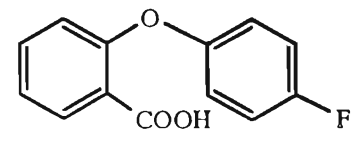
2-(3,5-dichlorophenoxy)benzoic acid (15)



The title compound was prepared from 3,5-dichlorophenol (2.38 g, 14.6 mmol) and diphenyliodonium-2-carboxylate (2 g, 5.84 mmol) using the procedure described in Method 2. The crude product was purified by column chromatography to give a white solid (0.3 g, 18%); mp 139.9-142.8°C; (Found M-H 280.9764 $C_{13}H_7Cl_2O_3$ requires 280.9772) ^{1}H NMR ($CDCl_3$) δ 8.14 (1 H, dd, J = 7.86, 1.65 Hz, C(6)H), 7.60 (1 H, td, J = 7.82, 1.68 Hz, C(4)H), 7.32 (1 H, t, J = 7.60 Hz, C(5)H), 7.14 (1 H, t, J = 1.57 Hz, C(4')H), 7.02 (1 H, d, J = 8.22 Hz, C(3)H), 6.89 (2 H, d, J = 1.71 Hz, C(6')H, C(2')H); ^{13}C NMR ($CDCl_3$) δ 166.0 (COOH), 158.0 (C2), 153.0 (C1'), 136.0 (C3', C5'), 135.2 (C4), 133.4 (C6), 125.3 (C4'), 123.9 (C5), 122.0 (C1), 121.4 (C3), 117.1 (C2', C6'); IR (KBr) νmax 3200-2850 (COOH), 1710 (C=O), 1600, 1500 cm^{-1}; MS (FAB) (m/e) 287 (10), 285 (46), 283 (79) (M+1), 269 (12), 267 (74), 265 (100), 121 (57).

The title compound was prepared using Method 1 in 32% yield.

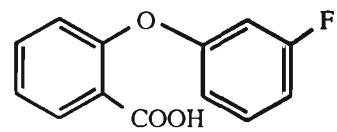
2-(4-fluorophenoxy)benzoic acid (16)



The title compound was prepared using Method 1 from 4-fluorophenol (2.24 g, 20.2 mmol) and 2-iodobenzoic acid (5 g, 20.2 mmol) in DMF (2.5 ml) 170°C for 2.5h. After evaporation of the solvent the mixture was diluted with water (4 ml), filtered and the product was precipitated with HCl and collected. The crude product was purified on a short silica column and recrystallised from aqueous ethanol to give a white solid

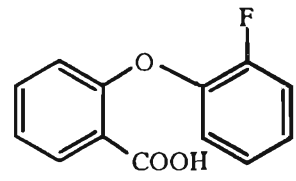
(2.30 g, 49%); mp 141.7°C (lit.[145] 142°C); ^{1}H NMR ($CDCl_3$) δ 8.12 (1 H, dd, J = 7.86, 1.74 Hz, C(6)H), 7.48 (1 H, td, J = 7.83, 1.77 Hz, C(4)H), 7.18 (1 H, t, J = 7.59 Hz, C(5)H), 7.05 (4 H, m, C(6')H, C(5')H, C(3')H, C(2')H), 6.84 (1 H, d, J = 8.38 Hz, C(3)H); ^{13}C NMR ($CDCl_3$) δ 168.1 (COOH), 157.9 (C2), 159.5 (d, J_{CF} = 276 Hz, C4'), 151.4 (C1'), 134.7 (C4), 133.3 (C6), 123.5 (C5), 121.2 (d, J_{CF} = 9 Hz, C2', C6'), 120.3 (C1), 118.3 (C3), 116.6 (d, J_{CF} = 22.5 Hz, C3', 5'); IR (KBr) νmax 3200-2800 (COOH), 1710 (C=O), 1620, 1500 cm^{-1}; MS (FAB) (m/e) 233 (100) (M+1), 232 (19), 215 (75), 185 (42), 121 (36).

2-(3-fluorophenoxy)benzoic acid (17)



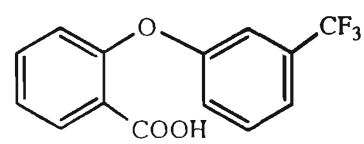
The title compound was prepared from 3-fluorophenol (2.24 g, 20.2 mmol) and 2-iodobenzoic acid (5.0 g, 20.2 mmol) using the modification of Method 1 described above. The crude product was purified using radial chromatography and finally recrystallised from aqueous ethanol to give a white solid (1.84 g, 39%); mp 128.5°C (lit.[145] 130°C); ^{1}H NMR ($CDCl_3$) δ 8.12 (1 H, dd, J = 7.86, 1.74 Hz, C(6)H), 7.52 (1 H, td, J = 7.83, 1.77 Hz, C(4)H), 7.32 (1 H, t, J = 7.29 Hz, C(5')H), 7.25 (1 H, td, J = 7.29, 0.96 Hz, C(5)H), 6.97 (1 H, dd, J = 8.28, 0.81 Hz, C(3)H), 6.82 (2 H, m, C(4')H, C(6')H), 6.74 (1 H, dt, $J_{H,F}$ = 9.78 Hz, $J_{H,H}$ = 2.32 Hz, C(2')H); ^{13}C NMR ($CDCl_3$) δ 169.6 (COOH), 163.4 (d, J_{CF} = 247 Hz, C3'), 158.1 (C1'), 156.3 (C2), 134.8 (C4), 132.9 (C6), 130.6 (d, J_{CF} = 9.7 Hz, C5'), 124.2 (C5), 121.5 (C1), 120.6 (C3), 114.1 (C6'), 110.5 (d, J_{CF} = 22 Hz, C4'), 106.3 (d, J_{CF} = 25 Hz, C2'); IR (KBr) νmax 3200-2800 (COOH), 1710 (C=O), 1600, 1500 cm^{-1}; MS (FAB) (m/e) 233 (99) (M+1), 232 (29), 215 (100), 121 (50).

2-(2-fluorophenoxy)benzoic acid (18)



The title compound was prepared from 2-fluorophenol (2.24 g, 20.2 mmol) and 2-iodobenzoic acid (5.0 g, 20.2 mmol) using the modification of Method 1 described above. The crude product was purified using radial chromatography and finally recrystallised from aqueous ethanol to give a white solid (1.97 g, 42%); mp 142.5°C (lit.[145] 140°C); ^{1}H NMR ($CDCl_3$) δ 8.18 (1 H, dd, J = 7.84, 1.74 Hz, C(6)H), 7.84 (1 H, td, J = 7.84, 1.8 Hz, C(4)H), 7.19 (5 H, m, C(5)H, C(6')H, C(5')H, C(4')H, C(3')H), 6.81 (1 H, d, J = 8.37 Hz, C(3)H); ^{13}C NMR ($CDCl_3$) δ 168.4 (COOH), 157.4 (C2), 154.2 (d, J_{CF} = 250 Hz, C2'), 142.6 (C1'), 134.9 (C4), 133.5 (C6), 126.3 (C5'), 125.2 (C4'), 123.7 (C5), 122.5 (C6'), 119.9 (C1), 117.5 (d, J_{CF} = 18 Hz, C3'), 117.3 (C3); IR (KBr) νmax 3200-2800 (COOH), 1700 (C=O), 1610, 1500 cm^{-1}; MS (FAB) (m/e) 233 (100) (M+1), 232 (20), 215 (99), 121 (44).

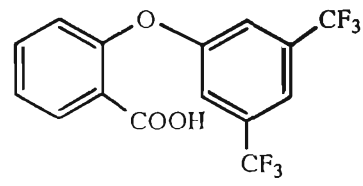
2-(3-trifluoromethylphenoxy)benzoic acid (19)



The title compound was prepared from 3-trifluoromethylphenol (3.33 g, 20.5 mmol) and 2-chlorobenzoic acid (1.28 g, 8.2 mmol) using the modification of Method 1 previously described. After cooling, the mixture was extracted with ether and the aqueous layer acidified with concentrated HCl and extracted with ether (3 x 100 ml). The combined ether layers were washed with sat. $NaHCO_3$ solution. The aqueous layer was acidified, and the resultant solid removed by filtration and dried. The crude product was recrystallised from cyclohexane and purified by column chromatography

(1.16 g, 52%); mp 85.4°C (lit.[88] 86-88°C); ^{1}H NMR ($CDCl_3$) δ 8.12 (1 H, dd, J = 7.86, 1.77 Hz, C(6)H), 7.54 (1 H, td, J = 7.78, 1.8 Hz, C(4)H), 7.46 (1 H, t, J = 7.89 Hz, C(5')H), 7.39 (1 H, d, J = 7.77 Hz, C(4')H), 7.25 (2 H, m, C(2')H, C(5)H), 7.16 (1 H, d, J = 7.77 Hz, C(6')H), 6.95 (1 H, dd, J = 8.29, 0.9 Hz, C(3)H); ^{13}C NMR ($CDCl_3$) δ 169.8 (COOH), 157.4 (C2), 156.2 (C1'), 135.0 (C4), 133.1 (C5'), 132.2 (q, J_{CF} = 25 Hz, C3'), 130.4 (C6), 124.4 (C5), 123.1 (q, J_{CF} = 273 Hz, CF_3), 121.8 (C1), 121.6 (C2'), 120.7 (C4'), 120.2 (C3), 115.4 (C6'); IR (KBr) νmax 3200-2750 (COOH), 1700 (C=O), 1620, 1500 cm^{-1}; MS (FAB) (m/e) 283 (51) (M+1), 265 (100).

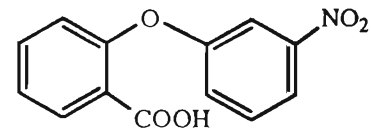
2-[3,5-bis(trifluoromethyl)phenoxy]benzoic acid (20)



The title compound was prepared from 3,5-bis(trifluoromethyl)phenol (4.50 g, 19.7 mmol) and 2-chlorobenzoic acid (1.23 g, 7.88 mmol) using the modified Method 1. After cooling, the mixture was extracted with ether, and the aqueous layer acidified with concentrated HCl and extracted with ether (3 x 100 ml). The combined ether layers were washed with sat. $NaHCO_3$ solution. The aqueous layer was acidified, and the resultant solid removed by filtration and dried. The crude product was recrystallised from cyclohexane (1.03 g, 37%); mp 128.4°C; (Found C,51.32; H,2.10; O,14.12; $C_{15}H_8F_6O_3$ requires C,51.44; H,2.30; O,13.71%) ^{1}H NMR ($CDCl_3$) δ 8.13 (1 H, dd, J = 7.83, 1.68 Hz, C(6)H), 7.64 (1 H, td, J = 7.80, 1.74 Hz, C(4)H), 7.58 (1 H, s, C(4')H), 7.37 (1 H, t, J = 7.62 Hz, C(5)H), 7.34 (2 H, s, C(2')H, C(6')H), 7.08 (1 H, d, J = 8.16 Hz, C(3)H); ^{13}C NMR ($CDCl_3$) δ 169.5 (COOH), 158.7 (C2), 154.7 (C1'), 135.5 (C4), 133.3 (C6), 132.9 (C3', 5'), 125.6 (C5), 123.0 (q, J_{CF} = 270 Hz, CF_3), 122.3 (C1), 121.9 (C2', C6'),

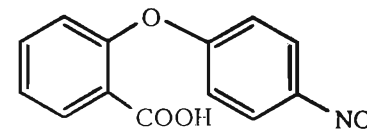
117.4 (C3), 116.3 (C4'); IR (KBr) νmax 3500 (COOH), 1710 (C=O), 1620, 1500 cm^{-1}; MS (FAB) (m/e) 351 (17) (M+1), 185 (100), 181 (90), 131 (69), 110 (75).

2-(3-nitrophenoxy)benzoic acid (21)



The title compound was prepared from 3-nitrophenol (3 g, 21.5 mmol) and 2-chlorobenzoic acid (3.05 g, 19.5 mmol) using the modification of Method 1 described above. The crude product purified on a column of silica and recrystallised from aqueous methanol to give a white solid (2.79 g, 55%); mp 136.4-137.9°C (lit.[119] 138-139°C); ^{1}H NMR ($CDCl_3$) δ 8.12 (1 H, dd, J = 7.87, 1.74 Hz, C(6)H), 7.95 (1 H, ddd, J = 8.16, 2.07, 0.87 Hz, C(4')H), 7.74 (1 H, t, 2.2 Hz C(2')H), 7.60 (1 H, td, J = 7.80, 1.8 Hz, C(4)H), 7.48 (1 H, t, 8.22 Hz, C(5')H), 7.33 (1 H, td, J = 7.65, 1.05 Hz, C(5)H), 7.29 (1 H, ddd, J = 8.25, 2.4, 0.81 Hz, C(6')H), 7.05 (1 H, dd, J = 8.23, 1.05 Hz, C(3)H); ^{13}C NMR ($CDCl_3$) δ 169.6 (COOH), 158.3 (C2), 155.2 (C1'), 149.2 (C5'), 135.2 (C4), 133.2 (C3'), 130.3 (C6), 125.2 (C2'), 123.7 (C5), 122.2 (C1), 121.8 (C3), 117.8 (C4'), 112.3 (C6'); IR (KBr) νmax 3150-2800 (COOH), 1700 (C=O), 1600, 1530, 1480, 1360 cm^{-1}; MS (FAB) (m/e) 260 (100) (M+1), 242 (98), 196 (67), 121 (57).

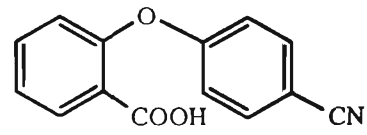
2-(4-nitrophenoxy)benzoic acid (22)



Using the modification of Method 1 described above, the title compound was prepared from 4-nitrophenol (3 g, 21.5 mmol) and 2-chlorobenzoic acid (3.05 g, 19.5 mmol). The crude product was purified on a column of silica and recrystallised from aqueous

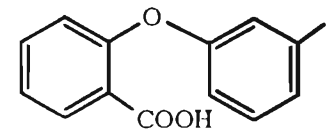
methanol to give a white solid (3.08 g, 61%); mp 160.4°C (lit.[146,147] 157°C, 153-155°C); ^{1}H NMR ($CDCl_3$) δ 8.20 (2 H, d, J = 9.21 Hz, C(3')H, C(5')H), 8.12 (1 H, dd, J = 7.87, 1.56 Hz, C(6)H), 7.66 (1 H, td, J = 7.78, 1.69 Hz, C(4)H), 7.37 (1 H, t, J = 7.59 Hz, C(5)H), 7.13 (1 H, d, J = 8.19 Hz, C(3)H), 6.96 (2 H, d, J = 9.21 Hz, C(2')H, C(6')H); ^{13}C NMR ($CDCl_3$) δ 169.6 (COOH), 163.4 (C2), 155.5 (C1'), 143.1 (C4'), 135.7 (C4), 133.5 (C6), 126.1 (C3', C5'), 126.0 (C5), 123.0 (C3), 122.8 (C1), 117.0 (C2', C6'); IR (KBr) νmax 3200-2750 (COOH), 1710 (C=O), 1610, 1512, 1500, 1360 cm^{-1}; MS (FAB) (m/e) 260 (100) (M+1), 244 (52), 196 (58), 185 (58), 121 (89).

2-(4-cyanophenoxy)benzoic acid (23)



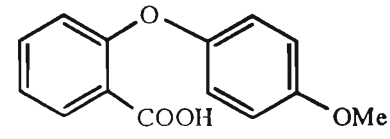
The title compound was prepared from 4-cyanophenol (2.28 g, 19.1 mmol) and 2-chlorobenzoic acid (2 g, 12.7 mmol) using the modified Method 1 at 150°C for 4.5 hrs. The crude product was recrystallised from aqueous methanol to give a white solid and purified by column chromatography (0.91 g, 30%); mp 64-64°C; (Found M-H 238.0494 $C_{13}H_8NO_3$ requires 238.0504) ^{1}H NMR ($CDCl_3$) δ 8.11 (1 H, dd, J = 7.45, 1.68 Hz, C(6)H), 7.61 (2 H, d, J = 8.67 Hz, C(3')H, C(5')H), 7.62 (1 H, td, J = 7.9, 1.77 Hz, C(4)H), 7.35 (1 H, td, J = 7.62, 1.02 Hz, C(5)H), 7.08 (1 H, d, J = 8.19 Hz, C(3)H), 6.95 (2 H, d, J = 9.2 Hz, C(2')H, C(6')H); ^{13}C NMR ($CDCl_3$) δ 167.1 (COOH), 166.1 (C2), 160.1 (C1'), 133.7 (C4), 131.4 (C6), 129.3 (C3', C5'), 128.2 (C4'), 124.8 (C5), 122.1 (C1), 116.9 (C3), 116.0 (C2', C6'); IR (KBr) νmax 3150-2800 (COOH), 2250 (CN), 1680 (C=O), 1610, 1495 cm^{-1}; MS (m/e) 240 (100) (M+1), 222 (65), 185 (35), 121 (46).

2-(3-iodophenoxy)benzoic acid (24)



The title compound was prepared from 3-iodophenol (2.57 g, 11.7 mmol) and diphenyliodonium-2-carboxylate (2 g, 5.85 mmol) using Method 2. The crude product was purified on a short silica column to give a white solid (0.22 g, 11%); mp 125.9-127.9°C; (Found C,46.33; H,2.67; O,13.74; I,37.11; $C_{13}H_9IO_3$ requires C,45.91; H,2.67; O,14.11; I,37.31%) ^{1}H NMR ($CDCl_3$) δ 8.14 (1 H, dd, J = 7.87, 1.65 Hz, C(6)H), 7.52 (2 H, m, C(4)H, C(4')H), 7.40 (1 H, s, C(2')H), 7.25 (1 H, t, J = 7.56 Hz, C(5)H), 7.10 (1 H, t, J= 7.96 Hz, C(5')H), 7.02 (1 H, d, J = 8.10 Hz, C(6')H), 6.92 (1 H, d, J = 8.31 Hz, C(3)H); ^{13}C NMR ($CDCl_3$) δ 169.6 (COOH), 157.3 (C1'), 156.6 (C2), 135.1 (C4), 133.2 (C4'), 133.1 (C6), 131.4 (C5'), 127.9 (C2'), 124.3 (C5), 121.6 (C1), 120.4 (C3), 118.4 (C6'), 94.5 (C3'); IR (KBr) νmax 3150-2800 (COOH), 1700 (C=O), 1600, 1500 cm^{-1}; MS (FAB) (m/e) 341 (100) (M+1), 196 (100), 121 (70).

2-(4-methoxyphenoxy)benzoic acid (25)

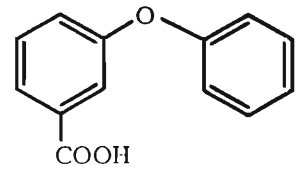


The title compound was prepared from 4-methoxyphenol (1.5 g, 11.7 mmol) and diphenyliodonium-2-carboxylate (2. g, 5.85 mmol) using Method 2 to give a white solid (0.15 g, 22%); mp 140.7°C (lit.[142,148] 140-141°C, 149-150°C); ^{1}H NMR ($CDCl_3$) δ 8.22 (1 H, dd, J = 7.77, 1.29 Hz, C(6)H), 7.42 (1 H, t, J = 7.74 Hz, C(4)H), 7.16 (1 H, t, J = 7.54 Hz, C(5)H), 7.07 (2 H, d, J = 9.12 Hz, C(3')H, C(5')H), 6.94 (2 H, d, J = 9.03, C(2')H, C(6')H), 6.76 (1 H, d, J = 8.52 Hz, C(3)H), 3.82 (3 H, s, OCH_3); ^{13}C NMR ($CDCl_3$) δ 165.9 (COOH), 158.1 (C2), 157.4 (C4'), 147.3 (C1'), 134.7 (C4), 133.5 (C6), 123.2 (C5), 121.8 (C2', C6'),

118.8 (C1), 116.5 (C3), 115.3 (C3', C5'), 55.7 (OCH_3); IR (KBr) νmax 3200-2800 (COOH), 1700 (C=O), 1610, 1500 cm^{-1}; MS (FAB) (m/e) 245 (100) (M+1), 244 (94), 227 (99), 185 (62), 121 (72).

5.3 PREPARATION OF 3-PHENOXYBENZOIC ACID

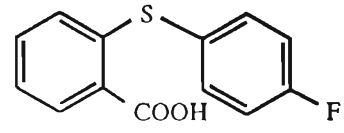
3-phenoxybenzoic acid (26)



Sodium phenoxide was prepared by dissolving phenol (3 g, 31.9 mmol) in a solution of sodium (0.73 g, 31.9 mmol) and methanol (10 ml). Removal of the methanol under reduced pressure gave sodium phenoxide as a paste. The potassium salt of 3-chlorobenzoic acid (2.5 g, 15.9 mmol) and copper powder were mixed together and added to the sodium phenoxide paste and heated at 190°C for 5h. The crude product was recrystallised from aqueous ethanol and petroleum ether (3.77 g, 56%); mp 148.2°C (lit.[143,148,149] 146.5°C, 150-153°C, 145-146°C); ^1H NMR ($CDCl_3$) δ 7.83 (1 H, dt, J = 7.71, 1.27 Hz, C(6)H), 7.71 (1 H, dd, J = 2.32, 1.63 Hz, C(2)H), 7.43 (2 H, t, J = 7.95 Hz, C(3')H, C(5')H), 7.37 (1 H, t, J = 7.95 Hz, C(5)H), 7.26 (1 H, ddd, J = 8.19, 2.5, 1.0 Hz, C(4)H), 7.17 (1 H, tt, J = 7.40, 1.05 Hz, C(4')H), 7.03 (2 H, dd, J = 7.56, 1.17 Hz, C(2')H, C(6')H); ^{13}C NMR ($CDCl_3$) δ 171.7 (COOH), 157.7 (C3), 156.6 (C1'), 131.1 (C1), 129.9 (C3', C5'), 129.8 (C5), 124.9 (C6), 124.1 (C4), 123.9 (C4'), 119.9 (C2), 119.3 (C2', C6'); IR (KBr) νmax 3150-2800 (COOH), 1700 (C=O), 1590, 1500 cm^{-1}; MS (FAB) (m/e) 215 (100) (M+1), 197 (69), 185 (99), 110 (58).

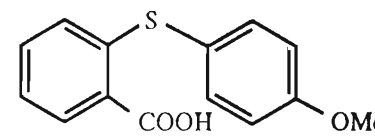
5.4 PREPARATION OF 2-ARYLTHIOBENZOIC ACIDS

2-(4-fluorophenylthio)benzoic acid (27)



4-Fluorothiophenol (1.98 g, 15.4 mmol) and KOH (3.1 g) in water (25 ml) was heated to 50°C and stirred for 15 minutes, copper powder (0.37 g) and 2-iodobenzoic acid (3.83 g, 15.4 mmol) were then added. The mixture was refluxed for 10h, and diluted with water (10 ml) and filtered over active charcoal. The filtrate was allowed to cool and acidified with hydrochloric acid and the precipitate filtered off and washed with cold water. The crude product was recrystallised from ethanol/water to give the title compound as a white solid (3.05 g, 80%); mp 201.6°C (lit.[150] 204-205°C); ^{1}H NMR (CD_3OD, $CDCl_3$) δ 8.12 (1 H, dd, J = 7.80, 1.5 Hz, C(6)H), 7.57 (2 H, dd, $J_{H,H}$ = 8.76 Hz, $J_{H,F}$ = 5.37 Hz, C(2')H, C(6')H), 7.29 (1 H, td, J = 7.70, 1.59 Hz, C(4)H), 7.15 (3 H, m, C(5)H, C(3')H, C(5')H), 6.75 (1 H, d, J = 7.62 Hz, C(3)H); ^{13}C NMR ($CDCl_3$) δ 167.8 (COOH), 162.9 (d, J_{CF} = 247.9Hz, C4'), 142.4 (C2), 137.2 (d, J_{CF} = 7.5Hz, C3', C5'), 131.5 (C4), 130.9 (C6), 125.9 (C5), 126.6 (C1'), 125.6 (C1), 123.6 (C3), 116.2 (d, J_{CF} = 22.6Hz, C2', C6'); IR (KBr) νmax 3200-2750 (COOH), 1695 (C=O), 1600, 1500 cm^{-1}; MS (m/e) 248 (90), 202 (59), 137 (100),

2-(4-methoxyphenlythio)benzoic acid (28)



The title compound was prepared from 4-methoxythiophenol (5 g, 35.6 mmol) and 2-iodobenzoic acid (8.04 g, 32.4 mmol) using the procedure described above. The product was filtered and recrystallised from a mixture of ethanol and benzene (5:1) to

give a white solid (5.45 g, 65%); mp 235.0-238.9°C (lit.[151] 240.5-243°C); ^{1}H NMR (d$_6$-DMSO) δ 8.02 (1 H, dd, J = 7.77, 1.5 Hz, C(6)H), 7.49 (2 H, d, J = 6.78 Hz, C(2')H, C(6')H), 7.25 (1 H, td, J = 7.68, 1.5 Hz, C(4)H), 7.12 (1 H, td, J = 7.51, 1.05 Hz, C(5)H), 6.98 (2 H, d, J = 6.93 Hz, C(3')H, C(5')H), 6.75 (1 H, d, J = 8.19 Hz, C(3)H), 3.87 (3 H, s, OCH_3); ^{13}C NMR ($CDCl_3$) δ 172.2 (COOH), 164.8 (C2), 148.3 (C1'), 141.7 (C3', C5'), 136.2 (C4), 135.4 (C6), 131.4 (C4'), 130.5 (C5), 128.0 (C3), 126.8 (C1), 119.7 (C2', C6'), 59.6 (OCH_3); IR (KBr) νmax 3200-2800 (COOH), 1690 (C=O), 1600, 1510 cm^{-1}; MS (FAB) m/e 261 (52) (M+1), 243 (47), 201 (36), 185 (100), 110 (51).

5.5 TTR ISOLATION

Human transthyretin (TTR) was isolated and purified using a modification of the method of Dwulet and Benson.[139] Blood plasma was obtained from the blood bank. A series of three columns were used, each monitored by a UV spectrometer at a wavelength of 254 nm. Approximately 177 ml of plasma was diluted with 920 ml of milli Q water and loaded onto the first column, a 5 cm x 50 cm, DEAE-Sephecel ion exchange column. The column was washed with starting buffer (0.15 M NaCl, 20 mM Tris, 0.02% NaN_3, pH 7.4) at 2 ml/min until the protein had ceased eluting. A second buffer of higher concentration of salt (2000 ml, 0.3 M NaCl, 20 mM Tris, 0.02% sodium azide, pH 7.4, 2.0 ml/min) was run which displaced TTR from the column. TTR in blood plasma is bound to the highly conjugated molecule retinol binding protein (RBP), hence the TTR in the collected fractions is identified by measurement of fluorescence. The appropriate fractions were pooled, concentrated and re-equilibrated with the low salt buffer using ultrafiltration (Amicon YM10). The crude TTR-RBP complex was applied to a dye affinity column (2.5 cm x 60 cm, Blue Sepharose CL-6B, Pharmacia) to cleave the TTR-RBP complex, and eluted with the low salt buffer (2 ml/min). The fractions containing protein as determined by UV

absorbance at 254 nm were concentrated by ultrafiltration and applied to a gel filtration column (2 cm x 100 cm Ultragel AcA-34, LKB) for final purification. The column was eluted under gravity with 100 mM KPO_4 pH 7.4. TTR eluted between 1400 and 1700 minutes. Fractions 25-42 were pooled, the buffer salts were removed by ultrafiltration with distilled water and the solution lyophilised to afford 23.0 mg of purified TTR. Protein purity was established using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This type of gel is run under denaturing conditions and therefore the TTR tetramer is cleaved into a dimeric form. This was evident as one major band with a molecular weight between 15 and 20 kDa. The identity of the isolated protein was confirmed by testing against compounds like flufenamic acid of known affinity.

Purification of TTR using three different columns successfully removed plasma proteins Albumin and TGB which would interfere with the TTR binding studies. The non-thyroidal hormone analogues prepared in this thesis, were tested for their ability to displace T_4 from TTR binding studies.

References

If I have seen further than others,
it is by standing upon the shoulders
of giants.

Isaac Newton

1. Fox, Stuart Ira., (1996), *Human Physiology*, WMC Brown Co. Publishers, 5th Edition, London.

2. Oppenheimer, J.H., (1983), *The Molecular Basis of Thyroid Hormone Action*, Academic Press, NY.

3. Thompson, J., Historical Notes. In: Smithers, D., ed. (1970), *Tumours of the Thyroid.*, **6**, Neoplastic Disease at Various Sites, Churchill Livingston, Edinburgh.

4. Parry, C.H., (1825), *Collections from the Unpublished Papers of the late Caleb Hilliel Parry*. London., **2**, 111.

5. Graves, R.J., (1835), *Med. Surg. J. London.*, **7**, 516.

6. King, T.N., (1836), *Guys Hosp. Rep.*, **1**, 429.

7. Reverdin, J.L., Reverdin, A., (1883), *Rev. Med.*, **3**, 169.

8. Kocher, T., (1883), *Arch. Klin. Chir.*, **29**, 254.

9. Horsley, V.A., (1885), *Proc. R. Soc. Lond.*, **33**, 5.

10. Baumann, E., (1895), *Z. Physiol. Chem.*, **21**, 319.

11. Oswald, A., (1899), *Z. Physiol. Chem.*, **27**, 14-49.

12. Kendall, E.C., (1915), *Trans. Assoc. Am. Physicians.*, **30**, 420-499.

13. Kendall, E.C., (1919), *J. Biol. Chem.* **39**, 125-149.

14. Harington, C.R., (1926), *Biochem J.*, **20**, 293.

15. Harington, C.R., Barger, G., (1927), *Biochem. J.*, **21**, 169-183.

16. Gross, J., Pitt-Rivers, R., (1954), *Recent Prog. Horm Res.*, **10**, 109-129.

17. Roche, J., Lissitzky, S., Michel, C.R., (1952), *Hebd. Seances Acad. Sci. Paris.*, **234**, 997-998.

18. Jorgenson, E.C., (1981), *Burger's Medicinal Chemistry*, 4th Ed., (Ed. M.E. Wolff) Chp. 31, John Wiley and Sons : New York , and references cited.

19. Munro, S.L.A., (1990), *PhD Thesis*, Victorian College of Pharmacy.

20. Mendell, C.R., Weisinger, R.A., and Jones, A.L., (1987), *Endocrinology.*, **120**, 1742-1749.

21. Robbins, J., (1991), *Thyroid Hormone Transport Proteins and the Physiology of Hormone Binding, In Werner's The Thyroid*, 6th Ed; Ingbar, S.H., Braverman, L.E., Eds; Lipponcot: Philadelphia, 111-125.

22. Taurog, A., (1991), *Hormone Synthesis: Thyroid Iodine Metabolism In Werner's The Thyroid*, 6th Ed; Ingbar, S.H., Braverman, L.E., Eds; Lipponcot: Philadelphia, 51-97.

23. Martin, I. Surks., (1991), *Treatment of Hypothyroidism. In Werner's The Thyroid*, 6th Ed; Ingbar, S.H., Braverman, L.E., Eds; Lipponcot: Philadelphia, 1099-1103.

24. Kohrle, Josef., Aesch, R.D., Leonard, J.L., (1991), *Intracellualr Pathways of Iodothyronine Metabolism, In Werner's The Thyroid*, 6th Ed; Ingbar, S.H., Braverman, L.E., Eds; Lipponcot: Philadelphia, 144-189.

25. Duggan, B.M., Craik, D.J., Munro, S.L.A., Conformations and Binding Interactions of Thyroid Hormone and Analogues, 1-50, unpublished data.

26. Ingbar, S. H., (1972), *Mayo Clin. Proc.*, **47**, 814.

27. Wolff, J., Chaikoff, I. L., (1948), *J. Biol. Chem.*, **174**, 555.

28. Bayliss, R.I.S., (1982), *Thyroid Disease. THE FACTS*: Oxford University Press, NY.

29. Davies, J. C., Hipkin, L. J., (1977), *Clinical Endocrine Pathology*, Blackwell Scientific Publications.

30. Danowski, T. S., (1968), *Outline of Endocrine Gland Syndromes*, 2nd Ed.

31. Green, W.L., (1991), *Antithyroid Compounds, In Werner's The Thyroid*, 6th Ed; Ingbar, S.H., Braverman, L.E., Eds; Lipponcot: Philadelphia, 322-335.

32. Greenspan, Francis, S., (1983), *Endocrinology*, Large Medical Book, 3rd Ed.

33. Christensen, L.K., (1959), *Nature.*, **183**, 1189-1190.

34. Wolff, J., Standaert, M.E., Rall, J.E., (1961), *J. Clin. Invest.*, **40**, 682-685.

35. Richardson, S.J., and Schreiber, G., (1997), *Comparative Biochemistry*, **116(2)**, 137-160.

36. Stockigt, J.R., Barlow, J.W., White, E.L., Csicsmann, J.M., (1982), *Influence of Altered Plasma Binding on Free and Total Thyroid Hormone Levels, In Free Hormones in Blood*; Albertini, A., Ekins, R.P., Eds; Elsevier: NY.

37. Cody, V., (1980), *Endocrine Rev.1*, 140-66.

38. Marshall, J.S., Pensky, J., Williams, S., (1973), *Arch. Biochem. Biophys.*, **156**, 456-462.

39. Loebermann, H., Tokuoka, R., Diesenhofer, K., Huber, R., (1984), *J. Mol. Biol.*,**177**, 531-555.

40. Jarvis, J.A., Munro, S.L.A., and Craik, D.J., (1992), *Protein Eng.*, 5, 61-67.

41. Jarvis, J.A., (1985), *PhD Thesis*, Victorian College of Pharmacy.

42. Andrews, P.R., Craik, D.J., and Munro, S.L.A., (1988), *NMR Spectroscopy in Drug Research,* Alfred Benzon Symposium., **26**, 20-36.

43. Blake, C.C.F., Geisow, M.J., Oatley, S.J., Rerat, B., Rerat, C., (1978), *J. Mol. Biol.*, **121**, 339-356.

44. Blake, C.C.F., Geisow, M.J., Swan, I.D.A., Rerat, C., Rerat, B., (1974), *J. Mol. Biol.*, **88**, 1-12.

45. He, X.M., Carter, D.C., (1992), *Nature.*, **358**, 209-215.

46. Tritsch, G.L., and Tritsch, N.E., (1963), *J. Biol. Chem.*, **238(1)**, 138-142.

47. Tabachnick, M., (1967), *J. Biol. Chem.*, **242(7)**, 1646-1650.

48. Benvenga, S., Cahnmann, H.J., and Robbins, J., (1993), *Endocrinology.*, **133(3)**, 1300-1305.

49. Andrea, T.A., Cavalieri, R.R., Goldfine, I.D., Jorgenson, E.C., (1980), *Biochemistry.*, **19**, 55-63.

50. McKinney, J.D., Chae, K., Oatley, S.J., Blake, C.C.F., (1985), *J. Med. Chem.*, **28**, 375-381. Somack, R., Andrea, T.A., Jorgenson, E.C., (1982), *Biochemistry.*, **21**, 163-170.

51. Blake, C.C.F., Burridge, M., Oatley, S.J., (1978), *Biochem. Soc. Trans.*, **6**, 1114-1118.

52. Craik, D.J., Munro, S.L.A., and Stockigt, J.R., unpublished.

53. Chalmers, D., (1993), *PhD Thesis*, Victorian College of Pharmacy.

54. Munro, S.L.A., Lim, C.F., Hall, J.G.,Barlow, J.W., Craik, D.J., Topliss, D.J., Stockigt, J.R., (1989), *J. Clin. Endocrinol. Metab.*, **68(6)**, 1141-1147.

55. Barlow, J.W., Raggat, L.E., Lim, C., Munro, S.L.A., Topliss, E.J., Stockigt, (1999), J.R., *Clin. Sci.*, in print.

56. Wojtczak, A., Luft, J.R., and Cody, V., (1993), *J. Biol. Chem.*, **268(9)**, 6202-6206.

57. Cody, V., Luft, J.R., Pangborn, W., (1995), *Proceedings of the Day Dream Island Peptide Conference*, S9-5.

58. Jorgenson, E.C., (1964), *Mayo. Clin. Proc.*, **39**, 560.

59. Cody, V., (1978), *Recent Progress in Hormone Research.*, **34**, 437-75.

60. Zenker, N., Jorgenson, E.C., (1959), *J. Am. Chem. Soc.*, **81**, 4643-7.

61. Gale, D.J., Craik, D.J., and Brownlee, R.T.C., (1988), *Magnetic Resonance in Chemistry.*, **26**, 275-280.

62. Jorgenson, E.C., (1978), Thyroid Hormone and Analogs. II. Structure-Activity Relationships, in Hormonal Proteins and Peptides, Ed. Li, C. H. Academic Press, NY.

63. Wagner, R.L., Apriletti, J.W., McGrath, M.E., (1995), *Nature.*, 378, 690-697.

64. Seibel, G.L., Kollman, P.A., (1990), *Comprehensive Medicinal Chemistry*, Hansch, C., Eds; Pergammon Press : Oxford, 125-138.

65. Cody, V., (1981), *Acta. Cryst.*, **B37**, 1685-1689.

66. Dietrich, S.W., Bolger, M.B., Kollman, P.A., (1977), *J. Med. Chem.*, 20, **7**, 863-880.

67. Andrea, T.A., Dietrich, S.W., Murray, W.I., Kollman, P.A., Jorgenson, E.C., Rothenberg, S., (1979), *J. Med. Chem.*, **22**, 221.

68. Jorgenson, E.C., (1976), *Pharmacol Ther [B].*, **2**, 661.

69. Selenkow, H.A., and Asper, S.P., (1955), *Physiol. Rev.*, **35**, 426.

70. Pittman, C.S., and Pittman, J.A., (1974), *Endocrinology.*, 3, Green, R.O., and Astwood, E.B. Eds., Thyroid, American Physiological Society, Washington, D.C, 233.

71. Jorgenson, E.C., and Lehman, P.A., (1961), *J. Org. Chem.*, **26**, 894.

72. Chopra, I.J., (1976), *J. Clin. Invest.*, **58**, 32.

73. Gavin, L.A., Hammond, M.E., Castle, J.N., and Cavalieri, R.R., (1978), *J. Clin. Invest.*, **61**, 1276.

74. Sakurada, T., Rudolph, M., Fang, S.L.L., Vagenakis, A.G., Braverman, L.E., and Ingbar, S.H., (1978), *J. Clin. Endocrinol. Metab.*, **46**, 916.

75. Gavin, L.A., Castle, J.N., McMahon, F., Martin, P., Hammond, M., and Cavalieri, R.R., (1977), *J. Clin. Endocrinol. Metab.*, **44**, 733.

76. Comite, F., Burrow, G.N., Jorgenson, E.C., (1978), *Endocrinology.*, **102**, 1670.

77. Kriz, B.M., Jones, A.L., and Jorgenson, E.C., (1978), *Endocrinology.*, **102**, 712.

78. Benson, M.C., Lin, J.P., Huang, Y.P., Burger, A., Rivlin, R.S., (1978), *Endocrinology.*, **102**, 562.

79. Selenkow, H.A., Plamondon, C.A., Wiswell, J.G., Asper, S.P., (1958), *Bull. John Hopkins Hosp.*, **102**, 94.

80. Jorgenson, E.C., Wiley, R.A., (1963), *J. Med. Chem.*, **6**, 459.

81. Jorgenson, E.C., Muhlhauser, R.O., Wiley, R.A., (1969), *J. Med. Chem.*, **12**, 689.

82. Duggan, B., (1986), PhD, Thesis, Victorian College of Pharmacy.

83. Pages, R.A., Burger, A., (1967), *J. Med. Chem.*, **10**, 435.

84. Harrington, C.R., (1948), *Biochem. J.*, **43**, 434.

85. Niemann, C., (1950), *Fort. Chem. Org. Naturst.*, **7**, 167.

86. Barker, S.B., Shimada, M., Makinchi, M., (1965), *Endocrinology.*, **76**, 115.

87. Jorgenson, E.C., Bertean, P.A., (1971), *J. Med. Chem.*, **14**, 1199.

88. Atkinson, D.C., Godfrey, K.E., Meek, B., Saville, J.F., Stillings, M.R., (1983), *J. Med. Chem.*, **26**, No. 10, 1361-64.

89. Seidlova, V., Pelz, K., Adlerova, E., Jirkovsky, I., Protiva, M., (1969), *Collect. Czech. Chem. Commun.*, **34**, 2258-2277.

90. Kmonicek, V., Pomykacek, J., Holubek, J., Svatek, E., Valchar, M., Metysova, J., Polivka, Z., (1991), *Collect. Czech. Chem. Commun.*, **56**, 2468-2481.

91. Beringer, F.M., and Gindler, E.M., (1955), *J. Am. Chem. Soc.*, **77**, 3203-3207.

92. Ullmann, F., Zlokasoff, B., (1905), *Berichte.*, **38**, 2111-19.

93. Ullmann, F., Engi, G., (1904), *Berichte.*, **37**, 2367-2373.

94. Goldberg, A.A., Walker, H.A., (1953), *J. Chem. Soc.*, 1438-1357.

95. Lockhardt, T.P., (1983), *J. Am. Chem. Soc.*, **105**, No. 7, 1940-1946.

96. Lindley, J., (1984), *Tetrahedron.*, **40**, No. 9, 1433-1456.

97. Scherrer, R.A., Beatty, H.R., (1980), *J. Org. Chem.*, **45**, 2127-31.

98. Caserio, M. C., Glusker, D. L., Roberts, J. D., (1959), *J. Am. Chem. Soc.*, **81**, 336.

99. Tuong, T. D., Hida, M., (1970), *Bull. Chem. Soc. Japan.*, **43**, 1763.

100. Tuong, T. D., Hida, M., (1974), *J. Chem. Soc. Perkin Trans. II.*, 674.

101. Williams, A. C., Kinnery, R. E., Bridger, R. F., (1967), *J. Org. Chem.*, **32**, 2501.

102. Weingarten, H., (1964), *J. Org. Chem.*, **29**, 3624.

103. Connor, J. A., Dubowski, D., Jones, A. C., Price, R., (1982), *J. Chem. Soc, Perkin Trans. I.*, 1143.

104. Beringer, Marshall., Bodlaender, Peter., (1969), *J. Org. Chem.*, **34**, 1981-85.

105. Bacon., Hill., (1964), *J. Chem. Soc.*, 1100.

106. Lewin, A. H., Goldberg, N. L., (1972), *Tetrahedron Lett.*, 491.

107. Whitesides, George. M., Sadowski, John. S., Lilburn, Jennifer., (1974), *J. Am. Chem. Soc.*, **96**, 2829-2835.

108. LeGoff, E., (1962), *J. Am. Chem. Soc.*, **84**, 3786.

109. Beringer, F. M., Huang, S. J., (1964), *J. Org. Chem.*, **29**, 445.

110. Fieser, L. F., Haddadin, M. J., (1973), in "**Organic Synthesis**"., Collect., **5**, Wiley, New York, 1037.

111. Graebe, C., and Lagodzinski, K., (1892), *Chem Ber.*, **25**, 1733; *Chem. Soc.*

Abstr., **62**, 1086.

112. Ullmann, F., (1903), *Chem. Ber.*, **36**, 2382; *Chem. Soc. Abstr.*, **84**, 692.

113. Kulkarni, V.S., Kulkarni, S.R., Lele, S.R., Hosangadi, B.D., (1988). *Tetrahedron.*, **44**, No. 16, 5145- 5149.

114. Sindelar, K., Holubek, J., Ryska, M., Svatek, E., Dlabac, A., Hrubantova, M., Protiva, M., (1982), *Collec. Czech. Chem. Commun.*, **47**, 72-87.

115. Deshpande, C.N., Sattur, P.B., and Nargund, K.S., (1957), *J. of Karnatak University.*, 33-42.

116. Galt, R.H.B., Horbury, J., Matusiak, Z.S., Pearce, R.J., Shaw, J.S., (1989), *J. Med. Chem.*, **32**, 2357- 2362.

117. Sammes, Peter.G., Thetford, Dean., Voyle, Martyn., (1988), *J. Chem. Soc. Perkin Trans. I.*, 3229-3231.

118. Mancini, M.L., Honek, J.F., (1989), *Synthetic Communications.*, **19(11&12)**, 2001-2015.

119. Koelsch, C.F., Lucht, F.J., (1949), *J. Am. Chem. Soc.*, **71**, 3556-3558.

120. Rewcastle, Gordon. W., Atwell, G.J., Palmer, B.D., Boyd, P.D.W., Baguley. B.C., Denny, W.A., (1989), *J. Med. Chem.*, **32**, 793-799.

121. Goldberg, A.A., (1952), *J. Chem. Soc.*, 4368.

122. Pauson, P.l., Segal, J.A., (1975), *J. Chem. Soc. Dalton Trans.*, 1677.

123. Goldberg, A.A., Wragg, A.H., (1958), *J. Chem. Soc. London.*, 4227-41.

124. Goldberg, A.A., and Kelly, W., (1946), *J. Chem. Soc.* 102; (1905), Ullmann, F.,

Kipper, H., *Chem. Ber.*, **38**, 2120.

125. Ullmann, F., (1907), *Justus Liebigs Ann. Chem.*, 355, 312; (1908).*Chem. Abstr.*, **2**, 87.

126. Sindelar, K., Holubek, J., Ryska, M., Dlabac, A., Valchar, M., Metysova, J., Protiva, M., (1984), *Collec. Czech. Chem. Commun.*, **49**, 2531-39.

127. Backtus, E, A., Hotelling, E. B., Neuworth, M. B., (1957), *J. Org. Chem.*

128. Cervena, I., Holubek, J., Svotek, E., Protiva, M., (1988), *Collec. Czech. Chem. Commun.*, **53**, 1307-16.

129. Kmonicek, V., Pomykacek, J.,Holubek, J., Svatek, E., Valchar, M., Metysova, J., Polivka, Z., (1991), *Collec. Czech. Chem. Commun.*, **56**, 2468-79.

130. Migita, T., Shimizu, T., Asami, Y., Shiobara, J., Kato, Y., Kosugi, M., (1980). *Bull. Chem. Soc. Jpn.*, **53**, 1385-1389.

131. Aldrich Catalogue of NMR Spectra

132. Oppenheimer, J.H., Surks, M.I., Smith, J.C., and Squef, R., (1965), *J. Biol. Chem.*, **240**, 173-180.

133. Raz, A., and Goodman, D. S. (1969), *J. Biol. Chem.*, 244(12), 3230-3237.

134. Branch, W. T., Robbins, J., and Edelhoch, H., (1971), *J. Biol. Chem.*, 246.

135. Cheng, S. Y., Cahnmann, H.J., Wilchek, M., (1975), *Biochemistry.*, **14(18)**, 4132-4136.

136. Ferguson, R. N., Edelhock, H., Saroff, H.A., (1975), *Biochemistry.*, **14(2)**, 282-289.

137. Cohn, E.J., Strong, L.E., Hughes, W.L., (1946), *J. Am. Chem. Soc.*, **68**, 459-475.

138. Tritsch, G L., (1972), *J Med.*, 3, 129-145.

139. Dwulet, F. E., Benson, M. D., (1986), *J Clin. Invest.*, 78, 880-886.

140. Sunde, M., Richardson, S.J., Chang, L.J., (1996), *Eur. J. Biochem.*, 491-449.

141. Cadogan, J.I.G., Hutchison, S.H., McNab, H., (1992), *Tetrahedron.*, **48**, No. 36, 7747-62.

142. Dippy, J.F.J., Lewis, R.H., (1937), *J. Chem. Soc. London.*, 1426-29.

143. Mustafa, A., Sobhy Ezz El-Din, M., (1955), *J. Am. Chem. Soc.*, **77**, 5124-26.

144. Allen, F.L., Koch, P., Suschitzky, H., (1959), *Tetrahedron.*, **6**, 315-18.

145. Brewster, R.Q., Strain, F., (1934), *J. Am. Chem. Soc.*, 117-20.

146. Mann, F.G., Turnball, J.H., (1951), *J. Chem. Soc.*, 747-756

147. Tomita, I, (1954), *J. Pharm. Soc. Japan.*, 1061, 1065.

148. Langham, W., Brewster, R.Q., Gilman, H., (1941), *J. Am. Chem., Soc.* 545-549.

149. Jilek, J.D., Metysova, J., Pomykacek, J., Protiva, M., (1968), *Collection Czech. Chem. Commun.*, **33**, 1831.

150. Pelz, K., Jirkovsky, I., Adlerova, E., Metysova, J., Protiva, M., (1968), *Collection Czech. Chem. Commun.*, **33**, 1895-1910.

The last link is broken,
That bound me to thee,
And the words thou hast spoken
Have rendered me free.

Fanny Steers