ABSTRACT

THYROXINE ANALOGS. THE SYNTHESIS AND ACTIVITY
OF
VARIOUS BRIDGED ANALOGS

Ellen Cheung Ngoi Yin
Ph.D. Dissertation
Department of Pharmaceutical Chemistry
School of Pharmacy
University of California
San Francisco

In this study, we report the synthesis of nine new thyroxine analogs, their in vivo antigoitrogenic activities and in vitro binding affinities to nuclear receptors and thyroxine binding prealbumin (TBPA). These analogs are designed to preserve the preferred skewed conformation which is essential for activity while varying the bridging element.

With a dihedral angle of 120°, the ketone bridged analogs of 3'-isopropyl-3,5-diiodo-DL-thyronine (T_2iPr) possess low but significant binding to the receptor and to TBPA. The methylene-bridged compounds of T_2iPr exhibit the strongest thyromimetic activities in the rat antigoiter experiment and exceptionally high binding affinities to the receptor. These observations might be explained in terms of a more acute dihedral angle (~114°) that the CH_2 bridge offers. The sp^3 carbon at the bridging position appears to have a

potentiating effect on hormonal activity when incorporated with a 3'-isopropy group.

Taking advantage of this combination, a methylene-bridged DIMIT molecule was synthesized and tested. The binding to nuclear receptor increased tenfold whereas the affinity to TBPA remained almost the same when compared with DIMIT. The increased hormonal activity and added lipophilicity offer a more potent DIMIT analog which should facilitate transport through the placental.

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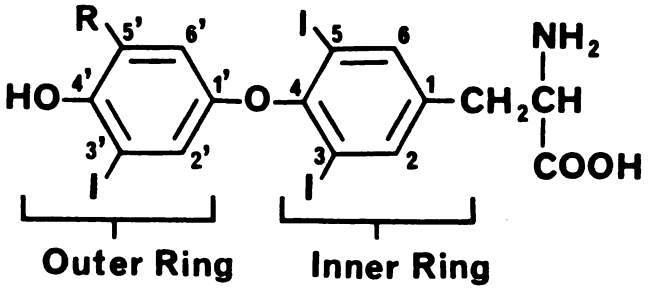
CHAPTER ONE

INTRODUCTION

The two thyroid hormones, thyroxine (T_4, 1-1) and 3,5,3'-triiodothyronine (T_3, 1-2), elicit a multitude of biological responses and are essential for normal growth and development (Pitt-Rivers and Tata,1959; Jorgensen 1980).



R = I, 1-1

R = H, 1-2

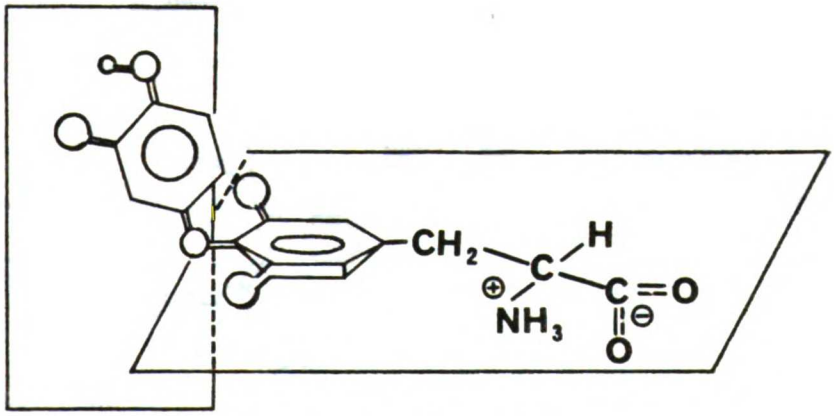
Numerous hypotheses were proposed for the mechanism of thyroid hormone action. Early studies tried to relate the observed metabolic stimulation to a direct involvement of the hormone in the energy-producing reactions. Nieman (1950) postulated the reversible oxidation of thyroxine to a quinoid form coupled with electron transfer to a substrate involved in energy-generating metabolism. Szent-Gyorgyi (1957) proposed that thyroxine serves as a carrier for iodine, and that the unique heavy atom properties of this element provide for the generation and transmittal of energy by way of the excited triplet state of iodine. Green (1941) suggested the direct interaction of hormone and enzymes.

These hypotheses were ruled out gradually as extensive studies on structure activity relationships were performed with many hormone analogs and with the detection of nuclear receptors from rat liver and kidney cells by Oppenheimer et al (1972). The excellent correlation observed between hormonal activities in vivo and the relative binding affinities of hormonal structural analogs to the nuclear receptor protein suggests that the binding of the hormone to its receptor is responsible for initiation of biological effects. The close association of the receptors with chromatin deoxyribonucleic acid (DNA) in the cell nucleus (Surks et al. 1973, Spindler et al. 1975) supports the proposal that the binding of T_3 (or T_4) to the nuclear receptor protein transmits a signal to a specific DNA template, which in turn stimulates the synthesis and release of specific messenger ribonucleic acids (mRNA). The mRNA stimulate the increased synthesis of proteins including those used for structural or functional purposes during growth and development, and those enzymes that mediate the physiological expressions of the hormones.

In view of these recent studies, attention is focused on the structural and stereochemical aspects of the hormone, as they relate to receptor interaction, with particular emphasis on: (a) the importance of the phenyl-X-phenyl conformation, as influenced by X and the 3, 5, 2' and 6' substituents; (b) specific hydrophobic, hydrogen bonding and steric interactions of the 3', 4' and 5' substituents with

receptors; and (c) the importance in transport, metabolism and receptor binding of the 1 position side chain length, conformation, stereochemistry, and associated charges. A number of physico-chemical studies of the hormones and analogs utilizing X-ray crystallography (Cody 1974,1975; Cody and Duax 1973 a,b; Cody et al. 1972), NMR spectroscopy (Lehman and Jorgensen 1965, Emmett and Pepper 1975), and theorectical molecular orbital calculations (Kollman et al. 1973, 1979), as well as analog structure-activity studies have consistently supported the structural and stereochemical dependence of thyromimetic activity.

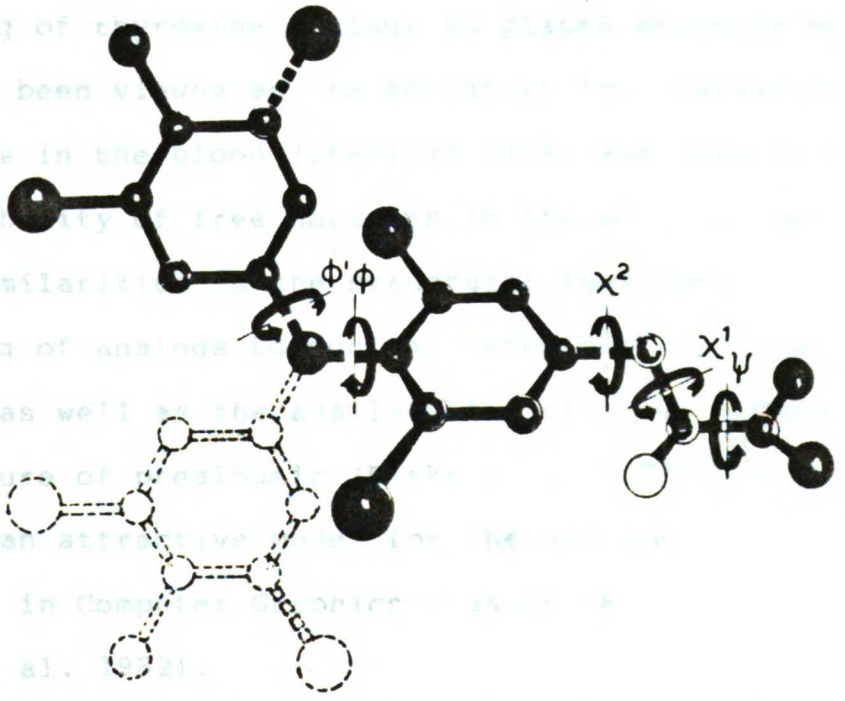
The unique, skewed conformation of the thyroxine molecule was first recognized by Jorgensen (1964) using space-filling models and subsequently confirmed by crystallographic studies.



Skewed Conformation of T_3

The bulk of the 3-, 5-iodines causes the phenolic ring (outer ring) to adopt a perpendicular and bisecting position with respect to the plane of the alanyl ring (inner ring).

In this preferred skewed conformation, rotation about the phenyl ether bonds is restricted and minimal steric interaction between the 3-, 5-iodines and the 2'-, 6'-hydrogens is achieved. X-ray crystallographic data on T_4 and T_3 show that the diphenyl ether link (C4-O4-C1') is angled at 120° (dihedral angle O) (Camerman and Camerman 1974, Cody 1974). The diphenyl ether conformation is further defined by the torsional angles ϕ (C5-C4-O4-C1') and ϕ' (C4-O4-C1'-C6'). Molecular orbital calculations and NMR studies at low temperatures indicated that the skewed conformation (ϕ/ϕ' = 90°/0°) is the preferred low energy conformation.



Torsion Angles: $\phi\ C_5C_4O_4C_{1'}$; $\phi'\ C_4O_4C_{1'}C_{6'}$; $\psi\ N_8C_8C_9O_{10}$; $\chi\ C_2C_1C_7C_8$; $\chi'\ C_1C_7C_8N_8$.

In vitro assays which measure the binding affinities of thyroid hormones and analogs to isolated rat hepatic nuclei (Koerner et al. 1975, Jorgensen et al. 1975), to solubilized rat hepatic nuclear non-histone proteins (Jorgensen et al. 1977, Samuels et al. 1974a,b), and to various purified plasma proteins (Synder et al. 1975, 1976; Andrea 1977) have contributed to the further understanding of the structure activity relationships for thyromimetic activity. The apparent binding constants reflect the thermodynamics of binding rather than the actual biological activity. *In vitro* measurements avoid the difficulties arising from distribution, metabolism and biological response. The *in vivo* binding of thyroxine analogs to plasma proteins has primarily been viewed as the mechanism for transport and storage in the blood (Sterling 1964) and determines the availability of free hormones in the whole animal. However, the similarities in the structural requirements for *in vitro* binding of analogs to nuclear receptors and to plasma proteins as well as the availability of X-ray crystallographic structure of prealbumin (Blake et al. 1974) have made prealbumin an attractive model for the nuclear receptor, especially in Computer Graphics studies (Blake et al. 1977, Blaney et al. 1982).

As part of an extensive study of structure-activity relationships, this work is devoted to examination of the effect on thyromimetic activity and binding of substitution of the diphenyl ether oxygen atom with either a more polar

carbonyl group or a more lipophilic, metabolically more stable, methylene moiety. Incorporation of these groups into the basic thyronine structure will alter the dihedral angle very slightly. The preferred skewed conformation is further ensured by the presence of the 3-, 5-iodines. The isopropyl group is chosen for the 3' position in preparation for the final syntheses of halogen-free hormone analogs. Chapter Two describes the synthesis of six ketone- and methylene-bridged compounds. The assessment of *in vivo* antigoitrogenic activity and *in vitro* binding to solubilized nuclear receptors and prealbumin are presented in Chapter Four, Three and Five, respectively. Promising results obtained for the halogenated methylene-bridged analogs prompted the further syntheses of a sequence of methylene-bridged, halogen-free compounds with the hope of improving on the existing transplacentally active 3,5-dimethyl-3'-isopropyl-thyronine (DIMIT). This is described in Chapter Six.

CHAPTER TWO

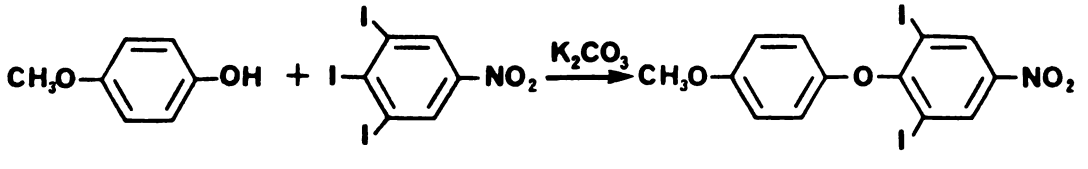
SYNTHESIS OF THYROID HORMONE ANALOGS

2.1 Synthetic Background

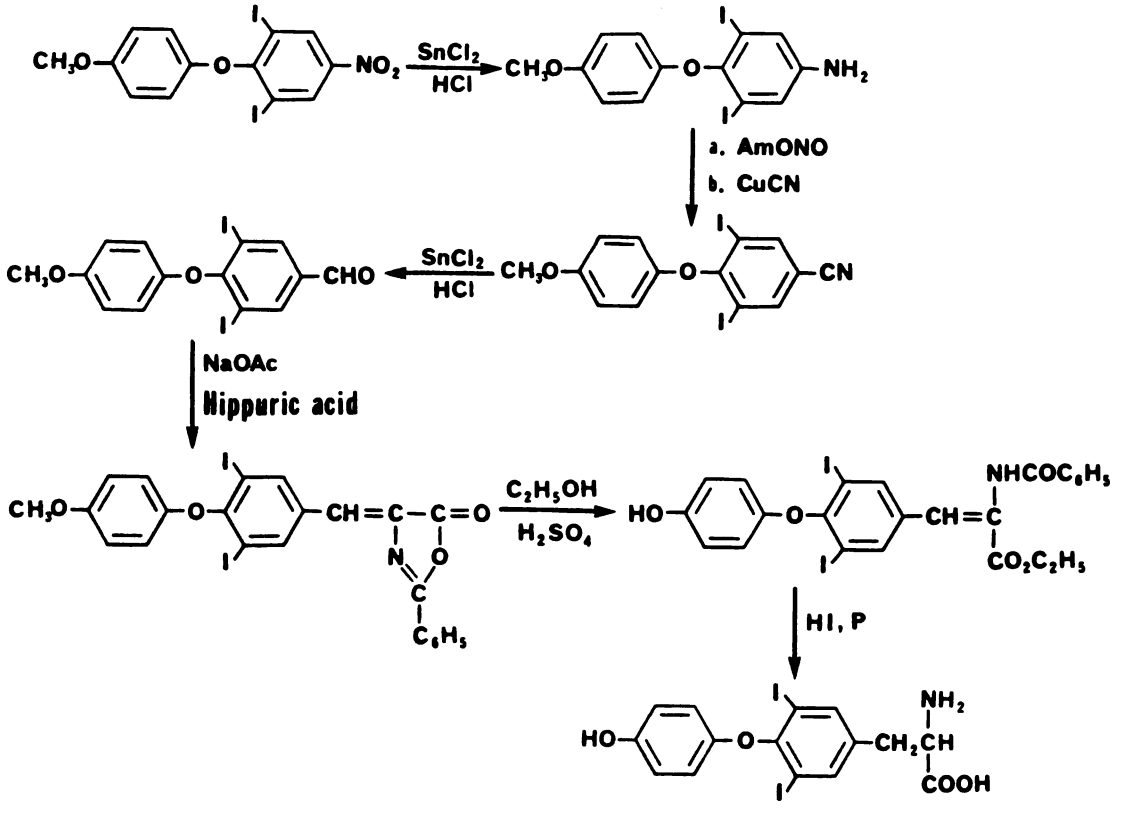
Syntheses of the thyroid hormones and of structurally related analogs vary primarily in the method used to establish the diphenyl ether nucleus. Secondarily, synthetic variations involve the nature of the groups present in the single-ring compounds used in forming the diphenyl ether nucleus, their subsequent conversion to the desired substituents, and the reactions used to introduce new groups into the preformed diphenyl ether. The synthetic methods for the formation of the diphenyl ether structure are of four basic types:

A. The Reaction of an Ortho or Para-Halonitrobenzene with a Phenol

Harington and Barger (1927) initiated the first synthesis of thyroxine by condensation of hydroquinone monomethylether with 3,4,5-triiodonitrobenzene, taking advantage of the high reactivity of the halogen para to the nitro group.

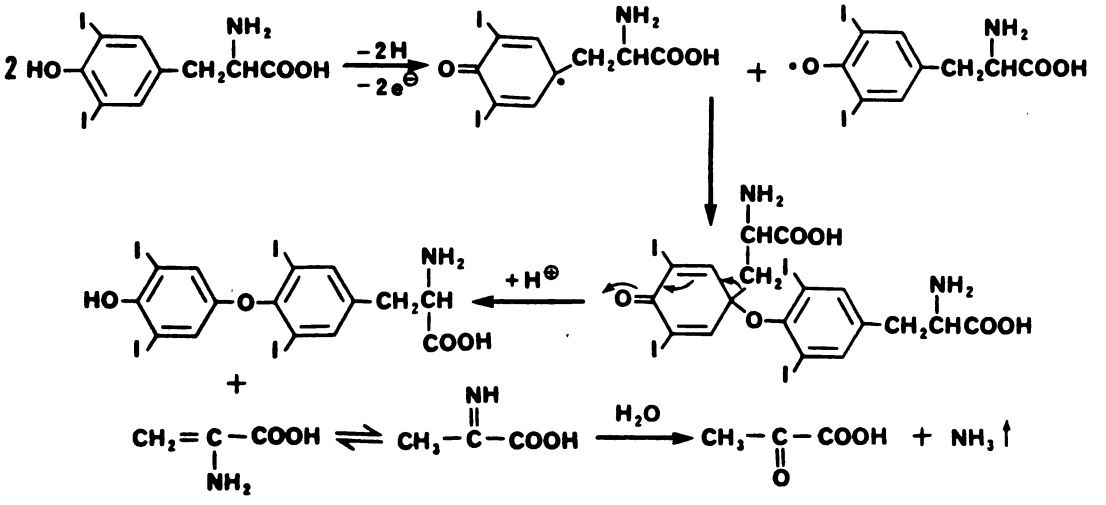


Formation of the diphenyl ether was followed by: reduction of the nitro group to the amine, Sandmeyer conversion of the amine to the cyano compound, conversion of the cyano group to the aldehyde, and azlactone synthesis to yield an N-protected α-amino cinnamic acid ethyl ester. Reduction of the double bond of the ester was affected by brief treatment with red phosphorus and hydriodic acid in acetic acid, followed by heating under reflux with hydrobromic acid to remove protective groups and to form the DL-alanine side chain.



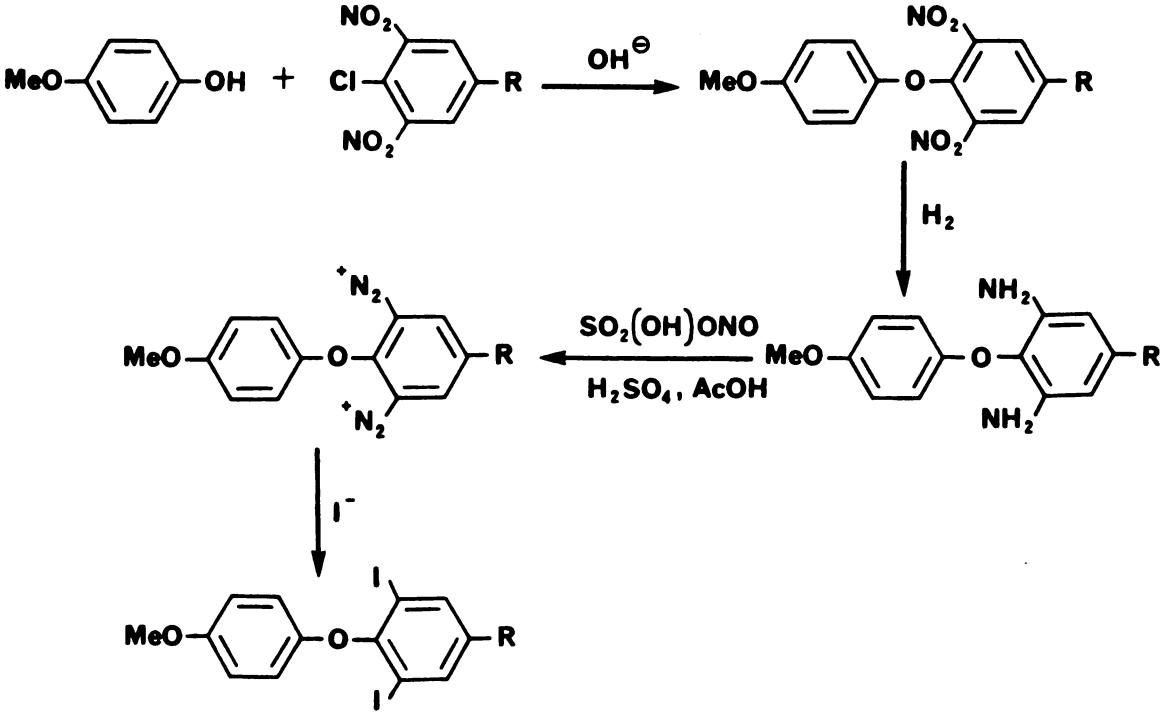
B. Biosynthetic-like Condensations of 3,5-Diiodotyrosine and related 2-Iodophenols

Incubation at 38°C of a weakly basic solution of 3,5-diiodotyrosine was reported by von Mutzenbecher (1939) to give rise to small amounts of thyroxine after a few days. Johnson and Tewkesbury (1942) postulated that in the conversion of diiodotyrosine to thyroxine, free radical intermediates condense to form a quinol ether intermediate, followed by loss of an alanine side chain, possibly as dehydroalanine. This dehydroalanine, in its iminopyruvic acid tautomeric form, would hydrolyze to ammonia and pyruvic acid, the latter being detected by the formation of indigo from reaction with o-nitrobenzaldehyde.



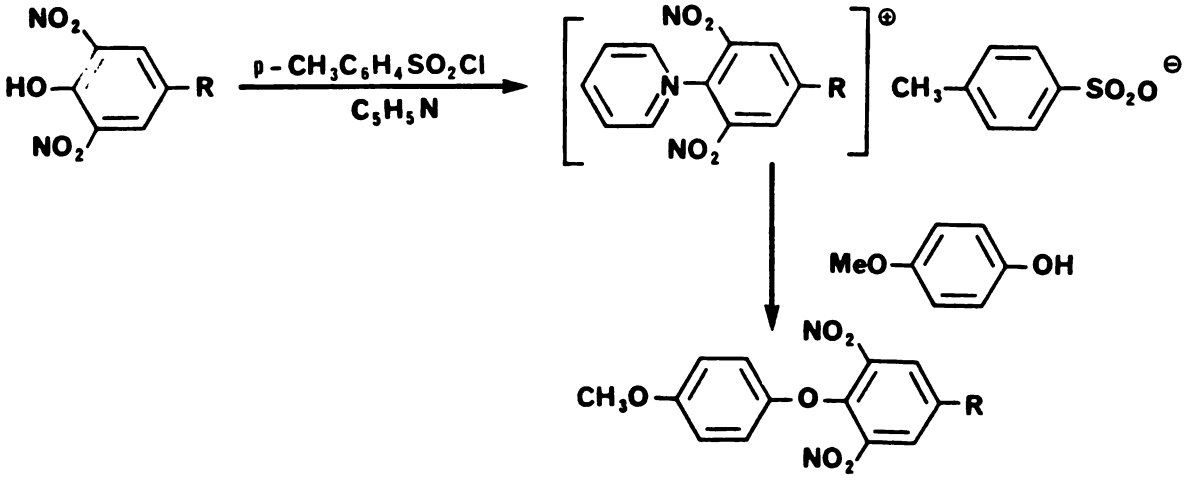
C. Condensation of a Phenol with a Dinitroaryl Pyridinium Salt

Aromatic halogen compounds activated by nitro groups in the ortho and para positions react readily with phenols to displace the halogen atom, forming nitrophenyl ethers. The dinitro groups are catalytically reduced to give the diamine. Borrows et al. (1949a) found that the bis-diazonium compound was formed by reaction with nitrosylsulfuric acid under anhydrous conditions, and that decomposition by aqueous KI yielded the desired diiodo compounds.



The limitation lies with the poor availability of halogeno-2,6-dinitrobenzenes substituted with groups readily converted to the desired alanyl side chain. However, con-

sideration of the reaction mechanism proposed by Borsche and Feske (1927), which emphasized the reactivity of pyridinium quaternary salts, led Borrows and coworkers (1949b) to attempt the direct reaction of dinitrophenol, after treatment with p-toluenesulfonyl chloride and pyridine, with 4-methoxyphenol. The desired dinitrodiphenyl ether was obtained.

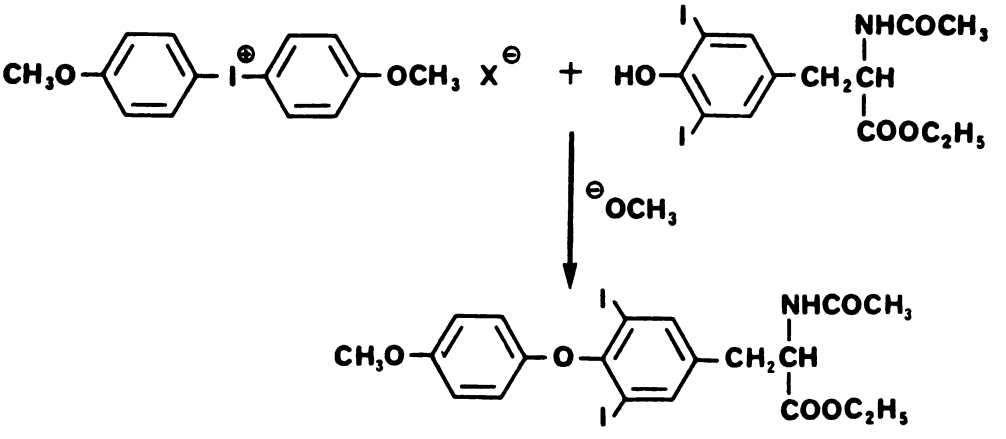


The dinitroaryl pyridinium salt condensation with the appropriate phenol has been widely applied in syntheses of thyroxine analogs (see Barnes et al. 1953a,b; Blank et al. 1963a, 1966b; Buess et al. 1965; Jorgensen and Lehman 1961; Jorgensen and Reid 1965; Jorgensen and Slade 1971; Kharasch et al. 1956; Zenker and Jorgensen 1959) and is often designated as the Glaxo method because of the place of origin and the diversity of authors who contributed to the synthesis. A useful modification was introduced by Meltzer and cowork-

ers (1957), who used methanesulfonyl chloride in place of p-toluenesulfonyl chloride. This variation provides a more rapid reaction under milder conditions and eliminates the need to isolate the reactive sulfonyl ester intermediate.

D. Condensation of Substituted Phenols with Diaryliodonium Salts

The method for the formation of 2,6-dihalodiphenyl ethers directly from a 2,6-dihalophenol was first reported by Hillman (1956, 1959), who described the reaction of 4,4'-dimethoxy-diphenyl iodonium bromide with N-acetyl-3,5-diiodo-L-tyrosine ethyl ester in methanol at reflux temperature for 80 hours in the presence of magnesium methoxide or sodium methoxide.



Bevilacqua et al. (1959) carried out the same reaction in a shorter time (23 hours) at room temperature, in the presence of triethylamine and a copper catalyst. The iodonium reaction has been applied in the preparation of a number of 3,5-

dihalothyronines or side-chain analogs with varied substituents in the phenolic ring (Blank et al. 1963a,b, 1966a; Jorgensen and Reid 1964, 1965; Jorgensen and Nulu 1969; Matsuura 1964) and for 3,5-dihalothyronines with different halogens in the same ring (Dibbo et al. 1961a). It has also been particularly useful in the synthesis of 3-halothyronines (Block 1967, 1976) from which other structural analogs are readily prepared.

Some special synthetic reactions have been applied in the preparation of analogs which were not accessible by the general methods described. Of particular interest are the synthetic procedures used in generating analogs containing bridging atoms other than oxygen, namely diphenylsulfides, diphenylamines, diphenylketones and diphenylmethanes.

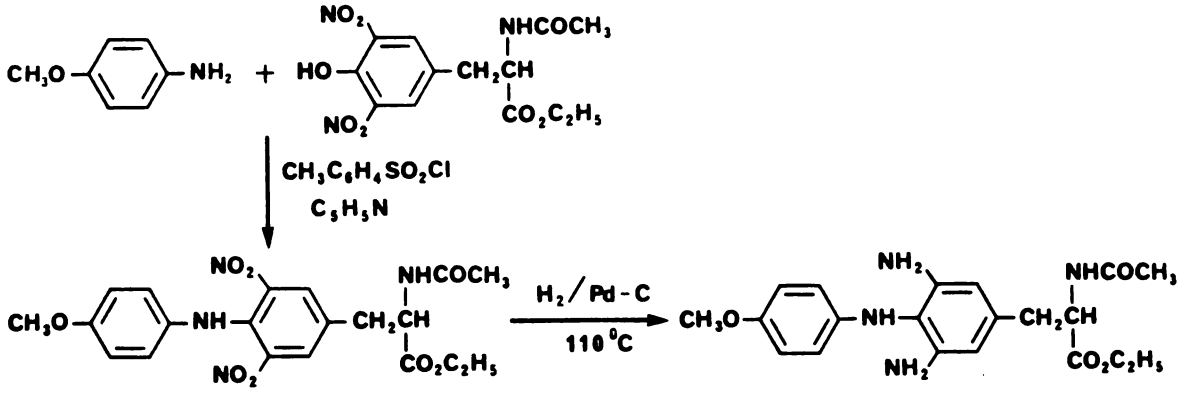
A. Diphenylsulfides

Synthesis of the sulfur-bridged analogs of thyroid hormones parallels that of the oxygen compounds and is, in most cases, effected by replacement of the phenol with thiophenol. Harington (1948) succeeded in synthesis of the diphenylsulfide analog of thyroxine by reacting 4-methoxythiophenol with 3,4,5-triiodonitrobenzene. The Glaxo method has been applied by Dibbo et al. (1961b). 4-Methoxythiophenol reacted with the appropriate o-dinitrophenol in the presence of pyridine and p-toluenesulfonyl chloride to give the protected sulfur

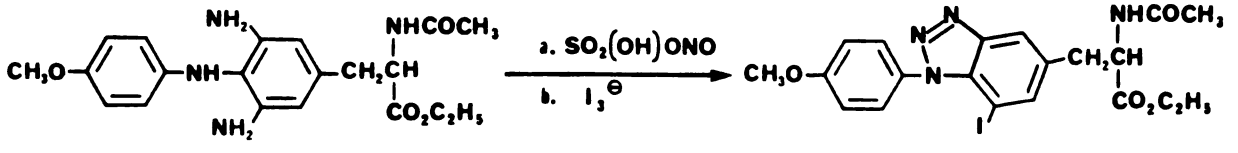
bridged analog.

B. Diphenylamines

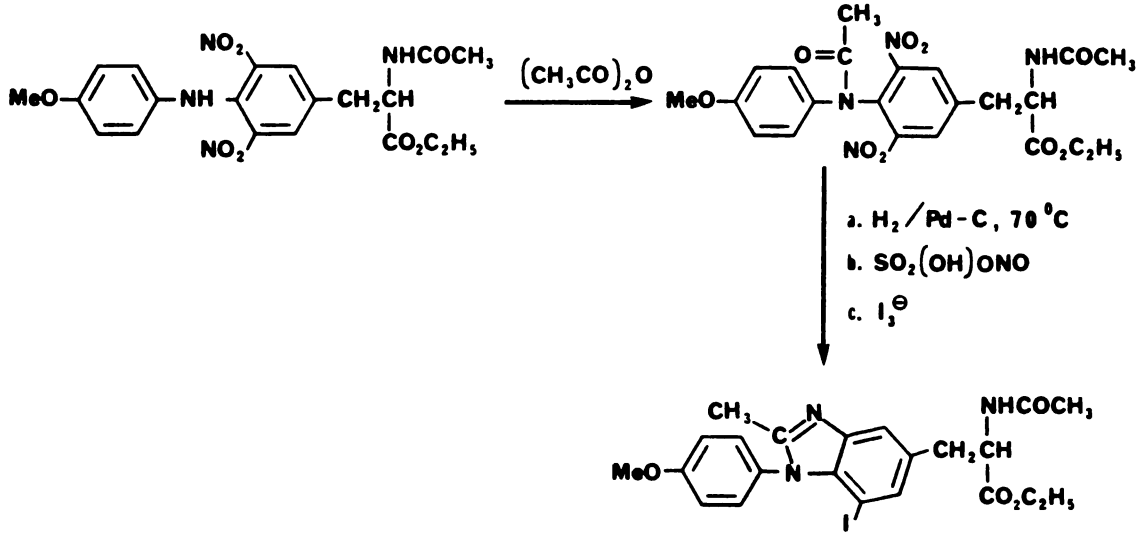
Several unsuccessful attempts have been made to synthesize diphenylamine analogs of the thyroid hormones. Cookson (1953) used the Glaxo method, replacing the phenolic component with p-anisidine to form the protected 3,5-dinitro-diphenylamine. This was reduced under elevated temperature and pressure to form the diamine.



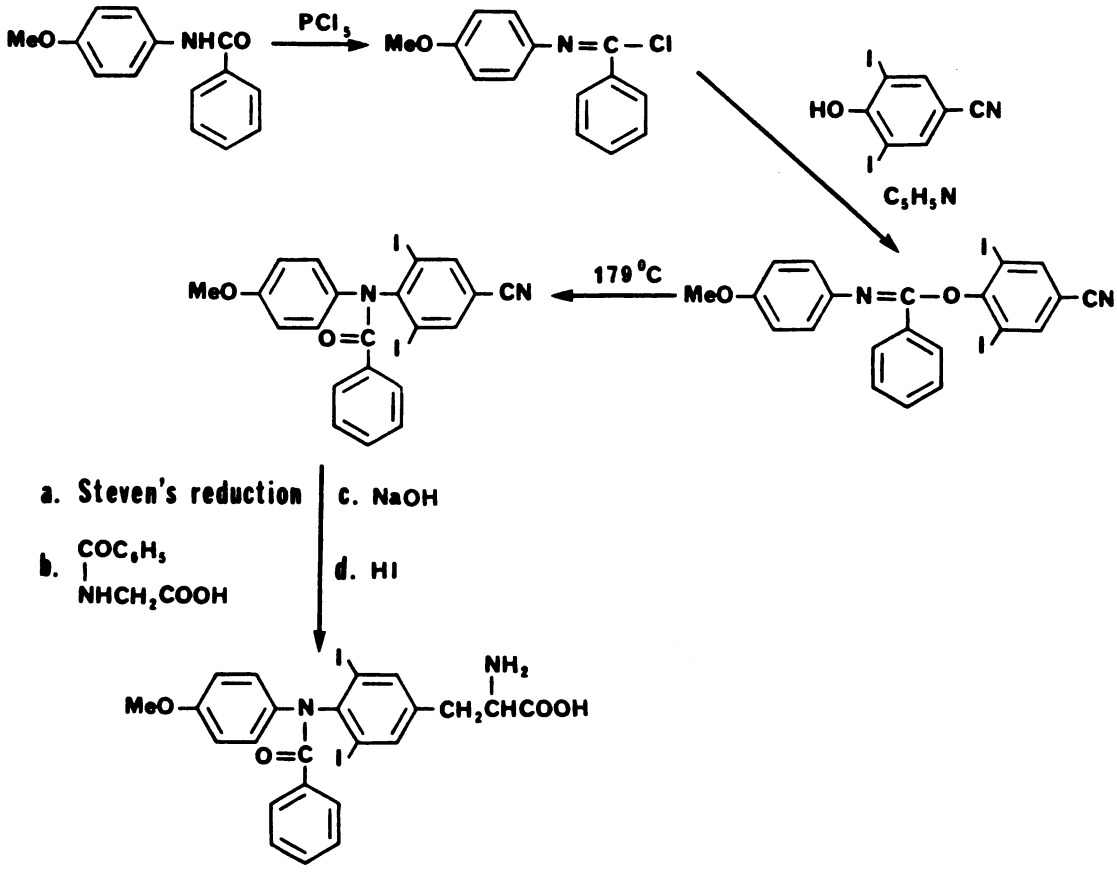
Bis-diazotization and treatment with triiodide led to replacement of only one diazonium group with an iodine atom. The second diazonium group reacted with the bridging nitrogen atom to form a benzotriazole.



To prevent triazole formation, Cookson (1953) protected the bridging nitrogen atom as the N-acetyl derivative. However, following reduction to the diamine, and under the strongly acidic conditions required for bis-diazotization, one of the amino groups reacted before diazotization with the bridging N-acetyl group to form the monoiodo 2-methylbenzimidazole.

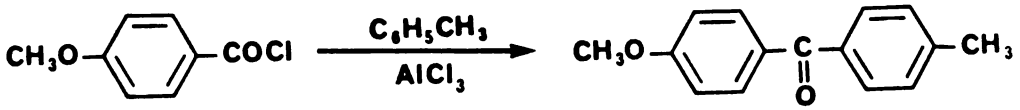


Following the leads established by Cookson (1953) using compounds containing methyl and carbomethoxy side chains, Mukherjee and Block (1971) further explored the Chapman rearrangement of benzimidates to form N-benzoyl-2,6-diiodo-diphenylamines as a route to diphenylamine analogs. Although the Chapman rearrangement and side chain modifications proceeded as expected, removal of both N-benzoyl and O-methyl protective groups led to destruction of the compound. This was apparently due to the extreme susceptibility to oxidation of the free p-hydroxydiphenylamine structure or to lability of the 3,5-iodine atoms.


MeO
NHCO
PCl5
MeO
N=C-Cl
HO
CN
C5H5N
MeO
N=C-O
CN
179°C
MeO
N
CN
O=C
a. Steven's reduction
b. COC6H5
NHCH2COOH
c. NaOH
d. HI
MeO
N
NH2
CH2CHCOOH
O=C

C. Diphenylketones and Diphenylmethanes

Horner and Medem (1952) prepared analogs containing carbonyl and methylene groups in place of the ether oxygen bridge. A Friedal-Crafts reaction of p-methoxybenzoyl chloride with toluene in the presence of aluminum chloride yielded the diphenylketone.



The amino acid side chain was developed by light-stimulated bromination of the methyl group, reaction of the resulting benzyl bromide with sodium N-phenylacetylaminocyanoacetic acid methyl ester, and hydrolysis of all protective groups with hydrobromic acid. Iodination in ammonium hydroxide yielded the carbonyl-bridged analog of 3',5'-diiodo-DL-thyronine. The methylene-bridged thyronine was prepared from the diphenylketone by reduction with hydriodic acid and red phosphorus at high temperature and pressure. Iodination again yielded the 3',5'-diiodo analog. Horner and Medem (1952) were unsuccessful in attempts to prepare carbonyl- and methylene- bridged analogs containing iodine atoms in the 3 and 5 positions.

Methylene- and carbonyl- bridged analogs of thyronines and thyroacetic acids iodinated in the 3 and 5 positions

have been prepared by Tripp and coworkers (1973). Their synthetic method, which was adapted for synthesis of analogs presented in this dissertation, will be discussed in the Synthetic Scheme section.

2.2 Synthetic Rationale

Numerous bridged analogs of triiodothyronine (T_3) have been synthesized in this study for the purpose of: (1) establishing the importance of structural conformation for thyromimetic activity, (2) exploring possible interactions with the nuclear receptor related to the bridging area of thyroxine, (3) summarizing all known activity requirements by incorporating the essential structures into one analog, and finally (4) altering the structure of an existing transplacental analog to maximize activity and facilitate passage through the placenta.

X-ray crystallographic studies show that the two phenyl rings of L-T_3 are oriented in a skewed conformation with a dihedral angle Θ, C4-O4-C1', of 120° and that the C4-O4, O4-C1' bonds are 1.37 Å and 1.43 Å respectively. (Cody 1974) Both Θ and the ether bridge length are well approximated by standard bond angles and standard single bond covalent radii. (The bond angle for a digonal oxygen is 110° and the calculated carbon-oxygen bond length obtained from the summation of the covalent radii of an sp^2 C (0.73 Å) and an oxygen atom (0.66 Å) is 1.39 Å. Applying similar approximations, we can predict that a methylene and a carbonyl

bridging group in place of the oxygen atom will give dihedral angles of about 110° and 120° respectively. The carbon-carbon bond lengths will be approximately 1.50 Å for the methylene bridge, and 1.46 Å for the ketone bridge (C-C, sp^3 = 0.77 Å, C-C, sp^2 = 0.73 Å). A recent study by Cody (1981) of 4-(α-Hydroxy-4-methoxybenzyl)-3,5-diiodobenzyl alcohol indicated that the bridging C-C-C angle for this compound is 114° with C-C bond lengths of 1.523 Å and 1.521 Å. Other studies on similar compounds bearing the basic diphenylmethane structure all showed bond angles ranging from 110° to 114°. (Hay and MacKay 1979, Tranqui et al. 1974, van der Haijden et al. 1975, Smith et al. 1977) Based on the above evidence, we predict that the carbon-bridged analogs that we propose to synthesize will mimic the conformation of T_3 with the dihedral angle slightly smaller than 120°.

Tripp and coworkers (1973) synthesized the methylene-bridged analog of T_3. This compound showed high activity similar to T_3 in both in vivo and in vitro (Jorgensen et al. 1974, Koerner et al. 1975, Frieden and Yoshizato 1974, Goldfine et al. 1976). In some test systems, this analog is more potent than T_3. Modification of the methylene-bridged T_3 (MB-T_3) by replacing the 3'- iodine with an isopropyl group gives 3,5-diiodo-4-(3'-isopropyl -4-hydroxybenzyl)-DL-phenylalanine, which we desired to synthesize. This compound possesses all the structural features summarized by Jorgensen (1978) that contribute to maximal activity. These

features are:

(1) An alanyl side chain at position 1 which provides ion paired association with the nuclear receptor via the carboxylate ion. Removal of the aminomethylene group gives the acetic acid side chain analog which usually has higher receptor affinity but lower in vivo activity due to decreased transport and more rapid metabolism and excretion.

(2) Two iodines on the inner ring at the 3 and 5 positions. These substituents provide the steric constraints that define the minimal energy conformation of the aromatic rings and also contribute by a size-limited hydrophobic associaton with the receptor.

(3) An isopropyl group at the 3' position provides maximal activity by contributing to weak associations with transport proteins, strong associations to nuclear receptors, and by not acting as a substrate for outer-ring deiodinase enzymes.

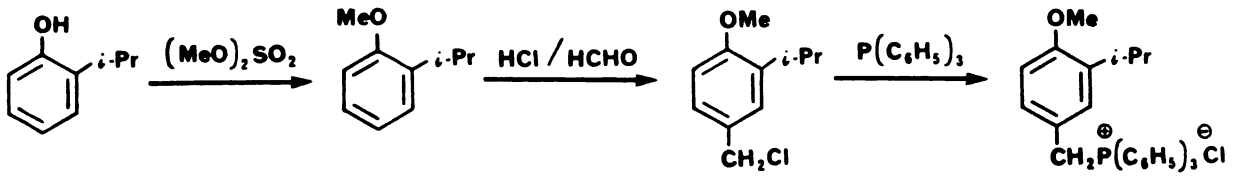
(4) A phenolic hydroxyl group at the 4' position that can hydrogen bond with the receptor. It is our prediction that 3,5-diiodo-4-(3-isopropyl-4-hydroxybenzyl)-phenylacetic acid will have the highest nuclear affinity whereas its corresponding alanyl side chained analog will be most active in vivo.

Failure of Tripp and coworkers (1973) to iodinate the outer phenolic ring prevented testing of any of their ketone-bridged analogs because without the outer ring 3' substituent, these compounds are virtually inactive. The replacement of the 3'-iodine with an isopropyl group allows

us to obtain hormonally active ketone-bridged analogs. Thyroid hormone analogs with a 3'-isopropyl group are not acted upon by the outer ring deiodinase enzymes and are therefore more active in vivo due to decreased metabolism. This consideration is important for our synthesis of an active halogen-free analog. The rationale and synthetic procedures for these halogen-free analogs are discussed in Chapter Six.

2.3 Synthetic Scheme

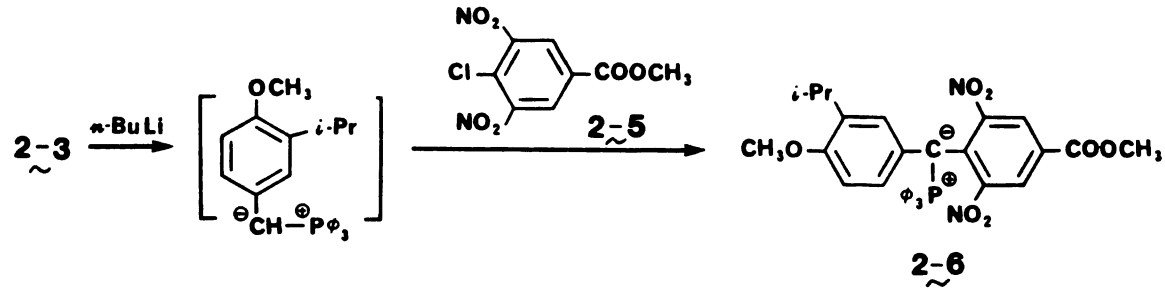
The diaryl ether linkage of synthetic thyroxines is generally formed by nucleophilic attack of a phenolate anion on an activated aromatic substrate. Using this reaction as a model, the coupling of the two aromatic rings was performed using a carbon nucleophile to displace the aromatic halogen: 2-isopropylanisole (2-1) was chloromethylated (Blank and Pfeiffer 1967) to 3-isopropyl-4-methoxybenzyl chloride (2-2) and reacted with triphenylphosphine using a similar method described by Ketcham et al. (1962).



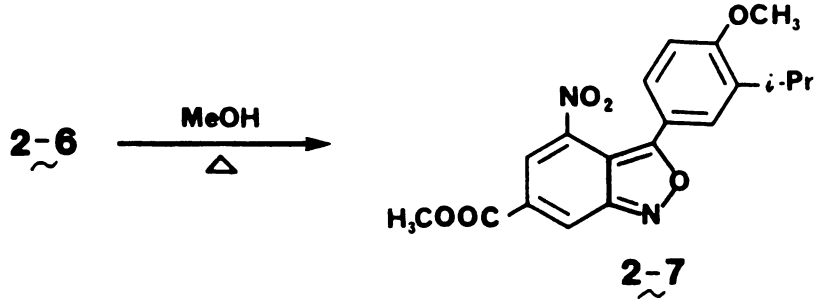
2-1 2-2 2-3

The action of n-butyllithium on 3-isopropyl-4-methoxybenzyltriphenylphosphonium chloride (2-3) generated the car-

bon nucleophile. Attack of this carbon anion on methyl 3,5-dinitro-4-chlorobenzoate (2-5) (Pappas and Gancher 1966) formed the ylid 2-6.



Decomposition of 2-6 in refluxing methanol resulted in an unexpected cyclization, giving the benzisoxazole 2-7.



Tripp et al. (1973) first reported an identical cyclization with an ylid of similar structure. They proposed that the reaction probably proceeded by methanolysis of the ylid to give a benzyl anion intermediate and cyclization occurred with the attack of the anion on the adjacent nitro group. (Figure 2.1)

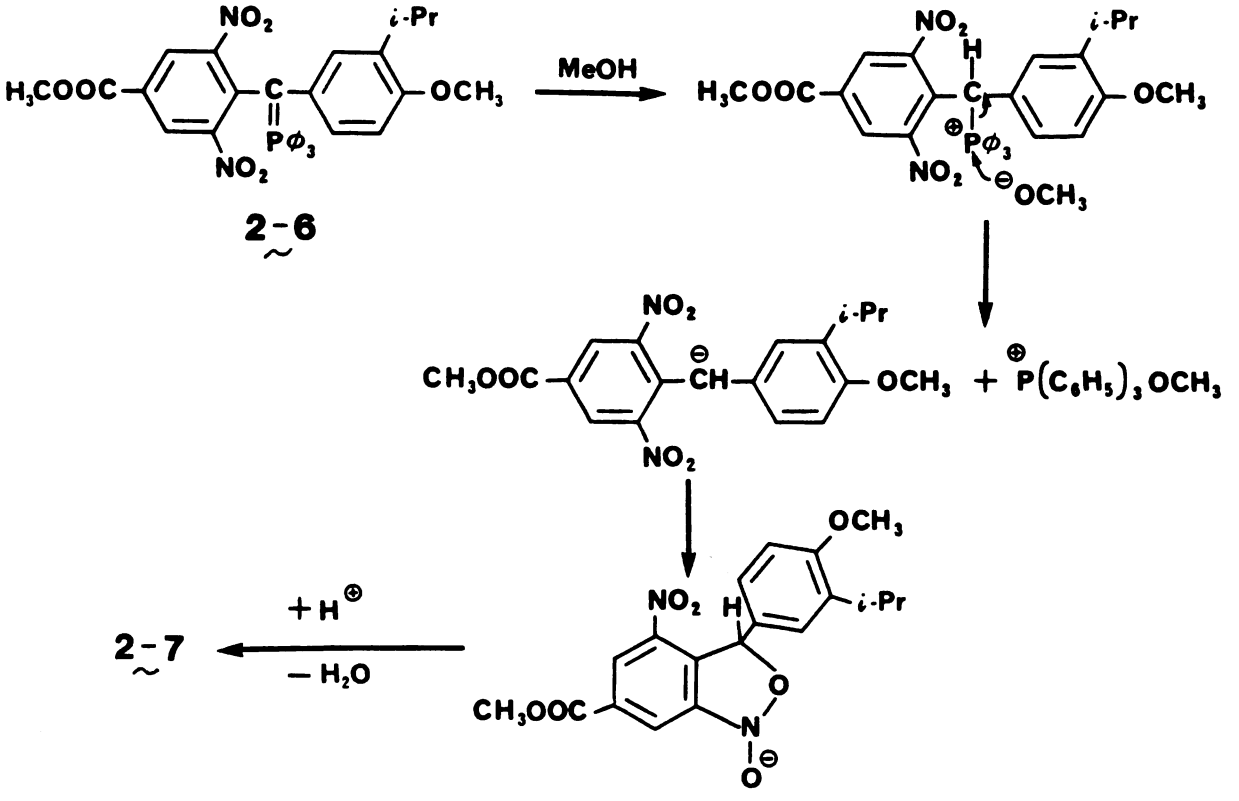


Figure 2.1 Cyclization of Ylid 2-6 (Tripp et al. 1973).

The presence of a methoxy group on ylid 2-6 and the possibility of extensive electron delocalization led us to propose an alternate mechanism with the para-methoxy function assisting the process of cyclization. (Figure 2.2)

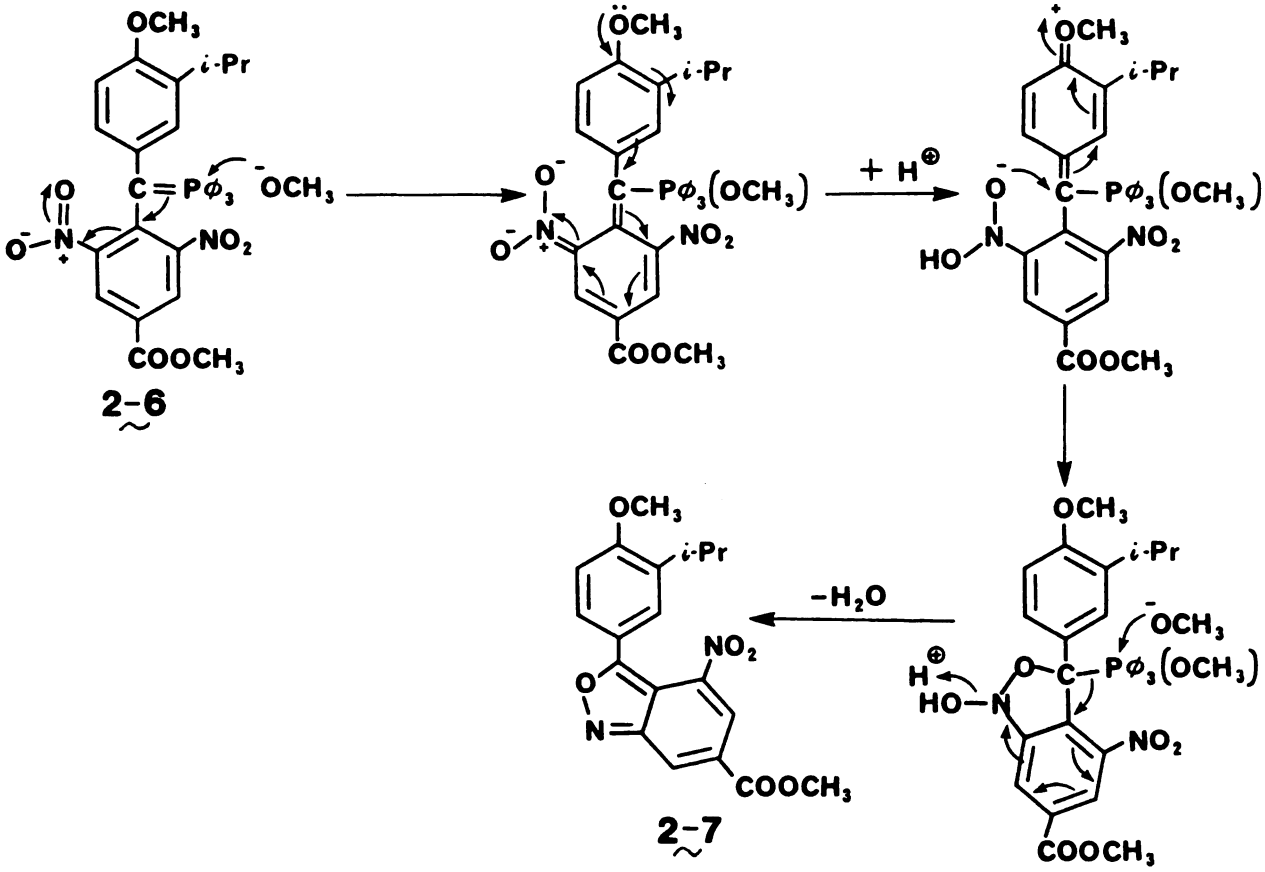
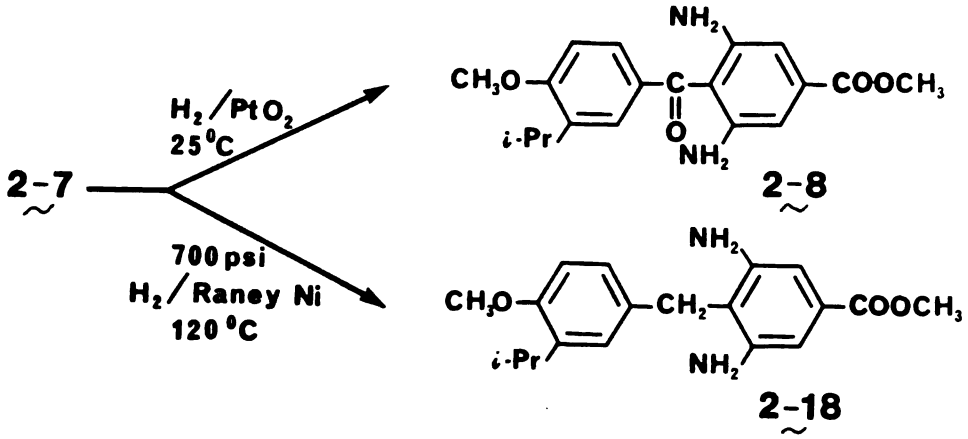


FIGURE 2-2

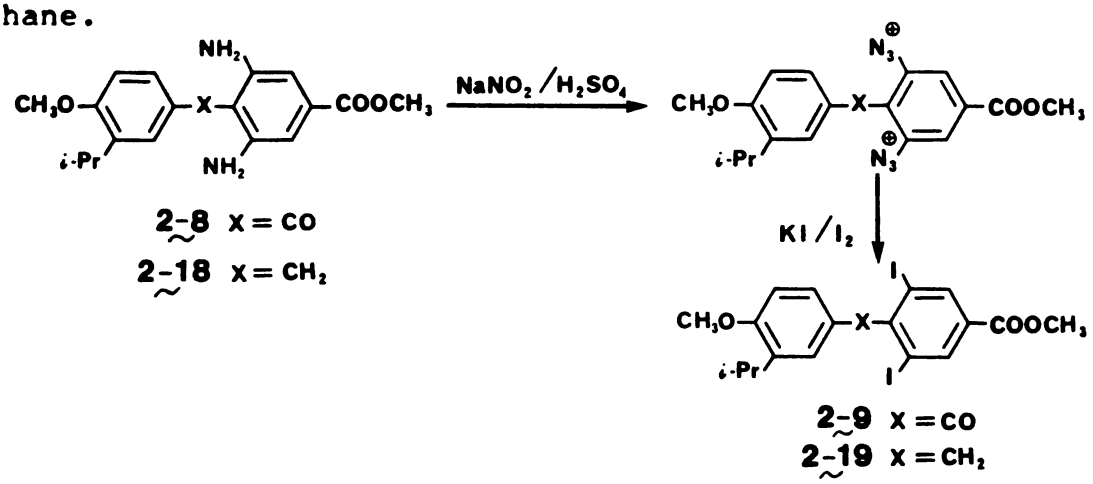
To distinguish between the two proposed mechanisms, cyclization of a similar ylid bearing no ortho or para electron donating groups might be attempted.

Under mild conditions, benzisoxazole 2-7 was reduced to the diamino-benzophenone 2-8 (Walker 1962); under vigorous

conditions, reduction produced the diamino-diphenylmethane 2-18.



Bis-diazotization under anhydrous conditions followed by treatment with triiodide ion formed the diiodo derivatives (2-9, 2-19) of both the diphenylketone and diphenylmethane.



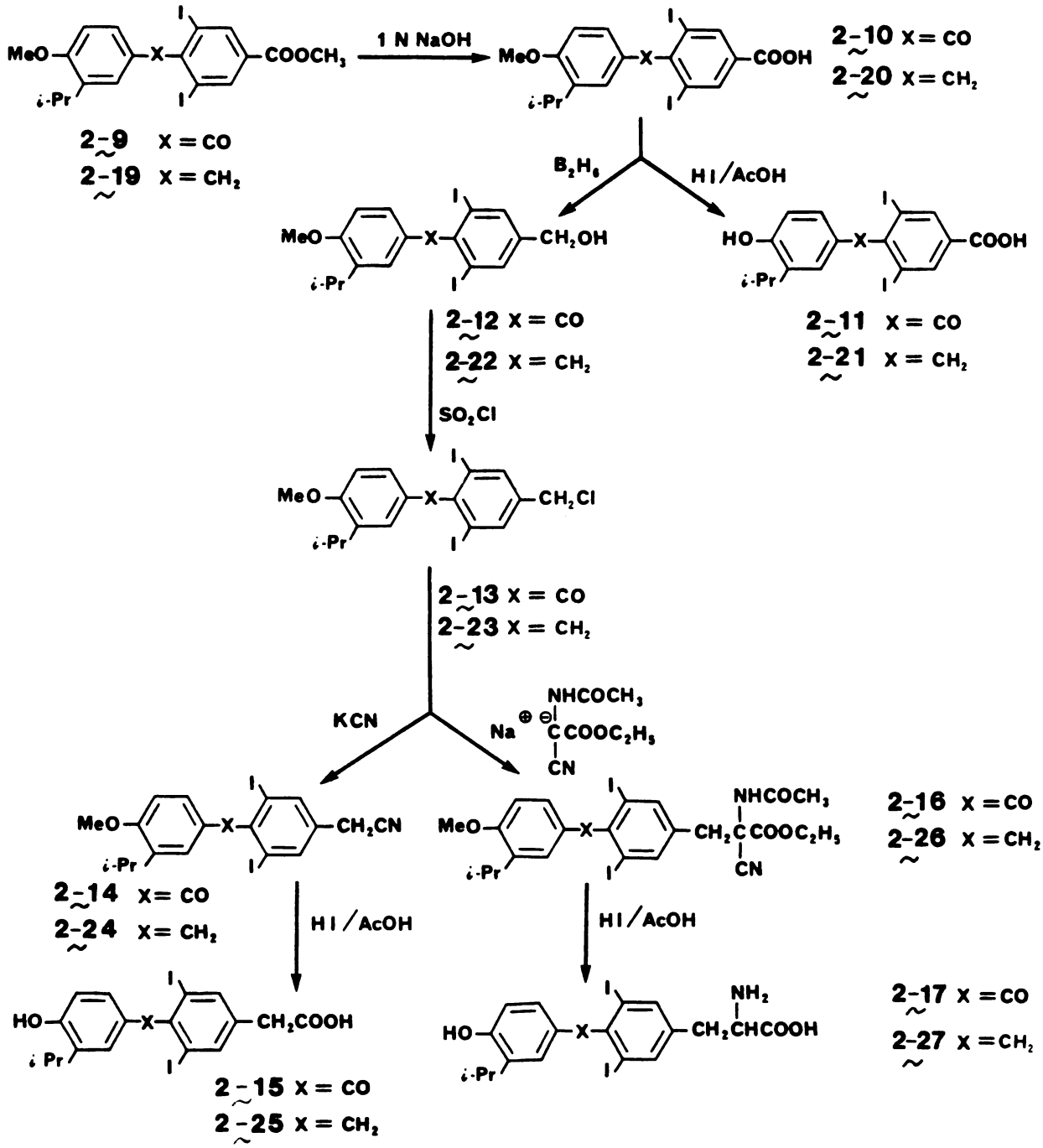
The carbomethoxy group in each series was converted via the benzyl chloride to either the formic acid, DL-alanyl or acetic acid side chains. Figure 2.3 shows the basic hydrolysis of the methyl esters to the free benzoic acids 2-10 and 2-20. Cleavage of the phenyl methyl ether bond in hydriodic acid gave the formic acid analogs 2-11 and 2-21. Diborane reduction of 2-10 and 2-20 in THF followed by

nucleophilic substitution with thionyl chloride yielded the benzyl chlorides 2~13 and 2~23, the common precursors that could be converted into either the acetic acid or alanine derivatives. Diborane reduction of 2~10 required very low temperature. At higher temperatures reduction tends to bring about reduction of the carbonyl bridge to the hydroxyl function. To prepare the acetic acid analogs, the benzyl chlorides were reacted with KCN in DMSO, hydrolyzed and demethylated simultaneously to 2~15 and 2~25. The DL alanine analogs, 2~16 and 2~26, were obtained by reaction of the benzyl chlorides with the sodium salt of ethyl acetamidocyanoacetate in ethanol and demethylation-hydrolysis with hydriodic acid.

2.4 Experimental Section

Melting points, obtained on a Thomas-Hoover Uni-Melt apparatus, are uncorrected. Nuclear magnetic resonance (Nmr) spectra were recorded on a Varian FT-80 spectrometer. Nmr chemical shift values are expressed in δ units (ppm) relative to either TMS or DDS (2,2-dimethyl-2-silapentane-5-sulfonate) internal standards. Electron impact mass spectra (EIMS) were performed on a Kratos MS-A25S spectrometer operated in the electron impact mode. Elemental analyses were performed by the Microanalytical Laboratory, University of California, Berkeley, California.

Figure 2.3


MeO
X
COOCH3
i-Pr
I
1 N NaOH
COOH
2-9 X = CO
2-19 X = CH2
2-10 X = CO
2-20 X = CH2
B2H6
HI/AcOH
CH2OH
HO
2-12 X = CO
2-22 X = CH2
2-11 X = CO
2-21 X = CH2
SO2Cl
CH2Cl
2-13 X = CO
2-23 X = CH2
KCN
Na
NHCOCH3
COOC2H5
CN
CH2CN
2-14 X = CO
2-24 X = CH2
2-16 X = CO
2-26 X = CH2
CH2COOH
2-15 X = CO
2-25 X = CH2
NH2
CH2CHCOOH
2-17 X = CO
2-27 X = CH2

2-Isopropylanisole (2~1). Method 1. 2-Isopropylphenol was methylated with dimethyl sulfate utilizing the general procedure of Dhami and Stothers (1966) for the O-methylation of hindered phenols. Thus, 2-isopropylphenol (312 gm, 2.29 moles; Aldrich Chemical Company, Inc.) was mixed with 420 ml of Claisen's alkali (Fieser 1957) with cooling, and added dropwise to 237 ml of chilled Me_2SO_4. After heating to reflux for 3 hours, the K_2SO_4 was separated and the filtrate extracted twice with 200 ml portions of petroleum ether (b.p. 30 - 60°C). The organic phases were combined and washed with equal volumes (150 ml) of 40% NaOH (twice) and H_2O. Drying (Na_2SO_4) and evaporation of the volatiles left a crude oil. Distillation in vacuo gave 152.9 gm (44.5%) of 2~1: b.p. 40° - 42°C/0.5 mm Hg; lit. b.p. 198° - 199°C/751 mm Hg (Heilbron 1965).

Method 2. 2-Isopropylanisole (2~1) was also prepared utilizing phase transfer catalysis as described by McKillop (1974). A mixture of 2.0 liters of dichloromethane, 2.0 liters of water, 101.1 gm (0.74 mole) of 2-isopropylphenol, 44.0 gm (1.1 moles) of NaOH, 147 ml (1.55 moles) of dimethyl sulfate and 23.0 gm (0.07 mole) of benzyltributylammonium chloride was agitated with an overhead mechanical stirrer at room temperature for 21 hours. The organic layer was separated and the aqueous layer extracted three times with 350 ml portions of methylene chloride. The organic fractions were combined and was stirred with 1.0 liter of 15% NH_4OH for 1 hour, separated from the aqueous phase and

concentrated under reduced pressure. The residue was taken up in 1.0 liter of ethyl ether, washed three times each with 100 ml portions of 15% NH_4OH, 2 N NaOH and saturated NaCl solution. After drying over Na_2SO_4, the solvent was evaporated and the residual anisole purified by vacuum distillation. 109.9 gm (99.0%) of 2-1 was obtained.

3-Isopropyl-4-methoxybenzyltriphenylphosphonium chloride (2-3). 2-Isopropylanisole (2-1) was chloromethylated using a modified method of Blank and Pfeiffer (1967). A sample of 2-1 (152.9 gm, 1.02 moles) was stirred in a mixture of 138 ml of formalin (40% $HCHO/H_2O$) and 55 ml of dimethoxyethane. Zinc chloride (22 gm, 0.16 mole) was added to the reaction mixture which was chilled in an ice bath. HCl gas was bubbled vigorously into the mixture for 1 hour at 0°C and for an additional 30 minutes at room temperature. The mixture was then poured into approximately 500 ml of ice and extracted twice with 200 ml portions of hexanes. The organic layer was washed twice with 100 ml of 5% Na_2CO_3 and dried over Na_2SO_4. In vacuo removal of solvents left 191.7 gm of 3-isopropyl-4-methoxybenzyl chloride (2-2). This crude oil was used without further purification.

The impure 2-2 was taken up in 512 ml of benzene and 278 gm (1.06 moles) of triphenylphosphine was added. After heating under reflux for 3 hours, a precipitate was obtained. The mixture, diluted with benzene to a total volume of 1.5 liters, was stirred at 60°C for 2 hours and

the precipitations collected via hot filtration to give 183 gm (39.2% from 2-1) of a white solid: m.p. 251° - 152°C. Calculated for $C_{29}H_{30}O_1P_1Cl_1$: C, 75.57; H, 6.52. Found: C, 75.30; H, 6.55.

3,5-Dinitro-4-chlorobenzoic Acid (2-4). In a solution of 100 gm (0.64 mole) of 4-chlorobenzoic acid (Aldrich Chemical Company, Inc.) in 2000 gm (1100 ml) of conc. H_2SO_4 at 70° - 100°C was added dropwise 300 gm (225 ml) of conc. HNO_3, the temperaure was then slowly raised from 120° to 140°C during 1.5 hours and stopped. The cooled solution was poured into ice whereby 2-4 separated and was collected by filtration, washed with water and air dried for several hours. Yield of 2-4, 108.9 gm (69%): m.p. 157° - 158.5°C; lit. m.p. 159°C (Ullman 1909).

Methyl 3,5-Dinitro-4-chlorobenzoate (2-5). 3,5-Dinitro-4-chlorobenzoic acid (2-4) (108.93 gm, 0.44 mole) was brought to reflux with 109 gm (60 ml) of conc. H_2SO_4 in 300 ml of methanol for 3 hours; the reaction proceeded with the precipitation of a yellow solid which was filtered and washed with water. Dilution of the filtrate with water afforded more product. Recrystallization in methanol gave 102.53 gm (89.2%) of 2-5: m.p. 102.5° -103.5°C; lit. m.p. 105°C (Ullman 1909).

Methyl 3-(3-Isopropyl-4-methoxyphenyl)-4-nitro-2,1-benzisoxazole-6-carboxylate (2-7). 3-Isopropyl-4-methoxybenzyltriphenylphosphonium chloride, 2-3, was

suspended in 200 ml of benzene and treated with 0.05 mole (31.15 ml) of n-butyllithium in hexane. After the reaction had been stirred for 1 hour, 6.16 gm (0.025 mole) of methyl 3,5-dinitro-4-chlorobenzoate, 2-5, in 75 ml of benzene was added. The solution, initially dark orange, rapidly turned purplish black, and was stirred overnight and filtered to remove the LiCl. Washing with warm benzene removed most of the dark color from the filter cake.

The filtrate and washings were combined and evaporated to give 3.79 gm of crude ylid (2-6) as a dark purple oil. The ylid 2-6 was taken up in 225 ml of methanol and refluxed for 1 hour. During which time the purple solution turned dark orange. Chilling and filtration afforded 4.26 gm (46.1%) of orange crystals. Recrystallization from benzene-methanol gave the analytical sample: m.p. 123° - 124°C. Caluculated for $C_{19}H_{18}N_2O_6$: C, 61.62; H, 4.86; N, 7.57. Found C, 61.77; H, 4.98; N, 7.43.

Methyl 3,5-Diiodo-4-(3-isopropyl-4-methoxy-benzoyl)-benzoate (2-9). To 6 gm (16.22 mmoles) of benzisoxazole (2-7) in 270 ml of ethyl acetate and 14 ml of acetic acid was added 0.4 gm of PtO_2 catalyzt. Hydrogenation was carried on at room temperature and atmospheric pressure for 24 hours, after which the catalyst was filtered and the orange solution evaporated in vacuo to give a dark orange oil. This crude diamino product 2-8 was tetraazotized and iodinated without further purification.

A solution of nitrosylsulfuric acid was prepared by addition of 9 gm (0.13 mole) of $NaNO_2$ to 75 ml of conc. H_2SO_4 kept at 40°C. After the solution cooled, 75 ml of AcOH was added and the whole added dropwise to a solution of the crude diamino compound in 127 ml of AcOH and 255 ml of H_2SO_4 at 0°C. The reaction mixture turned viscous and was stirred mechanically for an additional 45 minutes in an ice bath. This was added slowly to a well-chilled iodinating medium consisting of 36 gm of KI, 20 gm of I_2, 9.0 gm of urea in 600 ml of water and 300 ml of $CHCl_3$. Following overnight stirring at room temperature, the chloroform layer was separated and the aqueous layer was extracted twice with 300 ml of $CHCl_3$. The organic phase was washed with 10% $NaHSO_3$, 2 M $NaHCO_3$ and H_2O. Drying (Na_2SO_4) and evaporation in vacuo afforded 5.51 gm of a brown solid. Crystallization from CH_2Cl_2-MeOH gave 3.37 gm (40.4% from 2-7) of 2-9: m.p. 198° - 200°C. Nmr ($CDCl_3$) δ1.17(d, 6 H, iPr-CH_3); 3.27(m, 1 H, iPr-CH); 3.9(s, 3 H, CO-OCH_3); 3.95(s, 3 H, Ar-OCH_3); 6.75 - 7.75(5, 3 H, Ar-2',5',6'-H); 8.45(s, 2 H, Ar-2,6-H). Calculated for $C_{19}H_{18}O_4I_2$: C, 40.44; H, 3.19. Found: C, 40.6; H, 3.37.

3,5-Diiodo-4-(3-isopropyl-4-methoxybenzoyl)benzoic Acid (2-10). A sample of 2-9 (814 mg, 1.44 mmoles) was heated under reflux in 16 ml of 1 N NaOH for 5 hours. The reaction mixture was concentrated by evaporation in vacuo and 6 N HCl was added dropwise. The solid precipitate was filtered, washed with cold water and vacuum dried to give 766 mg

(96.8%) of 2-10: m.p. 242° - 246°C.

3,5-Diiodo-4-(3-isopropyl-4-hydroxybenzoyl)benzoic Acid (2-11). Removal of the protecting group on the phenolic function was carried on in a non-aqueous medium. The benzoic acid 2-10 (117.2 mg, 0.21 mmole) was dissolved in 4 ml of a 1:1 mixture of glacial acetic acid and hydriodic acid, and heated under reflux for 72 hours. Cooling to room temperature afforded a white precipitate. The reaction mixture, including solids, was concentrated in vacuo, filtered and washed with water. A second precipitation using 1 N HCl and 1 N NaOH gave 98.65 mg (86.4%) of 2-11: m.p. >270°C. Nmr (DMSO-d_6) δ1.09(d, 6 H, iPr-CH_3); 3.23(m, 1 H, iPr-CH); 6.25 - 7.05(5, 3 H, Ar-2',5',6'-H); 8.30(s, 2 H, Ar-2,6-H). Calculated for $C_{17}H_{14}O_4I_2$: C, 38.07; H, 2.61. Found: C, 37.89; H, 2.67.

3,5-Diiodo-4-(3-isopropyl-4-methoxybenzoyl)benzyl Alcohol (2-12). To 77 ml of freshly distilled THF under a nitrogen atmosphere was added 995.5 mg (1.81 mmoles) of 2-10. After cooling to -30°C, 5 ml (5 mmoles) of a 1 M solution of borane in THF stabilized with 5 mole % sodium borohydride was added. The reaction was brought to room temperature gradually and stirred for 9 hours. Enough methanol was added to destroy the excess diborane. The volatiles were removed by evaporation to give a yellow oil which was crystallized from MeOH, 911.2 mg (95%): m.p. 218° - 219°C. Nmr (DMSO-d_6) δ1.16(d, 6 H, iPr-CH_3); 3.92(s, 3 H,

Ar-OCH_3); 4.48(d, 2 H, Ar-CH_2); 5.41(t, 1 H, -OH); 7.01 - 7.7(5, 3 H, Ar-2',5',6'-H); 8.28(s, 2 H, Ar-2,6-H). Calculated for $C_{18}H_{18}O_3I_2$: C, 40.32; H, 3.38. Found: C, 40.15; H, 3.53.

3,5-Diiodo-4-(3-isopropyl-4-methoxybenzoyl)benzyl Chloride (2-13). To a suspension of the benzyl alcohol 2-12 (761 mg, 1.42 mmoles) in 25 ml of benzene was added 2 ml of thionyl chloride followed by a drop of DMF. The mixture was kept at 40°C for 5 hours and allowed to stand overnight at room temperature. Evaporation of the solvent gave a yellow oil. Trituration in hot methanol yielded a pale yellow solid. Crystallization from CH_2Cl_2-MeOH afforded 761.3 mg (96.7%) of crystalline product: m.p. 153° - 154°C. Nmr ($CDCl_3$) δ1.19(d, 6 H, iPr-CH_3); 3.29(m, 1 H, iPr-CH); 3.88(s, 3 H, Ar-OCH_3); 4.46(s, 2 H, Ar-CH_2Cl); 6.75 - 7.50(5, 3 H, Ar-2',5',6'-H); 7.85(s, 2 H, Ar-2,6-H).

3,5-Diiodo-4-(3-isopropyl-4-methoxybenzoyl)benzyl Cyanide (2-14).

The benzyl chloride 2-13 (2.17 gm, 3.92 mmoles) was added gradually to a slurry of 350 mg (5.38 mmoles) of KCN in 6 ml of dimethyl sulfoxide. The reaction mixture was stirred and warmed in a water bath at 40°C. After 4 hours, water was added and the resulting gummy solid was dissolved in CH_2Cl_2 and separated from the aqueous phase. The aqueous layer was washed twice with CH_2Cl_2 and the organic phases were combined. Extraction with water, 6 N HCl and water again

removed most of the coloration. Drying ($CaCl_2$) and evaporation of solvent gave 1.19 gm (89.6%) of a white solid. An analytical sample was obtained by preparative thin layer chromatography (silica gel, $CHCl_3$): m.p. 80° - 83°C. Nmr ($CDCl_3$) δ1.18(d, 6 H, iPr-CH_3); 3.27(m, 1 H, iPr-CH); 3.7(s, 2 H, Ar-CH_2CN); 3.89(s, 3 H, Ar-OCH_3); 6.75 - 7.75(5, 3 H, Ar-2',5',6'-H); 7.8(s, 2 H, Ar-2,6-H). Calculated for $C_{19}H_{17}N_1O_2I_2$: C, 41.85; H, 3.38; N, 2.57; I, 46.59. Found: C, 42.02; H, 3.38; N, 2.42; I, 46.16.

3,5-Diiodo-4-(3-isopropyl-4-hydroxybenzoyl)phenylacetic Acid (2-15). The benzyl cyanide 2-14 (2.14 gm, 3.92 mmoles) was hydrolyzed in 21 ml of a 1:1 mixture of AcOH and HI. After 4 hours of heating under reflux, the reaction mixture was allowed to cool overnight during which time the product precipitated from solution. The solid was filtered, washed well with cold water and vacuum dried to give 2.13 gm of 2-15 (98.8%): m.p. 234°C. Nmr (DMSO-d_6) δ1.13(d, 6 H, iPr-CH_3); 3.61(s, 2 H, Ar-CH_2); 6.8 - 7.6(5, 3 H, Ar-2',5',6'-H); 7.85(s, 2 H, Ar-2,6-H). Calculated for $C_{18}H_{16}O_4I_2$: C, 39.28; H, 2.91; I, 46.16. Found: C, 39.25; H, 3.01; I, 45.98.

3,5-Diiodo-4-(3-isopropyl-4-hydroxybenzoyl)-DL-phenylalanine (2-17). A pinch of NaI was added to a solution of sodium ethoxide (103 mg, 4.48 mmoles of sodium in 7 ml of absolute ethanol). The solution was brought to reflux and 762 mg (4.48 mmoles) of ethyl acetamidocyanoacetate was

added. After 20 minutes reflux, a suspension of 2.37 gm (4.28 mmoles) of the benzyl chloride 2-13 in 7 ml of warm ethanol was added in one portion. After continuous heating for 3 hours, the hot reaction mixture was poured into 72 ml of water and ice. The precipitate was filtered and washed with 5% $NaHCO_3$ and H_2O. The resulting yellow solid was chromatographed on silica gel (eluted with EtOAc:Hex 3:1). A first fraction contained unreacted starting material, the second fraction gave 1.05 gm of a glass showing two components by thin layer chromatography. No attempt was made to separate the components. Instead, the mixture was dissolved in 8 ml of AcOH and 5 ml of HI and heated under reflux for 8 hours and poured while still hot into 20 ml of cold water. 10% NaOH was added to adjust the pH to 5. The precipitate was collected and purified by dissolving it in 20 ml of ethanol, adding 6 N HCl to make it acidic and readjusting the pH to 5 with 10% NaOAc. Filtration, washing and vacuum drying afforded 664 mg (27.57% from 2-13) of a white solid: m.p. 210°C (dec). Tlc ($CHCl_3$:MeOH:NH_4OH 20:10:1, UV, ninhydrin); Nmr (MeOH-d_4) δ1.10(d, 6 H, iPr-CH_3); 4.25(t, 1 H, $CH(COO^-)NH_3^+$); 6.74 - 7.6(5, 3 H, Ar-2',5',6'-H); 7.86(s, 2 H, Ar-2,6-H). Calculated for $C_{19}H_{19}N_1O_4I_2$: C, 39.39; H, 3.28; N, 2.42; I, 43.85. Found: C, 39.18; H, 3.29; N, 2.22; I, 43.70.

Methyl 3,5-Diiodo-4-(3-isopropyl-4-methoxybenzyl)benzoate (2-19). Methyl 3-(3-isopropyl-4-methoxyphenyl)-4-nitro-2,1-benzisoxazole-6-carboxylate, 2-7,

(5.4 gm, 14.58 mmoles) was dissolved in 240 ml of methanol and approximately 3 gm of Raney Nickel (Aldrich Chemical Company, Inc.; finely divided powder, approx. pore size 50 u, surface area 80 - 100 m^2/g) was added as catalyzt. Hydrogenation in a rocker type pressure reactor (Parr Instrument Company, Bomb #4051, bomb size = 500 ml) was carried on under 600 pounds per square inch pressure at 110°C. After 18 hours, the catalyzt was filtered and the yellow solution evaporated in vacuo to give a yellow solid. This crude diamino compound 2-18 was stored under nitrogen at 0°C. Without further purification, 2-18 was bisdiazotized and iodinated. Thus, 3.3 gm (10.1 mmoles) of 2-18 was reacted and worked up by the previously described procedure. Crystallization from CH_2Cl_2-MeOH gave 1.43 gm (17.9% from 2-7) of 2-19: m.p. 81° - 85°C. Nmr ($CDCl_3$) δ1.08, 1.17(d, 6 H, iPr-CH_3); 3.2(m, 1 H, iPr-CH); 3,72(s, 3 H, CO-OCH_3); 3.85(s, 3 H, Ar-OCH_3); 4.43(s, 2 H, Ar-CH_2-Ar); 6.55 - 6.93 (3, 3 H, Ar-2',5',6'-H); 8.42(s, 2 H, Ar-2,6-H). Calculated for $C_{19}H_{20}O_3I_2$: C, 41.5; H, 3.6. Found: C, 41.32; H, 3.72.

3,5-Diiodo-4-(3-isopropyl-4-methoxybenzyl)benzoic Acid (2-20). The methyl ester 2-19, (600 mg, 1.09 mmoles), was hydrolyzed in 16 ml of 1 N NaOH. Following 4 hours of reflux, the reaction medium was made acidic by adding 6 N HCl and the resulting precipitate was filtered, washed with water, and dried to give 423.2 mg (71.82%) of 2-20: m.p. 174° - 175°C. Nmr (DMSO-d_6) δ1.08(d, 6 H, iPr-CH_3); 3.15(m,

1 H, iPr-CH); 3.74(s, 3 H, Ar-OCH_3); 4.38(s, 2 H, Ar-CH_2-Ar); 6.73 - 6.98(3, 3 H, Ar-2',5',6'-H); 8.34(s, 2 H, Ar-2,6-H).

3,5-Diiodo-4-(3-isopropyl-4-hydroxybenzyl)benzoic Acid (2-21). A sample of 2-20 (500 mg, 0.91 mmole) of 2-20 was taken into 10 ml of acetic acid and 10 ml of hydriodic acid. Heating at reflux was continued for 48 hours after which the reaction was cooled and water was added. Further cooling at 4°C afforded more solid which was filtered and washed with ice water. Drying under vacuum gave 503.4 mg of a crude product. An analytical sample was obtained by crystallization from methanol-water (451 mg, 92.5%). m.p. 201° - 205°C. Nmr (MeOH-d_4) δ1.16(d, 6 H, iPr-CH_3); 4.53(s, 2 H, Ar-CH_2-Ar); 6,68 - 6.96(3, 3 H, Ar-2',5',6'-H); 8.55(s, 2 H, Ar-2,6-H). Calculated for $C_{17}H_{16}O_3I_2$: C, 39.10; H, 3.07; I, 48.64. Found: C, 39.18; H, 3.20; I, 48.52.

3,5-Diiodo-4-(3-isopropyl-4-methoxybenzyl)benzyl Chloride (2-23). To 36 ml of freshly distilled THF was added 803.7 mg (1.50 mmoles) of 2-20, followed by 2.4 ml of 1 M diborane solution in THF. The whole was heated to reflux for 1.5 hours and stirred overnight at room temperature. Evaporation of all solvent gave 402.1 mg (53.1%) of a yellow oil which solidified on standing. No analytical sample was prepared for 2-22. m.p. 122° - 124°C. Nmr (MeOH-d_4) δ1.07(d, 6 H, iPr-CH_3); 3.74(s, 3 H, Ar-OCH_3); 4.39(s, 2 H, $ArCH_2OH$); 4.46(s, 2 H, Ar-CH_2-Ar); 6.73 - 6.89(3, 3 H,

Ar-2',5',6'-H); 7.86 (s, 2 H, Ar-2,6-H).

The crude oil, 2-22, was dissolved in 15 ml of benzene and treated with 0.6 ml of thionyl chloride followed by a few drops of DMF. The reaction was maintained at 40°C for 4 hours. Evaporation under vacuo gave a brown oil which was crystallized from CH_2Cl_2-MeOH to afford 382.1 mg (88.75%) of 2-23: m.p. 107° - 108°C. Nmr ($CDCl_3$) δ1.14(d, 6 H, iPr-CH_3); 3.79(s, 3 H, Ar-OCH_3); 4.45(s, 2 H, Ar-CH_2-Ar); 5.30(s, 2 H, ArCH_2Cl); 6.76 - 6.79(3, 3 H, Ar-2',5',6'-H); 7.91(s, 2 H, Ar-2,6-H). Calculated for $C_{18}H_{19}O_1I_2Cl_1$: C, 39.98; H, 3.52; I, 46.97. Found: C, 39.75; H, 3.89; I, 46.69.

3,5-Diiodo-4-(3-isopropyl-4-methoxybenzyl)benzyl Cyanide (2-24). A sample of 2-23 (1.64 gm, 3.03 mmoles) was added gradually to a slurry of 297 mg (4.56 mmoles) of KCN in 10 ml of DMSO. The reaction was stirred in a water bath kept at 40°C. After 4 hours, water was added and the oil that separated was taken into chloroform and separated. The aqueous phase was extracted twice with 20 ml portions of $CHCl_3$ and the combined organic solvent was washed with 6 N HCl and H_2O. Drying over $CaCl_2$ and evaporation under vacuo gave 1.65 gm of a yellow solid. The crude product was chromatographed on a silica gel column and eluted with $CHCl_3$ to give the analytical sample: 1.13 gm (70.26%) of a crystalline solid. m.p. 152° - 154°C. Nmr ($CDCl_3$) δ1.14(d, 6 H, iPr-CH_3); 3.27(m, 1 H, iPr-CH); 3.66(s, 2 H, Ar-CH_2CN);

3.79(s, 3 H, Ar-OCH_3); 4.46(s, 2 H, Ar-CH_2-Ar); 6.76 - 7.03(3, 3 H, Ar-2',5',6'-H); 7.86(s, 2 H, Ar-2,6-H). Calculated for $C_{19}H_{19}N_1O_1I_2$: C, 42.95; H, 3.58; N, 2.64; I, 47.82. Found: C, 42.77; H, 3.56; N, 2.56; I, 47.66.

3,5-Diiodo-4-(3-isopropyl-4-hydroxybenzyl)phenylacetic Acid (2-25). The benzyl cyanide 2-24, 1.13 gm (2.13 mmoles), was hydrolyzed and O-demethylated in 12 ml of a 1:1 mixture of AcOH/HI. After heating at reflux for 8 hours and standing overnight at room temperature, the solid was separated by filtration, washed liberally with water and dried. 1.08 gm (94.6%) of 2-25 was obtained, m.p. 154° - 155°C. Nmr (MeOH-d_4) δ1.10(d, 6 H, iPr-CH_3); 3.48(s, 2 H, Ar-CH_2COOH); 4.35(s, 2 H, Ar-CH_2-Ar); 6.56 - 6.84(3, 3 H, Ar-2',5',6'-H); 7.83(s, 2 H, Ar-2,6-H). Calculated for $C_{18}H_{18}O_3I_2.H_2O$: C, 39.00; H, 3.61; I, 45.83. Found: C, 40.06; H, 3.44; I, 45.42.

3,5-Diiodo-4-(3-isopropyl-4-hydroxybenzyl)-DL-phenylalanine (2-27). Using the method described by Blank and Pfeiffer (1967), ethyl 3,5-diiodo-4-(3-isopropyl-4-methoxybenzyl)benzylacetamidocyanoacetate (2-26) was formed from 2-23. To a well stirred, refluxing solution of sodium ethoxide (0.1 gm of sodium in 7 ml of absolute ethanol) was added 762 mg (4.48 mmoles) of ethyl acetamidocyanoacetate. Following an additional 20 minutes of heating to reflux, 739 mg (1.37 mmoles) of 2-23 suspended in 7 ml of absolute ethanol was added in one portion and again brought to reflux

for 3 hours. The reaction mixture was poured onto a mixture of ice and water and chilled overnight. The semisolid product was filtered, dissolved in $CHCl_3$ and chromatographed on a silica gel column. Elution with $CHCl_3$ afforded three distinct bands which corresponded to starting material 2-23, 2-26 and partially hydrolyzed 2-26. The starting material eluted first from the column and the second and third bands were combined for the final hydrolysis.

Heating to reflux for 4 hours in 12 ml of a 1:1 mixture of AcOH/HI brought about the hydrolysis of 2-26 (221 mg, 0.33 mmole). 10% NaOAc solution was added to bring the pH to 5 whereupon a precipitate formed which was separated, washed and purified by a second isoelectric precipitation. A sample of 2-27 (114.1 mg, 61.78% from 2-26) was obtained and the purity was determined by thin layer chromatography developed with a mixture of $CHCl_3$:MeOH:NH_4OH at the ratio 20:10:1. The chromatogram was sprayed with freshly prepared ninhydrin to indicate the location of the free amino acid. m.p. dec.~190°C. Calculated for $C_{19}H_{21}N_1O_3I_2$: C, 40.37; H, 3.72; N, 2.48; I, 44.94. Found: C, 40.48; H, 3.71; N, 2.31; I, 45.34. EIMS: m/e 565 (M^+).

CHAPTER THREE

ISOLATION OF CRUDE NUCLEAR RECEPTORS AND DETERMINATION OF BINDING AFFINITIES OF VARIOUS HORMONE ANALOGS

3.1 Introduction

Evidence for the probable existence of nuclear receptors for thyroid hormones was first reported by Oppenheimer and coworkers (1972). In their studies, rat liver and kidney cells were fractionated thirty minutes after *in vivo* injection of tracer doses of [^{125}I]-T_3 along with increasing quantities of nonradioactive hormone. The proportion of radiolabeled T_3 bound to the nuclear fraction was found to decrease with increasing unlabeled T_3 concentrations. A distinct reduction in nuclear binding of [^{125}I]-T_4 was also observed after injection of the animals with large amounts of T_4. A similar dose-response relationship could not be demonstrated for other subcellular fractions. Although these *in vivo* displacement studies have very low sensitivity because of the low specific activity of the [^{125}I]-labeled hormone, and the authors made no attempt to calculate an equilibrium constant or to determine binding capacity, the presence of limited-capacity binding sites in the nuclei for both T_3 and T_4 was demonstrated. The observation was also made that T_3 was more potent than T_4 in displacing [^{125}I]-T_3 from the binding sites.

Samuels and Tsai (1973, 1974) subsequently confirmed

the presence of high-affinity, limited capacity T_3 binding sites after incubation of hormone with intact rat pituitary GH_1 cells in culture, isolated G_1 cell nuclei, or rat liver nuclei. In each of these studies the total number of T_3 binding sites was similar (~8000/cell), and the estimated equilibrium dissociation constants were similar in the isolated GH_1 cell nuclei and rat liver nuclei (Samuels and Tsai 1974). The presence of putative nuclear receptors for thyroid hormones was also confirmed in a number of other laboratories (DeGroot el al. 1974, Kistler et al. 1975, Spindler et al. 1975).

Corroborative evidence supporting the hypothesis that the nuclear T_3 binding sites are receptors is summarized below.

(1). The relative binding affinities of a large number of thyroid hormone analogs are well correlated with their in vivo biological activities. This provides the strongest indication that the binding sites are receptors.

(2). In cultured cells and in rats, the concentrations of T_3 and certain of its analogs required for relative saturation of the receptors parallel the concentrations required for the biological response.

(3). The receptors are found only in thyroid hormone-responsive tissues. Further, the tissue concentration of receptors parallels the degree to which the hormone stimulates oxygen consumption.

(4). Receptor concentrations are correlated with changes in

the cellular sensitivity to the hormone.
(5). The localization of receptors in chromatin is consistent with the findings that these hormones regulate RNA concentrations.

Nuclear binding of T_3, T_4, and their analogs has been determined *in vivo* in the rat (Oppenheimer et al. 1973, DeGroot and Strausser 1974), in cells grown in tissue culture (Samuels and Tsai 1973, Papavasiliou 1977), in isolated nuclei incubated with the analogs *in vitro* (Koerner et al. 1974, 1975; DeGroot and Torresani 1975) and in solubilized nuclear extracts (Samuels et al.1974a, Thomopoulos et al. 1974, Torresani and DeGroot 1975, Latham et al. 1976, Jorgensen et al. 1977, Silva et al. 1977, Apriletti el al. 1980). In this work, the binding affinities of the newly synthesized analogs were determined using solubilized crude nuclear receptors. These binding studies were chosen to obtain information about the effect of the bridging group on the molecular interaction of the hormone and its receptor and the influence the bridging group exerts on the binding of other substituents. This chapter describes the process of isolating a soluble receptor preparation from frozen rat liver, and the detailed methods for measuring the *in vitro* binding affinity of thyroid hormone analogs.

3.2 Isolation of Receptors from Rat Liver Nuclei

The solubilization of protein from rat liver nuclei was performed by a modification of the procedure described by

Bolger (1977). Male Sprague-Dawley rats from Simonsen Laboratories weighing approximately 350 grams each were sacrificed by decapitation and their livers excised and stored immediately in a liquid nitrogen cryogenic storage container. All buffers required for the solubilization process were prepared in advance and refrigerated until used. The contents of each buffer are listed in Table 3.1.

TABLE 3.1 BUFFER SOLUTIONS FOR THE ISOLATION OF NUCLEAR RECEPTORS

	NAME	CONTENTS
1.	LIVER PREP. SOLUTION A	0.34 M Sucrose, 15 mM $MgCl_2$, 0.25 mM Spermine.4HCl.
2.	HOMOGENIZATION SOLUTION B	240 ml of 2.1 M Sucrose, 1.44 ml of 1 M $MgCl_2.6H_2O$, 0.25 ml of 0.1 M Spermine.4HCl.
3.	NUCLEAR PELLET WASHING BUFFER C	20 mM Tricine, 2 mM $CaCl_2.H_2O$, 1 mM $MgCl_2.6H_2O$, pH 7.6.
4.	RESUSPENSION BUFFER D	20 mM Tris (pH 8.0), 0.25 M Sucrose, 1.0 mM EDTA, 0.1 mM Dithiothreitol, 5% Glycerol.

Eighty grams of frozen liver was weighed into a mortar and quickly crushed with a pestle. The chunks of frozen liver were poured into 160 ml of warm (35°C) Liver Prep. Solution A and stirred until thawed to give a final temperature of about 4°C. The liver pieces were then drained using a coarse nylon mesh, and added to ice cold Homogenization

Solution B. The mixture was homogenized for three one-minute periods, separated by one minute of cooling, using a Tekmar Dispex Homogenizer (Cincinnati, Ohio) at maximum speed. This preparation, when stained with 1% crystal violet, was observed by light microscopy to contain cell debris, intact nuclei and no whole cells. The mixture was then filtered through one layer of Miracloth (Calbiochem) with kneading to obtain a homogeneous mixture without large pieces of connective tissue. Six Sorvall GSA-rotor bottles were filled with about 150 ml each and 125 ml of 2.1 M sucrose was added to the bottom of the bottles with a separatory funnel in order to float the liver mixture to the top. The nuclei were pelleted through the sucrose medium by centrifugation at 25,000 g for ninety minutes at $4^{o}C$ in a Sorvall RC-2B with a GSA rotor (diameter 14.61 cm). After centrifugation the cell debris and sucrose solution was removed with special care to avoid contamination of the nuclear pellet. The nuclear pellets were suspended in 50 ml of Nuclear Pellet Washing Buffer C containing 0.5% Triton X-100 and were repelleted by centrifugation at 500 g for ten minutes at $5^{o}C$ in an International Refrigerated Centrifuge. After decanting the supernatant, the final nuclear pellets were each resuspended in 20 ml of ice cold Resuspension Buffer D and 1.05 ml of saturated (4 M) ammonium sulfate was added to give a final concentration of 0.2 M. This suspension was sonicated two times for 15 seconds at 95 watts with a microtip, allowing for ice cooling between bursts. The

sonicated mixture was finally centrifuged at 48,000 g for thirty minutes at 4°C using a Sorvall RC-2 centrifuge. The supernatant, approximately 120 ml, designated as nuclear extract, was stored in 1 ml fractions at liquid nitrogen temperature in sealed plastic tubes. This extract does not lose binding activity on freezing or after prolonged storage (up to six months) in liquid nitrogen. Endogenous triiodothyronine in the nuclear extract was reported by Latham et al. (1976) to be less than 1 pM as measured by radioimmunoassay. Since the binding assays routinely used 0.1 nM hormone, 1 pM endogenous cold triiodothyronine would be insignificant. It was therefore considered unnecessary to thyroidectomize the rats to lower endogenous hormone levels.

3.3 Competitive Binding Assays

3.3.1 Theory

The *in vitro* binding affinities of thirteen thyroid hormone analogs were measured by competitive displacement of radiolabeled T_3 using the method of Bolger (1977). The relative binding constant for each analog was obtained using equation 3.1 which was derived from a two ligand, one binding site, model. Derivation of equation 3.1 and the assumptions applied are presented below:

Given $$P + T_3^* \rightleftharpoons PT_3^*$$

and

$$P + A \rightleftharpoons PA$$

According to the Law of Mass Action and Conservation of Mass,

$$P_t = P + PA + PT_3^* \qquad (3.2)$$

$$K_{aA} = \frac{(PA)}{(P)(A)} \qquad (3.3)$$

$$K_{aT_3} = \frac{(PT_3^*)}{(P)(T_3^*)} \qquad (3.4)$$

where P = unbound nuclear receptor protein

T_3^* = $[^{125}I]$-T_3

A = unlabeled analog

PT_3^* = receptor bound to T_3^*

PA = receptor bound to A

P_t = total moles per liter of binding sites

Substituting equation 3.2 into equation 3.3:

$$K_{aA} = \frac{(PA)}{(A)(P_t - PA - PT_3^*)} \qquad (3.5)$$

Rearrangement of equation 3.5 gives:

$$\frac{1}{K_{aA}} = \frac{(A)(P_t - PT_3^*)}{(PA)} - \frac{(A)(PA)}{(PA)} \qquad (3.6)$$

Dividing equation 3.6 by $(P_t - PT_3^*)$ and rearranging:

$$\frac{1}{K_{aA}(P_t - PT_3^*)} + \frac{(A)}{(P_t - PT_3^*)} = \frac{(A)}{(PA)} \qquad (3.7)$$

From equation 3.3 and equation 3.4:

$$\frac{(A)}{(PA)} = \frac{K_{aT_3}}{K_{aA}} \frac{(T_3^*)}{(PT_3^*)} \qquad (3.8)$$

Substituting equation 3.8 into equation 3.7:

$$\frac{\cancel{K_{aA}}}{K_{aT_3}.\cancel{K_{aA}}(P_t - PT_3^*)} + \frac{\frac{K_{aA}}{K_{aT_3}}(A)}{(P_t - PT_3^*)} = \frac{(T_3^*)}{(PT_3^*)} \qquad (3.9)$$

Since the concentration of the analog added is large compared to the tracer [^{125}I]-T_3, the first assumption made is that the free analog concentration, (A), is equal to the total concentration of analog added. The second assumption is (PT_3^*) becomes small compared to P_t as the concentration of analog added increases thus $P_t - PT_3^* \simeq P_t$. Therefore equation 3.9 can be simplified to give equation 3.1

$$\frac{T_3^*}{PT_3^*} = \frac{1}{K_{aT_3}(P_t)} + \frac{\frac{K_{aA}}{K_{aT_3}}\left[\begin{array}{l}\text{CONC. OF}\\ \text{ANALOG ADDED}\end{array}\right]}{P_t} \qquad (3.1)$$

which represents a straight line. The apparent binding constant of the analog relative to T_3 is obtained by dividing the slope by the y-intercept.

3.3.2 Solubilized Receptor Binding Assay Description

Two parallel sets of incubation tubes (12 x 75 mm) are prepared to determine (a) the equilibrium concentration of labeled hormone, [^{125}I]-T_3, that is bound to the receptor after competitive binding and (b) the amount of "non-specifically" bound radioactivity. The non-specific component is defined as non-saturable binding of [^{125}I]-T_3 to low affinity, high capacity binding sites and is detected as radioactivity which is bound even in the presence of a thousand-fold excess of unlabeled T_3.

To each duplicate tube was added increasing amount of unlabeled thyroid hormone analog followed by a tracer dose of [^{125}I]-T_3 (50 ul, 2.52nM). Incubation buffer (50 mM sodium phosphate at pH 7.6, 0.2 M ammonium sulfate, 1.0 mM dithiothreitol, 50% glycerol) was also added so that the final incubation volume was 0.5 ml when the volume of added nuclear extract is taken into account. To determine "non-specific" binding, 50 ul of a 1 x 10^{-3} M solution of unlabeled L-T_3 was added to the set of two tubes containing no analog. The radioactivity of the tubes was counted before the assay was started with the addition of 50 ul of nuclear extract. The tubes were mixed with a vortex mixer and incubated at 25^{o}C for two hours. After the incubation period,

the tubes were quickly cooled to 4°C with an ice bath. This low temperature slows the dissociation rate and allows for separation of bound and free counts without significant loss of bound counts in the gel filtration medium. A gel filtration procedure was employed to separate the bound and free hormone populations at 4°C. A small Sephadex G-25 column (bed volume = 2.0 ± 0.05 ml, equilibrated in Incubation Buffer) was prepared for each incubation tube from a five inch disposable Pasteur pipette with a cotton plug at the narrow end. A sample of incubation mixture (0.4 ml) was layered on these minicolumns followed by 0.4 ml of incubation buffer. The macromolecular-bound fraction was collected by elution with an additional 0.8 ml of buffer and the amount of radioactivity present was determined with a Searle Auto-Gamma spectrometer (76% efficiency).

3.4 Results

Experimental data are analyzed according to the method of Koerner et al. (1974) using equation 3.1. Specific binding, PT_3^* (i.e. high-affinity, limited-capacity), is calculated by subtracting the counts (cpm) for non-specific binding from the cpm for total binding. Free hormone, T_3^*, is calculated by subtracting the cpm for total binding from the cpm for total labeled hormone added. The relative binding affinity to the nuclear receptor for each thyroid hormone analog is compared to L-T_3 and is expressed on a percentage basis where L-T_3 is taken as 100%. The results are

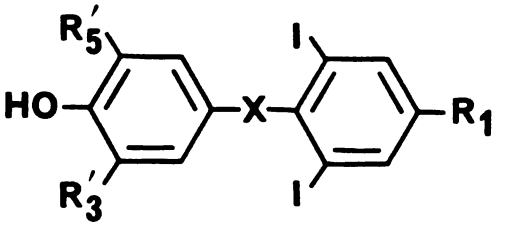
presented in Table 3.2. Typical plots of the competitive binding assay for two cold analogs are seen in Figure 3.1.

3.5 Discussion

The results presented in Table 3.2 show very high affinities of the methylene bridged analogs to the nuclear receptor. Comparison of MB-T_3 to L-T_3 and MB-T_2iPr to L-T_2iPr indicates a thirteen to sixteen-fold increase in binding affinity. In these two cases, only the DL- mixtures are available for testing. We expect the L-isomers of these analogs to be even more tightly bound. The acetic acid side chain analogs generally have higher binding activity than the corresponding alanyl compounds. In this study, we found the methylene-bridged carboxylic acid analogs to be three to four times more active than their corresponding oxygen bridged compounds. The increase in activity due to a CH_2 bridge was also reported by Koerner and coworkers (1975). In their *in vivo* binding studies, where animals (rats) were injected with radiolabeled T_3 and increasing amount of hormone analogs, they found the (DL-MB-T_3) binds two to five times better to whole nuclei than L-T_3. The combination of an isopropyl group at the 3' position and a methylene moiety at the bridging area appears to increase binding more than predicted by the sum of the individual modifications.

The lower binding of the (DL)-MB-T_4 analog to the nuclear receptor when compared to L-T_4 is surprising but not

TABLE 3.2 NUCLEAR RECEPTOR BINDING AFFINITIES FOR THE THYROID HORMONE ANALOGS

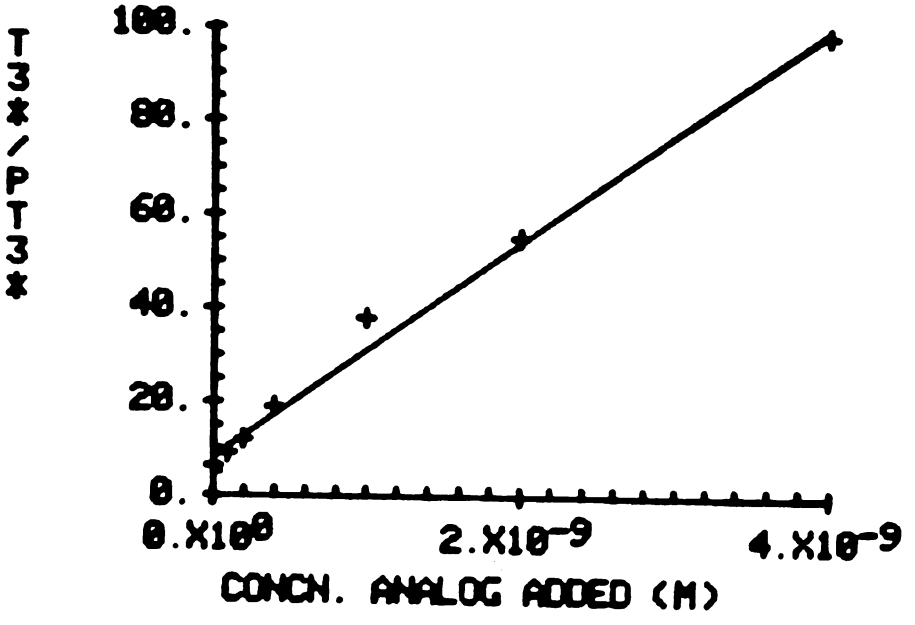


	COMPOUND	$R_{3'}$	$R_{5'}$	X	R_1	% L-T_3
1.	L-T_3	I	H	O	L-ALA	100
2.	MB-T_3	I	H	CH_2	DL-ALA	1342.66
3.	L-T_4	I	I	O	L-ALA	6.50
4.	MB-T_4	I	I	CH_2	DL-ALA	3.92
5.	L-T_2IPR	IPR	H	O	L-ALA	96.50
6.	MB-T_2IPR	IPR	H	CH_2	DL-ALA	1590.91
7.	CO-T_2IPR	IPR	H	CO	DL-ALA	1.81
8.	TRIAC	I	I	O	CH_2COOH	860.14
9.	IPRDIAC	IPR	H	O	CH_2COOH	1664.34
10.	MB-IPRDIAC	IPR	H	CH_2	CH_2COOH	6538.46
11.	CO-IPRDIAC	IPR	H	CO	CH_2COOH	16.12
12.	IPRI_2FORM	IPR	H	O	COOH	3.53*
13.	MB-IPRI_2FORM	IPR	H	CH_2	COOH	9.55
14.	CO-IPRI_2FORM	IPR	H	CO	COOH	0.78

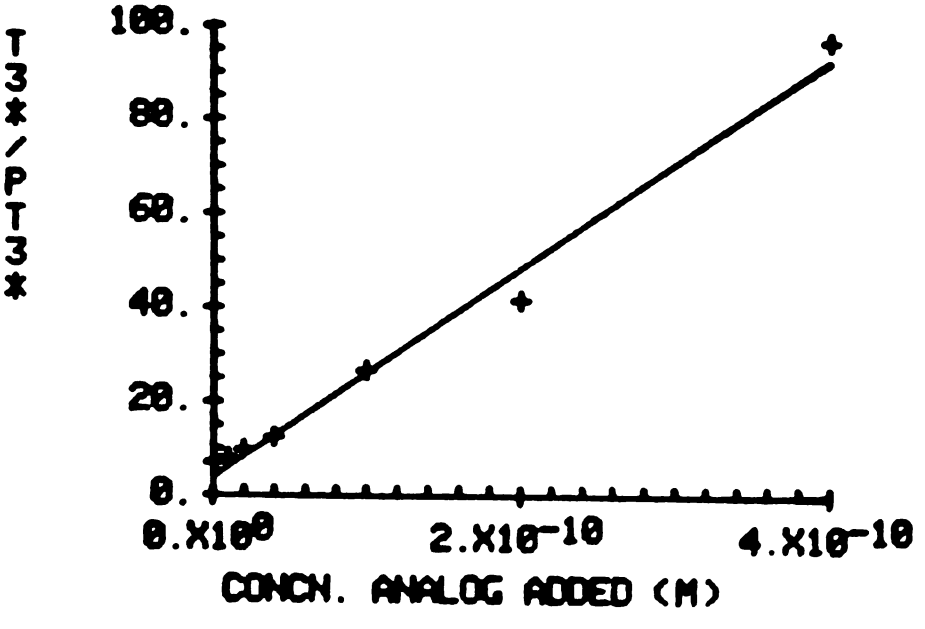
*VALUE FROM BOLGER (1977)

Figure 3.1

A. L-T_3



B. MB-T_2iPr



unreasonable. Koerner (1975) measured the *in vivo* binding of (DL)-MB-T_4 and obtained a value of 2.6% (L-T_3 = 100%) whereas L-T_4 is 12.5%. We attribute the decrease in binding to the presence of a 5' iodo group. The binding site on the nuclear receptor is less tolerant of a bulky 5' substituent. The decrease in activity may be caused by interference with the hydrogen bonding of the 4' hydroxyl to the receptor or by direct steric interference with the receptor (Jorgensen 1978). The methylene-bridged analogs possess a smaller dihedral angle of 114^{o} and, with the 5' substitution, may have created a worse situation by placing the 5' group more in the direction of the 4' hydroxy binding site and by bringing the 5' iodine even closer to the protein, causing a bigger steric interference ("collision" with the protein).

As a result of the failure by Tripp and coworkers (1973) to iodinate the outer ring of the carbonyl-bridged hormone analogs, the activities of these analogs are not measurable. In this study, three 3'-isopropyl carbonyl-bridged compounds are tested for binding with the receptor. They possess low but significant binding affinities. A polar group such as the carbonyl function appears to decrease binding to the receptor.

CHAPTER FOUR

IN VIVO BIOLOGICAL ACTIVITIES OF THE KETONE- AND METHYLENE- BRIDGED THYROID HORMONE ANALOGS

4.1 Introduction

In vivo biological test systems have been developed over the years for a large number of the various biological responses that the thyroid hormones and analogs elicit. These assays include: (1) increase of guinea pig body weight (Abelin 1934, Bovarnick et al. 1939); (2) elevation in oxygen consumption (Gaddum 1930, Maclagan and Sheahan 1950, Baker et al. 1965, Smith et al. 1947); (3) induction of amphibian metamorphosis (Kendall 1919, Gaddum 1927, Bruice et al. 1954, Tomita and Lardy 1956, Frieden and Westmark 1961, Wahlborg et al. 1964, Frieden and Yoshizato 1974); (4) goiter prevention (Dempsey and Astwood 1943, Reiineke and coworkers 1945, Mussett and Pitt-Rivers 1957, Comite 1976, Comite et al. 1976,1978; Dietrich 1976); (5) increase in the activity of enzymes such as α-glycerophosphate dehydrogenase (Westerfield et al. 1965, Van Pilsum et al. 1970); (6) decrease in plasma or tissue cholesterol levels (Duncan and Best 1958, Boyd and Oliver 1960, Greenberg el al. 1961, 1963; Herrman et al. 1961); (7) increase in heart rate (Baker et al. 1965) and (8) suppression of radioactive iodide uptake by the rat thyroid (Money et al. 1959).

The antigoiter assay in the rat has been used to test the relative activities of hundreds of analogs, and this represents the largest and best single body of *in vivo* data available. Current practice generally follows the procedures originally developed by Dempsey and Astweed (1943), or the extensions and modifications of Reineke and coworkers (1945) and Mussett and Pitt-Rivers (1957). To ensure optimal reproducibility and accuracy, Dietrich (1976) recommended some useful guidelines. A detail description of the antigoiter bioassay performed in this study is presented below.

4.2 Rat Antigoiter Assay

The oxidative reactions involved in the biosynthetic formation and conversion of the iodinated tyrosines into the thyroid hormones are inhibited by a variety of thioamides such as thiourea, thiouracil, and propylthiouracil. Daily administration of the thioamide drug, usually in the food, leads to decreased levels of circulating thyroid hormone. The pituitary gland responds by release of the glycoprotein hormone, thyrotropin, which stimulates the thyroid gland, producing a three- to five- fold increase in thyroid gland weight (goiter) over a ten day period. Concomitant administration of T_3, T_4, or of a thyromimetic analog, inhibits thyrotropin release and blocks goiter formation. Cortell (1949) showed that hormone antagonists could be detected by their ability to reverse the antigoitrogenic effects of T_4.

Following the guidelines suggested by Dietrich (1976), three of the thyroid hormone analogs synthesized for this study were tested for their thyromimetic, antigoitrogenic activities. The assay also served as a preliminary screening for the potential antagonist.

4.2.1 Assay Guidelines (Dietrich 1976)

1. The goitrogen should be supplied well-mixed in the food, rather than in the drinking water. Low water solubility limits the dose, and the bitter taste of thiouracil discourages the rat from drinking, resulting in uneven dosage.
2. Analogs and reference compounds should be administered by subcutaneous injection, rather than by admixing with the food, in order to ensure adequate doses.
3. Compounds should be administered on a dose schedule that is adjusted to increasing body weight. (ug or umoles per 100 grams body weight)
4. Activities should be estimated on the basis of relative molar amounts of drugs required to produce 50% inhibition of goitrogen induced goiter. Full log dose versus thyroid weight curves are preferred.
5. L-T_3 is recommended as the standard reference compound. Variations in deiodination rates <u>in vivo</u> of T_4 to form the more potent T_3 and the essentially inactive reverse-T_3 (3,3',5'-T_3) could lead to greater individual variations in response when T_4 is used as the reference standard.

4.2.2 Assay Procedure

Solutions of the analogs were made up a day or two before the injections were to begin and were prepared so that they could be compared to the reference compound on a molar basis. An arbitary molar ratio value of 1.00 was assigned to the dose of L-T_3 containing 0.25 ug of L-T_3 per 100 grams of rat body weight. Solutions were prepared so that the dose administered to a 100-gram rat was contained in 0.125 ml of solution. Samples were weighed on a Cahn Electrobalance; stock solutions were prepared by dissolving in a solvent containing 0.1 N NaOH and 0.9% NaCl. Compounds that were insoluble in this solvent were dissolved in ethanol and diluted with normal saline (0.9% NaCl). Injection solutions were stored in sample vials fitted with a septum and were refrigerated when not in use.

Sprague-Dawley rats, weighing between 80 and 100 grams, were purchased from Simonsen Laboratories and housed three to a cage. All were fed a normal diet of powdered Simonsen Rat Maintenance Diet (S/L white) for two days prior to the start of the assay. This was done in order to allow them to "settle in" and to establish a regular feeding regimen and hence insure a fairly constant intake of thiouracil when incorporated into their feed. Groups of six rats were used for each reference compound and analog dose. The thiouracil and normal control groups each contained five rats. The normal control received a normal diet and all other animals

received 0.3% thiouracil in their feed. Diets were begun one day before the injections were started. The normal and thiouracil controls were injected subcutaneously with 0.1 N NaOH in normal saline. The other groups were injected with L-T_3 or analogs at the predetermined dose levels. The volume of solution injected was determined on the basis of daily weighing. (Table 4.1)

After ten days of injection, the animals were sacrificed by chloroform inhalation and weighed. The thyroid glands were excised, kept moist with normal saline, cleaned of extraneous tissue under a dissecting microscope and weighed to the nearest 0.1 mg. The thyroid weights were converted to milligrams per hundred grams body weight and the means were calculated for each dose level.

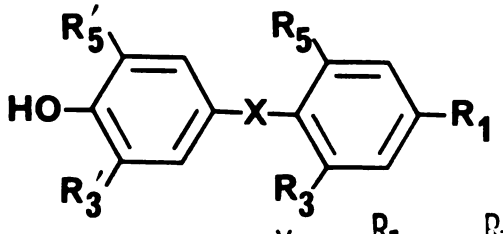
4.3 Results

The compounds tested for their thyromimetic activities in the rat antigoiter bioassay are listed in Table 4.2. The detailed results of the assay are presented in Table 4.3. The statistical analysis of the data was performed using the PROPHET computer system, Chemical/Biological Information Handling Program, National Institutes of Health, Bethesda, MD.

TABLE 4.1

RAT WEIGHT (GM)	VOLUME OF SOLUTION (ML)
80 - 99	0.100
100 - 119	0.125
120 - 139	0.150
140 - 159	0.175
160 - 179	0.200
180 - 199	0.225
200 - 219	0.250

TABLE 4.2 THYROXINE ANALOGS TESTED IN THE RAT ANTIGOITER BIOASSAY



COMPOUND	ABBREVIATION	X	R_1	$R_3 = R_5$	$R_{3'}$	$R_{5'}$
4-1	L-T_3	O	L-ALA	I	I	H
4-2	MB-T_2IPR	CH_2	(DL)-ALA	I	$CH(CH_3)_2$	H
4-3	MB-IPRDIAC	CH_2	CH_2COOH	I	$CH(CH_3)_2$	H
4-4	CO-T_2IPR	CO	(DL)-ALA	I	$CH(CH_3)_2$	H

TABLE 4.3 RAT ANTIGOITER BIOASSAY OF THYROID HORMONE ANALOGS

COMPOUND INJECTED	DAILY DOSE (μG/100 GM BODY WT.)	MOLAR RATIO	# OF RATS	MEAN THYROID WEIGHT (μG/100 GM BODY WT.) ± S*
(NORMAL)				
SALINE			5	8.50 ± 0.60
(THIOURACIL)				
SALINE			5	31.57 ± 2.20
L-T_3 (4-1)	0.0625	0.25	5	27.90 ± 2.81
	0.1250	0.50	5	17.71 ± 5.21
	0.2500	1.00	4	12.43 ± 3.07
	0.5000	2.00	2	8.00 ± 1.07
MB-T_2IPR (4-2)	0.0108	0.05	6	30.66 ± 6.70
	0.0434	0.20	6	18.79 ± 4.94
	0.2169	1.00	6	7.26 ± 1.71
MB-IPRDIAC (4-3)	0.0210	0.10	6	28.26 ± 4.87
	0.0421	0.20	6	26.98 ± 5.08
	0.2104	1.00	6	12.02 ± 4.23
CO-T_2IPR (4-4)	2.2230	10.00	6	29.41 ± 4.18
	11.1130	50.00	6	15.28 ± 3.16
	22.2260	100.00	6	10.81 ± 2.92

* S = STANDARD DECIATION

The arithmatic mean, $\bar{X}$; variance, s^2; and standard deviation, s; are computed according to the following equations:

$$\bar{X} = \sum_{i=1}^{n} \frac{X_i}{n}$$

X_i = thyroid weight/100 g body weight for individual animal

n = number of observations

= number of animals per group

$$s^2 = \sum_{i=1}^{n} \frac{(X_i - \bar{X})^2}{n-1}$$

n - 1 = degrees of freedom

$$s = \sqrt{s^2}$$

The mean $\bar{X}$ is the average value of a group at a particular dose level or of the control group, and is expressed as thyroid weight (mg)/100 g body weight. The variance of the sample, s^2, provides information about the variability or spread of the data. s^2 is an unbiased estimator of σ^2, the population variance. If an indefinitely large number of samples of size n were drawn from a single population, in the long run the mean of the s^2 so obtained would equal the true population variance, σ^2. The standard deviation is the positive square root of the variance.

To establish the significance of the results obtained, the mean of the individual dose level of L-T_3 or analogs ($\bar{X}_{cpd}$) is compared to the mean value of the thiouracil control ($\bar{X}_{thio}$) using the unpaired t-test. The first step in the unpaired t-test is to test for the equality of variances. This is done with the F-test. The F-statistic is defined as

$$F = \frac{s^2_{thio}}{s^2_{cpd}}$$

where s^2_{thio} is greater than s^2_{cpd} (otherwise the order is simply reversed). The degrees of freedom are $(n_{thio} - 1)$ and $(n_{cpd} - 1)$. The null hypothesis is that the variances are not different. If the calculated F value exceeds the critical F-value, the null hypothesis is rejected; if not, it is accepted. When it is concluded that the variance are equal, i.e. normal distribution for both populations, the appropriate t-statistic with degrees of freedom (DF) = $n_{thio} + n_{cpd} - 2$, is

$$t = \frac{\bar{X}_{thio} - \bar{X}_{cpd}}{\left[s^2_p \left(1/n_{thio} + 1/n_{cpd}\right)\right]^{\frac{1}{2}}}$$

where s_p^2, the pooled estimate of the population variance σ^2, is

$$S_p^2 = \frac{(n_{thio}-1)s_{thio}^2 + (n_{thio}-1)s_{cpd}^2}{n_{thio} + n_{cpd} - 2}$$

The calculated t-value indicates whether the hypothesis that the population means are equal should be rejected. When the critical t-value is met or exceeded by the calculated t-value, the null hypothesis is rejected and the confidence level is determined from the tabulated probability (Pr) value. Conversely, when the calculated t-value is less than the critical t-value, the null hypothesis is accepted based on insufficient evidence to reject it.

Results of the F-test and t-test are presented in Table 4.4. All the data points are normally distributed and the mean thyroid weight for the lowest dose level approaches the thiouracil control value; in these cases the null hypothesis of equal means is accepted. The difference between $\bar{X}_{cpd}$ for other dose levels and $\bar{X}_{thio}$ is established at confidence levels of at least 99.9%. This shows the effect of L-T_3 or the analogs in the thiouracil treated animals is significant. The lowest dose level is basically not effective in decreasing thyroid weight; as graded higher doses are given, the thyroid weight decreases and in all cases approaches the value for the normal control animals. (Table 4.3) The analogs 4-2, 4-3 and 4-4 are thyromimetic.

TABLE 4.4 STATISTICAL ANALYSIS FOR THE RAT ANTIOGOITER BIOASSAY

I. F DISTRIBUTION

COMPOUND	MOLAR RATIO	N	s^2	F	DF (N/D)	NULL HYPOTHESIS*
THIOURACIL		5	4.85			
L-T_3 (4-1)	0.25	5	7.88	1.624	4/4	ACCEPT
	0.5	5	27.13	5.587	4/4	ACCEPT
	1.00	4	9.42	1.941	3/4	ACCEPT
MB-T_2IPR (4-2)	0.05	6	44.79	9.224	5/4	ACCEPT
	0.20	6	24.38	5.021	5/4	ACCEPT
	1.00	6	2.92	1.664	4/5	ACCEPT
MB-IPRDIAC (4-3)	0.10	6	23.69	4.878	5/4	ACCEPT
	0.20	6	25.80	5.315	5/4	ACCEPT
	1.00	6	17.86	3.677	5/4	ACCEPT
CO-T_2IPR (4-4)	10.00	6	17.46	3.596	5/4	ACCEPT
	50.00	6	10.01	2.062	5/4	ACCEPT
	100.00	6	8.53	1.758	5/4	ACCEPT

* NULL HYPOTHESIS = EQUAL VARIANCES

TABLE 4.4 STATISTICAL ANALYSIS FOR THE RAT ANTIOGOITER BIOASSAY

II. T-TEST ON UNPAIRED DATA (EQUAL VARIANCE)

COMPOUND	MOLAR RATIO	N	$t_{CAL'D}$	D.F.	NULL HYPOTHESIS*	PR
THIOURACIL		5				
L-T_3 (4-1)	0.25	5	2.297	8	ACCEPT	
	0.50	5	5.482	8	REJECT	0.001 (99.9%)
	1.00	4	10.932	7	REJECT	0.0001 (99.99%)
MB-T_2IPR (4-2)	0.05	6	0.288	9	ACCEPT	
	0.20	6	5.326	9	REJECT	0.0005 (99.95%)
	1.00	6	20.650	9	REJECT	0.0001 (99.99%)
MB-IPRDIAC (4-3)	0.10	6	1.397	9	ACCEPT	
	0.20	6	1.868	9	ACCEPT	
	1.00	6	9.292	9	REJECT	0.0001 (99.99%)
CO-T_2IPR (4-4)	10.00	6	1.035	9	ACCEPT	
	50.00	6	9.684	9	REJECT	0.0001 (99.99%)
	100.00	6	13.056	9	REJECT	0.0001 (99.99%0

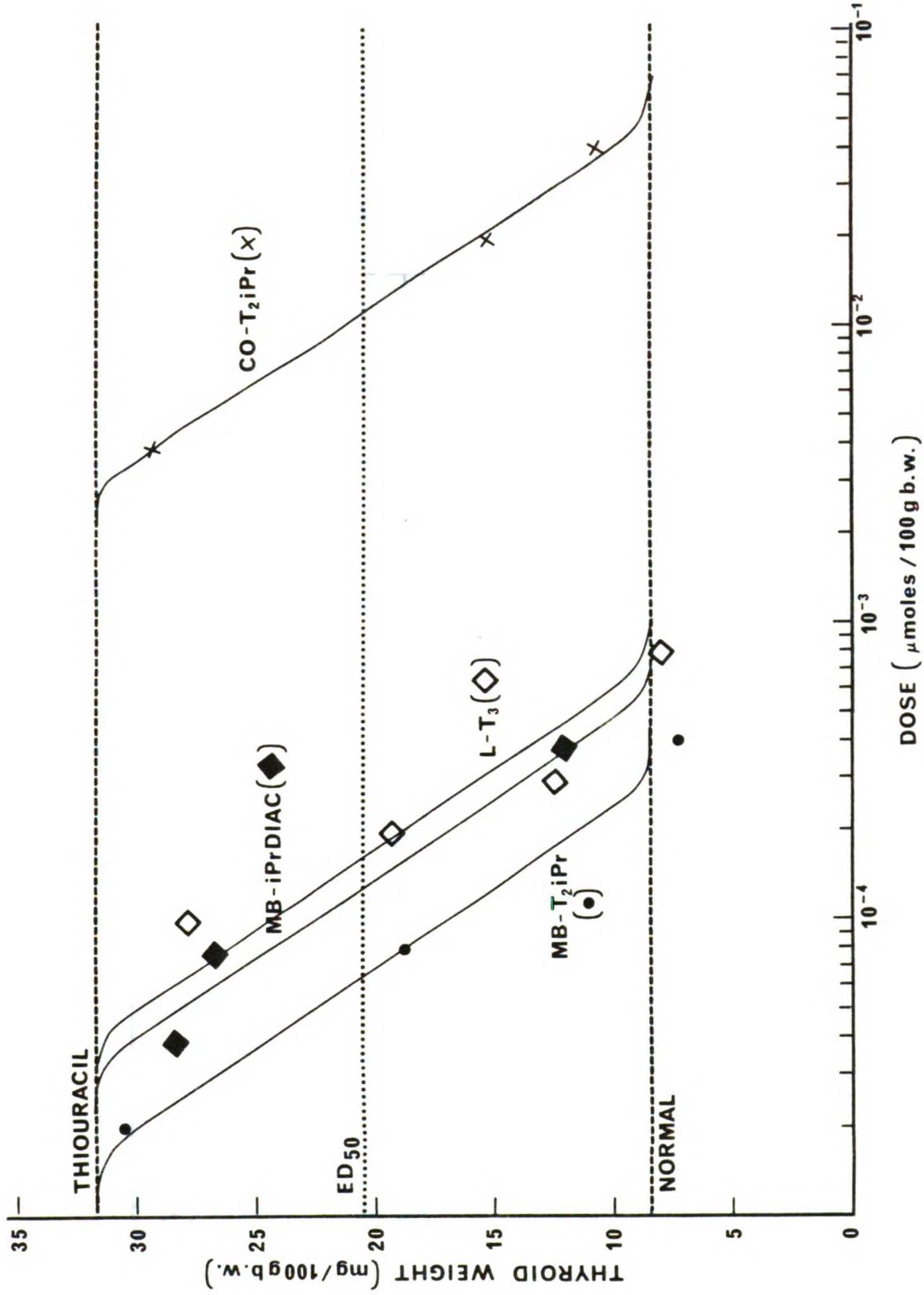
* NULL HYPOTHESIS = EQUAL MEANS

4.4 Discussion

The biological response observed after administration of the thyroid hormone analogs is the decrease in thyroid weight as compared to the thiouracil control animals. 50% inhibition of goiter formation is defined as the value half-way between the maximum and minimum responses. The maximum value being given by the mean thyroid weight per 100 grams body weight of the thiouracil control group ($\bar{X}_{thio}$) and the minimum is the mean for the normal control group. The biological activities of the compounds tested are estimated on the basis of either dose or of relative molar amount required to produce 50% inhibition of goiter. Figures 4.1 and 4.2 are the log dose versus thyroid weight, and log molar ratio versus thyroid weight curves. The dose and the molar ratio obtained at the 50% inhibition level are compared to the corresponding values of the reference compound, L-T_3. The biological activities of the analogs are expressed in percent relative to L-T_3 where L-T_3 = 100%.

The log molar ratio versus thyroid weight plot is prepared following the Dietrich (1976) assay guidelines so as to be consistent when comparison with literature values is necessary. The log. dose versus thyroid weight curve is obtained graphically and also by analysis of covariance. The statistical analysis relating all the dose levels of each analog to that of L-T_3 is a more powerful tool than the

FIGURE 4.1


THYROID WEIGHT (mg/100g b.w.)
35
30
25
20
15
10
5
0
THIOURACIL
ED50
NORMAL
CO-T2iPr (x)
MB-iPrDIAC (◆)
L-T3 (◇)
MB-T2iPr (●)
10-4
10-3
10-2
10-1
DOSE (μmoles / 100 g b.w.)

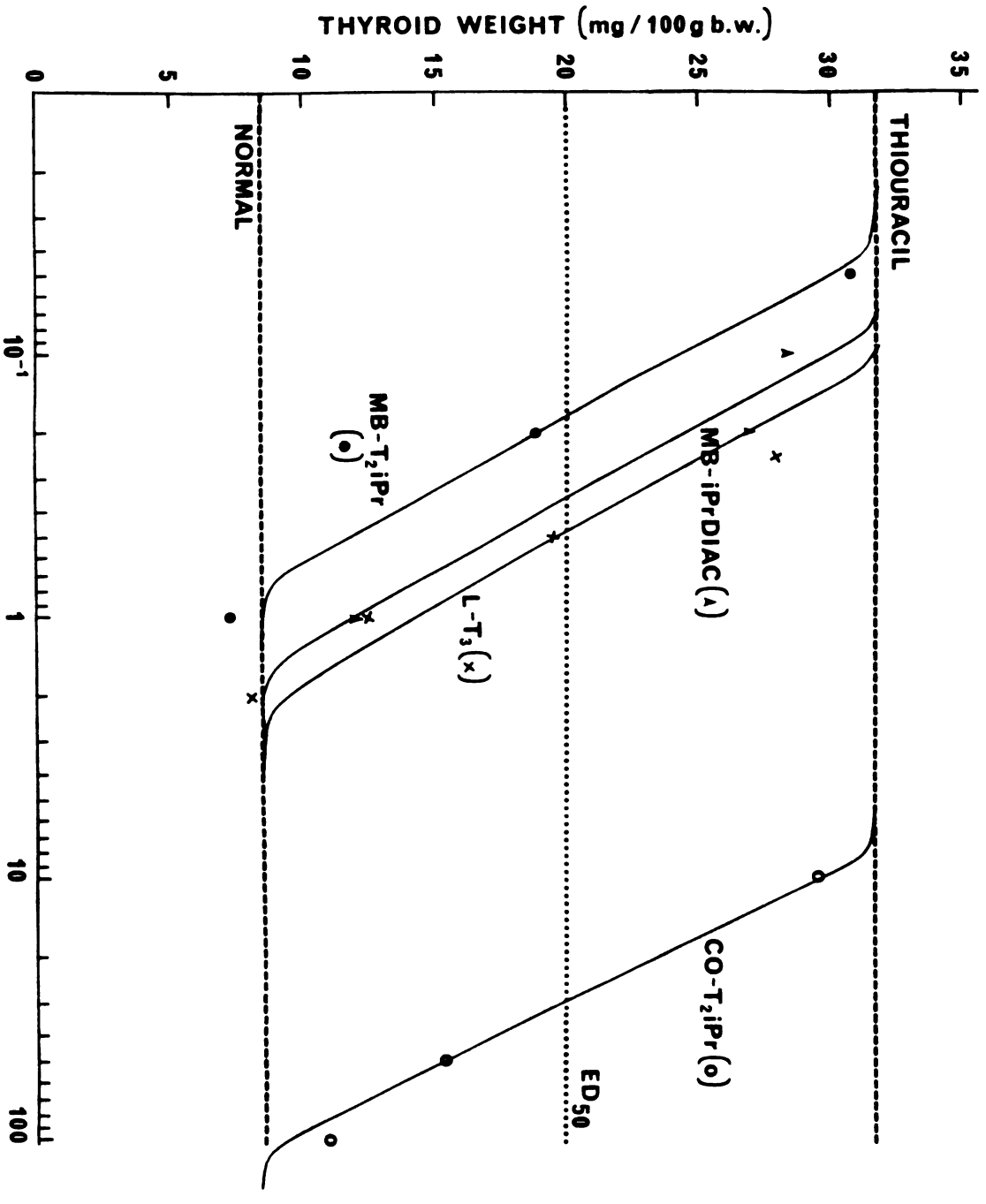

THYROID WEIGHT (mg / 100g b.w.)
35
30
25
20
15
10
5
0
THIOURACIL
NORMAL
MB-iPrDIAC(▲)
MB-T2iPr (●)
L-T3(×)
CO-T2iPr(○)
ED50
10-1
1
10
100
MOLAR RATIO

FIGURE 4.2

t-test which compares only the mean of the individual dose to that of the standard.

The covariance analysis is a combination of analysis of variance and regression analysis. It is designed to investigate and compare several regressions simultaneously; such comparison is often useful for removing the effect of one or more unwanted factors. The experimental observations for each compound ($L\text{-}T_3$ and analogs) are first fitted using non-linear regression to the curve denoted by the equation:

$$\text{EFFECT} = \text{NODRUG} - \frac{(\text{NODRUG} - \text{MAXDRUG}) \times \text{DOSE}}{ED_{50} + \text{DOSE}}$$

where EFFECT is the thyroid weight (mg)/100 g. body weight (b.w.)

NODRUG is the thyroid weight (mg)/100 g.b.w. for the thiouracil control group

MAXDRUG is the thyroid weight (mg)/100 g.b.w. for the normal control group

DOSE is the amount of compound (mmoles)/100 g.b.w. administered daily

and ED_{50} is the effective dose to bring about 50% inhibition of goiter formation.

The constraints applied for the regression analysis are:

(1) $\text{MAXDRUG} \geq 0$,

implying that the thyroid weight at 100% inhibition

should be larger or equal to 0; and

(2) NODRUG > MAXDRUG,

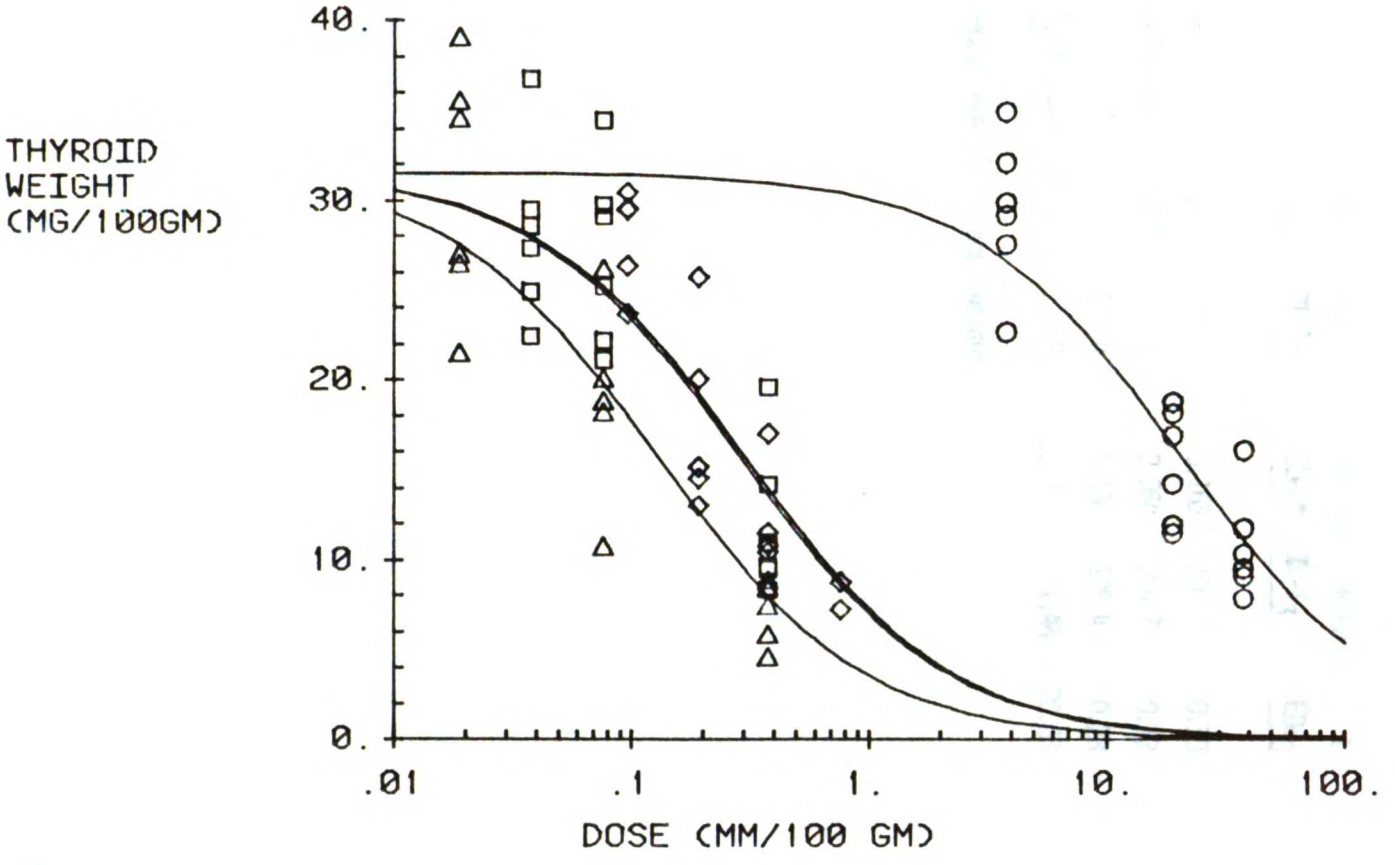
which sets the maximum value for thyroid weight per 100 grams body weight and assumes thyromimetic activity for the analogs.

The regression lines for L-T_3, 4~2, 4~3 and 4~4 are then compared on a semilogarithmic scale to give the ED_{50} values (Figure 4.3).

Table 4.5 shows the relative activities obtained using either molar ratios or doses in millimoles. For ease of comparison, the values determined on the basis of molar ratios are quoted in this section, though covariance analysis should give more reliable results.

DL-3,5-Diiodo-4-(3-isopropyl-4-hydroxybenzyl)-phenylalanine (4~2) is 2.7 times as active as L-T_3. Adjusting for the difference in biological activity between the DL-mixture and the L-isomer (Dietrich 1976), the L isomer of 4~2 would be 4.6 times more active. This unusually high activity parallels the result obtained in the <u>in vitro</u> nuclear receptor binding study and is unique even when compared to L-T_2iPr which is the only thyroxine analog to have a higher activity in the rat antigoiter assay than L-T_3 (Table 4.6). L-T_2iPr is about 1.5 times as active as L-T_3 whereas L-MB-T_2iPr is 4.6 times more active. This increase in activity is again apparent when MB-iPrDIAC is compared to iPrDIAC. The acetic acid side chain analogs possess lower

Figure 4.3



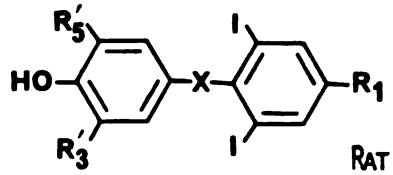
◇ L-T_3 ; △ MB-T_2iPr ; □ MB-DIAC ; o CO-T_2iPr

TABLE 4.5 THYROMIMETIC ACTIVITIES OF THE THYROID HORMONE ANALOGS

	LOG MOLAR RATIO		LOG DOSE (GRAPH)		LOG DOSE (COVARIANCE)	
COMPOUND	ED_{50}	%L-T_3	ED_{50}*	%L-T_3	ED_{50}*	%L-T_3
L-T_3 (4-1)	0.46	100	0.163	100	0.300 ± 0.082	100
(DL)MB-T_2iPr (4-2)	0.17	270.6	0.064	254.7	0.128 ± 0.041	234.5
MB-iPrDIAC (4-3)	0.35	131.4	0.130	125.4	0.286 ± 0.080	104.9
(DL)CO-T_2iPr (4-4)	28.5	1.61	11.3	1.44	20.639 ± 6.035	1.45

* ED_{50} MMOLES PER 100 GM BODY WEIGHT

TABLE 4.6



COMPOUND	$R_{3'}$	$R_{5'}$	X	R_1	RAT ANTIGOITER %L-T_3	TADPOLE METAMORPHOSIS %L-T_3	%L-T_4
L-T_3	I	H	O	L-ALA	100	100	
L-T_4	I	I	O	L-ALA	13[A], 12.5[C], 20[D] 18.1[E]	10[B]	100
(DL)MB-T_3	I	H	CH_2	DL-ALA	54.25[F], 38[H,I]		1200[G]
* L-MB-T_3	I	H	CH_2	L-ALA	91.95[F]		
(DL)MB-T_4	I	I	CH_2	DL-ALA			150[G]
(DL)T_2IPR	IPR	H	O	DL-ALA	54[J]		
L-T_2IPR	IPR	H	O	L-ALA	142[E], 125[K], 180[J]	300[B]	
(DL)MB-T_2IPR	IPR	H	CH_2	DL-ALA	270.6		
* L-MB-T_2IPR	IPR	H	CH_2	L-ALA	458.6		
IPRDIAC	IPR	H	O	CH_2COOH	16[J]	50[B]	
MB-IPRDIAC	IPR	H	CH_2	CH_2COOH	131.4		
(DL)CO-T_2IPR	IPR	H	CO	DL-ALA	1.61		
* L-CO-T_2IPR	IPR	H	CO	L-ALA	2.73		

A STASILLI (1959)
B WAHLBORG (1964)
C KOERNER (1975)
D GOLDFINE (1976)
E JORGENSEN (1977)
F JORGENSEN (1974)
G FRIEDEN (1974)
H MUSSETT (1957)
I JORGENSEN (1970)
J GREENBERG (1963)
K WOOL (1966)

* ACTIVITY OF L-ANALOG = ACTIVITY OF DL-ANALOG /0.59

activity *in vivo* than their corresponding amino acid analogs due to decreased transport, increased metabolism and excretion (Jorgensen 1978). The activity of 3,5-diiodo-4-(3-isopropyl-4-hydroxybenzyl)phenylacetic acid (MB-iPrDIAC) is 1.3 times that of L-T_3 as compared to 0.16 for the corresponding oxygen bridged analog (iPrDIAC).

Methylene-bridged analogs of T_3 and T_4 were tested by several investigators in different biological systems and their results are also presented in Table 4.6. The methylene-bridged L-T_3 (L-MBT_3) is almost as active as L-T_3 in the rat antigoiter assay and about 1.2 times more active in the tadpole metamorphosis assay. The combination of a CH_2 group between the two aromatic rings and an isopropyl group at the 3' position enhances activity in the rodent more than what might have been expected. The ketone bridged analog (CO-T_2iPr) exhibits very low activity. The results obtained from the rat antigoiter bioassay are consistent with the results from the competitive nuclear binding assay.

CHAPTER FIVE

IN VITRO BINDING OF THYROXINE ANALOGS TO THYROXINE-BINDING PREALBUMIN

5.1 Introduction

The serum proteins present for the transport of thyroid hormones are: thyroxine-binding globulin (TBG), thyroxine-binding prealbumin (TBPA) and human serum albumin (HSA). Their binding affinities for thyroxine (T_4) differs, with TBG (K_a = 2.5 x 10^9 M^{-1}, Synder et al. 1976) > TBPA (K_a = 4.9 x 10^7 M^{-1}, Andrea 1977) > HSA (K_a = 1.5 x 10^6 M^{-1}, Tabachnick 1964). The relative abundance of these proteins in blood serum follows the reverse order. HSA is present in high concentration in blood and binds a variety of plasma constituents, including free fatty acids. Despite its low affinity for the thyroid hormones, its high concentration leads to its carrying about 10 - 15% of the plasma T_4. TBG is the primary carrier and displays specific structural requirements for ligand binding (Snyder et al. 1976, Dietrich et al. 1977). TBPA is the secondary carrier for T_4 in the blood. Although T_4 binds to TBPA with only about 1% the affinity with which it binds to TBG, the high plasma concentration of TBPA relative to that of TBG makes it important as a hormone carrier. The information available on the structure of TBPA and the specificity required for hormone binding makes it a useful model for the study of the nature of, and the structural requirements for, binding

interactions with the less accessible receptor proteins.

5.2 Thyroxine-Binding Prealbumin (TBPA)

Human plasma TBPA is as yet the only fully characterized hormone-binding protein. It is a nonglycosidic linear protein consisting of four identical subunits each containing 127 amino acids (Kanda et al. 1974). The subunits are associated in an ellipsoid shape with a channel running completely through the long axis of the tetramer. X-ray crystallographic studies by Blake and his associates (1978) with resolution to 1.8 $\mathring{A}$ and affinity labeling studies (Cheng et al. 1977a) have fixed the primary binding site in one arm of the channel. Affinity of T_4 to the identical second site is reduced 100-fold by binding of the first molecule.

Several molecular characteristics make TBPA an attractive preliminary model for the nuclear receptor. Both proteins show similar molecular weights (TBPA = 54,980, nuclear receptor = 55,00 to 65,000) and ellipsoid shapes as estimated from diffusion studies (Kanda et al. 1974, Surks et al. 1975, Latham et al. 1976). In addition to a specific deep-seated binding site for the thyroid hormones, TBPA possesses two identical surface depressions that are remarkably complementary to the overall size and shape of a segment of DNA (Blake 1977). The nuclear receptor resides on the chromatin DNA, which implies that it possesses a highly specific DNA binding region. The major difference between the proteins is that the receptor binds T_3 with about ten

times the affinity of T_4, while the converse is true for TBPA. This difference disappears when the crude nuclear receptor is purified to remove the associated basic histone proteins. The partially purified histone-free receptor, like TBPA, binds T_4 more strongly than T_3 (Eberhardt 1979). A parallel situation exists for TBPA. In plama, TBPA is associated with the strongly polar retinol-binding protein that in turn carries the vitamin retinol (Rask 1979). It is possible that the nuclear histones contribute to the high binding affinity and specificity of the receptor for T_3 by constraining the hormone binding site in a highly defined and semirigid conformation. One attractive hypothesis is that TBPA and the nuclear receptor are derived from a common ancestral gene and protein, and that the hormone binding regions have retained considerable homology as has the general shape of the so called DNA binding region of TBPA. In TBPA, however, the binding functionality to DNA has not been retained since it plays no known role in the plasma transport process.

Molecular interactions between thyroxine and TBPA are approximated by X-ray crystallographic studies (Blake and associates 1978) and are further refined by computer graphic docking experiments (Jorgensen et al. 1980). The binding of the hormone to TBPA occurs in one arm of the central channel, with the carboxyl and ammonium groups of the alanine side chain associated by ion pairing with Lys-15 and Glu-54 residues in the channel mouth. Deep within the channel the

phenolic hydroxyl group is associated with the hydroxyl groups of paired Ser-117 and Thr-119 residues, with this hydrogen bonding being mediated by a crystallographically well-defined water molecule.

Identical hydrophobic pockets of TBPA lined with methyl groups of Leu-17C, Thr-106A, Ala-108A, Val-121A, and the polymethylene side chain of Lys-15A hold the 3 and 5 iodine atoms of T_4 or T_3. The 3' and 5' iodine atoms of the outer ring of T_4 each fit into a pocket with quite different characteristics. The 5' iodine atom, distal to the inner ring, fits into a pocket lined with side chain and peptide backbone atoms of residue Ala-108C, Ala-109C, Leu-110C, Ser-117C and Thr-119C. The 3' iodine atom, proximal to the inner ring, fits into a pocket defined by residues Leu-17A, Ala-108A, Ala-109A, Leu-110A, and the polymethylene side chain of Lys-15A.

The amino acid residues around the bridging area of T_4 are mainly non hydrophobic. In this study the binding affinities to TBPA are determined for nine newly synthesized analogs. The results will provide information on:

(a) possible interaction of the new bridging groups with TBPA;

(b) how well the new bridging group is tolerated in comparison with the nuclear receptor; and finally

(c) how tightly these analogs are bound to transport proteins, knowledge of which is crucial for the design of a

transplacental analog.

5.3 In Vitro Binding to TBPA

A procedure similar to the gel filtration method (Chapter Three) for L-T_3 receptor binding was developed by Somack and coworkers (1982) for studying L-T_4-prealbumin binding. In this assay, the affinity constants of L-T_4 for the two binding sites of prealbumin are determined by Scatchard analysis and unlabeled analogs are tested for their ability (relative to L-T_4) to displace [^{125}I]-L-T_4 from only the high affinity binding site. The justification in only focusing attention on competition occurring at that level was discussed by Andrea (1977).

5.3.1 Materials

L-T_4 and L-T_3 were from Nutritional Biochemicals. [^{125}I]-L-T_4 was purchased from New England Nuclear Corporation. The specific activities were 800 - 1200 uCi/ug. Methylene-bridged T_3 and T_4 analogs were obtained from Dr. Spencer L. Tripp (Ciba-Geigy Corporation, N.Y.). 3'-Isopropyl-3,5-diiodo-L-thyronine and its acetic acid side chain analog were prepared by Dr. August Gallo of this research group. Trizma base, ethylenediamine tetracetic acid (EDTA) and Sephadex G-25 (bead size: 50 - 150 u) were purchased from Sigma Chemical Company. Prealbumin was obtained from Dr. R.C. Hevey, Behring Diagnostics, Somerville, N.J., and had an immunological purity of 98%.

5.3.2 Buffer and Stock Solutions

The incubation buffer (Buffer I) contains 0.1 M Trizma base, 0.1 M sodium chloride and 1 mM EDTA. The pH was titrated to 8.0 by addition of concentrated HCl. A 1×10^{-7} M solution of prealbumin was prepared by dissolving the protein in Buffer I. The assumed molecular weight was 55,000 (Blake et al. 1978). The purchased [^{125}I]-L-T_4 solution was diluted one hundred fold with Buffer I to give a stock solution with concentration of approximately 3×10^{-9} M. To obtain concentrated solutions (1×10^{-3} M) of the analogs, samples were weighed on a Cahn Electrobalance and dissolved with methanol. For analogs not entirely soluble in methanol, a few drop of base was added (50% NaOH solution). Sequential dilution were made with Buffer I as solvent.

5.3.3 Experimental Procedures

Two parallel sets of incubation tubes (12 x 75 mm) are set up for each binding experiment. Graded concentrations of either L-T_4 or hormone analogs are present in each tube along with a tracer dose of [^{125}I]-L-T_4 (50 ul of stock solution to give a final reaction concentration of approximately 3×10^{-10} M and total bound counts of appromiximately 100,000), 50 ul of unlabeled L-T_4 (final reaction concentration = 1.25×10^{-8} M) and Buffer I in a final total volume of 0.5 ml. (The final volume includes the volume of TBPA to be added.) The radioactivity of the tubes is counted (Searle Auto-gamma Spectrometer, 76% efficiency) before the

assay is started in order to have an accurate measure of the initial concentration [^{125}I]-L-T_4. To start the assay, 50 ul of the TBPA stock solution is added (final concentration of TBPA = 1 x 10^{-8} M). The tubes are then mixed with a vortex mixer and incubated at 25 ^{o}C. After 30 minutes, the tubes are quickly cooled to 4^{o}C with an ice bath. This low termperature slows the dissociation rate and allows for separation of bound and free counts without significant loss of bound counts in the gel filtration medium. Only fourteen tubes are processed for each run to further insure rapid separation.

The bound and free hormone populations are separated on a small Sephadex G-25 column (bed Volume = 2.0 $\pm$ 0.05 ml, equilibrated in Buffer I at 4 ^{o}C). A sample of incubation mixture (0.4 ml) is layered on these minicolumns with a Pipetman^{R} digital pipette. Incubation Buffer I (0.4ml) is added to start the elution of bound counts. The bound protein fraction is then collected with 0.8 ml of additional buffer and the amount of ^{125}I is determined with gamma spectrometry.

5.4 Results

For Scatchard analysis, the concentration of unlabeled T_4 was varied from 5 to 4000 nM. The data were fitted to

Scatchard's model for two distinguishable and independent sites. (Equation 5.1, Tanford 1961)

$$\frac{\bar{\nu}}{T_f} = \frac{N_1 K_1}{1 + K_1 T_f} + \frac{N_2 K_2}{1 + K_2 T_f} \qquad (5.1)$$

where $\bar{\nu}$ is the average number of L-T_4 molecules bound per prealbumin molecule, T_F is the concentration of free L-T_4 in moles per liter; N_1 and N_2 are the numbers of high- and low-affinity sites, respectively; and K_1 and K_2 are the affinity constants of L-T_4 at the high- and low-affinity sites, respectively.

The dependent variables, $\bar{\nu}$ and T_F, were calculated from experimental data with the following equations:

$$S.A. = \frac{C_T \times 1000}{T_T}$$

$$C_f = C_T - C_B$$

$$T_f = \frac{C_f \times 2000}{S.A.}$$

$$T_B = \frac{C_B \times 2000}{S.A.}$$

$$\bar{\nu} = \frac{T_B}{P_t}$$

where S.A. = specific activity

C_T = total counts

T_T = total concentration of unlabeled L-T_4

T_B = concentration of bound L-T_4

C_B = bound counts

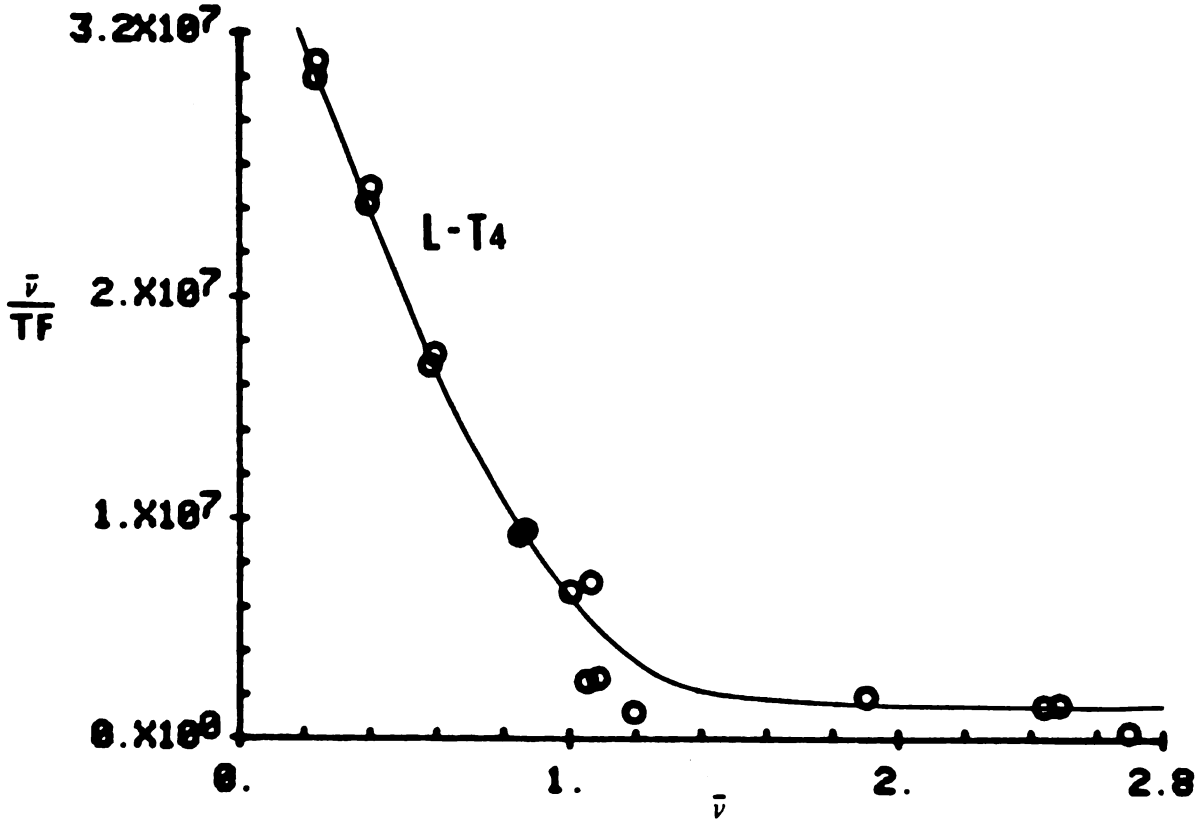
C_F = free counts

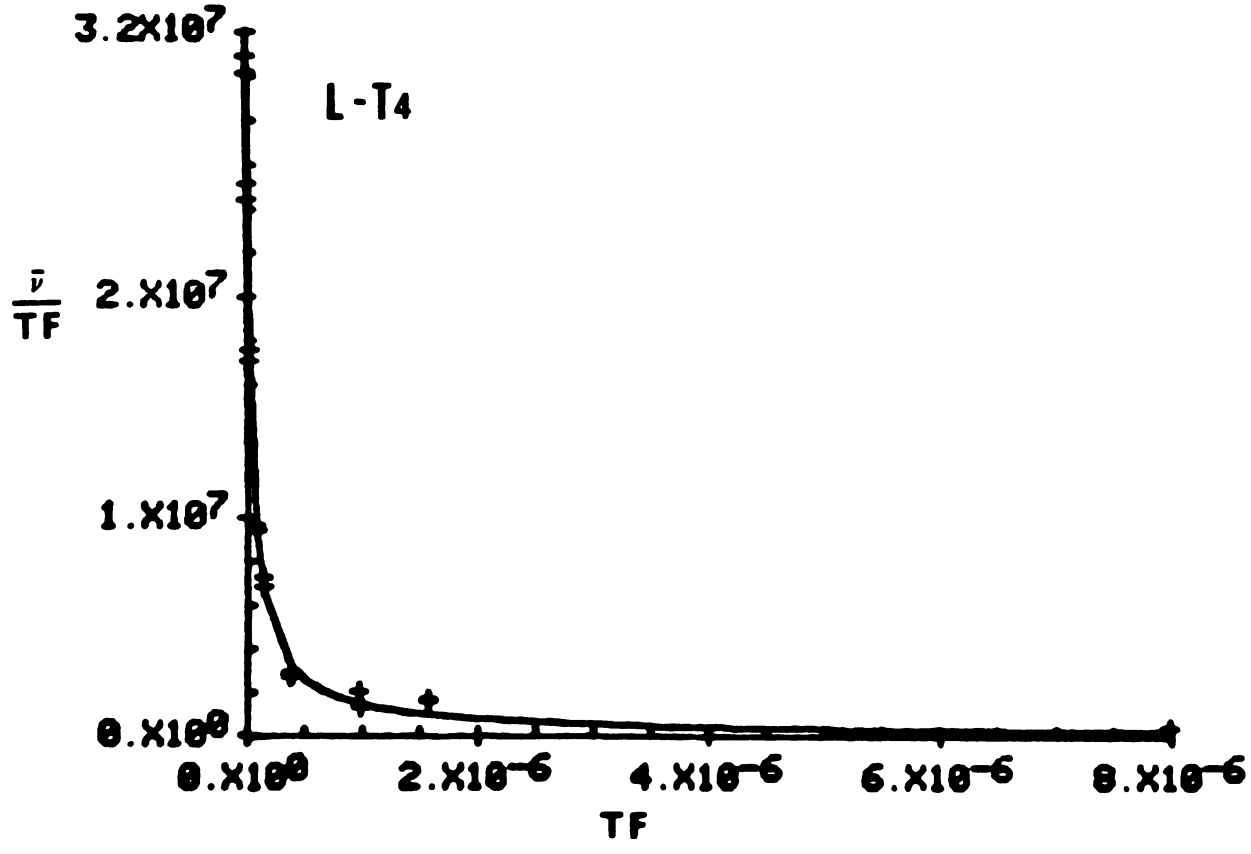
T_F = concentration of free L-T_4

P_t = protein concentration

Curve fitting was done by the Marquardt-Levenberg procedure for non-linear least squares using Public procedure FITFUN on the PROPHET computer system, Chemical/Biological Information Handling Program, National Institutes of Health, Bethesda, MD.. The values of the model parameters as determined in 0.1 M Tris, 0.1 M NaCl, and 1 mM EDTA buffer at pH 8.0 and 25°C. were N_1 = 1 ($\pm$ 0.11), N_2 = 1 ($\pm$ 1.08), K_1 = 3.78 ($\pm$ 0.39) x 10^7 M^{-1}, and K_2 = 1.03 ($\pm$ 1.95) x 10^6 M^{-1}. Figure 5.1 shows the Scatchard plots of the data and the best fit curves. The corresponding affinty constants measured by other investigators were K_1 = 1.0 x 10^8 M^{-1} and K_2 = 9.5 x 10^5 M^{-1} (Ferguson et al. 1975) and K_1 = 1.6 x 10^8 M^{-1} and K_2 = 2.5 x 10^6 M^{-1} (Cheng et al. 1977b) in 0.05 M phosphate, 0.1 M NaCl, and 1 mM EDTA buffer at pH 7.4 and 25°C. In an identical bufferred system at pH 8.0 and 37°C, using equilibrium dialysis, Andrea et al. (1980) obtained values of 3.53 x 10^7 M^{-1} and 2.57 x 10^6 M^{-1} for K_1 and K_2 respectively. Using gel filtration competitive binding assay, Somack (1982) reported values of K_1 6.6 x $10^7 M^{-1}$ and K_2 =

Figure 5.1


3.2X10⁷
2.X10⁷
1.X10⁷
0.X10⁰
$\frac{\bar{\nu}}{TF}$
L-T4
0.
1.
2.
2.8
$\bar{\nu}$


3.2X10⁷
2.X10⁷
1.X10⁷
0.X10⁰
$\frac{\bar{\nu}}{TF}$
L-T4
0.X10⁰
2.X10⁻⁶
4.X10⁻⁶
6.X10⁻⁶
8.X10⁻⁶
TF

$1.0 \times 10^{6} M^{-1}$. The binding affinities of L-T_4 to prealbumin measured in this study are similar to previously recorded values.

The binding affinity of unlabeled analogs to prealbumin is measured in terms of their ability (relative to L-T_4) to displace [^{125}I]-L-T_4 from the high affinity binding sites. A PROPHET computer program (Bolger 1980) is employed to analyzed the binding data according to the method of Oppenheimer et al. (1974) using equation 5.2,

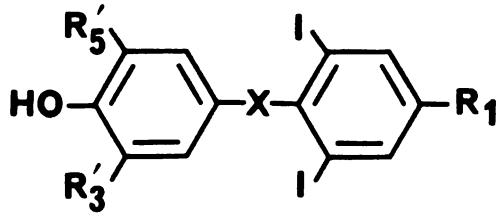
$$\frac{\text{FREE}}{\text{BOUND}} = \frac{1}{(K_{1,T_4})(P_t)} + \frac{\frac{(K_{1,A})}{(K_{1,T_4})}\left[\text{CONC. OF ANALOG}\right]}{P_t} \qquad (5.2)$$

where $K_{1(T4)}$ and $K_{1(A)}$ are the affinity constants for L-T_4 (obtained from a Scatchard plot) and unlabeled analog respectively, and P_t is the concentration of hormone binding sites. Derivation of equation 5.2 for two competitive ligands and one binding site was presented in Chapter Three. The results of binding of the methylene- and ketone-bridged analogs to prealbumin is presented in Table 5.1. Figure 5.2 shows two examples of the actual plots obtained in this study.

5.5 Discussion

The higher binding of the free carboxylic acid analogs to prealbumin is consistent with results obtained by Andrea

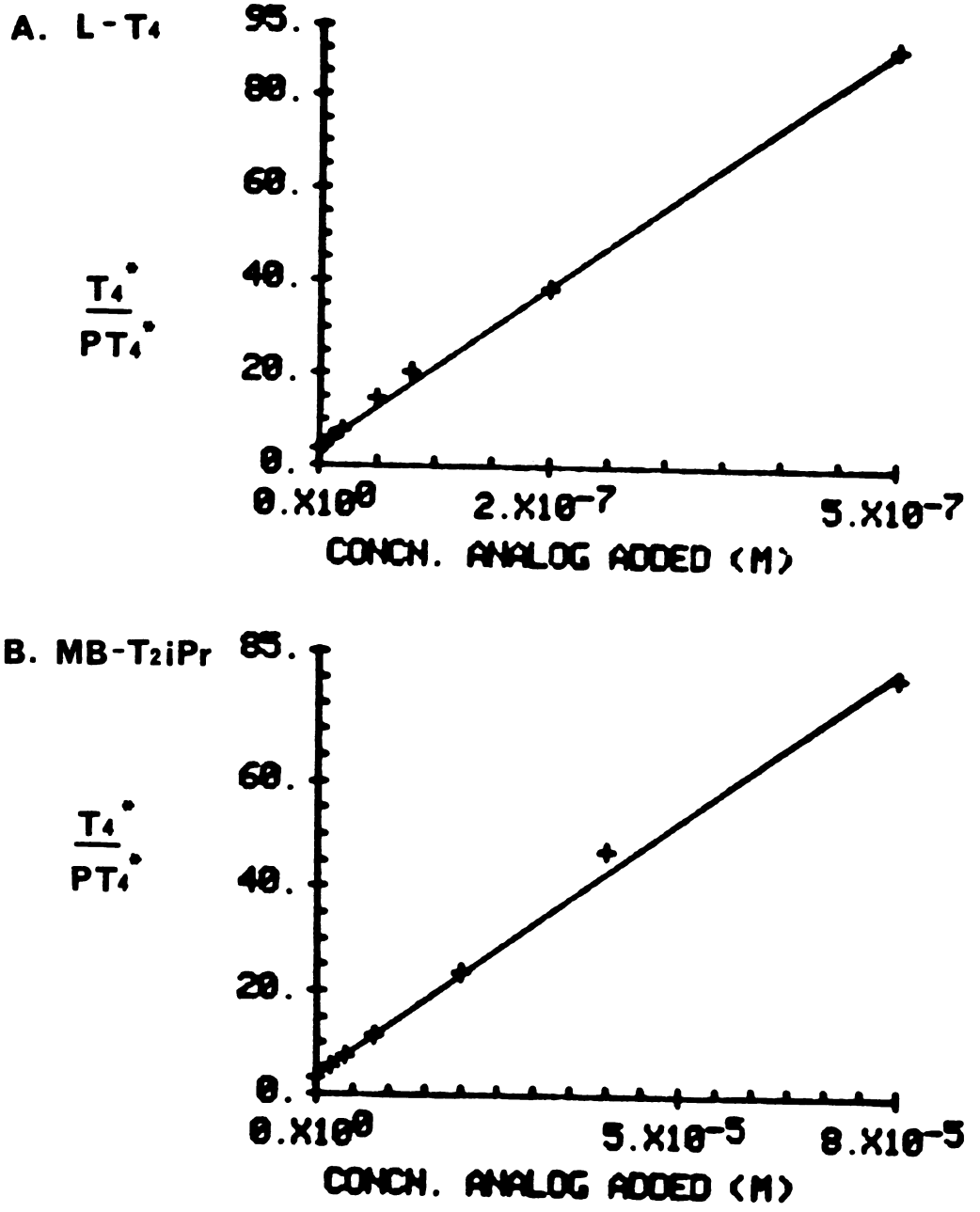
TABLE 5.1 BINDING OF THYROXINE ANALOGS TO PREALBUMIN



	COMPOUND	$R_{3'}$	$R_{5'}$	X	R_1	BINDING AFFINITY
1.	L-T_4	I	I	O	L-ALA	100
2.	MB-T_4	I	I	CH_2	DL-ALA	21.45
3.	L-T_3	I	H	O	L-ALA	3.67 *
4.	MB-T_3	I	H	CH_2	DL-ALA	1.01
5.	L-T_2IPR	IPR	H	O	L-ALA	1.16
6.	MB-T_2IPR	IPR	H	CH_2	DL-ALA	0.64
7.	CO-T_2IPR	IPR	H	CO	DL-ALA	0.55
8.	TETRAC	I	I	O	CH_2COOH	254 *
9.	TRIAC	I	H	O	CH_2COOH	11.9 *
10.	IPRDIAC	IPR	H	O	CH_2COOH	9.08
11.	MB-IPRDIAC	IPR	H	CH_2	CH_2COOH	1.02
12.	CO-IPRDIAC	IPR	H	CO	CH_2COOH	1.85
13.	T_4FORM	I	I	O	COOH	229 *
14.	MB-T_2IPRFORM	IPR	H	CH_2	COOH	0.23
15.	CO-T_2IPRFORM	IPR	H	CO	COOH	0.75

* VALUE FROM ANDREA (1977)

Figure 5.2


A. L-T4
95.
80.
60.
40.
20.
0.
T4*
PT4*
0.X10^0
2.X10^-7
5.X10^-7
CONCN. ANALOG ADDED (M)
B. MB-T2iPr
85.
60.
40.
20.
0.
T4*
PT4*
0.X10^0
5.X10^-5
8.X10^-5
CONCN. ANALOG ADDED (M)

(1977) using equilibrium dialysis. The carboxylate ion interacts with Lys 15 in the entrance of the binding channel of TBPA. Andrea (1977) pointed out that the addition of a positively charged ammonium group to form the alanine moiety would introduce electrostatic repulsion to the carboxylate-lysine ammonium ion pair, and would decrease binding, as observed.

Replacement of the 3' iodine with an isopropyl group decreases binding by a factor of two and can be explained in terms of the effect of 3' substitution on the pK_a of the 4' hydroxyl group. The availabilty of the phenolate ion for hydrogen bonding with the water molecule within the binding channel could account for the difference observed.

The substitution of the ether linkage of T_3 and T_4 with a methylene group decreases binding to prealbumin. Though the ketone-bridged analogs of T_4 and T_3 are not available, comparison of the ketone-bridged isopropyl analog (Table 5.1, #7) to L-T_2iPr shows a similar decrease. The ketone-bridged analogs have comparable and, in the case of a free carboxylic acid side chain, superior binding ability to prealbumin than the corresponding methylene bridged-compounds.

This observed binding behaviour to TBPA differs greatly from the crude nuclear receptor binding, where the methylene bridged analogs exhibit strong binding which exceeds that of the oxygen bridged compounds and the ketone bridged analogs

are relatively poor binders. Both the methylene and carbonyl groups contribute negatively to binding to prealbumin. The magnitude of this effect is small relative to that observed with the crude nuclear receptor. Prealbumin accomodates the bridging groups more easily. The relative ease of dissociation of L-T_4 from TBPA suggests a wider binding channel in TBPA than the crude receptor and would tolerate larger bridging groups. The contribution to binding with these groups, whether positive or negative, would be less apparent due to longer distances and thus weaker interactons between the protein residues and bridging moiety.

The unexpectedly low binding affinity of the methylene-bridged analogs can be explained by a decrease in molecular interaction from slight displacement of the 3' substitutent from its binding pocket when the dihedral angle between the two aromatic rings is more acute. The angle is 114^{o} as compared to 120^{o} for both the carbonyl (Cody et al. 1982) and oxygen bridged analogs (Cody 1974). This decrease in interaction could account for the lower binding affinity of the methylene relative to the ketone bridged compounds. The poor binding to serum proteins implies a higher concentration of free hormones available for interaction with target cells and for metabolic degradation.

CHAPTER SIX

HALOGEN-FREE ANALOGS: 3,5-DIMETHYL-4-(3-ISOPROPYL-4-HYDROXYBENZYL)-DL-PHENYLALANINE AND RELATED COMPOUNDS

6.1 Background

The substituents on the outer ring of 3,5,3'-triiodothyronine (T_3) have been widely varied. The 3'-iodine atom may be replaced with an alkyl group without loss of hormonal ativity. In a homologous series of 3' alkyl analogs maximal activity is expressed by the isopropyl substituent (Bolger 1977). In a variety of *in vivo* test systems, 3,5-diiodo-3'-isopropylthyronine (T_2iPr) is the most potent analog yet prepared, more active than T_3. In a novel clinical application, T_2iPr has been used for hormone replacement therapy in a hypothyroid patient who had become allergic to T_3 or T_4 from natural or synthetic sources (Burger et al. 1980). Strong allergic reaction was also present with ortho-iodophenols, such as an iodine-containing food dye, and this is a structural feature of the phenolic ring of T_3 and T_4. The ortho-isopropyl phenolic group of T_2iPr contributes to strong hormone receptor binding, is a metabolically stable group relative to iodine, and produces long lasting hormone replacement on oral administration, free of allergic reactions.

On the inner ring of T_3, bromine and chlorine atoms

have replaced the 3 and 5 iodine substituents with diminished but appreciable effectiveness. These substituents appear to be involved in both direct receptor binding and the maintenance in both of an active conformation of the diphenyl ether nucleus. Small alkyl groups such as methyl and ethyl can replace the 3 and 5 iodine atoms, but activity is lost with larger substituents such as isopropyl or sec-butyl.

To date the most active combination of substituents other than halogen is present in 3,5-dimethyl-3'-isopropylthyronine (DIMIT). This halogen-free analog is about 20% as potent as T_4 in a variety of tests in vivo. Because DIMIT shows very weak binding to the plasma transport proteins TBG and TBPA, good hormone receptor binding affinity, and resistance to fetal deiodinase enzymes, it exerts a selective hormonal effect on the fetus when administered to pregnant experimental animals (Comite et al. 1978). DIMIT produces dramatic changes in the subcellular structures and enzyme activities of cells of fetal organs (Kriz 1978) and increases the production of lung surfactant in the fetal rabbit lung when administered in the maternal circulation (Ballard et al. 1980). DIMIT displays prolonged thyroid hormonal effects on oral administration to the human male (Tamagna et al. 1979). Thus, DIMIT, or a more potent DIMIT-like analog, is a candidate for use in fetal hypothyroidism and in the prevention of respiratory distress syndrome in premature birth.

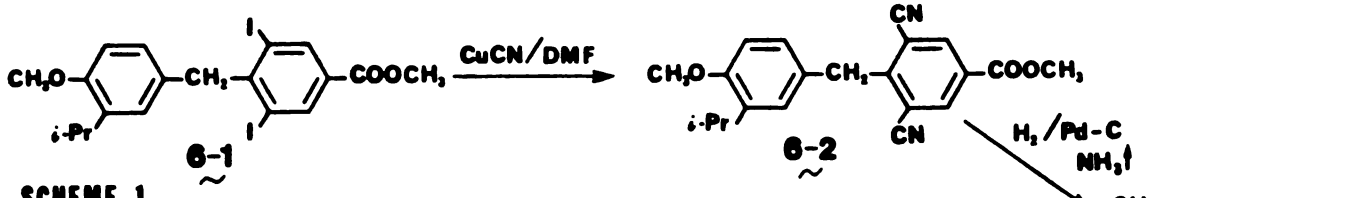
6.2 Synthetic Rationale

As presented in early chapters, the ether bridge of the thyronine structure can be replaced by the CH_2 group. Molecular gerometry is retained, as is hormonal activity. The methylene-bridged analogs synthesized in this study exceed their corresponding oxygen-bridged compounds in activity both *in vivo* and *in vitro*. 3,5-Diiodo-4-(3-isopropyl-4-hydroxybenzyl)-DL-phenylalanine (MBT_2iPr) is 2.5 times more active than T_2iPr in the antigoiter assay and binds 15 times stronger to the nuclear receptor protein. From our observations, we concluded that replacement of the oxygen bridge by the more lipophilic, more metabolically stable CH_2 group contributes to increasing activity *in vivo* and *in vitro* and decreasing binding to transport proteins. The unusual augmentation in activity by the presence of a CH_2 bridge and a 3' isopropyl group suggested a possible modification of the DIMIT molecule to increase its thyromimetic activity.

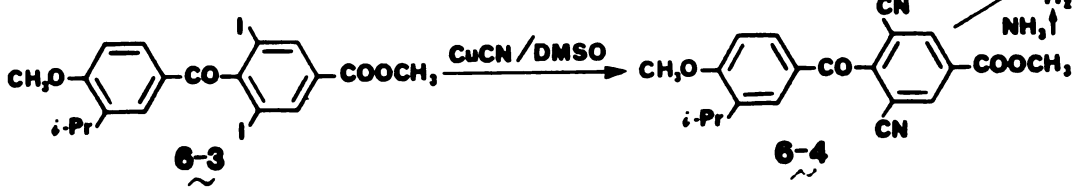
In this chapter, we report the syntheses and activities of the methylene-bridged DIMIT analog (3,5-dimethyl-4-(3-isopropyl-4-hydroxybenzyl)-DL-phenylalanine) and two other related compounds. We expect the poor affinity to transport protein and the added lipophilicity of these compounds to be advantageous in passage through the placenta. The stability and contribution to activity of the 3' isopropyl group as compared to the 3' iodine has been discussed.

6.3 Synthetic Scheme

To synthesize the methylene-bridged DIMIT (MB-DIMIT) we planned two independent routes. The first method is by replacement of the 3, 5 iodines in methyl 3,5-diiodo-4-(3-isopropyl-4-methoxybenzyl)benzoate with methyl groups and subsequent modification of the methyl benzoate side chain to give the formic acid, the alanyl, and the acetic acid analogs. Because the synthesis of our desired starting material (6-1) proceeded with low yield, we experimented with another method. Methyl 3,5-diiodo-4-(3-isopropyl-4-methoxybenzoyl)benzoate (6-3) was obtained in fairly good yield in our syntheses and using it as the starting material, we hope to replace the iodines with cyano groups followed by reduction to the dimethyl compound. The drastic condition employed in the reduction of C≡N to CH_3 could reduce the carbonyl bridge to a methylene moiety.



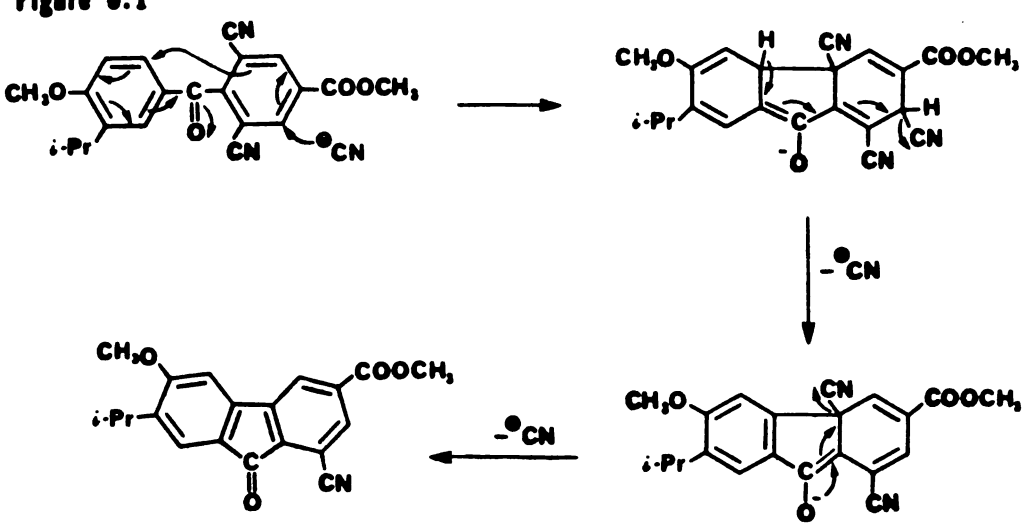
SCHEME 1



SCHEME 2

Nucleophilic substitution using cuprous cyanide of 6~3 in refluxing dimethylformamide or pyridine failed to produce the dicyano compound 6~4. The product obtained from both reactions was crystalline, intensely yellow and melts at 216° - 219°C. Its molecular weight, determined by high resolution mass spectrometry, is 335.115480 mass units, with the elemental composition assigned as $C_{20}H_{17}O_4N_1$. The NMR spectrum shows an aromatic absorption pattern characteristic of a fluorenone structure. We suspected that the nucleophilic displacement of the iodo groups on an aromatic ring activated by an ortho carbonyl group proceeds rapidly. Attack by another cyanide ion at the 2 position which is flanked by two electron withdrawing groups, the methoxycarboxyl and the cyano, led to the formation of a third ring as shown in figure 6.1.

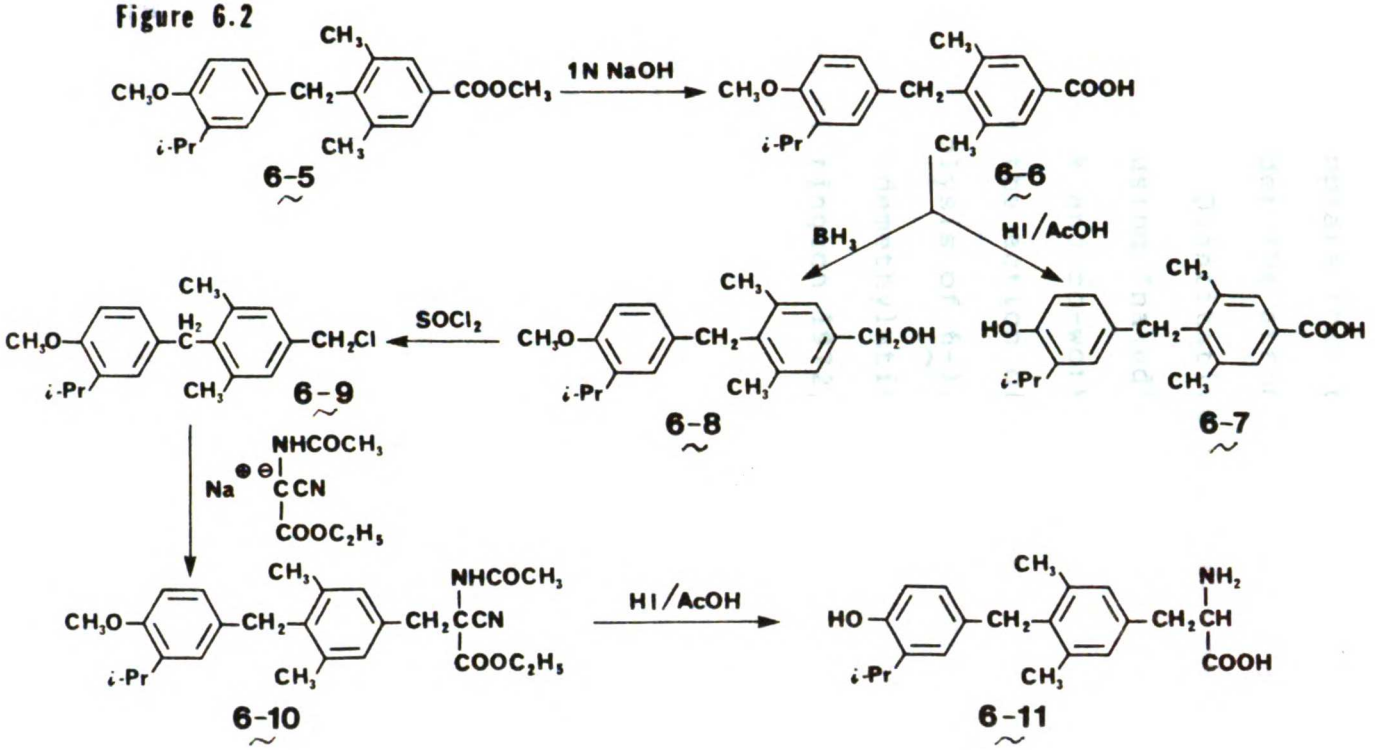
Figure 6.1



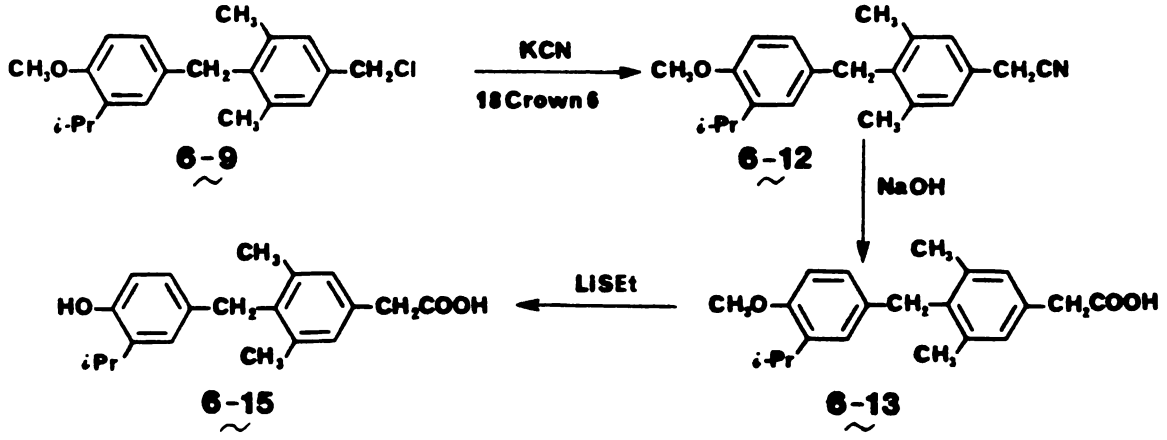
To avoid cyclization, conditions similar to those of Friedman et al. (1960, 1961) were used. The diiodo compound 6-3 was mixed with a slurry of CuCN and DMSO, heated to reflux for a very short time (30 min.) and the copper complex degraded in acidic ferric chloride solution. The products obtained contained mostly the dicyano compound 6-4 and a small amount of the cyclized product as discerned by thin layer chromatography. Reduction in refluxing p-cymene was effective in converting the 3, 5- cyano groups to methyl groups as evidenced by the titration of the ammonia formed by 1 N HCl. However, the bridging CO group was reduced to probably very unstable free radical intermediates. We were unable to isolate any stable product from the reaction. We abandoned scheme 2 after several trials and pursued the synthesis using scheme 1.

Displacement of the iodo groups of 6-1 was effected with cuprous cyanide in refluxing DMF (Friedman and Shechter 1961). Hydrolytic reduction of 6-2 with hydrogen using 10% palladium on carbon as catalyst in refluxing p-cymene (176°C - 178°C) provided the 3,5-dimethyl diphenylmethane 6-5 with evolution of ammonia (Kindler and Luhrs 1967). Careful purification and drying of the p-cymene (Block and Coy 1972) and anhydrous conditions were found to be extremely critical for this reaction. The slightest moisture or impurities in the p-cymene very effectively poisoned the catalyst. Conversion of the methyl benzoate side chain to DL-alanyl system is shown in Figure 6.2.

Figure 6.2



Conversion of the benzyl chloride to the benzyl nitrile using KCN in DMSO proceeded with very low yield and subsequent HI/AcOH hydrolysis failed to provide the acetic acid analog. It appears that the phenolic product obtained decomposed under the strong acidic conditions employed for the reaction. Quantitative yield of the benzyl nitrile 6-12 was obtained using "naked" cyanide ion according to the method of Cook and co-workers (1974). The "naked" anion was generated by the action of 18-crown-6 on KCN in acetonitrile. Hydrolysis of 6-12 was performed in basic conditions followed by O-demethylation using lithium thioethoxide (Feutrill and Mirrington 1972, 1970; Kelly et al. 1977).



6.4 Results and Discussion

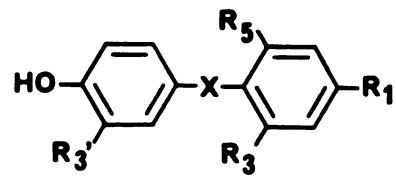
The dimethyl diphenylmethane analogs 6-7, 6-11 and 6-14 were tested for their binding affinities to nuclear receptor proteins and Thyroxine Binding Prealbumin (TBPA) using the procedures described in Chapter Three and Five. The results

are presented in Table 6.1 and 6.2.

The affinity of 3,5-dimethyl-4-(3-isopropyl-4-hydroxybenzyl)-DL-phenylalanine, 6~11 (DL-MB-DIMIT), to nuclear receptor is ten times highter than L-DIMIT. Adjusting for the differences in binding to the receptor for stereoisomers, L-MB-DIMIT would associate approximately twenty times better than L-DIMIT. Association affinity of the analogs *in vitro* to the receptor protein reflects the relative thyromimetic activity *in vivo* without the complication of absorption, distribution, metabolism and excretion.

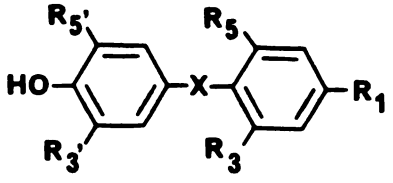
Binding to plasma transport proteins determines the abundance of free hormone in the whole animal. Potency of hormone analogs is correlated with the amount of free hormones available in the plasma, which implies that weak binding to transport proteins contributes to thyromimetic activity. DL-MB-DIMIT, 6~11, binds TBPA with the same affinity as L-DIMIT. The very weak binding to the plasma transport proteins, the good hormone receptor binding affinity, the transplacental ability, and the resistance to fetal deiodinase enzymes are factors which distinguished DIMIT from other analogs as the candidate for treatment of fetal hypothyroidism and prevention of respiratory distress syndrome in premature birth. The MB-DIMIT analog synthesized and tested in this study has all the characteristics of DIMIT and shows superior receptor binding ability. The more lipophilic CH_2 bridge should facilitate the transplacental

TABLE 6.1 NUCLEAR RECEPTOR BINDING ASSAY



COMPOUND	X	R_1	$R_3 = R_5$	$R_{3'}$	K_A	% L-T_3
L-T_3	O	L-ALA	I	I	$1.63 \pm 0.23 \times 10^9$	100
MBIPR(ME)$_2$ALA	CH_2	DL-ALA	CH_3	IPR	$1.08 \pm 0.12 \times 10^7$	0.663
MBIPR(ME)$_2$AC	CH_2	CH_2COOH	CH_3	IPR	$1.40 \pm 0.62 \times 10^7$	0.962
L-DIMIT	O	L-ALA	CH_3	IPR	$1.03 \pm 0.35 \times 10^6$	0.063
MBIPR(ME)$_2$FORM	CH_2	COOH	CH_3	IPR	$1.03 \pm 0.34 \times 10^6$	0.063

TABLE 6.2 THYROXINE BINDING PREALBUMIN BINDING ASSAY



COMPOUND	X	R_1	$R_3 = R_5$	$R_{3'}$	$R_{5'}$	K_A	% L-T_4
L-T_4	O	L-ALA	I	I	I	$4.69 \pm 0.35 \times 10^7$	100
MBIPR(ME)$_2$AC	CH_2	CH_2COOH	CH_3	IPR	H	$3.42 \pm 0.44 \times 10^4$	0.073
MBIPR(ME)$_2$ALA	CH_2	DL-ALA	CH_3	IPR	H	$3.16 \pm 0.37 \times 10^4$	0.067
L-DIMIT	O	L-ALA	CH_3	IPR	H	$3.80 \pm 1.06 \times 10^4$	0.081
MBIPR(ME)$_2$FORM	CH_2	COOH	CH_3	IPR	H	$2.57 \pm 0.01 \times 10^4$	0.056

process. We are hopeful that the methylene bridged DIMIT will prove to have better therapeutic utility due to its increased potency and lipophilicity.

6.5 Experimental Section

Melting points, obtained on a Thomas-Hoover Uni-Melt apparatus, are uncorrected. Nuclear magnetic resonance (Nmr) spectra were recorded on a Varian FT-80 spectrometer. Nmr chemical shift values are expressed in δ units (ppm) relative to either TMS or DDS (2,2-dimethyl-2-silapentane-5-sulfonate) internal standards. Electron impact mass spectra (EIMS) were performed on a Kratos MS-A25S spectrometer operated in the electron impact mode. Elemental analyses were performed by the Microanalytical Laboratory, University of California, Berkeley, California.

Methyl 3,5-Dicyano-4-(3-isopropyl-4-methoxybenzyl)-benzoate (6-2). The preparation of nitriles from aryl halides using dimethylforamide as solvent and improved isolation techniques were reported by Friedman and Schechter (1961). With few modifications, the diiodo compound 6-1 (2.64 gm, 4.8 mmoles) was mixed with 1.02 gm (11.4 mmoles) of cuprous cyanide in 9.4 ml of DMF. Heating under reflux for 4 hours afforded a dark solution which was poured while hot into an aqueous solution of $FeCl_3.6H_2O$ (3.74 gm) in 47.6 ml of H_2O and 10.2 ml of conc. HCl. The copper complex was degraded in this medium maintained at 60° - 70°C with stirring for 20 minutes. Separation of the solid, liquid phase

afforded a dark brown solid. Crystallization from CH_2Cl_2 - MeOH gave 963.5 mg (57.7%) of 6~2. m.p. 150° - 152°C. Nmr ($CDCl_3$) δ 1.14(d, 6 H, iPr-CH_3); 3.24(m, 1 H, iPr-CH); 3.78(s, 3 H, Ar-OCH_3); 3.97(s, 3 H, Ar-$COOCH_3$); 4.40(s, 2 H, Ar-CH_2-Ar); 6.7 - 7.32(m, 3 H, Ar-2',5',6'-H); 8.46(s, 2 H, Ar-2,6-H). Calculated for $C_{21}H_{20}N_2O_3.H_2O$: C, 68.85; H, 6.01; N, 7.65. Found: C, 68.82; H, 5.88; N, 7.30. EIMS: m/e 348 (M+).

Methyl 3,5-Dimethyl-4-(3-isopropyl-4-methoxybenzyl) Benzoate (6~5). The hydrogenation was carried out under the conditions described by Block and Coy (1972) and used by Jorgensen et al. (1974). p-Cymene (99+%, Aldrich Chemical Company, Inc.) was purified exactly as described by Block and Coy (1972) and was stored under nitrogen. A three-necked flask (100 ml) was fitted with a thermometer, gas dispersion tube, and reflux condenser, the tip of which led to a second dispersion tube, dipping beneath the surface of water (91 ml) containing methyl-red indicator. 3,5-Dicyano-4-(3-isopropyl-4-methoxybenzyl)benzoate, 6~2 (898.0 mg, 2.58 mmoles), was dissolved in 46 ml of freshly purified p-cymene containing 2.4 gm of 10% Pd/C, the reaction system having been flushed with nitrogen. Hydrogen was bubbled through from a lecture bottle while the temperature was maintained at 168° - 171°C. The ammonia evolved was absorbed and titrated against 0.1 N HCl. The reaction became slower near its end and virtually ceased when 98% of the theoretical amount of acid had been neutralized (1.75

hours). After allowing to cool to 60°C, the catalyst was removed by filtration through celite and washed copiously with $CHCl_3$. The volatile solvents were removed in vacuo and the p-cymene vacuum distilled to give a brown oil. Purification using column chromatography on silica gel matrix and eluting with CHl_3 gave 79 mg (89%) of 6-5 in the form of an oil. Nmr ($CDCl_3$) δ 1.09, 1.17(d, 6 H, iPr-CH_3); 2.28(s, 6 H, Ar-CH_3); 3.17(m, 1 H, iPr-CH); 3.70(s, 3 H, Ar-$COOCH_3$); 3.86(s, 3 H, Ar-OCH_3); 4.15(s, 2 H, Ar-CH_2-Ar); 6.65 - 7.28(m, 3 H, Ar-2',5',6'-H); 7.92(s, 2 H, Ar-2,6-H).

3,5-Dimethyl-4-(3-isopropyl-4-methoxybenzyl)benzoic Acid (6-6). The methyl ester 6-5 (1.19 gm, 3.65 mmoles) was hydrolyzed in 55 ml of 1 N NaOH. After reflux for 8 hours, conc. HCl was added dropwise with cooling to bring the pH to ~4 which caused a precipitate to form. The mixture was chilled overnight at 0°C, filtered, washed with water and vacuum dried to give 1.03 gm (90.24%) of a white powder, m.p. 206° - 209°C. Nmr ($CDCl_3$) δ 1.09(d, 6 H, iPr-CH_3); 2.28(s, 6 H, Ar-CH_3); 3.73(s, 3 H, Ar-OCH_3); 3.97(s, 2 H, Ar-CH_2-Ar); 6.60 - 6.86(3, 3 H, Ar-2',5',6'-H); 7.73(s, 2 H, Ar-2,-H). Calculated for $C_{20}H_{24}O_3$: C, 76.92; H, 7.69. Found: C, 76.84; H, 7.96. EIMS: m/e 312 (M+).

3,5-Dimethyl-4-(3-isopropyl-4-hydroxybenzyl)benzoic Acid (6-7). Hydrolysis and O-demethylation of methyl 3,5-dimethyl-4-(3-isopropyl-4-methoxybenzyl)benzoate, 6-5, was carried out simultaneously in hydriodic acid. Thus, 871.5

mg (6.67 mmoles) of 6-5 was heated under reflux in 7 ml of glacial acetic acid and 14 ml of HI for 8 hours. Volatile materials were evaporated under vacuo and water was added to the remaining solution. The solid obtained was filtered and washed well with cold water to give 556.6 mg (70.04%) of a white solid, 6-7. m.p. 159° - 160°C. Nmr ($CDCl_3$) δ 1.14(d, 6 H, iPr-CH_3); 2.28(s, 6 H, Ar-CH_3); 3.10(m, 1 H, iPr-CH); 3.96(s, 2 H, Ar-CH_2-Ar); 6.52 - 6.83(3, 3 H, Ar-2',5',6'-H); 7.73(s, 2 H, Ar-2,6-H). Calculated for $C_{19}H_{22}O_3$: C, 76.51; H, 7.38. Found: C, 76.55; H, 7.40. EIMS: m/e 298 (M+).

3,5-Dimethyl-4-(3-isopropyl-4-methoxybenzyl)benzyl Alcohol (6-8). A sample of dried 6-6 (695.7 mg, 2.23 mmoles) was dissolved in 100.5 ml of freshly distilled THF. Under a nitrogen atmosphere, 23 ml of 1 M diborane in THF was added and was brought to reflux for 7 hours. Methanol was added cautiously to the cooled reaction solution to destroy the excess diborane. Evaporation of all solvents left a brown oil which was taken into $CHCl_3$ and extracted three times with water. After drying over $CaCl_2$, removal of all solvents gave 658 mg of a crude oil, 6-8. This material, nearly homogenous to tlc (EtOAc/$CHCl_3$ = 1:3), was dried and used without further purification. Nmr ($CDCl_3$) δ 1.03(d, 6 H, iPr-CH_3); 2.12(s, 6 H, Ar-CH_3); 3.57(s, 3 H, Ar-OCH_3); 3.81(s, 2 H, Ar-CH_2-Ar); 4.40(s, 2 H, Ar-CH_2OH); 6.47 - 6.95(4, 5 H, Ar-H). EIMS: m/e 298 (M+).

3,5-Dimethyl-4-(3-isopropyl-4-methoxybenzyl)benzyl Chloride (6~9). The crude oil 6~8 (658 mg), in 39.6 ml of benzene, was treated with 1.5 ml of thionyl chloride followed by a few drops of DMF. The reaction was stirred and kept warm in a 40°C water bath for 4 hours. In vacuo removal of solvents gave a brown oil which was chromatographed on a silica gel column and eluted with $CHCl_3$. The yellow oil (560 mg, 79.34% from 6~6) obtained solidified on freezing. Nmr ($CDCl_3$) δ 0.99(d, 6 H, iPr-CH_3); 2.08(s, 6 H, Ar-CH_3); 3.15(m, 1 H, iPr-CH); 3.54(s, 3 H, Ar-OCH_3); 3.80(s, 2 H, Ar-CH_2-Ar); 4.31(s, 2 H, Ar-CH_2Cl); 6.48 - 7.15(4, 5 H, Ar-H). EIMS: m/e 317(M+).

3,5-Dimethyl-4-(3-isopropyl-4-hydroxybenzyl)phenyl-DL-alanine (6~11). A pinch of NaI was added to a solution of sodium ethoxide (150 mg, 3.75 mmoles of Na in 10 ml of absolute ethanol). The solution was brought to reflux and 1.10 gm (6.46 mmoles) of ethyl acetamidocyanoacetate was added. After 20 minutes reflux, 716 mg (2.26 mmoles) of the benzyl chloride 6~9 was added and heated under reflux for an additional 3 hours. The reaction mixture was poured onto a mixture of ice and water and the gummy solid which formed was extracted into $CHCl_3$, dried over Na_2SO_4 and chromatographed on a silica gel column. Elution with $CHCl_3$ gave the unreacted, starting material 6~9, and with a 1:1 mixture of EtOAc/$CHCl_3$, 6~10 (403.3 mg) was obtained. Nmr ($CDcl_3$) δ 1.02, 1.10(d, 6 H, iPr-CH_3); 1.18(t, 3 H, $COOCH_2\underline{CH_3}$); 2.14(s, 6 H, Ar-CH_3); 3.16(m, 1 H, iPr-CH); 3.64(s, 3 H,

Ar-OCH_3); 3.82(s, 2 H, Ar-CH_2-Ar); 4.15(q, 2 H, $COO\underline{CH_2}CH_3$); 6.27 - 6.77(3, 5 H, Ar-H).

Removal of the protecting groups of 6-10 was carried on in 5 ml of HBr and 5 ml of glacial acetic acid. After reflux for 1.5 hours, the reaction mixture was cooled to room temperature and poured into 10 ml of ice. 2 N NH_4OH solution was added dropwise to bring the pH to 5 which caused a solid to form. The mixture was refrigerated, filtered and washed liberally with water. An analytical sample (638.0 mg, 82.8% from 6-9) was prepared by preparative thin layer chromatography (silica gel plate, Whatman PLK5F; solvent system, $CHCl_3$/MeOH/NH_4OH = 20:10:1), m.p. 191°C (dec.). Nmr (MeOH-d_6) δ 1.15, 1.24(d, 6 H, iPr-CH_3); 2.28(s, 6 H, Ar-CH_3); 3.05(m, 1 H, iPr-CH_3); 3.94(s, 2 H, Ar-CH_2-Ar); 6.56 - 7.01(3, 5 H, Ar-H). Calculated for $C_{21}H_{27}N_1O_3.0.5H_2O$: C, 72.00; H, 8.00; N, 4.00. Found: C, 72.39; H, 7.99; N, 3.83. EIMS: m/e 341 (M+).

3,5-Dimethyl-4-(3-isopropyl-4-methoxybenzyl)benzyl Cyanide (6-12). The benzyl chloride 6-9 was converted to the benzyl cyanide using the method described by Cook et al. (1974) to generate the "naked" cyanide anion. A sample of 6-9 (359 mg, 1.13 mmoles) was mixed with a solution of 24.40 mg (0.092 mmoles) of 18-crown-6 (Aldrich Chemical Company, Inc.) in 0.63 ml of acetonitrile and poured over 147.17 mg (2.26 mmoles) of KCN. Heating and stirring for 1.5 hours resulted in a brown mixture. Water and chloroform (25 ml

each) were added to bring all insoluble materials into solution. The two phases were separated and the aqueous layer was washed twice with 15 ml of $CHCl_3$. The organic layers were combined and dried over $CaCl_2$. Evaporation to dryness gave a brown oil which was separated on a silica gel column and eluted with $CHCl_3$. A yellow oil (282 mg, 81.29%) was obtained. This oil solidified on freezing and was homogenous to tlc ($CHCl_3$). Nmr ($CDCl_3$) δ 1.03(d, 6 H, iPr-CH_3); 2.15(s, 6 H, Ar-CH_3); 3.18(m, 1 H, iPr-CH); 3.55(s, 2 H, Ar-CH_2CN); 3.66(s, 3 H, Ar-OCH_3); 3.87(s, 2 H, Ar-CH_2-Ar); 6.56 - 6.91(4, 5 H, Ar-H). EIMS: m/e 307 (M+).

3,5-Dimethyl-4-(3-isopropyl-4-methoxybenzyl)-phenylacetic Acid (6-13). The benzyl cyanide 6-12 (291.2 mg, 0.95 mmole) was heated under reflux in 2 ml of 40% NaOH and 1 ml of MeOH for 24 hours. After removal of volatile solvent under vacuo, the reaction mixture was acidified with 6 N HCl. The resulting precipitate was filtered, washed with water and dried to give 272.5 mg (88.0%) of 6-13. This product was used without further purification. Nmr ($CDCl_3$) δ 0.99, 1.07(d, 6 H, iPr-CH_3); 1.95(s, 6 H, Ar-CH_3); 3.21(s, 2 H, Ar-$\underline{CH_2}COOH$); 3.54(s, 3 H, Ar-OCH_3); 3.68(s, 2 H, Ar-CH_2-Ar); 6.40 - 6.82(3, 5 H, Ar-H).

3,5-Dimethyl-4-(3-isopropyl-4-hyroxybenzyl)phenylacetic Acid (6-14). A sample of 6-13 (322.2 mg, 0.99 mmole) was dissolved in 3 ml of hexamethylphosphoric triamide and 644.6 mg (9.49 mmoles) of lithium thioethoxide was added. After

heating for 3 hours at 120°C, and stirring overnight at room temperature, water and ethyl ether (25 ml each) were added to dissolve all solids and the two layers were separated. The organic phase was washed with 15 ml of 1 N HCl twice to destroy excess lithium thioethoxide and extracted three times with 15 ml of water. Drying over anhydrous Na_2SO_4 and evaporation of solvent gave a brown oil which was chromatographed on silica gel and eluted with a 1:1 mixture of EtOAc/$CHCl_3$. Evaporation of the fracton collected gave 258 mg (83.6%) of a glass. Nmr ($CDCl_3$) δ 1.15, 1.23(d, 6 H, iPr-CH_3); 2.20(s, 6 H, Ar-CH_3); 3.13(m, 1 H, iPr-CH); 3.55(s, 2 H, Ar-$\underline{CH}_2$COOH); 3.92(s, 2 H, Ar-CH_2-Ar); 6.52 - 6.95(2, 5 H, Ar-H). EIMS: m/e 312 (M+).

CHAPTER SEVEN

CONCLUSION

In this study, we report the syntheses of nine new thyroxine analogs. These analogs are designed to preserve the diphenyl ether conformation of the hormone while varying the bridging element. Both the methylene and ketone bridged compounds are biologically active indicating that the stereochemical configuration is the important factor for receptor binding and therefore the initiation of hormonal effect.

Following the preliminary results obtained by Tripp and co-workers (1973) on their methylene bridged T_3 analog, we synthesized a series of methylene bridged 3'-isopropyl analogs for the dual purposes of: (1) incorporating all known structural features contributing to maximal activity into one compound and (2) obtaining a biologically more potent analog than T_2iPr. 3,5-Diiodo- 4-(3-isopropyl-4-hydroxybenzyl)phenylacetic acid was designed based on previous structure activity studies and exhibits maximal in vitro receptor binding activity. Its receptor affinity far exceeded our initial estimate and indicates that the more acute dihedral angle in combination with a 3' isopropyl group have a potentiating effect on hormonal activity. 3,5-Diiodo-4-(3-isopropyl-4- hydroxybenzyl)phenylalanine, the methylene bridged T_2iPr, is superior in receptor binding

and in *in vivo* activity to T_2iPr. These two analogs, synthesized in this work, possess the highest *in vivo* and *in vitro* activity of all the known thyroid hormone analogs including native hormones T_3 and T_4.

In spite of their high binding to nuclear receptor protein, the methylene bridged analogs bind only weakly to transport proteins. Their binding affinities to TBPA are similar to those of the corresponding oxygen compounds, and in some cases, lower. This result offers a solution to the search for a more potent DIMIT compound. By incorporation of a methylene bridge into DIMIT, we obtained an analog with ten times the affinity of DIMIT for the receptor and yet just as weak an association with TBPA. It is to be hoped that, with more pharmacological studies, MB-DIMIT can be shown to be more useful than DIMIT in clinical applications.

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