THYROID HORMONE ANALOGS:

THE ROLE OF HALOGEN IN HORMONAL ACTIVITY

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ABSTRACT

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Ph. D. Dissertation

Halogen-free derivatives of 3,5-dimethyl-L-thyronine substituted in the 3'- or 3',5'- positions are shown to possess the following activities in the rat antigoiter assay (L-thyroxine = 100): 3'-methyl (3%), 3',5'-dimethyl (2%), 3'-isopropyl (18%). The methylene-bridged analog of 3,5,3'-triiodo-DL-thyronine was three times as active as L-thyroxine. Activity of these compounds in other assay systems (tadpole metamorphosis, jellyfish strobilation, cell-culture uptake of cycloleucine and in vitro nuclear displacement) all show parallel activity. 3,5-Dimethyl-3'-isopropyl-L-thyronine was unambiguously synthesized and comparison of its pmr, ir, uv and mass spectral data show the previously reported DL-compound to be a biphenyl isomer. Pmr and mass spectral evidence show that the compound previously reported as 3,5,3',5'-tetramethyl-DL-thyronine was an isomer of that structure. Molecular orbital calculations on thyroxine analogs indicate that the minimum energy conformation for 3,5-disubstituted compounds is an important structural feature determining biological activity. The proximal conformation of T_3 is predicted to be very slightly (0.2 kcal/mol) more stable than the distal. The representation of the valence electrons of Cl, Br and I with 2s- and 2p-like atomic orbitals appears to give a reasonably satisfactory

representation of the electronic structure of these halogens. The alkyl thyronines show that halogen is not an essential feature for thyromimetic activity. The high activity of the methylene-bridged analog invalidates the Niemann quinoid hypothesis of thyroid hormone action. The non-polar ring substituents appear to stabilize a semi-rigid three dimensional structure which is responsible for initiating the thyroid hormonal response.

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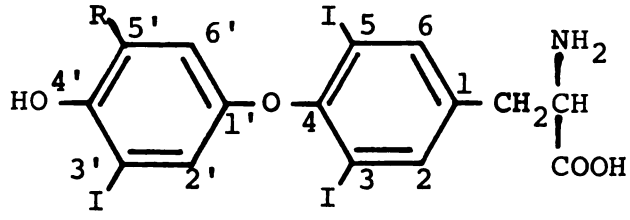
CHAPTER ONE: INTRODUCTION

The thyroid hormones, thyroxine (T_4; 1-1) and 3,5,3'-tri-



1-1, R = I

1-2, R = H

iodothyronine (T_3; 1-2), are two closely related iodinated α-amino acids. They are produced by the thyroid gland and are released by that gland into the general circulation. This synthesis and release is under the control of a well-defined, physiological negative feedback system operating between circulating levels of hormones, the hypothalmus and pituitary glands and the thyroid gland. The major roles of the hormones appear to be their necessity in regulating the body's production and utilization of energy and their requirement for maintaining normal growth and maturation in the young[1].

The hormones possess relatively simple structures and this has given impetus to the synthesis and testing of many analogs in an attempt to define those features essential for thyroxine-like activity. Selenkow and Asper in 1955 reviewed the activity of 152 structural analogs[2]. But these early studies suffered many drawbacks, chief of which was their lack of a systematic approach. For example, some analogs may have had more than one chemical function altered, preventing any correlation of activity to a particular structural change. Also, the mode of testing may have been inadequate. The decade following that time saw increased systemization in the changes in structural analogs and a general agreement as to the assays by which thyromimetic activity was to be measured. As a consequence, in the past decade, much information regarding the molecular components required for activity has been provided. Jorgensen[3] has summarized these requirements as follows:

(1) a diaryl ether or sulfide nucleus

(2) an aliphatic side chain containing a carboxyl amino or similar polar group or metabolic precursor in the 1-position; an L-alanyl group giving greatest activity

(3) a phenolic group, amino group or a group capable of being metabolically transformed into a phenolic group at the 4'-position

(4) halogen or methyl substituents in the 3,5-positions, and

(5) for maximal activity a variety of halogen atoms or aryl groups in the 3'- or 3',5'- positions which, in the case of 3'-substitution at least, follow a Hansch-

type relationship.

If one believes in a rational approach to drug design such as that developed by Hansch, one would predict that a coupling of elements (4) and (5) above, i.e. incorporation of alkyl groups into both the phenol- and alanine- bearing rings would produce a hormonally active, halogen-free derivative. When this was first attempted[5], the frustrating result was inactivity. Following these results, interest in thyroxine analogs began to decline. This was the situation at the time this study began.

An unambiguous approach to the synthesis of 3,5-dimethyl thyronines[6] and a preliminary investigation[7] showing that perhaps halogens were not required for activity opened new paths of investigation. Chapter Two recounts this episode and shows how previous synthesis were erroneous.

The quantum mechanical study of Chapter Three was started to determine the steric effect of the halogens and methyl groups and to see how closely the conformational energy maps of thyroxine analogs paralleled their biological activity. The methylene bridged analog was included since it was shown to be equipotent to T_3 in biological activity.

In addition a number of new biological assay procedures were introduced in the course of this study. We submitted the alkylated thyronines and other analogs to some of the assays. Chapter Four describes the results and assesses how closely they compare with the standard rat anti-goiter and tadpole metamorphosis assays.

Chapter Five describes some preliminary studies into photochemical and nitration reactions on the iodinated thyronine

nucleus. The analogs of triiodothyronine produced in these reactions provide additional useful information on the electronic and stereochemical requirements for hormonal activity.

As these studies conclude, information regarding the thyroid hormones and their analogs seems to be burgeoning. Further investigations into nuclear binding sites and cell-culture systems showing responses paralleling that of in vivo tests give evidence that we are on the verge of elucidating at least a portions of the events characterizing the molecular basis of action of the thyroid hormones.

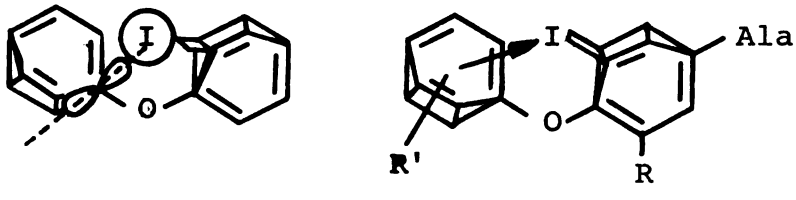
CHAPTER TWO: THYROMIMETIC ACTIVITY OF HALOGEN-FREE DERIVATIVES OF 3,5-DIMETHYL-L-THYRONINES

The thyroid hormones are unique in that they are the only naturally occurring iodine-containing molecules known to possess definite biological functions; namely, their necessity in maintaining normal growth and metabolism in a variety of organisms[8]. The presence of iodine in the thyroid gland has been known since 1895 when Baumann, a German biochemist, accidently introduced nitric acid into a thyroid preparation and observed the violet fumes of I_2[9]. Subsequently, Baumann proposed that dietary deficiency of iodine might be the cause of some of the ailments associated with the thyroid gland and suggested supplementary iodide salts as a cure.

Speculation that the biological activity of the hormones might be due to the atomic properties of iodine was not advanced until 1957. Then Szent-Gyorgi postulated[10] that the heavy-atom perturbation effect[11] of iodine may stabilize the triplet-state of certain molecules and that this is somehow related to the role of the thyroid hormones in regulating energy metabolism. Its role in uncoupling oxidative phosphorylation was specifically

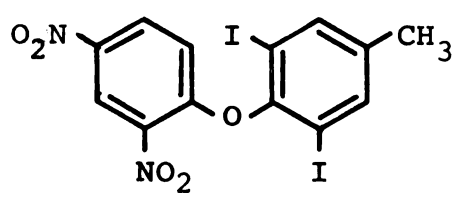
noted. Cilento and Berenholc[12] working independently under the same hypothesis experimentally determined that spin-forbidden processes[13] ($T \leftarrow S$ transitions) in iodophenols were strongly perturbed due to the presence of iodine. They also noted that 3-iodotyrosine and 3,5-diiodotyrosine (MIT and DIT), and by inference thyroxine also, form molecular complexes (charge-transfer complexes) with Mulliken acceptors. These phenomenon indicated that teses molecules should be very efficient in transferring triplet-state energy and led them to conclude that one function of thyroxine may be to act in intermolecular energy transfer.

However, as Lehmann points out[14], if the above were true then DIT and MIT should exhibit thyromimetic properties; but they do not. He proposed instead that conformational restrictions in combination with iodine perturbation are necessary for thyromimetic activity. He proposes that the minimum energy conformation in which both aromatic rings are twisted from coplanarity 37° in opposite directions places one of the iodine atoms of one aromatic ring above the other ring. In this conformation the halogen atom closest to the other ring lies directly over the C_1 carbon atom of that ring (approximately 3.5 A) placing its outer orbitals in the densest part of the



π-electron cloud. In analogy to the well documented[15] intermolecular interaction between aromatic compounds and halogen he visualized an intermolecular, electron donor-acceptor, charge transfer complex (intramolecular π-complex). He says furthermore formation of this intramolecular π-complex extends the heavy atom effect of the iodine to the phenolic or outer ring, causing a "loosening" of one of the π-electrons. This allows its spin to be reversed at much lower energy, thus increasing the probability of triplet state formation as well as lengthening the life time of the biradical intermediate. This low energy biradical of the thyroid hormones could then interact with the biochemical machinery, for example by easily giving up an electron resulting in a high reduction potential.

Lehmann's conjecture is based on calculations of the dipole moment of 3,5-diiodo-1-methylphenyl-2',4'-dinitrophenyl ether in various conformations assuming bond dipole additivity.



The measured dipole moment was 6.55 Debye compared to a calculated value of 6.64 D for the conformation in which the rings are twisted 37°. However, from Lehmann's results, it is not clear whether the observed dipole represents the twisted conformation or is an average over a number of nearly equal energy conformations. The calculated value for the conformation in which both rings are mutually perpendicular (see Chapter 3) is

itself reasonably close (7.26 D) to the experimental value. In view of the uncertainty in the bond additivity calculation for the dipole moment it could still be the energetically favored species.

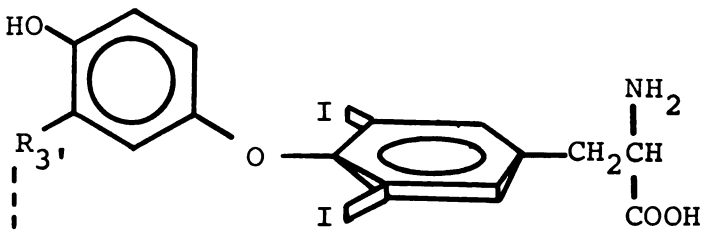
Other proposals which center on the iodine atom as the active functionality are those in which the thyroid hormones are viewed as carriers for a reactive form of iodine[16]. These hypotheses postulate thyroxine as associating with the membrane structure,whereupon it is deiodinated to give $I^{\cdot}$ or I^{+}. This species in turn interacts with the membrane altering its physicochemical properties, e.g. reacting with the lipid layer causing a lowering of the electrical resistance of the membrane, thus, converting it from an insulator to a semi-conductor which may account for some of the physiological effects attributed to the hormones.

Structure-activity studies on the thyroid hormones[3] have shown that partial replacement of halogen atoms on the thyronine nucleus by aryl or alkyl groups is possible[17]. In the phenolic ring the 3'- position can be occupied by halogens, alkyl and aryl groups (figure 2-1) with the isopropyl analog, 3,5-diiodo-3'-isopropyl-L-thyronine, showing maximal activity (3 to 8 times that of L-thyroxine.) Using Hansch π-values which are a measure of lipophilicity[18] and plotting biological activity as a function of these values, Jorgensen has obtained a parabolic relationship[3] (figure 2-2).

However, in the alanine-bearing ring only methyl groups have been effective in completely replacing halogens in the 3,5- positions with retention of hormonal activity. Thus

Figure 2-1

Thyroxine-like Activities of 3'-Substituted Analogs



F	8	OH	1
Cl	30	H	10
Br	140	Me	125
I	550	Et	500
		i-Pr	900
		t-Bu	120
		s-Bu	60
		Phe	30

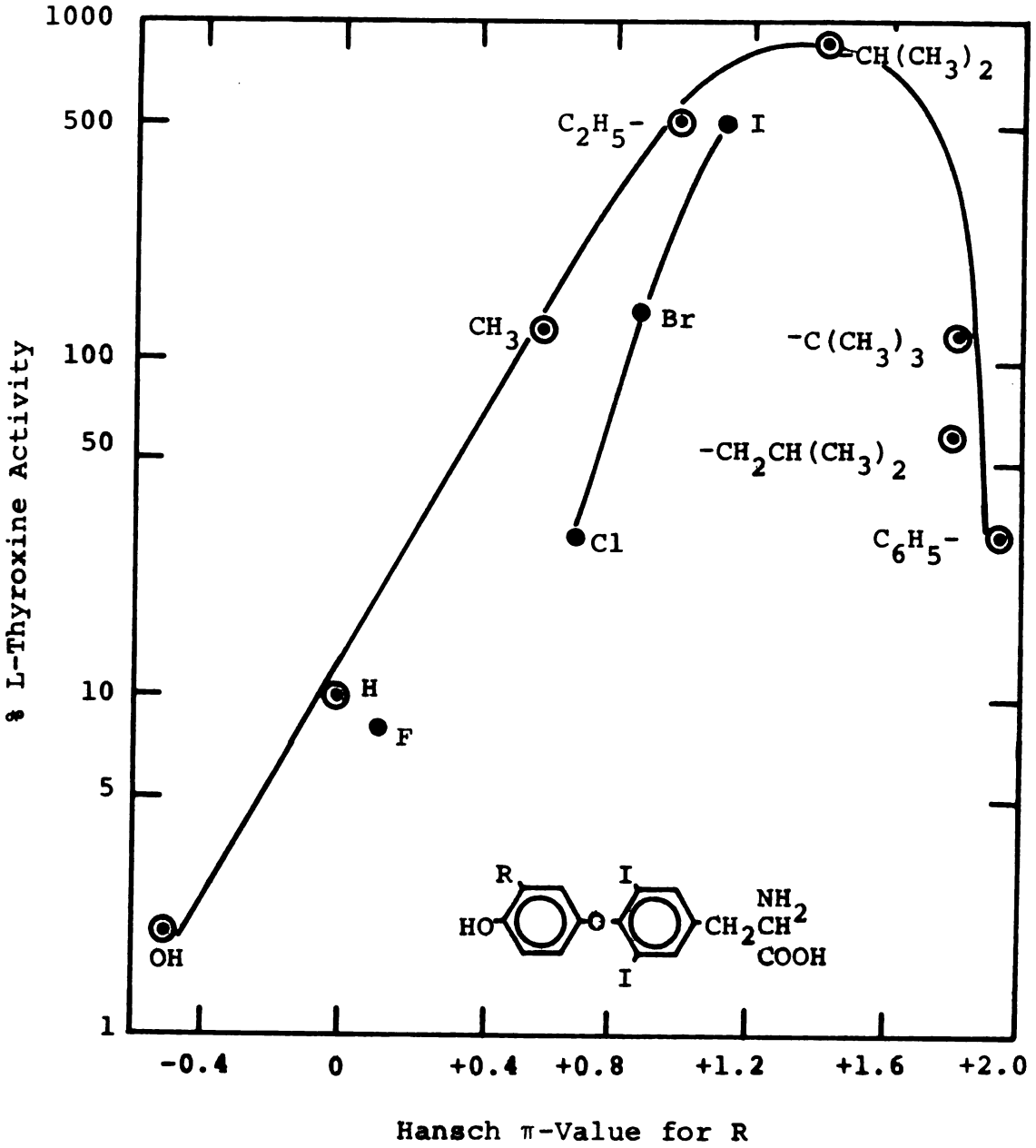
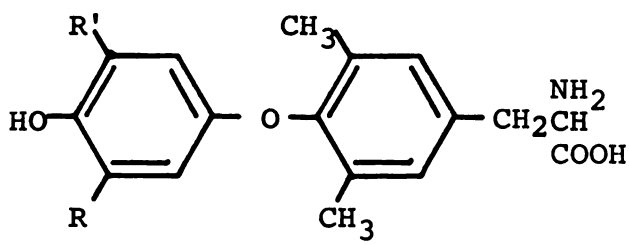


Figure 2-2

Parabolic Relationship of 3'-Substituents

3,5-dimethyl-3'-iodo-DL-thyronine showed an activity 3-5% that of L-thyroxine in the antigoiter test[19]. Other alkyl substitutions (isopropyl or sec-butyl) in the 3,5- positions gave inactive compounds[5a].

It has also been shown that the iodine atoms of the thyroid hormones can be completely replaced by bromine[20] or a combination of bromine and alkyl groups[21] with retention of hormonal activity. But the activity of other halogenated thyroxines does not preclude hypotheses ascribing unique functional roles to the halogen atom. Support for these theories are derived from the fact that all attempts at complete replacement of halogen atoms on the thyronine nucleus have led to total loss of activity[5]. Especially supportive are the studies reporting the inactivity of 3,5,3',5'-tetra-methyl-DL-thyronine[17f] (DL-Me_4 2-1) and 3,5-dimethyl-3'-isopropyl-DL-thyronine[5a] (DL-Me_2iPr, 2-2)



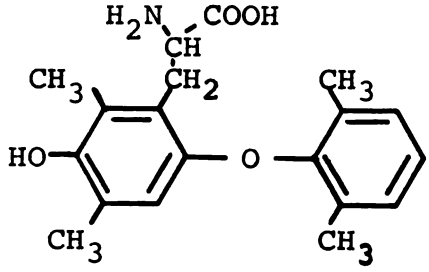
2-1, R = R' = $-CH_3$

2-2, R = iPr; R' = -H

2-3, R = $-CH_3$; R' = -H

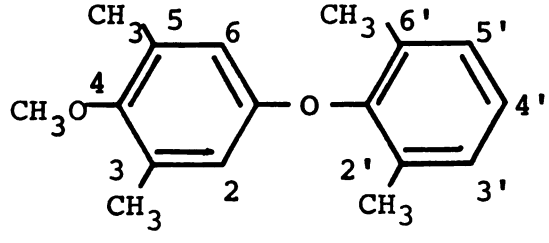
ERRONEOUS SYNTHESIS OF 3,5,3',5'-TETRAMETHYLTHYRONINE

A recent study[22] by Hamilton and Blanchard on the chloromethylation reaction used by Bielig[23] to introduce the amino acid chain onto the tetramethyldiphenyl ether nucleus indicated that substitution most likely occurred meta to the phenolic group rather than in the desired position para to the ether oxygen to yield the isomeric tetramethyl compound, 2,4-dimethyl-3-hydroxy-6(2',6'-dimethyl) phenoxy-DL-phenylalanine (i-DL-Me_4,2-4).



2-4, i-DL-Me_4

Their determination of structure was based on a proton magnetic resonance (PMR) study of 4-methoxy-3,5,2'6'-tetramethyldiphenyl ether 2-5 (parent anisole) and its chloromethylated and brominated derivatives. Figure 2-3 shows the reactions carried out by them and their assignments of the PMR spectra



2-5

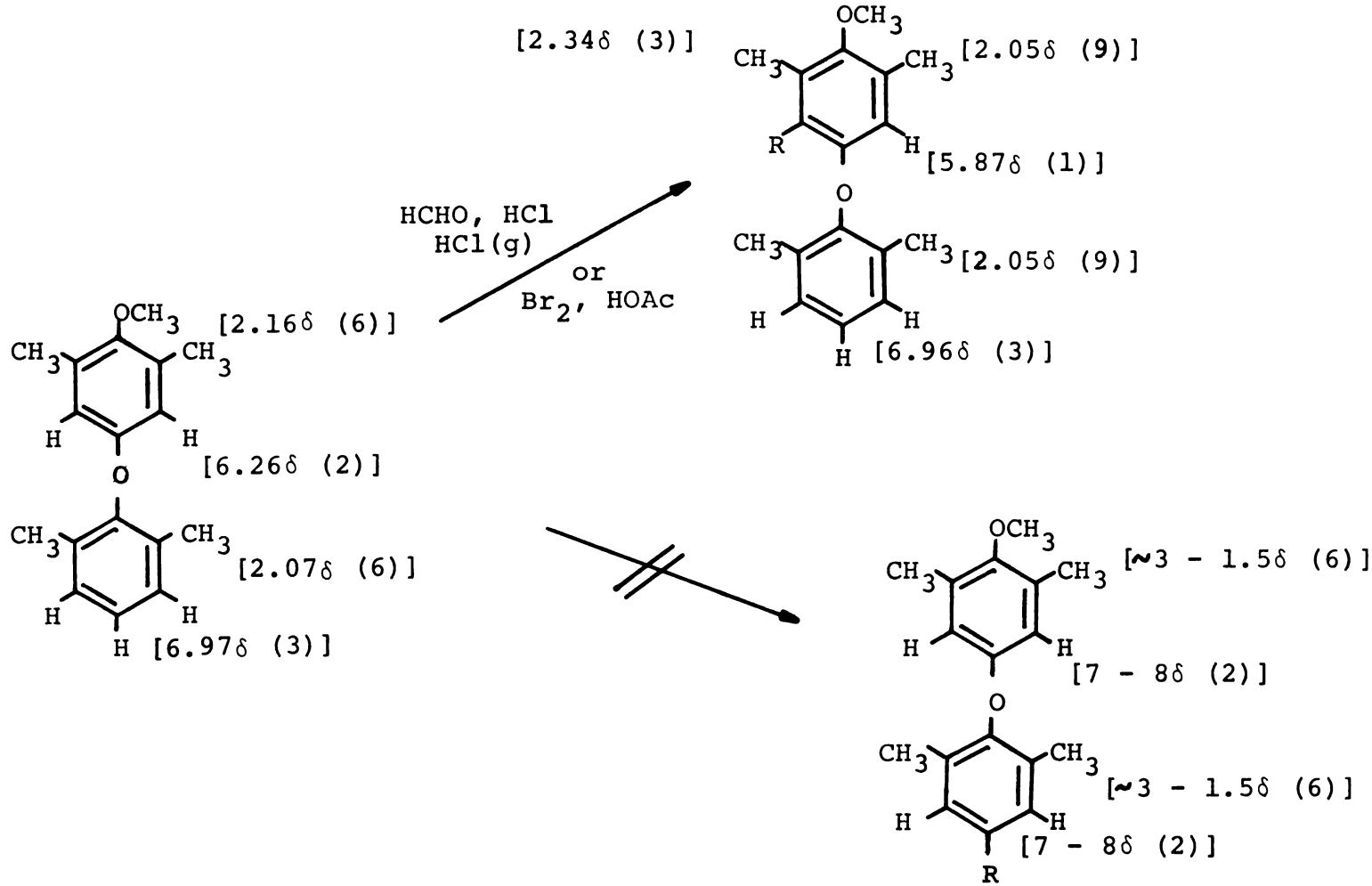


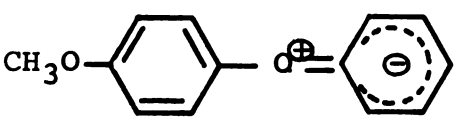
Figure 2-3. PMR assignments of electrophilic substitution reactions of sterically hindered diphenyl ethers[22].

for the arylmethyl and aromatic protons. The integration values are shown in parenthesis.

If the chloromethylation or bromination had gone as expected to give the desired products, one should have observed spectra which showed 2ArCH_3 singlets between 1.5 and 3.0δ integrating for 6 protons each and sets of equivalent Ar-H bands between 6-7δ integrating for 4 protons. Instead the products obtained showed: 2 ArCH_3 absorption peaks at 2.05δ and 2.34δ, the former integrating for 9 protons and the latter for 3; one aromatic proton with an abnormal upfield shift at 5.87δ and aromatic absorption at ca.6.96δ integrating for 3 protons. This could be accounted for only if the substitution had occurred at the 2-position.

The abnormal shift of the 6-proton is caused by the bulky 2-substituent hindering rotation of the anisole ring thus positioning the 6-proton directly over the phenoxy ring resulting in a shielding of that proton[24].

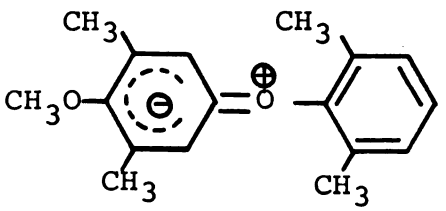
The electrophilic substitution at the 2-position was accounted for as follows. Resonance structure 2-6 largely contributes toward the reactivity of unsubstituted diphenyl ethers



2-6

because of the coplanarity which can occur between the phenoxy ring and the oxygen atom. The methoxy group at the 4-position

stabilizes such a structure. Electrophilic reactions in these compounds would therefore be expected to occur predominantly at the 4'-position, which they do. However, in tetrasubstituted diphenyl ethers such coplanarity is sterically prohibited, thus causing resonance structure 2-7 to contribute more.



2-7

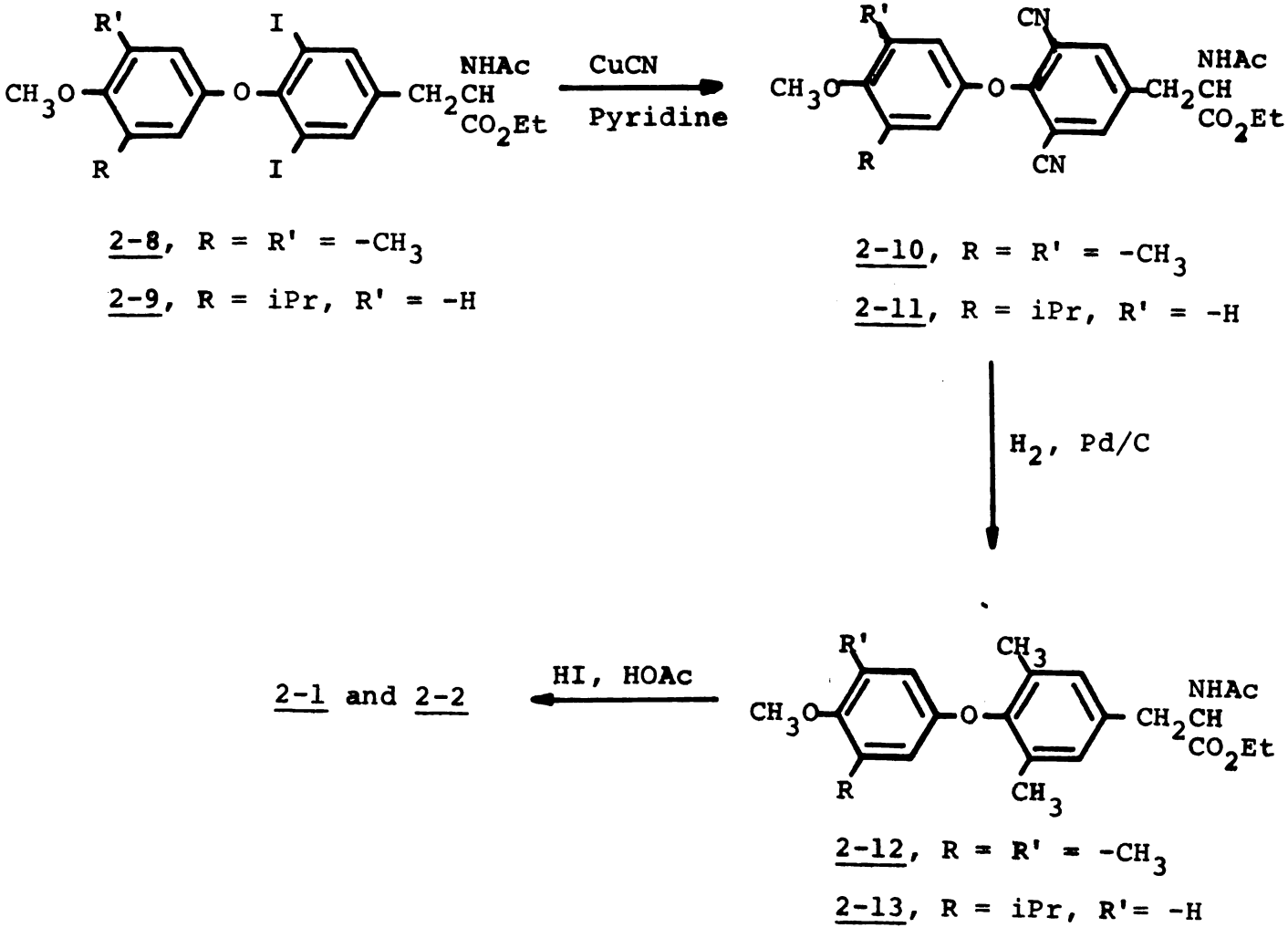
The sterically enforced predominance of 2-7 activates the anisole ring while similtaneously deactivating the phenoxy ring to electrophilic substitution because of the inductive, electron-withdrawing effect of the electropositive oxygen. The 3,5-methyl groups also contribute toward activating the 2-position.

Block[6], using the synthetic scheme in figure 2-4 which assured the position and optical activity of the alanine side chain, reported the synthesis of 3,5,3',5'-tetramethyl-L-thyronine (L-Me_4, 2-1). Starting with the protected 3,5-diiodothyronine, he converted this to the 3,5-dicyano intermediate, 2-10 followed by reduction to the 3,5-dimethyl compound, 2-12, and finally cleavage of the protective groups to the desired amino acid, 2-1. Because of the study of Hamilton and Blanchard both Bielig's and Block's reported tetramethylthyronines were subjected to the PMR and mass spectral comparisons given in Section 4.

In virtually all examples of active thyroid hormone analogs the 3,5,3'-trisubstituted thyronine is more active than the

Figure 2-4.

Synthetic pathway to the alkylated thyronines.



corresponding 3,5,3',5'-tetrasubstituted compound[25]. Some evidence has been presented in support of L-T_3 as the active hormone formed, in part by deiodination of L-T_4[26]. Since demethylation of an aromatic ring is an unlikely metabolic pathway, the trimethyl analog of L-T_3,3,5,3'-trimethyl-L-thyronine (L-Me_3, 2-3) was selected as a better candidate than the tetramethyl analog, 2-1, in the bioassays (Chapter 4).

ERRONEOUS SYNTHESIS OF 3,5-DIMETHYL-3'-ISOPROPYL-DL-THYRONINE

A preliminary bioassay[7] indicating the activity of L-Me_3 (2-3), made it desirable to reinvestigate the reported[5a] inactivity of DL-Me_2iPr (2-2), since the 3'-isopropyl substituent in the 3,5-diodo series shows greater activity than does either 3'methyl or 3'-iodine. 3,5-Dimethyl-3'-isopropyl-L-thyronine (L-Me_2iPr, 2-2) was prepared as described in figure 2-4 and its chromatographic and spectral characteristics were compared with the previously reported "DL-2".

The previous attempted synthesis[5a] of DL-Me_2iPr (DL-2-2) via the protected intermediate 2-11 was carried out as shown in figure 2-5, route A. PMR data (see Section 4, Physical Measurements) indicated the possibility that route B might have produced instead the protected biphenyl, 2-14, which was hydrolyzed to form 2-15, i-DL-Me_2iPr. The protected intermediates 2-13 and 2-14and the free amino acids, L-2-2 and DL-2-15 from the previous[5a] and present synthesis were compared by a variety of physical measurements which showed that the present synthesis yielded the desired 3,5-dimethyl-3'-isopropyl compounds L-2-13 and L-2-2, while the previous synthesis produced the isomeric biphenyls, DL-2-4 and 2-15.

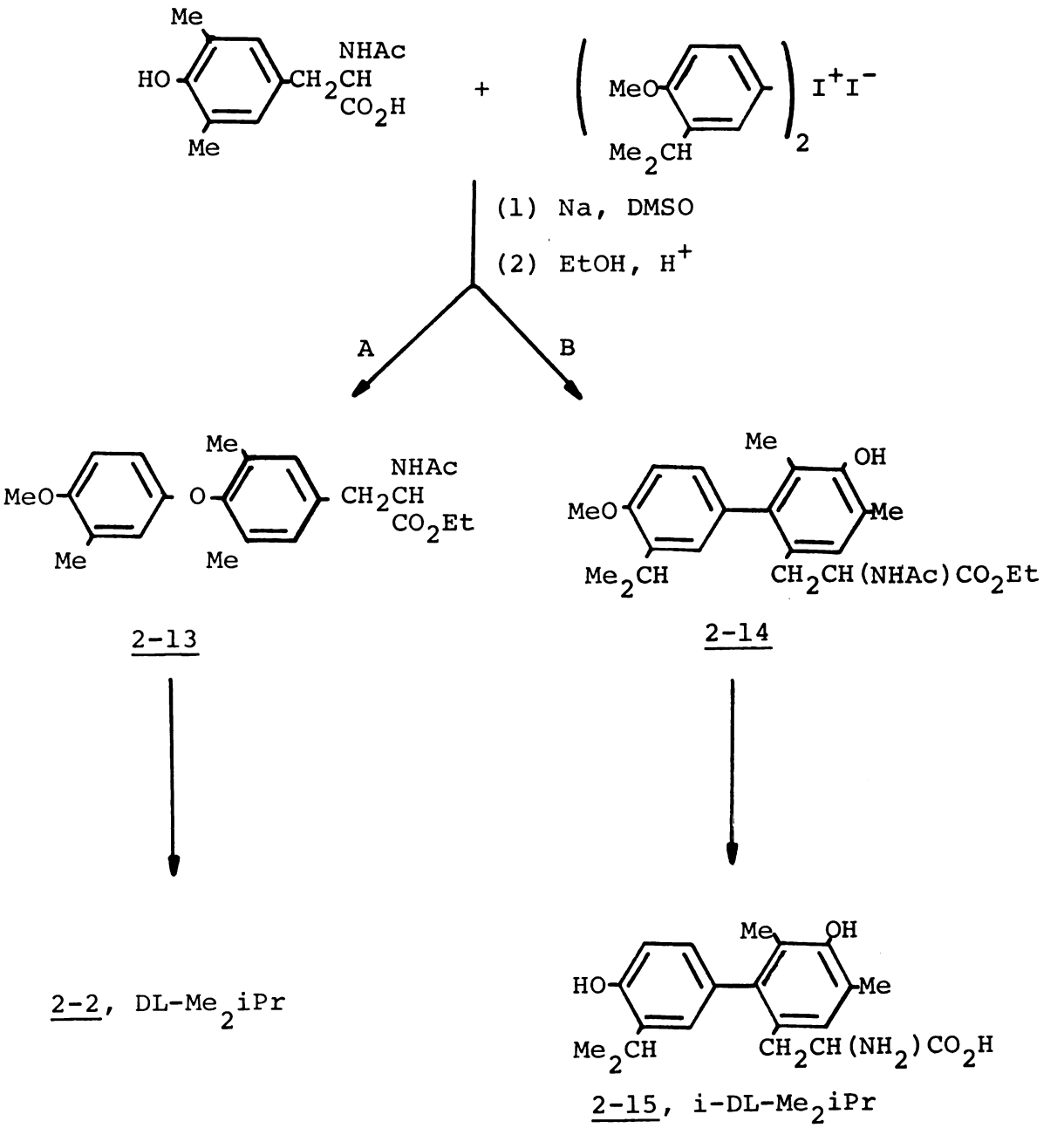
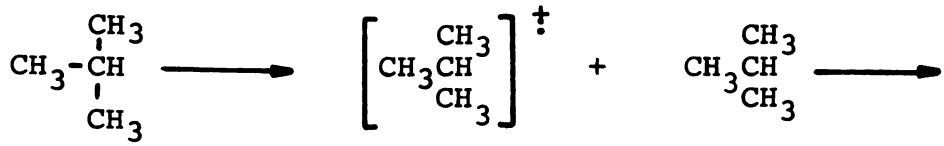


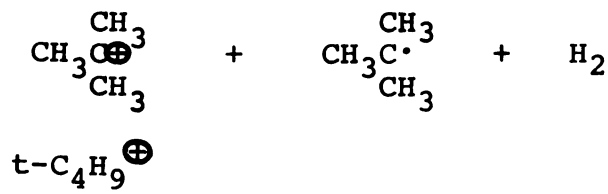
Figure 2-5. Erroneous synthesis of 3,5-dimethyl-3'-isopropyl-DL-thyronine.

PHYSICAL MEASUREMENTS

Table 2-1 summarizes the PMR spectra of the thyroid hormones and their alkyl analogs. In most cases a D_2O/NaOD solvent system was used with sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as the internal standard. Small amounts of t-butanol were found necessary to keep DSS in solution. In some cases trifluoroacetic acid was used as the solvent with tetramethylsilane use as internal reference. The amino acids were used at concentrations of 20-50 mg per ml.

Mass spectral data were obtained using the method of chemical ionization mass spectrometry (CIMS)[27]. CIMS was developed by Munson and Field[28] and consists in allowing a reactant gas, isobutane in these studies, to undergo the following ion-molecule reaction in a mass spectrometer ion source operated at a relatively high pressure





The t-butyl carbonium ion represents 90% of the reactive ions formed in this ion-molecule reaction. $t\text{-}C_4H_9^+$ has strong acidic properties and reacts mainly by proton transfer (a Bronsted acid with the molecule under investigation to give the quasi-

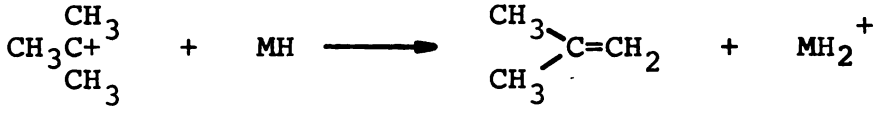
Table 2-1 PMR Spectra of Thyroid Hormones and Analogs[a]

Compound	Non-phenolic ring		Phenolic ring		iPr	
	ArH	3,5-Me	ArH	Me	CH	CH_3
L-T_2	7.76 (s,2H)	-	6.58 (s,4H)	-	-	-
L-T_3	7.75 (s,2H)	-	7.08-6.48 (comp m,3H)	-	-	-
L-T_4	7.72 (s,2H)	-	7.20 (s,2H)	-	-	-
L-Me_2	7.00 (s,2H)	2.10 (s,2H)	6.55 (s,4H)	-	-	-
L-Me_3 (2-3)	7.05 (s,2H)	2.14 (s,6H)	6.60-6.46 (comp m,3H)	2.11 (s,3H)	-	-
L-Me_4 (2-1)	6.99 (s,2H)	2.05 (s,6H)	6.39 (s,2H)	2.09 (s,6H)	-	-
i-DL-Me_4 (2-4)	7.03 (s,3H)	2.07 (s,6H)	5.91 (s,1H)	2.24 (s,3H) 1.92 (s,3H)	-	-
L-Me_2iPr (2-2)	7.08 (s,2H)	2.15 (s,6H)	6.70-6.39 (comp m,3H)	-	3.50(m)	1.11(d,J=7,6H)
L-Me_2iPr (2-2)[b]	7.20 (s,2H)	2.20 (s,6H)	6.95-6.85 (comp m,3H)	-	3.41(m)	1.25(d,J=7,6H)
i-DL-Me_2iPr (2-15)[b,c]	7.28 (s)	2.38 (s,3H)	7.13 (s)	-	3.38(m)	1.34(d,J=6,6H)

Footnotes for Table 2-1

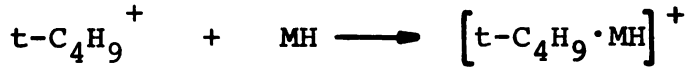
[a]In D_2O-NaOD at concentrations of 20-50 mg per ml. Chemical shifts are expressed in δ values (ppm) relative to DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate). Small amounts of t-BuOH were found necessary to keep DSS in solution. The following notations are used: s = singlet, d = doublet, m = multiplet and comp m = complex multiplet. In all cases the α-CH proton of the alanine sidechain appeared as a broad multiplet between 3.40 and 3.50 δ while the β-CH_2 protons appear as a broad multiplet between 2.80 and 3.00 δ. [b]In trifluoroacetic acid with the TMS as internal reference. [c]See text for biphenyl structure.

molecular ion, MH_2^+ or $(M+1)^+$ and isobutene. It may also act to a lesser extent as a hydride abstractor (a Lewis acid) with the sample molecule to give M^+ or $(M-1)^+$ species.



$(M+1)^+$ will fragment according to easily rationalized reactions to yield the appropriate fragmentation pattern[27]. Because the energetics involved with CIMS are much less than with electron impact mass spectrometry (ca 15 ev vs. 70 ev) a much simpler and in the case of amino acids more meaningful spectra is obtained. No electron impact reactions occur with the sample molecule because the reactant gas is usually in a 1000-fold excess.

The fragmentation pattern of an amino acid will typically show molecular-ion species having a molecular weight higher than $(M+1)^+$. This occurs when the reactant ions react with electron-rich compounds to form collision-stabilized complexes[29].



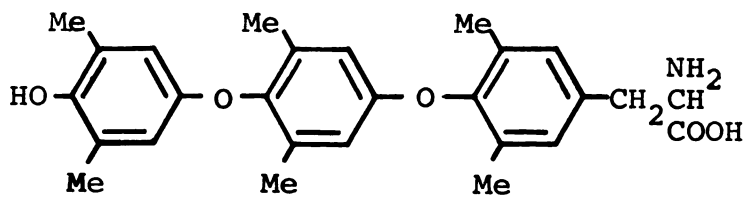
Such a complex accounts for the $(M + 57)^+$ species seen in the spectra in Appendix I. Other complexes observed are $(M + 43)^+$ and $(M + 29)^+$ which may be accounted for as products of the less abundant reactant ions $C_3H_7^+$ and $C_2H_5^+$.

A compilation of the PMR, CIMS, infra-red and ultra-violet spectra are given in Appendix I. PMR spectra (Table 2-1) of the thyroid hormones and of their alkyl analogs confirmed what was implied by previous studies[22] of intermediates; that L-Me_4 (2-1) prepared by Block[6] was the desired 3,5,3',5'-tetramethyl-thyronine, and that the compound prepared by Bielig[23] was the isomeric i-DL-Me_4 (2-4). The Bielig preparation, 2-4, contained 3 equivalent aromatic protons in the non-phenolic ring and two non-equivalent methyl groups in the phenolic ring. Three bulky groups (3,5-dimethyl, 2'-alanyl) flanking the ether link constrain the diphenyl ether structure of 2-4 to a conformation in which the 6'-proton is positioned in the ring current on the non-phenolic ring. As a result its chemical shift is observed at an abnormally high field (5.91 δ). The PMR spectra of the Block preparation, 2-1, and of the related compounds prepared by the same procedure, 2-4, 2-11 and 2-12 are completely consistent with the assigned structures.

As is consistent for the aromatic amino acids[27], CIMS of the alkyl thyronines show loss from the alanine side chain of the protonated molecular ion $(MH)^+$ of NH_3 (17), H_2O (18), CO_2H_2 (46) and of $CH(NH_2)=C(OH)_2$ (75), the latter creating charged benzylic fragments. The Bielig compound, 2-4, shows the MH^+, and has the stable benzylic ion, 2-16, as its base peak. In addition it undergoes fragmentation to yield 2-17

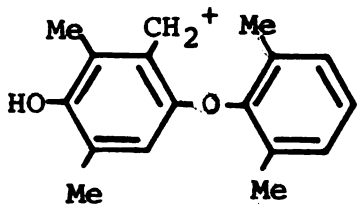
which is unique to this isomer in that the alanine side chain is attached to a ring bearing two oxygen atoms. The Block compound, 2-1, shows the charged benzylic fragment 2-18 as its base peak, but in this isomer 2-18 undergoes further cleavage to form 2-19 and 2-20. No fragment corresponding to 2-17 was observed thus confirming the specific isomeric natures of 2-1 and 2-4.

High resolution CI mass spectrometry of the alkyl thyronines showed that none of the minor peaks of mass greater than MH^+ contained iodine. In the spectrum of L-Me_4 (2-1), a small amount of three-ring compound, 2-21, was identified by its exact mass and secondary fragmentation pattern.

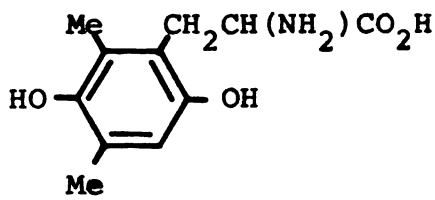


2-21

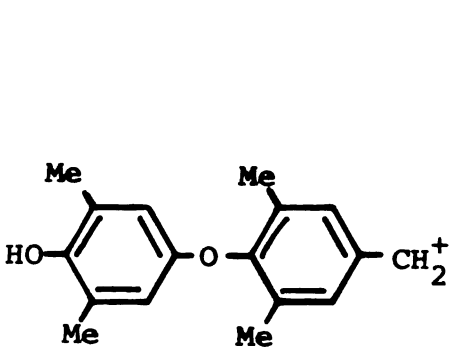
The previously reported[5a] "DL-2-2" (now recognized as 2-15) and its protected intermediate 2-14 showed PMR spectra [Table 2-1, i-DL-Me_2iPr (2-15), Experimental Section (2-14)] inconsistent with the diphenyl ether structures. Both showed 2 non-equivalent methyl groups, and 4 instead of the expected 5 aromatic protons. The spectra of L-Me_2iPr (2-2) and of its protected intermediate (2-10) were consistent with the assigned structure.



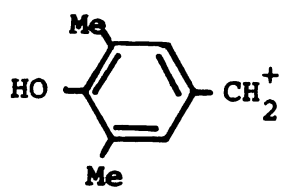
2-16, 255 m/e (100)



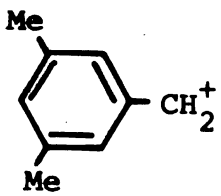
2-17, 225 m/e (20)



2-18, m/e 355

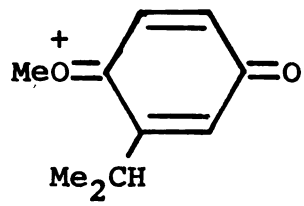


2-19, 135 m/e (14)



2-20, 117 m/e (11)

By CI mass spectrometry the protected intermediates L-2-11 and DL-2-14 both showed MH^+ at 428. Spectral differences included a fragment corresponding to 2-22 for the present preparation 2-11, which was absent in the previous preparation 2-14.



2-22, m/e 165

A large abundance of 269 mass peak, corresponding to the loss of $CH_2CH(NHAc)CO_2Et$ was present in 2-11 and virtually absent in 2-14, while a large abundance of a mass 295 peak, corresponding to the loss of both NHAc and COOEt was present in 2-14 but absent in 2-11. These differences can be rationalized by the presence in 2-11 of an oxygen atom para to the alanine side chain and its absence in 2-14.

The free hydroxyl group in 2-14 was shown to be present by uv and ir. Thus all physical data is consistent with the desired diphenyl ether structures for 2-11 and 2-2, and with

the biphenyl structures for 2-14 and for the previously tested and hormonally inactive 2-15.

RESULTS AND DISCUSSION

Figure 2-6 summarizes the dose-response data for the alkylated analogs L-Me_3, L-Me_4 and L-Me_2iPr in the rat anti-goiter assay[30]. Details regarding the biological evaluation in a number of assays are given in Chapter Four. It is clear that halogen is not required for thyroid hormone activity. Previously, it had been shown that methyl groups may replace iodine in the 3 and 5- positions of the thyronine nucleus and that alkyl groups such as methyl and isopropyl may replace halogen in the 3' or 3',5'- positions. These substitution patterns have been combined in these studies to give active halogen-free hormone analogs. Potencies within the 3,5-dimethyl-L-thyronine series are those predicted from the 3,5-diiodo series in which the 3'-substituent contributes to activity in the order iPr > I > Me. From the data for L-Me_4 and L-Me_3, however, we cannot state that 3',5'-disubstitution is less active than 3'-monosubstitution. Comparing these two groups using the F-test (Appendix II) we cannot separate them into two different populations, i.e. they appear to be equipotent. If this is so, it casts some doubt on recent studies[26] showing that for the iodinated, endogenous hormones the 3'-monosubstituted T_3 is the actual molecule responsible for thyroid hormone activity. More of this will be said in Chapter Four.

The activity of L-Me_4 in the thyroidectomized rats[31] indicates that the effect of the halogen-free analogs is pri-

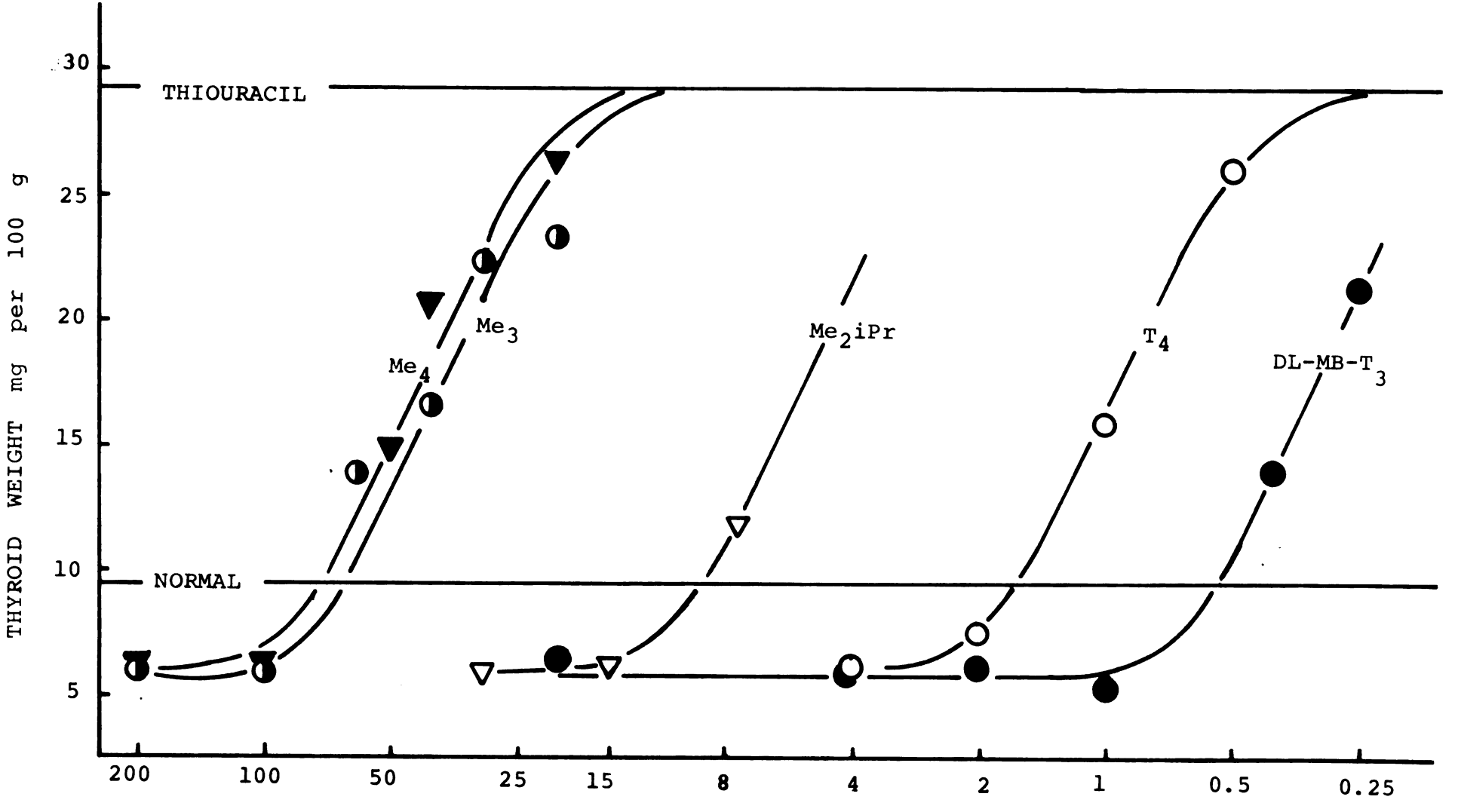

THYROID WEIGHT mg per 100 g
30
25
20
15
10
5
THIOURACIL
NORMAL
Me4
Me3
Me2iPr
T4
DL-MB-T3
200
100
50
25
15
8
4
2
1
0.5
0.25
DOSE - MOLAR RATIO

Figure 2-6

marily a direct one. That is, its action does not appear to be due to secondary effects caused by the alkyl analogs displacing the hormones from storage sites such as the plasma proteins or because the analogs are inhibiting the metabolism of the thyroid hormones. Additional biological studies of binding affinities of the 3,5-dimethyl-L-thyronines with plasma proteins and of any effects on the metabolism of the endogenous thyroid hormones would provide valuable supplementary data.

The halogen-free alkyl thyronines show appreciable hormonal activity in a variety of test systems (Chapter Four). Therefore, hypotheses proposing a functional role for iodine cannot be valid. Alkyl groups cannot participate in the heavy-atom perturbation effect involved in the hypothesis of hormonal action proposed by Szent-Gyorgyi[10] and Cilento[12]. Because of the low order of electronic inductive effect of methyl groups and their lack of any significant amount of polarizable electrons it is difficult to envision the occurrence of an intramolecular donor-acceptor π-complex as proposed by Lehmann[14]. The activity of the halogen-free analogs would seem to totally eliminate any hypothesis postulating an active form of halide as being responsible for initiating hormonal activity.

A biosynthetically simple and convenient route for organic incorporation of iodide ion is via the iodinated tyrosines. Taurog[32] has reviewed the oxidative steps and the enzymatic system necessary to convert iodide to an "active" form and the ease with which tyrosines are mono- and di- iodinated. It is easy to imagine the evolutionary steps progressing from invertebrates, e.g. jelly fish, to vertebrates which make use of

this convenient route using precursors and reactions available in the primative environment. Cahnmann and others[33] have studied the ease with which the coupling of two iodinated tyrosines occurs to give the iodinated thyronines. In fact it occurs spontaneously in near neutral solution at room temperature and in the presence of oxygen[34], although at a more rapid rate in the ordered environment of thyroglobulin.

The biological activities of the alkylated thyronines, coupled with the discussion in Chapter Three on the steric role of the 3,5-substituents using molecular orbital calculations and the biological activities of a number of analogs, especially the methylene-bridged analog, in Chapter Four turn our attention away from the concept of a particular functional portion of the molecule being involved in the hormonal action. Instead they redirect our attention to the concept of the hormone molecule acting as a structurally specific matrix. We will develop this concept in subsequent chapters.

EXPERIMENTAL SECTION

Melting points were determined in a Thomas-Hoover Uni-melt stirred oil capillary tube melting point apparatus and are uncorrected. Proton magnetic resonance spectra were determined at 60 MHz with a Varian Model A-60A PMR spectrometer and at 100 MHz with a Jeolco-JMN-100 PMR spectrometer. The chemical shifts are expressed in δ values (parts per million) relative to either a TMS or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DDS) internal standard. In the presentation of the PMR spectra the following notations are used: s = singlet, d = doublet,

m = multiplet and comp m = complex multiplet. Mass spectra were obtained with an Associated Electronic Inc. Model MS 902 double focus mass spectrometer equipped with a direct inlet system and modified to do chemical ionization mass spectra. Isobutane was used as the reactant gas. Optical rotations were measured with a Perkin Elmer Model 141 Polarimeter. The microanalyses were performed by the Microanalytical Laboratory, University of California, Berkeley, Calif. The infrared spectra were measured with a Perkin Elmer 337 infrared spectrophotometer. The ultraviolet spectra were recorded with a Cary Model 15 Recording Spectrophotometer.

Thin layer chromatography was used routinely as a check for purity of samples as well as an aid in determining the progress of reactions. Pre-coated silica gel plates with flourescent indicator (E. Merck Laboratories, Inc.) were used. Plates spotted with protected amino acids (as their N-acetyl, O-methyl ethyl esters) were developed in either ethyl acetate or 4:1 ethyl acetate-chloroform. The free amino acids were developed in both 4:1 i-propanol-ammonia (28%) and 10:1:1 i-propanol-glacial acetic acid water.

Di-(3-isopropyl-4-methoxyphenyl)iodonium Iodide. Iodine triflouroacetate was prepared from iodine following the procedures of Beringer[35] et.al and Jorgensen and Reid[36]. To a 100 ml, 3-necked round bottomed flask fitted with a pressure equalizing dropping funnel, thermometer and drying tube, equipped with a magnetic stirrer and immersed in a NaCl ice bath is placed 28 ml (30.0 g) of acetic anhydride. After cooling to -15°, 20.0 ml of red fuming nitric acid (Baker, sp. gr. 1.6)

was added drop-wise such that the temperature never rose above -10°. At the conclusion of the HNO_3 addition, 10.0 g of powdered iodine was added in one portion followed by the addition of 20 ml of trifluoroacetic acid. A dark red solution developed followed by the evolution of brown fumes. The mixture was heated in a 50° water bath with stirring until all the iodine dissolved. The solvents were removed under vacuum (5 mm) while kept in a 40° water bath to give a yellowish waxy solid. The solid was redissolved in 100 ml of acetic anhydride and the iodonium salt was prepared from this mixture. To the solution of iodine trifluoroacetate in acetic anhydride cooled to -10° was added drop-wise, and with stirring, a solution of 42.0 g of o-isopropylanisole, in 100 ml of acetic anhydride and 20 ml of trifluoroacteic acid. The addition was made in such a manner that the temperature never rose above 0°. Following the addition of o-isopropylanisole solution, the reaction mixture was stirred continuously in an ice bath and slowly allowed to come to room temperature overnight. The solvents were removed <u>in vacuo</u> (5 mm) giving a red oil which was subsequently dissolved in 400 ml of methanol. The methanolic solution was treated with 100 ml of 10% sodium bisulfite followed by slow addition of 140 g of potassium iodide in 800 ml of H_2O. This treatment gave a yellow precipitate. After stirring at room temperature for two hours the yellow precipitate was collected, washed with water and air dried. Two recrystallizations from tetrahydrofuran/hexanes gave 27.0 g (64.5%) of yellowish crystals mp 148-149°. PMR spectra in $CDCl_3$: δ 1.14 (d, J = 6.5 Hz, CH_3), 3.02-3.50 (m, J = 5.0 Hz, CH), 3.81 (s, OCH_3), 6.75 - 7.98 (comp m,

6H, arom. H). Final purification was accomplished by conversion into the water soluble sulfate and reprecipitation by iodide, as described by Block[37]. 27.0 g of recrystallized product and 7.1 g of silver sulfate were stirred for two hours in 150 ml of H_2O containing a suspension of activated charcoal. Filtration through filter aid and addition of 8 g KI in 20 ml water to the filtrate yielded a white precipitate. 22.9 g of product was recovered (mp 153 - 154°; lit 164 - 166[17d], 153 - 154[38]) upon filtration and drying.

N-Acetyl-3,5-diiodo-4-(3'-isopropyl-4'methoxy-L-phenyl-alanine Ethyl Ester (2-9). 22.9 g of di-(3-isopropyl-4-methoxyphenyl)iodonium iodide (.053 mol) prepared above and 10.0 g of N-acetyl-3,5-diiodo-L-tyrosine ethel ester (.04 mol) were dissolved in 100 ml of anhydrous methanol containing 3 ml of triethylamine and 0.21 g of copper powder. The mixture was stirred for 24 hours in a 250 ml round bottomed flask equipped with condenser and drying tube, magnetic stirrer and water bath maintained at 45°. The mixture was filtered to remove insoluble material and the organic solvents were removed in vacuo (water aspirator). The resultant brown, oily residue was taken up in 300 ml of benzene and 100 ml of 5% HCl. The benzene layer was spearated and washed succesively with 100 ml of 5% HCl, 100 ml water, two times with 100 ml 5% K_2CO_3 and 100 ml of water. The organic layer was collected and dried over anhydrous sodium sulfate. Following filtration, the benzene was removed by distillation under reduced pressure giving a brownish oil. The addition of 200 ml of petroleum ether (bp 30 - 60°) to this residue gave a whitish precipitate. Refrig-

eration followed by filtration and drying yielded 5.3 g (40.5%) of white solid. One recrystallization from aqueous ethanol gave 4.6 g, mp 132 - 132.5° (lit. 129 - 131°[41], EtOH); $[\alpha]_D^{25}$ + 3.74 (2.0 ethanol); CI mass spec [MH^+] 650; PMR ($CDCl_3$) δ 1.19 (d, J = 6.0 Hz, 6H, iPr-CH_3), 1.26 (t, J = 7.5 Hz, 3H, Et-CH_3), 2.05 (s, 3H, Ac-CH_3), 3.10 (2, 2H, β-CH_2), 3.30 (m, H, iPr-CH), 3.80 (s, 3H, OCH_3), 4.25 (q, J = 7.5 Hz, 2H, Et-CH_2), 4.87 (m, H, α-CH), 6.26 (d, J = 8.0 Hz, 1H, NH), 6.30 - 6.80 (7, 3H, Ar-2',5',6' H), 7.70(s, 2H, Ar-2,6 H).

N-Acetyl-3,5-dicyano-4-(3'-isopropyl-4'-methoxy phenoxy)-L-phenyalanine Ethyl Ester (2-11), 3.0 g of the diiodo compound (2-9) (4.6 mM), 2.0 g of cuprous cyanide (22.4 mM) and 20 ml of dry pyridine in a 50 ml round bottomed flask equipped with reflux condenser, stirring bar and Variac controlled heating mantle were heated under reflux for 14 hours, collected by filtration and washed with cold water. The solid was transferred to a beaker with 100 ml of 2N NH_4OH and 70 ml of $HCCl_3$. After vigorous stirring for 30 minutes the mixture was filtered through filter aid and the brown $HCCl_3$ layer was separated from the Prussian blue aqueous layer. The $HCCl_3$ was washed successively with 100 ml of 2N NH_4OH, 100 ml of H_2O, 100 ml of 2N HCl, 100 ml of H_2O and dried over anhydrous soduim sulfate. The brown solution was reduced to a thick syrup under water aspirator distillation, which became solid upon standing. The solid was recrystallized twice from aqueous ethanol to give 1.76 g (78.2%) of white powder. Mp 143 - 144°; $[\alpha]_D^{25}$ + 50.5 (c 2.0 $CHCl_3$); CI mass spec [MH^+] 450; PMR ($DCCl_3$) δ 1.20 (d, J = 6.0 Hz, 6H, iPr-CH_3), 1.30 (t, J = 7.5 Hz, 3H, Et-CH_3), 2.02 (s, 3H, Ac-

CH_3), 3.19 (d, 2H, β-CH_2), 3.26 (m, 1H, iPr-CH), 3.82 (s, 3H, OCH_3), 4.25 (q, J = 7.5 Hz, 2H, Et-CH_2), 4.85 (m, 1H, α-CH), 6.38 (d, J = 8.0 Hz, 1H, NH), 6.80 - 7.00 (m, 3H, Ar-2',5',6'H), 7.69 (s, 2H, Ar-2,6 H).

N-Acetyl-3,5-dimethyl-4-(3'-isopropyl-4'-methoxy)phenoxy-L-phenylalanine Ethyl Ester (2-13). This compound was prepared from the dicyano compound (2-11) following the procedure of Block[6]. A 100 ml 3-necked flask was equipped with gas dispersion tube, reflux condenser and oil bath. The top of the reflux condenser led to a second dispersion tube which dipped beneath 100 ml of H_2O having methyl red indicator and contained in a 200 ml 3-necked flask which was further equipped with a 10.0 ml burette and ventilating tube. The dicyano compound (2-11) [1.35 g, 3 mM] which was shown by mass spectra to contain no iodinated organic compounds was dispersed in 35 ml of purified p-cymene (250 ml Eastman #83, purified by washing with 50 ml portions of concentrated H_2SO_4 until the yellow color disappears, then successively with 10% K_2CO_3 and H_2O. Following drying over anhydrous magnesium sulfate and filtration, distillation and collection of the fraction distilling over at 174° yields 186.4 g of p-cymene). 0.6 g of 10% palladium on activated charcoal was added to the mixture. H_2 gas was bubbled through the refluxing mixture while the oil bath was maintained at 195°. The reaction was monitored by titrating the evolved ammonia against 1.0 N HCl. The reaction was ended when 97.5% of the theoretical amount of acid had been neutralized (5 hours). The hot mixture was filtered through a sintered glass filter containing filter aid and washed with hot ethyl acetate. The solvents were removed under

reduced pressure (water aspirator followed by 5 mm pump). The oily residue was treated with 100 ml of petroleum ether (bp 30 - 60°) and refrigerated overnight. Filtration followed by recrystallization in petroleum ether gave 0.9 g (87%), mp 94 - 96.5°, $[\alpha]_D^{25}$ + 17.6 (c 1.0, EtOH); tlc, single UV absorbing spot, R_f (using silica gel plate and 4:1 EtOAc-$CHCl_3$ solvent system)).34; CI mass spec [MH^+] 428; PMR ($DCCl_3$) δ 1.19 (d, J = 6.0 Hz, 6H, iPr-CH_3), 1.26 (t, J = 7.5 Hz, Et-CH_3), 2.02 (s, 3H, Ac-CH_3), 2.10 (s, 3H, Ar-CH_3), 3.09 (d, 2H, β-CH_2), 3.30 (m, 1H, iPr-CH), 3.80 (s, 3H, OCH_3), 4.24 (q, J = 7.5 Hz, 2H, Et-CH_2), 4.89 (m, 1H, α-CH), 6.17 (d, J = 8.0, 1H, NH), 6.30 - 6.82 (7, 3H, Ar-2',5',6' H), 6.91 (s, 2H, Ar-2,6 H). Analysis: Found C, 70.26; H, 7.59; N, 3.27; Calculated C, 70.23; H, 7.78; N, 3.27.

3,5-Dimethyl-3'-isopropyl-L-thyronine (2-2). The protected amino acid (2-13) [275 mg (.645 mM)] was dissolved in 10 ml of glacial acetic acid in a 50 ml, 3-necked round bottomedflask equipped with a reflux condenser, N_2 inlet dispersion tube, pressure equalizing dropping funnel and an oil bath maintained at 125°. The mixture was purged with N_2 for 15 minutes and then heated to reflux. 3 ml of 47% hydriodic acid was added drop-wise to the refluxing mixture under a N_2 atmosphere. Following HI addition, heating at reflux was continued for 8 hours. The acid mixture was transferred to a 100 ml round bottomed flask and the acid solvents were removed in vacuo (5 mm). Water was added 8 times in succession to the distilling flask to remove as much HI as possible. Following this, 2N NaOH was added to the mixture resulting in a white precipitate followed by

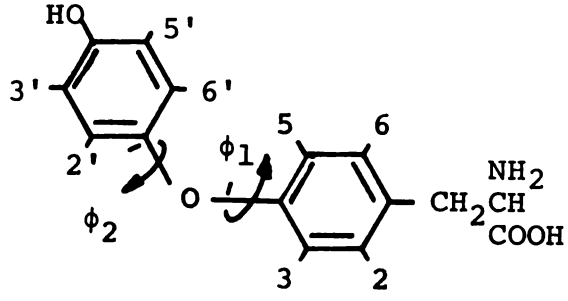
dissolution upon further addition of base. The solution was transferred to a centrifuge tube with washing and glacial acetic acid was added to give a beige precipitate. Following centrifugation, another isoelectric precipitation was performed, followed by an aqueous ethanol recrystallization. This afforded 101 mg of white solid (44.5%). Mp 210 - 212°, $[\alpha]_D^{25}$ = 12.4 (c, 2.0, 0.1N HCL in 50% EtOH). Tlc (UV, ninhydrin) R_f [silica gel, i-PrOH-concd NH_4OH (4:1)] 0.39] R_f [cellulose, n-BuOH-t-BuOH-H_2O (10:1:1)] 0.76; R_f [silica gel], i-PrOH-HOAc-H_2O (10:1:1)] 0.36 (separated from 2-15, R_f 0.45). CI mass spec [MH^+] 344. Analysis: Found C, 69.67; H, 7.17; N, 4.06; Calculated C, 70.0; H, 7.34; N, 4.08.

N-Acetyl-2-(3-isopropyl-4-methoxyphenyl)-3,5-dimethyl-DL-tyrosine Ethyl Ester (2-14). Previously prepared and reported as DL-2-11[5a]. Ir (KBr pellet) 3455 cm^{-1} (phenolic OH); UV $\lambda_{max}^{EtOH,OH^-}$ 276, 282 s (ε 3300); $\lambda_{max}^{EtOH,OH^-}$ 300 (ε 4000); Tlc (UV) R_f [silica gel, EtOAc-$CHCl_3$ (4:1)] 0.33; PMR ($DCCl_3$) δ 1.17 (t, J = 7.5 Hz , 3H, Et-CH_3), 1.23 (d, J = 6.0 Hz, 6H, iPr-CH_3), 1.90 (s, 3H, Ac-CH_3), 2.70 (q, 2H, β-CH_2) 3.40 (7, 1H, iPr-CH), 3.92 (s, 3H, OCH_3), 4.08 (q, J = 7.5 Hz, Et-CH_2) 4.58 (m, 1H, α-CH), 5.0 (s, 1H, OH, 5.65 (d, J = 6 Hz, 1H, NH), 7.00 (broad s, 3H, Ar-H), 7.07 (broad s, 1H, Ar-H). CI mass spec $[MH]^+$ 428.

2-(3-Isopropyl-4-hydroxyphenyl)-3,5-dimethyl-DL-tyrosine (2-15). Previously prepared and reported as DL-2[5a]. Tlc (UV, ninhydrin) R_f [silica gel, i-PrOH-HOAc-H_2O (lo:1:1)] 0.45 separated from 2-2, R_f 0.36. PMR, see Table 2-1, CI mass spec [MH^+] 344.

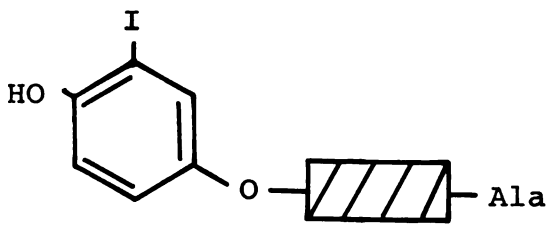
CHAPTER THREE: MOLECULAR ORBITAL STUDIES AND THE STEREOCHEMICAL ROLE OF HALOGEN

Having shown that halogen is not essential for hormonal activity (Chapter Two), we looked at a feature which appeared to be common to all the active analogs and hormones. It seemed obvious that 3,5-disubstitution on the thyronine nucleus formed a regular pattern in active compounds and equally obvious, when looking at space-filling molecular models, that bulky

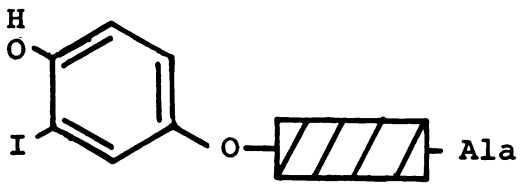


groups occupying these 3,5-positions hindered rotation of the phenol-bearing ring ("outer" ring) about the ether C-O bond. One objective was to quantitate this feature and to determine a manner in which this could be correlated to biological activity.

Earlier, Zenker and Jorgensen[39] studied the stereochemical nature of the 3,5-diodothyronine nucleus. They noted that bulky groups lying ortho to the ether linkage in one ring might favor the formation of a preferred conformation in the diphenyl ether moiety wherein the planes of the aromatic rings would be mutually perpendicular. In such a conformation ($\phi_1 = 90^\circ$, $\phi_2 = 180^\circ$) the 3'- and 5'- positions become non-equivalent with respect to the alanine-bearing ("inner" ring).



Proximal



Distal

If there were a single substituent, such as iodine in triiodothyronine (T_3), at either the 3'-, or 5'- position a degree of asymmetry would be imparted to the molecule which would not be present in a symmetrically 3'-,5'- disubstituted molecule such as T_4. T_3 for example could exist in two distinct conformations, one in which the 3'-iodine is oriented proximally, or toward, the alanine-bearing ring and another in which it is oriented distally, or away from, the inner ring. They reasoned that though hydrogens in the 2',6'- positions might not prevent rotation about the ether bond, a substituent such as methyl in the 2'- position would be forced to occupy a position distal to the

inner ring. Any group lying ortho to the 2'- substituent would now be locked into a 3'- distal position while groups para to the 2'- substituent would be fixed in the 5'- proximal position. Synthesizing and testing[17b] the 3,5-diiodo-2',3'-dimethylthyronine (distally oriented model) and the 3,5-diiodo-2',5'-dimethylthyronine (proximally oriented model) respectively, it was found that the distal analog was 100 times more effective than the proximal analog in the rat goiter-prevention assay.

Schussler[40], using the same analogs as above, found results consistent with the requirement for a distally oriented T_3 when he showed in binding studies of T_3 to thyroxine binding globulin (TBG) that the distally-fixed isomer had a two-fold greater binding affinity for TBG than the proximally-fixed isomer.

Recently, the x-ray data presented by the Camermans[41] for T_3 and ethyl 3,5,3'-triiodothyropropionate has shown the 3'-iodine to be the proximal position relative to the inner ring. Cody and Duax observed that the 3'-iodine of 3,5,3'-triiodothyroacetic acid[42a] and of T_3[42b] were in the distal position under their conditions of crystallization.

These findings suggest that the energy differences in the crystalline state between the proximal and distal forms might be a function mainly of the intermolecular interactions rather than any minimum energy conformation of an isolated molecule. Also, these x-ray studies indicate that the energy differences between the proximal and distal forms of the 3'-iodine is much smaller than the 132 kcal found in Camerman's extended Huckel calculations[41]. Examining energy differences between the proximal and distal conformations was one of the reasons for these

studies.

Molecular orbital calculations offered us an opportunity to quantitate the steric effect of 3,5-disubstitution on the thyronine nucleus. By looking at the total energy, E_T, of the molecular system as a function of conformation, e.g. the effect of rotating one of the aryl rings about the C-O ether bond while keeping the other ring fixed, we believed we could obtain evidence on minimum energy conformations, energy barriers to rotation and conformational maps of various substituted thyronines. Furthermore, by comparing information calculated for the endogenous hormones and a highly active methylene-bridged analog, 3,5-diiodo-4-(4'-hydroxy-3'-iodo)benzyl-DL-phenylalanine (MB-T_3 see Chapter Four), with less active analogs, 3,5-diiodo-4-(3'-iodo-4'-amino)phenoxy-DL-phenylalanine (4'-NH_2) and 3,5,3'-trimethyl-L-thyronine (L-Me_3) might reveal those characteristics of the structural features which would be important in the functional role of the hormones.

QUANTUM MECHANICAL CONSIDERATIONS

Many texts[43] and reviews[44] have been written on molecular orbital theory and the approximate methods, empirical and semi-empirical, developed to cope with the complex mathematics and time-consuming calculations involved in treating molecular structure in terms of the Schroedinger equation

$$H^T\Psi = E_T\Psi \qquad (3\text{-}1)$$

where H^T is the complete hamiltonian for a system, the complete wave function and E_T, the total energy.

By assuming a molecular system of stationary nuclei the total energy is separated into two components, the electronic energy, ε, and the electrostatic internuclear repulsion energy given by $\sum_{A<B} e^2 Z_A Z_B r_{AB}^{-1}$ such that

$$E_T = \varepsilon + \sum_{A<B} e^2 Z_A Z_B r_{AB}^{-1} \qquad (3\text{-}2)$$

The evaluation of the energy for nuclear-nuclear interaction is a trivial electrostatic calculation. Evaluation of the energy due to the motion of electrons about fixed nuclei requires a modified Schroedinger equation

$$H\Psi = \varepsilon\Psi \qquad (3\text{-}3)$$

where H is the electronic hamiltonian operator and Ψ becomes the electronic wavefunction. It is with this form that molecular orbital theory is concerned.

The second version of the complete neglect of differential overlap (CNDO/2) molecular orbital method was used in these calculations. Details of this method may be found elsewhere[45], but a review of the salient features of the approach used in developing approximate self-consistent field (SCF) molecular orbital methods and, especially, CNDO/2 follows.

In SCF methods the many-electron wavefunction, Ψ, is constructed as a product of functions each of which is dependent upon the coordinates of one electron only. These one-electron functions, Ψ_i, are called orbitals and not only must they specify the spatial distribution of electrons but they must also take into account the electron properties of spin and antisymmetry.

By taking into account the spin states, α and β, of an

electron and the property of antisymmetry the wavefunction is most simply represented as a normalized Slater determinant of orthonormal spin orbitals having the form

$$\psi_1(1,2,\ldots,2n) =$$

$$\frac{1}{(2n)!}\begin{vmatrix} \psi_1(1)\alpha(1) & \psi_1(1)\beta(1) & \psi_2(1)\alpha(1) & \ldots & \psi_n(1)\beta(1) \\ \psi_1(2)\alpha(2) & \psi_1(2)\beta(2) & \ldots & & \\ \ldots & \ldots & \ldots & & \ldots \\ \psi_1(2n)\alpha(2n) & & & & \psi_n(2n)\beta(2n) \end{vmatrix} \tag{3-4}$$

which written in contracted notation is

$$\psi(1,2,\ldots,2n) = |\psi_1\bar{\psi}_1\ldots\psi_n\bar{\psi}_n| \tag{3-5}$$

where 2n is the number of electrons.

The linear variation principle makes it advantageous to represent the molecular orbitals as linear combinations of basis functions. The simplest approximation is to use atomic orbitals, ϕ_i, as the basis functions (LCAO) and represent the molecular orbitals as

$$\psi_i = c_1\phi_1 + c_2\phi_2 + \ldots = \sum c_r\phi_r \tag{3-6}$$

The variational approach to calculations of approximate solutions of the Schroedinger equation involves solving for the expectation value of the energy

$$\varepsilon = \frac{\int \Psi^* H \Psi}{\int \Psi^* \Psi} \tag{3-7}$$

The electronic hamiltonian operator is separated into two com-

ponents, the one- and two- electron terms

$$H = \sum_i H^c(i) + \sum_{i<j} e^2 r_{ij}^{-1}. \qquad (3\text{-}8)$$

$H^c(i)$, the one electron term is called the core hamiltonian. It corresponds to the motion of an electron in the field of a nucleus, A, having charge, Z_A. It consists of the kinetic energy operator for an electron and the potential energy between an electron and all atomic cores of the molecule where M is the total number of nuclei.

$$H^c(i) = \frac{-h^2}{2m}\nabla_i^2 + \sum_{A=1}^{M} \nabla_A \qquad (3\text{-}9)$$

if all electrons are specifically included in the calculation, then V_A is the nuclear-electron potential energy equal to $-Z_A e^2/r_{iA}$.

The two-electron term, $\sum e^2 r_{ij}^{-1}$, corresponds to the potential energy of repulsion between the electrons.

The evaluation of the expectation value for the electronic energy gives us

$$\varepsilon = \int \psi_i^*(1) \left[\frac{h^2}{2m}\nabla_1^2 - \sum_{A=1}^{M} Z_A e^2/r_{1A} \right] \psi_i(1) d_1 + \sum_{i,j=1}^{n} (2J_{ij} - K_{ij}) \qquad (3\text{-}10)$$

The term $\sum_{i,j=1}^{n} (2J_{ij} - K_{ij})$ arises from the treatment of the two-electron hamiltonian. J_{ij} are the coulomb integrals and give the value that the total electron-electron repulsion would be if all electrons moved independently in the orbitals to which they were assigned. They take the form

$$J_{ij} = \int\int \psi_i^2(1) \frac{e^2}{r_{12}} \psi_j^2(2) d\tau_1 d\tau_2 \qquad (3\text{-}11)$$

K_{ij} are called the exchange integrals and their effect is to reduce the energy of interaction between the electrons in different orbitals ψ_1 and ψ_2 having parallel spin. Their form is

$$K_{ij} = \int\int \psi_i(1)\psi_j(2) \frac{e^2}{r_{12}} \psi_i(2)\psi_j(1) d\tau_1 d\tau_2 \qquad (3\text{-}12)$$

Rewriting the molecular orbitals in terms of the atomic orbitals (equation 3-6) our expression for the electronic energy becomes

$$\varepsilon = \sum_{i=1}^{2n} \sum_{r,s}^{m} c_{r_i} c_{s_i} \int \phi_r(1) \left(-\frac{h^2}{2m}\nabla_1^2 - \sum_{A}^{m} \frac{z_A e^2}{r_{1A}}\right) \phi_s(1) d\tau_1$$

$$+ \sum_{i,j=1}^{n} \sum_{r,s,t,u=1}^{m} c_{r_i} c_{s_i} c_{t_j} c_{u_j} (2J_{rstu} - K_{rtsu})$$

$$= \sum_{i=1}^{2n} \sum_{r,s}^{m} c_{r_i} c_{s_i} \langle r|H_{rs}|s\rangle$$

$$+ \sum_{i,j=1}^{n} \sum_{rstu=1}^{m} c_{r_i} c_{s_i} c_{t_j} c_{u_j} [2(rs|tu) - (rt|su)] \qquad (3\text{-}13)$$

where

$$\langle r|H_{rs}|s\rangle = \phi_r(1) H^c(i) \phi_s(1) d\tau_1 \qquad (3\text{-}14)$$

$$J_{rstu} = \int\int \phi_r(1)\phi_s(1) \frac{e^2}{r_{12}} \phi_t(2)\phi_u(2) d\tau_1 d\tau_2 = (rs|tu) \qquad (3\text{-}15)$$

$$K_{rtsu} = \int\int \phi_r(1)\phi_t(1) \frac{e^2}{r_{12}} \phi_s(2)\phi_u(2) d\tau_1 d\tau_2 = (rt|su) \qquad (3\text{-}16)$$

Having now determined the proper form of the energy expression in terms of the atomic (basis) orbitals, we now use the variation principle to find the MO coefficients c_{ri} which minimize the energy.

The Hartree-Fock (SCF) method is used to minimize the energy. By setting up the condition to obtain a stationary value for the total energy, i.e. $d\varepsilon/dc_{ri} = 0$, we obtain the secular equations

$$\sum_s \{c_{s_i} \langle r|H_{rs}|s\rangle + \sum_j^{occ} \sum_{stu} c_{s_i} c_{t_i} c_{u_i} [2(rs|tu) - (rt|su)]\}$$

$$= \sum_j \varepsilon_{ij} \sum_j c_{s_i} S_{rs} \qquad (3\text{-}17)$$

where ε_{ij} are the orbital energies and S_{rs} is the overlap integral for atomic functions ϕ_r and ϕ_s,

$$S_{rs} = \int \phi_r(1) \phi_s(1) d\tau_1 \qquad (3\text{-}18)$$

Choosing the off-diagonal lagrangian multipliers to be zero, thus assuring unique specification of the molecular orbitals ($\varepsilon_{ij} = 0$ unless $i = j$), the secular equation takes the form

$$\sum (F_{rs} - \varepsilon_i S_{rs}) c_{si} = 0 \qquad (3\text{-}19)$$

where the elements of the matrix representation of the Hartree-Fock hamiltonian operator F are

$$F_{rs} = (r|H_{rs}|s) + \sum_{i=1} \sum_{tu} c_{tu} c_{ui} [2J_{rstu} - K_{rstu}]$$

$$= H_{rs} + \sum_{tu} P_{tu} [(rs|tu) - {}^1/_2(rt|su)] \qquad (3\text{-}20)$$

where

$$P_{tu} = 2 \sum_{i}^{occ} c_{ti} c_{ui} \qquad (3\text{-}21)$$

P_{tu} is the bond order or density matrix written as a summation over all occupied molecular orbitals, χ_l.

There is one equation (3-19) for each atomic orbital, ϕ_r, in the set. To find their solutions the allowed energies are first determined by equating the secular determinant to zero

$$|F_{rs} - \varepsilon_i S_{rs}| = 0 \qquad (3\text{-}22)$$

Then each energy is substituted into (3-19) to determine the appropriate set of coefficients.

Once the elements F_{rs} and the overlap integrals, S_{rs}, are known the SCF orbitals can be obtained by overcoming some difficulties. First, F_{rs} depends on the bond orders and by definition these can be calculated only when the orbitals, i.e. the solutions of (3-19) and (3-22), are known. The equations, therefore, need to be solved iteratively. A rough estimate is made of the coefficients, c_{ti} (usually from a Huckel calculation), which then allows an estimation of the bond orders and consequently, the F_{rs} integrals. The secular equations are solved giving improved values of the coefficients. The cycle of calculations is repeated until the coefficients obtained by solving the secular equations are the same as those used to construct F_{rs}, i.e. the input and output coefficients are self-consistent.

The bottlenecks in solving SCF equations lie in the evaluation of the integrals involved in F_{rs}, particularly those two electron integrals (3-15) in which the four orbitals are all on

different atomic orbitals. To get around this bottleneck the zero-differential overlap (ZDO) method was developed by Pariser and Parr[46] and Pople[47].

Returning to expression (3-17) we see that unless there is some region in space where ϕ_r and ϕ_s are also simultaneously non-zero, then $(rs|tu) = 0$. Assuming zero overlap of ϕ_r and ϕ_s is sufficient to make this integral zero and to make $S_{rs} = 0$. If we are going to make the approximation that $S_{rs} = 0$ or $S_{tu} = 0$ for orbitals on different atoms, then to be consistent we should make $(rs|tu) = 0$.

In the ZDO approximation if ϕ_r and ϕ_s are different orbitals then only one electron repulsion integral arises from the summation over r and s in (3-20) and that happens when r = t and s = u, to give the integral

$$(rr|ss) \equiv \gamma_{rs} \tag{3-23}$$

represented by the symbol, γ_{rs}. The off-diagonal elements of the F-matrix therefore have the form

$$F_{rs} = H_{rs} - \tfrac{1}{2} P_{rs}\gamma_{rs} \tag{3-24}$$

The core matrix is from (3-13)

$$H_{rs} = \int \phi_r(-h^2\nabla_1^2/2m + V_M + V_N + \sum_A{}'' V_A)\phi_s dv \tag{3-25}$$

Here the core potentials V_M and V_N of atoms M and N (the nuclear centers of dr and ds, respectively) are separated from the rest i.e. $A \neq M, N$. If we were to use a zero overlap model stringently, then this integral should also be zero. But if this is done the very essential bond forming term in molecular orbital theory is lost since H_{rs} represents the energy of attraction

of the overlap cloud (between ϕ_r and ϕ_s) for the positively charged core. We assume therefore, that there is sufficient overlap of ϕ_r and ϕ_s, if they are neighboring atoms, to give a non-zero integral. In order to have a parameter which we see as characteristic of the M-N bond we assume that the potentials of distant cores, $\sum_A'' V_A$, make a negligible contribution to integral (3-25). If we define a "resonance integral", β, as

$$\beta_{rs} = \int \phi_r (-\tfrac{1}{2}\nabla^2 + V_M + V_N)\phi_s dv \qquad (3\text{-}26)$$

then the off-diagonal matrix elements of F from (3-24) is

$$F_{rs} = \beta_{rs} - \tfrac{1}{2}P_{rs}\gamma_{rs} \qquad (3\text{-}27)$$

The diagonal elements of the F-matrix (r = s) in the ZDO approximation is

$$F_{rr} = H_{rr} + \sum_t P_{tt}(rr|tt) - \tfrac{1}{2}P_{rr}(rr|rr) \qquad (3\text{-}28)$$

By treating the core hamiltonian, H_{rr}, in a manner similar to above we obtain

$$H_{rr} = \int \phi_r (-\frac{\hbar}{2m}\nabla_1^2 = V_M + \sum_A' V_A)\phi_r dv \qquad (3\text{-}29)$$

$$= U_{rr} + \sum_A' \int \phi_r V_A \phi \, dv \qquad (3\text{-}30)$$

where $A \neq M$ and

$$U_{rr} = \int \phi_r (-\frac{\hbar}{2m}\nabla_1^2 + V_M)\phi_r dv \qquad (3\text{-}31)$$

which is the energy of the orbital, ϕ_r, for the appropriate valence state of isolated M and can be calculated from spectroscopic energies[48].

When atoms A and M are far apart the integral

$$\int \phi_r V_A \phi_R dv \equiv V_{A,rr} \tag{3-32}$$

is approximately equal to $- Z_A e^2/R_{AM}$ where $Z_A e$ is the net charge of atomic core A. Similarly, the two-center electron repulsion integrals are dependent on R_{AM} at large separation of the two atoms.

$$\gamma_{rt} = (rr|tt) = e^2/R_{AM} \tag{3-33}$$

where orbital ϕ_A is on atom A. Therefore,

$$V_{A,rr} = - f(R) Z_A \gamma_{rt} \tag{3-34}$$

where f(R), the penetration function, allows for deviation of $V_{A,rr}$ and γ_{rt} from R_{AM}^{-1} at small R_{AM}. For most ZDO theories the penetration integral is ignored, i.e. f(R) = 1.

Substituting (3-30) and (3-34) into (3-28) gives

$$F_{rr} = U_{rr} + \tfrac{1}{2} P_{rr}\gamma_{rr} + \sum_{t \neq r} (P_{tt} - Z_A)\gamma_{rt} \tag{3-35}$$

and expressions (3-27) and (3-35) define the elements of the SCFZDO method.

The SCF approximation in which all integrals (rs|tu) are neglected (unless r = s and t = u) is called CNDO. However, to insure that the conditions of rotational and hybridization invariance hold, i.e. calculated energies and electron distribution remain the same no matter how the coordinate axes are chosen we require that integrals like (rr|tt) are the same for all valence orbitals r on atom M and t on atom A and this integral is

$$(rr|tt) = \gamma_{AM} \tag{3-36}$$

which is the average electronic repulsion between any electron on A and any electron on M.

In the CNDO method the resonance integrals, β_{rs}, are taken to be proportional to the overlap integrals, S_{rs} (the Mulliken approximation[49]).

$$\beta_{rs} = \beta^{0}_{AM} S_{rs} \tag{3-37}$$

this semiemprical approach satisfies the invarience conditions and we obtain a new constant β^{0}_{AM}, which depends only on the nature of atoms A and M.

The expressions for the F-matrix elements in CNDO approximation have a similar form as that of the ZDO method. From expression (3-27) we have

$$F_{rs} = \beta^{0}_{MN} S_{rs} - \tfrac{1}{2} P_{rs} \gamma_{MN}) \tag{3-38}$$

For the diagonal elements we start from expression (3-28) noting however the orbital ϕ_t may be on the same atom as ϕ_r or on a different atom ($A \neq M$)

$$F_{rr} = H_{rr} + \tfrac{1}{2} P_{rr} \gamma_{rr} + \sum_{t(M)} P_{tt} \gamma_{rt} + \sum'_{t(A)} P_{tt} \gamma_{rt} \tag{3-39}$$

Since γ_{rt} depends only on the nature of the two atoms, we combine together the sums over t on the same atom and introduce a net atom charge by

$$\sum_{t(A)} P_{tt} = P_{AA} \qquad (3\text{-}40)$$

The core matrix can also be split as in (3-30) to give the final expression

$$F_{rr} = U_{rr} - \tfrac{1}{2} P_{rr}\gamma_{rr} + P_{MM}\gamma_{MM} + \sum_{A}{}' (P_{AA}\gamma_{MA} - V_{MA}) \qquad (3\text{-}41)$$

In the first version of CNDO[45a] the integrals V_{MA} and $_{MA}$ were evaluated separately using Slater orbitals. Two modifications were added later[45b]. The first dealt with the evaluation of U_{rr} (see next section) and the second was to use (3-41) with zero penetration so that (3-41) was a form similar to (3-35)

$$F_{rr} = U_{rr} - \tfrac{1}{2} P_{rr}\gamma_{MM} + \sum_{A}{}' (P_{AA} - Z_A)\gamma_{MA} \qquad (3\text{-}42)$$

where $V_{MA} = Z_A\gamma_{MA}$. (3-43)

Once a set of CNDO coefficients, c_{ri} and a corresponding density matrix, P_{rs}, have been obtained, the total energy can be found from

$$\varepsilon_T = \tfrac{1}{2}\sum_{rs} P_{rs}(H^c_{rs} + F_{rs}) + \sum_{A<M} Z_A Z_M / R_{AM} \qquad (3\text{-}44)$$

using appropriate expressions for H_{rs} and F_{rs}.

CHOICE OF PARAMETERS FOR CNDO/2

A full specification of a CNDO/2 calculations requires values for the overlap integrals, S_{rs}, the core hamiltonian elements, U_{rr}, the electron repulsion integrals, γ_{MA}, and the bonding parameters, β^0_{MA}. The evaluation of the integrals U_{rr}

and γ_{AA} represent the critical stage in parameterization since these terms determine the energy levels of the separate atoms and the molecular energies will not be of the right order of magnitude unless the atomic energies are approximately correct.

The integral U_{rr} represents the energy of a single electron occupying an orbital, ϕ_r, in the field of the core; it is usually estimated from atomic spectroscopic data. In molecular orbital theory we wish to account satisfactorily for the tendency of an atomic orbital to both gain and lose electrons. In CNDO/2 the procedure adopted was to take the mean of the ionization potential, I_r, and electron affinity, A_r, according to the expression

$$U_{rr} = -\tfrac{1}{2}(I_r + A_r) - (Z_M - \tfrac{1}{2})\gamma_{MM} \qquad (3\text{-}45)$$

where Z_M is the core charge of atom M.

Using expressions (3-38), (3-41) and (3-45), the basic expression for the F-matrix are written

$$F_{rr} = -\tfrac{1}{2}(I_u - A_u) + [(P_{MM} - Z_M) - \tfrac{1}{2}(P_{rr} - 1)]\gamma_{MN} + \sum_{A\neq M}(P_{AA} - Z_A)\gamma_{MA} \qquad (3\text{-}46)$$

$$F_{rs} = \beta^0_{MN} S_{rs} - \tfrac{1}{2} P_{rs}\gamma_{MA} \qquad (3\text{-}47)$$

For the case where all atoms are neutral ($P_{MM} = Z_M$; $P_{AA} = Z_A$) and orbital ϕ_r contains one electron ($P_{rr} = 1$) the diagonal element F_{rr} reduces to

$$F_{rr} = -\tfrac{1}{2}(I_r + A_r) \qquad (3\text{-}48)$$

showing the relationship of F_{rr} to the Mulliken electronegativity for the element.

The bonding parameters β^0_{MA}, occurring in the resonance integrals are taken to be the average of constants β^0_M and β^0_A which depend only on the nature of atoms M and A respectively.

The overlap integrals, S_{rs}, are evaluated explicitly using Slater-type atomic orbitals. Details may be found in reference 43a, appendix B. The electron repulsion integrals, γ_{MA}, representing the average interaction between electrons in valence atomic orbitals on atoms M and A can be calculated by a variety of empirical relationships[43b] or as the two-center coulomb integral involving valence s-functions[43a].

Standard molecular geometries (bond length, bond angles and dihedral angles) were used except as noted[50]. A computer program[51] was employed to construct the three-dimensional molecule given bond lengths, bond angles and dihedral angles. The output was put directly as input to the CNDO/2 program[45c].

For diphenyl ethers the energy was considered as a function of two torsional angles (Figure 3-1): $\phi_1(R_1\text{-}C_2\text{-}C_{11}\text{-}O_{12})$ and $\phi_2(O_{12}\text{-}C_{13}\text{-}C_{14}\text{-}H_{15})$. [The torsion angle ϕ(A-B-C-D) between the bonded atoms A-B-C-D represents the angle between the planes ABC and BCD. Viewed from the direction of A, positive rotations of ϕ are clockwise and negative rotations are counterclockwise, the far end rotating with respect to the near end. The value $\phi = 0^0$ corresponds to the cis-planar arrangement of the bonds AB and CD].

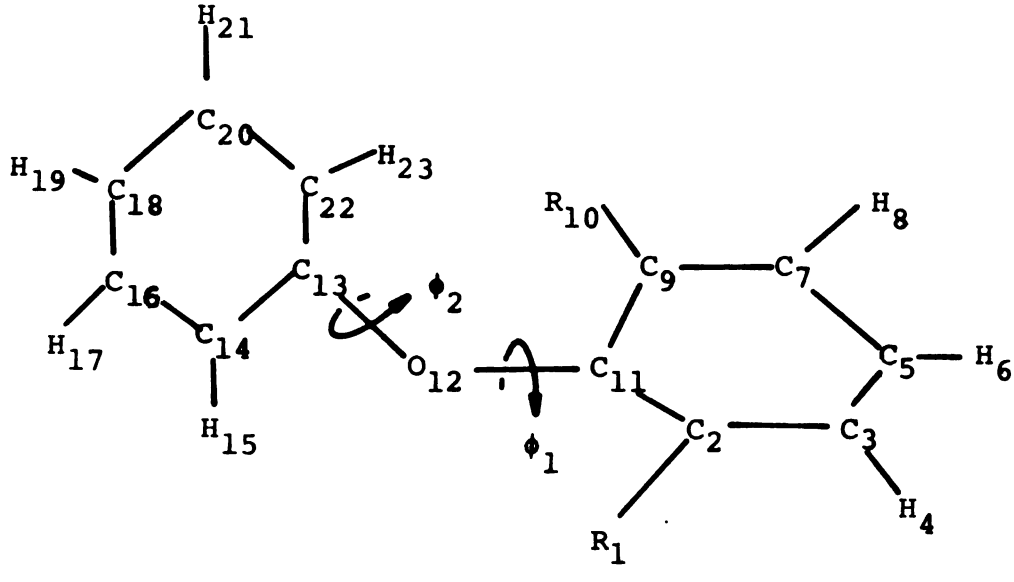


Figure 3-1. Torsional angles ϕ_1 and ϕ_2.

PARAMETERIZATION OF THE HALOGENS (F, Cl, Br, I)

When extending the CNDO methods to heavier atoms it seems intuitively obvious that a satisfactory description of the valence electronic structure would require at least d-atomic orbitals in the basis set of Slater orbitals. Deb and Coulson however formulated a treatment for the interhalogens wherein they used Slater-type s- and p- nodeless valence orbitals of quantum no. n = 3[52]. We adopted their procedure for choosing the orbital exponent ζ, as an arithmetic mean of ζ-values which fit the SCF values of the mean radial distance <r> for neutral atomic valence s and p orbitals respectively. However, we chose nodeless s- and p- orbitals of quantum no. n = 2 to represent the AO's of the halogens. The valence state ionization potentials and electron affinities, core matrix elements and bonding parameters, β_A^0, were taken directly from their work.

These approximations are clearly quite drastic. To determine whether it was worthwhile proceeding further we examined calculations on model systems and these results are tabulated in Table 3-1.

The first system we examined was CH_3-X. A search for a minimum energy C-I bond length led to a prediction of a bond distance of 2.08Å[53]. A similar geometry search in iodobenzene found R_{C-I}= 2.09Å, compared to the experimental R_{C-I} of 2.08Å[53]. The dipole moments predicted for these compounds are not so close to the experimental value, but the atomic populations on the various halogens reflect the smaller polarity of the C-X bond as we go down the periodic table.

Pauling's van der Waals radius for iodine[54] is 2.15 Å so

Table 3-1 Calibration Calculations

A. Halogen Parameters
$\frac{1}{2}(U_s + I_s)$, $\frac{1}{2}(U_p + U_p)$

X	β,eV	eV	eV	ζ
F	39.0	31.88	12.18	2.40
Cl	24.1	19.24	9.38	1.47
Br	21.5	18.28	8.40	1.30
I	18.0	15.69	8.10	1.09

B. Geometry Searches for "Best"
Carbon-Halogen Bond Distances

Compound	$R(C-X)_{calcd}$,Å	$R(C-X)_{exptl}$Å
H_3C-F	1.365	1.3315
H_3C-Cl	1.685	1.670
H_3C-Br	1.83	1.86
H_3C-I	2.080	2.14
Ph-I	2.086	2.09

C. Dipole Moments and Charge Distribution

Compound	μ_{calcd}, D	μ_{exptl}, D	Atomic pop on halogen
CH_2F	1.62	1.808	9.192
CH_3Cl	2.01	1.86	17.079
CH_3Br	2.07	1.78	35.051
CH_3I	2.50	1.64	53.044
C_2H_5F(staggered)	1.92	1.96	9.217
C_2H_5Cl	2.25	2.04	17.109
C_2H_5Br	2.24	2.03	35.077
C_2H_5I	2.57	1.90	53.061
PhI	2.72	1.70	53.067

Table 3-1 continued

D. Rotational Barriers-C_2H_5X

$(E_{eclipsd} - E_{staggered})$ (kcal/mol)

X	ΔE_{calcd}	ΔE_{exptl}
H	1.76	2.75
F	2.26	3.30
Cl	3.45	3.56
Br	3.70	3.57
I	4.26	3.2 ±0.5

E. Orbital Energies (in eV)

	Calculated HOMO energy	Lowest $\pi \rightarrow \pi^*$ transition (exptl)
PhH	13.6	7.76
PhOH	12.9	7.60
PhI	11.7	7.55

if we get much inside this value, we expect repulsion. Bringing a methane and a methyl iodide molecule together in a linear fashion [$H_3C...H-CH_3$], one finds a repulsion of 20 kcal/mole ar R_{I-H}= 2 Å. This shows that we should get a reasonable representation of van der Waals repulsion from our iodine containing compounds.

Finally, we have examined the rotational barrier in substituted ethanes (C_2H_5-X: X = H, F, Cl, Br, and I) and these results are presented in Table 3-1 (D). The calculated results are in reasonable agreement with the experimental[55] and give us some confidence that the rotational barriers we calculate for the thyronine system will be reasonable. It may be however, that the mechanism for the barrier in the two systems (ethanes and diphenyls) is quite different; thus, our reasonable success with the model calculations should not make us overconfident of the quantitative accuracy of our thyronine results.

RESULTS AND DISCUSSION

Before proceeding with calculations on the thyroxine analogs, we examined some model diphenyl ether systems, since the rotational properties of these will probably be similar to those observed in the thyronine system itself. The results of these calculations are summarized in Table 3-2. For diphenyl ether itself a 90^0, 90^0 ($\phi_1 = 90^0$ and $\phi_2 = 90^0$) conformation was calculated to be the lowest in energy. This result is not in agreement with the experimental results of Katon, et al[56] who found that a skew conformation, with only one plane of symmetry, was the preferred structure. However, the energy differ-

Table 3-2

Rotational Energetics (kcal/mol) of Disubstituted Diphenyl Ethers as a function of ϕ_1 and ϕ_2

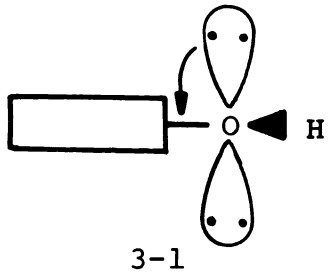
ϕ_2	ϕ_1 0°	30°	60°	90°	45°
			R = H		
0	-	1.13	1.82	0.73	
30	1.13	1.58	0.72	0.42	
60	1.77	0.77	0.33	0.21	
90	0.62	0.34	0.27	0.0	
			R = F		
0	-	4.34	2.20	1.35	
30	182	1.28	1.03	0.80	
60	1.89	0.44	0.58	0.66	
90	1.11	0	0.65	0.47	
			R = Cl		
0	-	-	3.38	0.13	
30	-	20.3	0	0.48	
60	-	10.0	0.21	1.73	
90	36.4	24.4	15.5	0.38	
			R = Br		
0	-	-	5.91	0	
30	-	42.2	1.06	0.91	
60	161	25.8	2.26	4.16	
90	78.5	71.0	40.5	3.34	
			R = I		
0	-	-	14.4	0	
30	-	106	5.1	2.06	
60	-	76.5	12.0	11.9	
90	216	237	115	15.5	
			R = CH_3		
0	-	-	-	0	6.45
30	-	-	0.30	0.43	6.1
45	-	-	0.05	22.9	3.42
60	-	-	0.1	6.05	0.3
90	39.8	-	2.8	10.3	23.8

ences are quite small, and it has been well documented[43a,44a] that CNDO/2 underestimates repulsion effects. If repulsion were not underestimated, we might expect some deviation from the 90^0, 90^0 conformation in order to relieve H-H repulsion. The 90^0, 0^0 conformation is the one that would be expected on steric grounds for very bulky R groups and our calculations do find this conformation to be the minimum energy for R = I and R = Br, with the rotational barrier in the iodine compound calculated to be 15.5 kcal/mole. For the chlorine-substituted compound the calculations find a minimum energy near 60^0, 30^0. The energies of the fluorine compound conformation map look somewhat like those for the H compound, but the minimum energy occurs for a skew (near 30^0, 90^0) conformation.

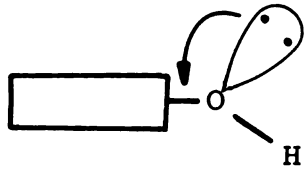
The R = Me compound appears to have a minimum energy near 90^0, 0^0 but the energies for some of the skew conformations are very similar to this and it is likely that the absolute minimum occurs for a skew conformation near 60^0, 45^0. In any case the conformation map for the compound R = Me looks more like Cl than I, Br, F or H. Interestingly enough, this is consistent with the hypothesis that the relative biological activities of the 3,5- I, Br, Cl, F, H and CH_3 compounds are determined by the minimum energy conformations.

In addition to the steric effect imposed upon the diphenyl ether system by ortho substituents, the effect of the <u>para</u> hydroxy group on the rotation of the aromatic ring about the C-O bond was also examined. Radom, <u>et al.</u>[57] studied the effect of <u>para</u> substituents on the rotational barrier of the C-O bond of phenol using <u>ab initio</u> molecular orbital calculations. They

found that phenol prefers to exist in a planar conformation 3-1 as opposed to the non-planar rotamer 3-2.



3-1



3-2

This can be attributed to the stabilization of 3-1 by delocalization of the p-type lone pair of oxygen. Electron donating groups, such as OH, in the para position are observed to decrease the rotational barrier for moving the hydrogen from inplane (3-1) to perpendicular (3-2). This can be rationalized as due to the electron donation of the two groups opposing each other, thus decreasing the double-bond character of the C-O bond. Using CNDO/2 we obtained similar results to reference 26 for phenol and its para-hydroxy homolog. However, our calculated barriers for phenol (1.55 kcal/mole) and dihydroxybenzene (1.40 kcal/mole) are considerably underestimated when compared to the experimental values and the ab initio calculated barriers (Table 3-3).

Turning our attention to the effect of the para-hydroxy group on the rotational barrier of diphenyl ethers, we obtain results which can be similarly rationalized. The 90^0, 90^0 conformation is preferred in both diphenyl ether and 4-hydroxy diphenyl ether. We find that it is 0.2 kcal/mole easier to rotate the phenol bearing ring to the perpendicular conformation

Table 3-3
Rotational Energy Barriers (kcal/mol)

	CNDO/2	ab initio	Exptl
Phenol	1.55	5.16	3.56
Dihydroxyphenol	1.40	4.21	2.69

than it is to rotate the unsubstituted ring to a similar perpendicular arrangement. This is theoretically what one would predict since in the $\phi_1 = 90^0$ and $\phi_2 = 0^0$ conformation the phenolic OH and ether oxygen would be opposing each other (the p-type lone pair of oxygens competing for delocalization), decreasing the C-O double bond character and causing a lower rotational barrier.

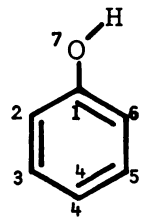
However, it can be clearly seen from the above barriers in Tables 3-2 and 3-3 that the effect of the hydroxy group on the C-O rotational barrier is small when compared to the steric effect of bulky ortho substituents.

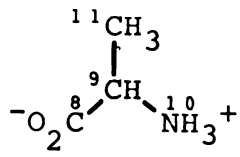
Kier and Hoyland used Extended Hückel Theory (EHT) to study the rotational barrier on 3,5,3'-trisubstituted thyronines[58]. They rationalized leaving out the alanine side chain in their calculations on the rotational barriers, and we agree with their argument that the alanine group is too far away from the ether linkage to significantly affect the rotational barrier. We have also carried out calculations to show that even the zwitterionic form of alanine in the gas phase has only a small effect on the electronic structure of the phenolic ring to which it is attached. The results of calculations comparing the Mulliken populations and orbital energies of alanine, phenol and the two bonded together (tyrosine in its extended conformation) are summarized in Table 3-4.

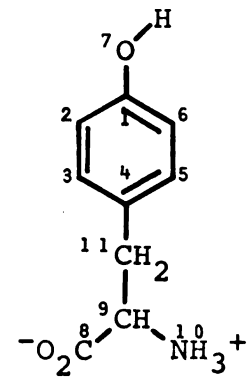
Table 3-5 presents the calculations of the rotational barrier on T_3 and T_4 analogs leaving out the alanine side chain. When we compare the rotational barrier of the phenol bearing ring in T_3 (15.1 kcal/mole) and T_4 (15.2 kcal/mole) we find

Table 3-4

Mulliken Populations and Orbital Energies of Alanine, Phenol and Tyrosine







	Phenol	Alanine	Tyrosine
	Atomic Populations on Atoms		
1	5.84		5.85
2	6.04		6.03
3	5.98		5.99
4	6.01		5.99
5	5.98		6.01
6	6.03		6.02
7	8.25		8.25
8		5.63	5.63
9		5.99	5.99
10		6.99	7.00
11		6.02	6.00
	Highest Occupied Orbital Energies		
1	-0.5221(π)		-0.5230(π)
2	-0.5408(n)		-0.5364(n)
3	-0.4762(π)		-0.4768(π)
4		-0.3922	-0.3907
5		-0.3588	-0.3555
6		-0.3383	-0.3378

Table 3-5
Rotational Barriers of Thyroid Hormone Analogs (kcal/mol)

ϕ_1	ϕ_2	T_3	$T_3(\theta=122^0)$	MB-T_3	T_4	Me_3	4'-NH_2-T_3	4'-F-T_3
90	0	0	0	0	0	0	0	0
90	90	15.1	11.0	10.1	15.2			
90	180	0.2	0.2	0.1	0	0.03	0.1	0.1
84	19		0.3					
84	199		1.0					

very nearly the same value as that for 2,6-diiodophenyl phenyl ether (15.5 kcal/mole). The small differences of 0.4 to 0.3 kcal/mole less energy needed for rotation in T_3 and T_4 than the model compound can probably be attributed to the presence of the para-hydroxy group and somewhat, perhaps, to the meta-iodo substituents. But the most important hindrance to rotation is the presence of the ortho iodine atoms.

Our calculations on the diphenyl ether diiodide compound (Table 3-2) cause us to conclude that the conformation proposed by Lehmann[14] to form the intra-molecular π-complex (37^0, 37^0) is far too high in energy even when one considers dispersion attractions not included in SCF-level calculations.

Recently, a number of 3,5,3'-triiodo diphenyl ethers have been studied by x-ray crystallography[42] and the ϕ_1 and ϕ_2 angles of a number of T_3 analogs determined. The largest deviation from the 90^0, o^0 conformation was the 84^0, 19^0 conformation observed for 3,5-diiodo-L-thyronine-N-methylacetamide. There was also a considerable difference in the C-O-C angle observed by x-ray in these T_3 analogs and the standard angle we chose (108^0), so we repeated our barrier calculations on T_3 with the C-O-C angle 122^0.

As expected, this had very little effect on the relative proximal and distal energies but lowered the rotational barrier from 15.2 to 11.0 kcal/mole. We also examined the energy of the 84^0, 19^0 and 84^0, 199^0 conformations and the energy of the 84^0, 19^0 conformation was only 0.3kcal/mole higher than the 90^0, 0^0.

Since the methylene-bridged analog of triiodothyronine,

DL-MB-T_3, showed such a high biological activity, we thought it would be informative to study the effect of the methylene bridge on the rotational barrier. The calculated value of 10.7 kcal/mole comes very close to the value of T_3 where $\theta = 122^0$ The 2'-H, 3-I distance at the top of the barrier is 2.57 Å in the O-bridged and 2.48 Å in the CH_2-bridged case.

It is evident that substituents ortho to the atom or group bridging the aryl rings is the major contributor toward inhibiting rotation. Therefore, to a first approximation one could use the rotational barrier calculated for the corresponding disubstituted diphenyl ether as a measure of the rotational barrier of the thyroid hormone analog, e.g. 3,5,3'-trimethylthyronine could be approximated by the 3,5-dimethylphenyl ether in Table 3-2 (both have barriers of 10.3 kcal/mole).

Since iodine has a relatively large 5s and 5p energy separation, we examined the effect of the barrier of varying the orbital exponent in our iodine functions to reproduce <r> for the Hartree-Fock 5p function, rather than reproducing the average <r> for the 5s and 5p functions. This led to a choice of orbital exponent of 1.0 instead of 1.09, and CNDO/2 calculations employing this exponent (1.0) predict a barrier of 18.8 kcal/mole barrier predicted by the calculations using an exponent of 1.09 (θ_{C-O-C}= 122 in both cases). These functions are compared with the Herman-Skillman[59] Hartree-Fock iodine 5p orbital in Figure 3-2. As one can see the function with $\zeta = 1.00$ more accurately reproduces the maximum in the accurate SCF orbital, but both functions die off too slowly at a long distance from the nucleus. This will cause the calculated barrier to rotation

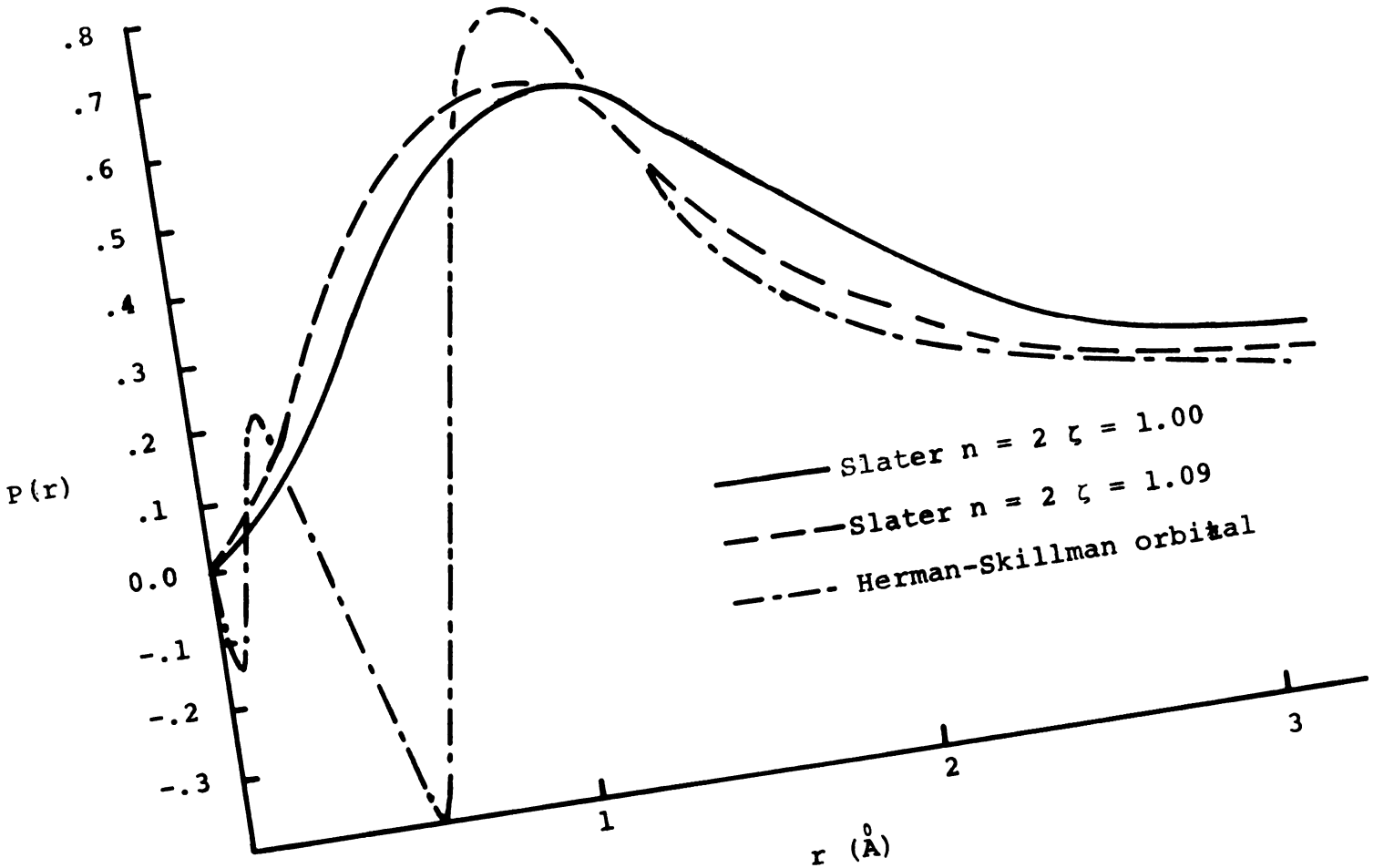


Figure 3-2. Iodine 5p orbitals.

to be too large, but the use of a fixed geometry during rotation and the CNDO/2 tendency to underestimate repulsions[43a] makes a more precise estimate of the accuracy of the calculated rotational barrier difficult. Clearly, the size of the barrier is sensitive to choice of orbital exponent, but ζ = 1.09 gives a better representation of the long range behavior of the H-S function[59] than does the ζ = 1.00 function.

The atomic populations of the atoms in T_3, Me_3 and DL-MB-T_3 (all in the distal orientation) are presented in Figure 3-3. The atomic populations for the proximal structure are similar. As one can see, the presence or absence of an O-bridge or I does not seem to have a drastic effect on the electronic structure. 4'-NH_2 and 4'-F substitutions (not shown in the figure) have a neglible effect on the electron densities at all positions except $C_{4'}$. Iodine containing rings appear to be better electron donors than those without (see Table 3-6), but 4'-NH_2 substitution also helps raise the orbital energies of the T_3 analogs. The distal and proximal conformations have very similar highest occupied molecular orbital energies, but in the 90°, 90° conformation of T_3 the top three occupied orbital energies are raised to - 0.4232, - 0.4103 - 0.4008.

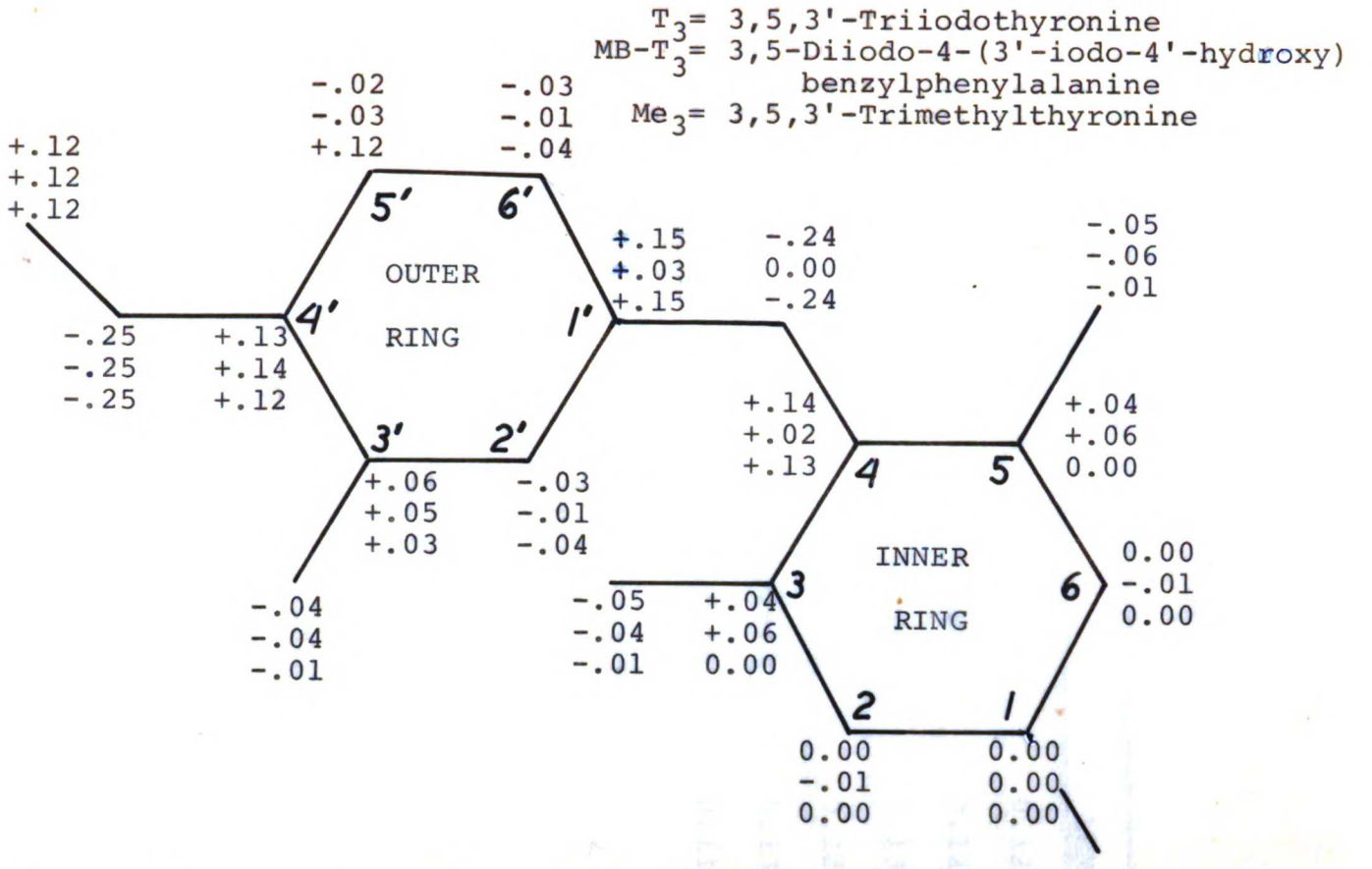


Figure 3-3. Atomic population difference of thyroid hormone analogs.

Table 3-6
Orbital Energies for T_3 Analogs
($\phi_1 = 90^0$ and $\phi_2 = 0^0$)

	HOMO			LEMO
T_4	-0.4340	-0.4288	-0.4184	0.1227
T_3	-0.4344	-0.4306	-0.4166	0.1251
MB-T_3	-0.4311	-0.4258	-0.4162	0.1314
Me_3	-0.4692	-0.4539	-0.4360	0.1254
4'-NH_3	-0.4295	-0.4241	-0.4073	0.1306
4'-F	-0.4362	-0.4319	-0.4238	0.1197

SUMMARY AND CONCLUSIONS

These calculations and the biological activity of the 3,5,3'-trialkylated thyronines and methylene-bridged analogs have served to reemphasize the steric specificity of thyromimetic agents, i.e., a semirigid structure of two mutually perpendicular aromatic systems insulated from one another by an appropriate bridge. Not yet totally resolved is the functional role of substituents located at the various positions of the aromatic rings. As has been suggested[60] aliphatic substitution in the 1-position is probably critical both in terms of binding at receptor sites and for their contribution toward pharmacodynamic properties such as movement through membranes and transport properties, since the L-amino acid is several times as potent as the D isomer. Our studies indicate that whatever effect changing the side chain has on the biological activity is due to the intrinsic properties of the side chain and not due to any effect the side chain has on the rings.

Role of 3,5-Substitution in the Biological Activity.

These calculations have given us a more precise picture of the conformational map of thyroxine analogs than has previously existed. The size of the barrier decreases as the size of $R_3 = R_5$ decreases in the order I > Br > CH_3 > Cl > F > H with the latter two having minimum energy conformations significantly different from 90^0, 0^0 or 90^0, 180^0 found in the iodine calculations. As stated previously, the biological activity follows this order, which is support for the importance of conformational fit with the receptor in determining biological activity . Another important factor in biological activity

may also be "dispersion-force" binding of the 3,5 groups to points on the receptor, which would be expected to be in the order I > Br > Cl > CH_3 > F > H.

The lack of biological activity of the $R_3 = R_5$ = F or H can be rationalized on the basis of the significantly different minimum energy conformation found for these molecules; thus they would not fit properly into the hypothesized thyroxine receptor. One can understand the biological activity of the methylene bridged analogs on the basis of the fact that one would expect their conformational profiles to be very similar to O compounds. On this basis, NH-bridged compounds would have similar activity, but S-bridged compounds might lose activity faster as $R_3 = R_5$ became less bulky, since a sulfur group could keep the rings further apart.

One would like to be able to rationalize the inactivity of the $R_3 = R_5$= isopropyl derivative[5b] since one would expect it to have a similar conformational map (with methyls pointing away from the outer ring) to the $R_3 = R_5 = CH_3$ derivative. There are two possible simple explanations for this: one is that the isopropyl groups, when pointing up toward the outer ring, prevent the rotation of the outer ring and perhaps "lock" the outer ring into the proximal orientation; the other obvious explanation is that the isopropyl groups are two bulky to fit the appropriate receptor site for the inner ring and thus prevent the thyronine from biological activity. One can distinguish between these two possibilities by carrying out biological studies with 3,5-diisopropyl-2',3'-dimethyl-L-thyronine, since this molecule should be locked into the outer ring distal

conformation and its inactivity could only be rationalized with the second explanation.

It is clear that substituents placed in the 3,5 positions play a major role in determining geometric orientation of the two aryl groups, but do they perform their function well? The shape of these groups appear to be highly critical since groups such as I, Br, and CH_3 which are about the same size and which are coplanar to the aromatic ring are active, whereas groups such as isopropyl or sec-butyl which are bulkier and lack coplanarity are inactive.

Proximal (3') and Distal (5') Substitutions

Our calculations clearly show that the proximal and distal T_3 analogs are of nearly equal energy and thus that small perturbations, such as effect of the amino side chain, solvent effects or interaction with the biological receptor can affect this equilibrium. For $R_3 = R_5 = I$ the x-ray structures on the biphenyl ethers find small deviations from 90^0, 0^0 or 90^0, 180^0 conformations, but these can be rationalized either on the basis of solvent effects or some small relief of repulsion (see $R_3 = R_5 = Cl$ conformational profile, Table 3-2).

Having a methyl group in the 2'-position raises the rotational barrier of the phenolic ring about the ether C-O bond to 37 kcal/mole. This is further proof that the distal and proximal conformations can be selectively isolated and gives added support to the finding[17b,39] that the distal conformation is the biologically active species.

Substitution in the 3'-position has been correlated[3] with the distributive properties of the substituents as given by the

Hansch π parameter. Apparently, the primary contribution of groups in this position is toward transport and distribution.

It has been noted that 3',5' disubstitution leads to less active compounds; from this general observation it has been proposed[61] that substituents in the 5' position sterically block the molecule from entering the receptor site. More will be said of this in Chapter Four.

The hormonal response is mediated by the binding and transport of T_4 and T_3 to thyroxine binding globulin (TBG), and this has been attributed to the greater ionization of the T_4 phenolic hydroxyl at physiologic pH 7.4[62]. Schussler[40] has pointed out that the preference of the TBG binding results as indicating that the proximal conformer is 0.85 kcal/mole more stable than the distal conformer, which is qualitatively consistent with the proximal-distal energy difference we calculate. The neglect of dispersion forces in our calculations, and the influence of the binding protein on the relative energies of the two conformations, make a more precise comparison difficult.

4' Substitution.

A free phenolic group or the functionally equivalent amino group at the 4' position has been postulated as essential for activity. Our calculations with a 4'-fluoro analog and comparisons of its effect on atomic populations with the 4'-hydroxy and 4'-amino analog show that, except for the hydrogen of OH and NH_2, the O, N and F have the same relative effect on the electron density of neighboring groups. It is critical to test the 4'-F analog to establish the necessity of the phenolic OH, since the result would have important implications as to the

functionality of the 4' group. A 4'-F would also be less susceptible to metabolic attack than 4'-OH or 4'-NH_2.

Other Positions on the Rings.

Substitution at the 2,6 position has not been systematically studied but has the possibility of revealing further details about the nature of the receptor site[63]. It would be interesting to examine the effect of small hydrophobic, e.g. CH_3 or polar groups (F) substitutions at these positions.

Substitution at the 2'(6') position has been used to lock the thyronine nucleus into either proximal or distal conformations. Groups as large as iPr do not diminish the biological activity; thus, one might profitably place even larger groups at 2' (with or without hydrophilic tails) to examine the limits of bulk and polarity for 2' substitution.

Future Physical Studies.

A number of NMR experiments suggest themselves from these studies. One might examine the temperature dependence of the proton or the C^{13} at the 2' outer ring position as a function of the $R_3 = R_5$ to see if one could determine quantitatively the rotational barrier of at least one of the 3,5-disubstituted diphenyl ethers. This would be a calibration point for the barriers in the other 3,5-disubstituted molecules. Relative peak heights for the 6'-H when it is proximal and distal should enable one to determine more precisely the energy differences between these two conformations.

Implications for CNDO/2 - MO Studies.

These studies indicate that one can reproduce reasonable qualitative features of group VII atoms with a simple CNDO/2

basis set of only 2s- and 2p- like functions. There are hopes that this procedure may extend itself to other heavy non-transition elements as well.

CHAPTER FOUR: BIOLOGICAL ACTIVITY OF SOME THYROID HORMONE ANALOGS

The multiplicity of physiological effects caused by the thyroid hormones has given rise to a variety of assay systems which may be used to assess the potency of the hormones or their analogs[8]. However, in terms of frequency of use and availability of data three bioassay procedures are principally employed: (1) the amphibian metamorphasis test; (2) the measurement of metabolic effects on thyroidectomized animals; and (3) the goiter-prevention assay.

The amphibian metamorphosis assay is based on the effect of the thyroid hormones on the growth and differentiation of lower vertebrates. The hormones accelerate the metamorphosis of tadpoles in a logarithmic relationship to the dose administered. The biological responses are usually measurements of anatomical changes such as tail size or the appearance of limb-buds. More recently, Derby[64] has quantitated the resorption or shrinkage of pieces of disks of tadpole dorsal tail fins cultured in varying concentrations of thyroxine. By measuring the amount a disc shrinks per day he obtains an accurate dose-response relationship between percent shrinkage per day and

concentration (levels of from 3 to 750 parts per billion of T_4). Frieden has adapted this technique to studies of analogs[65]. In addition Frieden also measures the effect of the hormones and analogs by studying the changes in nitrogen metabolism as metamorphosis progresses[66]. As the tadpole shifts from an aquatic to a terrestrial habitat, there is a parallel transition from ammonotelism to ureotelism. All the activities of enzymes of the urea cycle are significantly increased during normal or induced metamorphosis and a correlation can be made between the degree of metamorphosis and urea metabolism.

It is well established that the thyroid hormones elevate basal metabolic rate. Measurements of the ability of thyromimetic agents to stimulate oxygen consumption reflect their potency on peripheral metabolism. The effect on heart rate and the growth of certain organs generally parallel the effect on basal metabolic rate and these parameters are usually measured simultaneously or following the measurement of oxygen consumption. By using thyroidectomized rats, indirect effects such as might be produced when analogs displace hormones from plasma protein binding sites or if analogs protect hormones from metabolism are eliminated.

Because of the technical ease and the expertise developed over many years in this laboratory, this study utilizes the goiter-prevention assay as a measure of hormonal activity. It is based on the ability of circulating levels of thyroid hormones via a physiological feedback mechanism to supress both the pituitary secretion of thyrotropin (TSH) and the hypothalamic secretion of thyrotropin releasing hormone (TRH). This

method has been shown to be sensitive and reproducible and results with it correlate quite well with results from the tadpole metamorphosis assay and oxygen consumption test.

Spangenberg has recently shown that an invertebrate organism also responds to physiological levels of thyroxine[67]. She finds that strobilation in jellyfish (Aurelia) may be induced by thyroxine as well as by iodide ion and iodinated tyrosines. Strobilation is the process through which jellyfish scyphistomae (polyps) first lose their tenacles, then form segments which ultimately metamorphose to give rise to young medusae (jellyfish). (Figure 4-1)

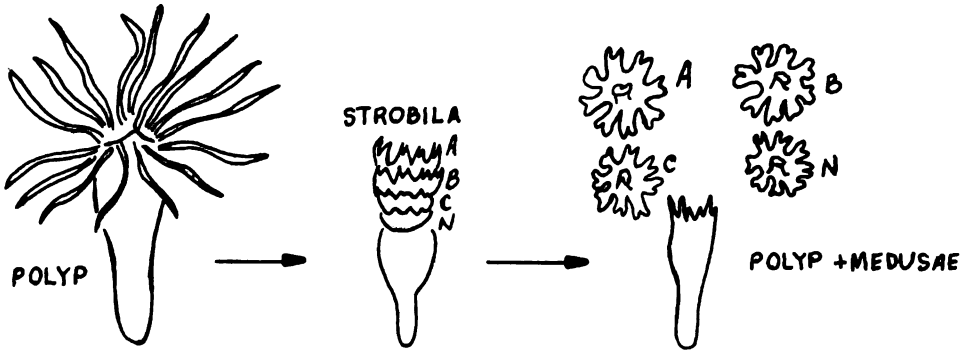


Figure 4-1

This segmentation process sets the metamorphosis of jellyfish apart from metamorphosis of higher animals in that segmentation leads to numerous new organisms, a feat not generally possible in higher organisms. Induction of strobilation by thyroxine represents the first clear-cut morphological response of a coelenterate to a hormone of known biological significance in higher organisms. It has important implications not only in studying the evolutionary nature of such metamorphic processes, but could also lead to valuable information regarding growth and differentiation both in lower and higher organisms.

In vivo studies are important and necessary tools in evaluating the potency of the hormones and analogs on entire organisms. But they can tell us nothing at all of the process occurring at the cellular, subcellular or molecular levels. If one could devise an *in vitro* system to study the effects of the thyroid hormones, it would offer significant advantages in studying the mechanism of action of T_4 and T_3 at the molecular levels. Until now such an invitro system has eluded investigators. Studies utilizing the thyroid hormones and their analogs to induce biological effects in subcellular components such as mitochodria or enzymes have required hormonal concentrations ranging from 10^4 to 10^6 times the physiological concentration of the free hormones[68]. Such effects are more often than not pharmacological or toxicological in nature.

Samuels[69] has recently described a cell-culture system responsive to physiological concentrations of thyroid hormones. Employing cells from a rat pituitary tumor cell line which are cultured in a medium containing serum from a thyroidectomized calf, they obtain dose-response relationships for a variety of thyronine derivatives which indicated a specificity of response similar to that observed *in vivo*. Measuring glucose utilization rates, which they assume to parallel rates of cell growth, they find that half-minimal responses are induced by free hormone concentrations of 10^{-12} M T_3 and 10^{-10} M T_4.

Goldfine, *et al.*[70] also studied the effects of thyroid hormones and analogs on a cell-culture system. They used an isolated rat thymocyte system and measured the effect of hormones on the uptake of the amino acid cycloleucine. Though the con-

centrations of the thyronine derivatives they studied were on the order of 10^{-4} to 10^{-6} M, the effects produced indicate that this may be a valuable system from which to gain information about the mechanism of action of the hormones at a cellular level. Initially, they incorporated albumin into the cell-culture medium. They have since employed an albumin-free medium[70c]. They found that whereas formerly they obtained results showing L-T_4 having 10% the activity of L-T_3, the new medium shows L-T_4 possessing 30% the activity of L-T_3. Since albumin binds T_4 to a greater extent than T_3 and thus less free T_4 would be available in the albumin-containing medium, they attributed the enhancement in activity to this effect.

Studies have shown that T_3 is metabolically more active than thyroxine[71]. This has given rise to speculations as to the extent of extrathyroidal conversion of T_4 to T_3. In 1955 the claim that T_4 was converted to T_3[72] was subsequently retracted[73] and the likelihood of metabolically significant conversion of T_4 to T_3 was generally discounted.

Recently, however, many groups have reported that indeed there is a sizeable conversion of T_4 to T_3 in athyreotic human subjects[74], normal human volunteers[75] and in the rat[76]. In addition, specific binding sites for T_3 have been reported in nuclei of rat liver and kidney cells[77] and in the anterior pituitary of the rat[78]. This evidence and the widely recognized generalization that 3,5,3'-trisubstituted thyronines are more active than the corresponding 3,5,3',5'-tetrasubstituted compounds[25] strongly suggest that T_3 is the primary hormone. Testing the trimethyl- and tetramethyl-thyronines and the 3'-methyl-

and 3'5'-dimethyl-3,5-diiodo-L-thyronines gave us an opportunity to see if this characterization carries over to the alkylated thyronines.

Biological Evaluations

As mentioned in the previous section, the bioassay method employed in our studies is the goiter prevention assay developed by Dempsey and Astwood[79] and modified by Jorgensen and Slade[30]. The assay is based on the following principles.

Animals are given thiouracil (0.3%) in their feed. This prevents the production of thyroid hormones by the thyroid gland probably by inhibiting the formation of an active form of iodine from iodide ion, thus blocking the iodination of tyrosine residues, and also by preventing oxidative coupling of iodotyrosine residues[80]. In doing so, blood levels of thyroid hormone fall. The lowered level of circulating thyroid hormones reduces the negative feedback to the hypothalamus and pituitary causing increased release of thyrotropin. This in turn stimulates the thyroid gland to achieve greater efficiency in concentrating iodide ion resulting in a proliferation of cells and increased vascularization which ultimately leads to hyperplasia of the gland (goiter). Goiter formation is well achieved within ten days, the length of an experimental assay.

Concommitant with the introduction of the thiouracil diet, subcutaneous injections of either L-thyroxine or the analog to be tested are administered daily. L-Thyroxine in varying doses is administered to some animals to establish control responses while other groups receive analogs in graded dose levels to test for efficacy. If an analog is thyromimetic, i.e. can mimic the

action of the thyroid hormones on the pituitary gland, no excess thyrotropin will be produced and, thus, no goiter will form.

In the first assay solutions of compounds to be tested were dissolved in aqueous 0.9% NaCl containing 0.01 N NaOH. It was noted that the solutions of 3,5,3',5'-tetramethyl-L-thyronine (L-Me_4), especially the solution of highest concentration, immediately developed a blue color which gradually became blue-green during the 10-day injection period. Also, no significant reversal of thiouracil-induced goiter was shown at a molar ratio of 100 times that of L-T_4. This dose response was out of line with the dose responses obtained with less concentrated solutions.

Suspecting that the alkaline solutions may have chemically altered L-Me_4, possibly by a free radical uncoupling to the corresponding 2,6-dimethylphenol (or quinone) and 3,5-dimethyl-L-tyrosine[81], we subsequently changed the aqueous vehicle. Stock solutions of all compounds (ranging from 1 to 28 mg) were prepared in 10 ml of absolute ethanol, a drop of water being added when necessary to effect solution. Aliquots (1 to 5 ml) were diluted to 25 ml with aqueous 0.9% NaCl. No colors developed and L-Me_4 was found to be clearly active.

Solutions of analogs were made up so that they could be compared on a molar basis with L-T_4. This was accomplished by assigning a value of 1 to the dose of L-T_4 containing 1 ugm of L-T_4 per 100 gm of rat body weight. Solutions were made up so that the dose administered to a 100 gm rat was contained in 0.125 ml of solution (the calibrated volume unit of a tuber-

culin syringe). Weighing of samples prior to dissolution was done either on a Mettler Semimicro Balance for samples weighing 10 mg or more or on a Cahn Electrobalance for samples weighing between 1 to 10 mg. Following the regimen stated above for preparation, the solutions were transferred by decantation to 50 ml multiple dose vials, fitted with a septum, capped and stored in the refrigerator when not in use.

Male Long-Evans rats, obtained from Simonsen Laboratories, Gilroy, California, were used in these studies. The animals weighed between 80 and 100 gm when obtained and were housed three to a cage. All were started on a normal diet of powdered Simonsen Rat Maintenance Diet two days prior to the start of the assay.

The experimental design was as follows. Groups of six rats were used. The "normal control" were maintained on the normal diet and were injected daily with an appropriate amount of the vehicle used to prepare solutions. All other animals received 0.3% thiouracil in their feed (thiouracil procured from the Nutritional Biochemicals Corporation and incorporated into the normal feed by the Pharmaceutical Technology Laboratory, School of Pharmacy, University of California). Diets were begun one day before an injection regimen was started. The thiouracil control were injected with the vehicle. The thyroxine controls (three groups) were injected with 1.0, 2.0 and 4.0 μgm of L-T_4 per 100 gm body weight in the first assay. The second assay used 0.5, 1.0 and 2.0 μgm of L-T_4 per 100 gm to obtain a better "spread" on the dose-response curve. The other groups were injected with analogs in dose levels ranging from 0.25 to 200

times the molar ratio of thyroxine. The weight of the animals was determined daily, and the amount of solution to be given was determined as follows:

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

The injections were carried out daily for ten days. On the eleventh day the animals were sacrificed by chloroform-ether inhalation. Their weights were recorded and the thyroid glands excised, kept moist with 0.9% NaCl solution on a saturated filter paper, cleaned under a dissecting microscope, blotted on a filter paper (Whatman #1) and weighed to the nearest 0.1 mg on a Roller-Smith torsion balance.

All thyroid weights were expressed in mg/100 gm body weight. The statistical treatment of the data has been discussed elsewhere[25] and the standard definitions and symbolic notations are employed. Appendix II, Section A gives the program run on a Hewlett-Packard Calculator used to calculate the critical ratio (C.R.)

$$C.R. = \frac{\bar{x} - \bar{y}}{\frac{1}{n_x} - \frac{1}{n_y} \frac{\sum(x-\bar{x})^2 + \sum(y-\bar{y})^2}{n_x + n_y - 2}}$$

The student "t" test was employed and the usual test for significance was applied, i.e. if the critical ratio values exceed tabular values of "t", then the mean values of the groups we are comparing are significantly different. Confidence levels of 99%, 95% and 90% were utilized. The statistical results of Assays 1 and 2 are given in Appendix II, Section B. Except for the discrepancy in the L-Me_4 (2-1) data for the first assay, the data between the two assays were so similar they were incorporated as a combined assay, the statistical evaluation of which is also included in Appendix II, Section B.

Figure 2-6 has presented the dose-response data (thyroid weight in mg/100 gm body weight plotted against log molar ratio of L-T_4) for the analogs as compiled by combining the results of assays 1 and 2. Table 4-1 summarizes the relative potencies of the analogs in the goiter prevention system. A number of these analogs have been tested in other bioassay systems so that a cross-comparison between assays is possible. Frieden[82] has tested the effects of the alkyl thyronines [L-Me_4 (2-1), L-Me_2-iPr (2-2), L-Me_3 (2-3) and L-Me_2] by injection in the tadpole metamorphosis assay (Rana catesbeiana). His results are summarized in Table 4-1 as well. As can be seen, he found activities relative to L-T_4 either equal to or greater than those found in the antigoiter assay.

Pittman[31] found that L-Me_4 possesses about 1% the activity of L-T_4 on the metabolic function of thyroidectomized rats, as shown by his results on oxygen consumption, heart rate, growth and pituitary size.

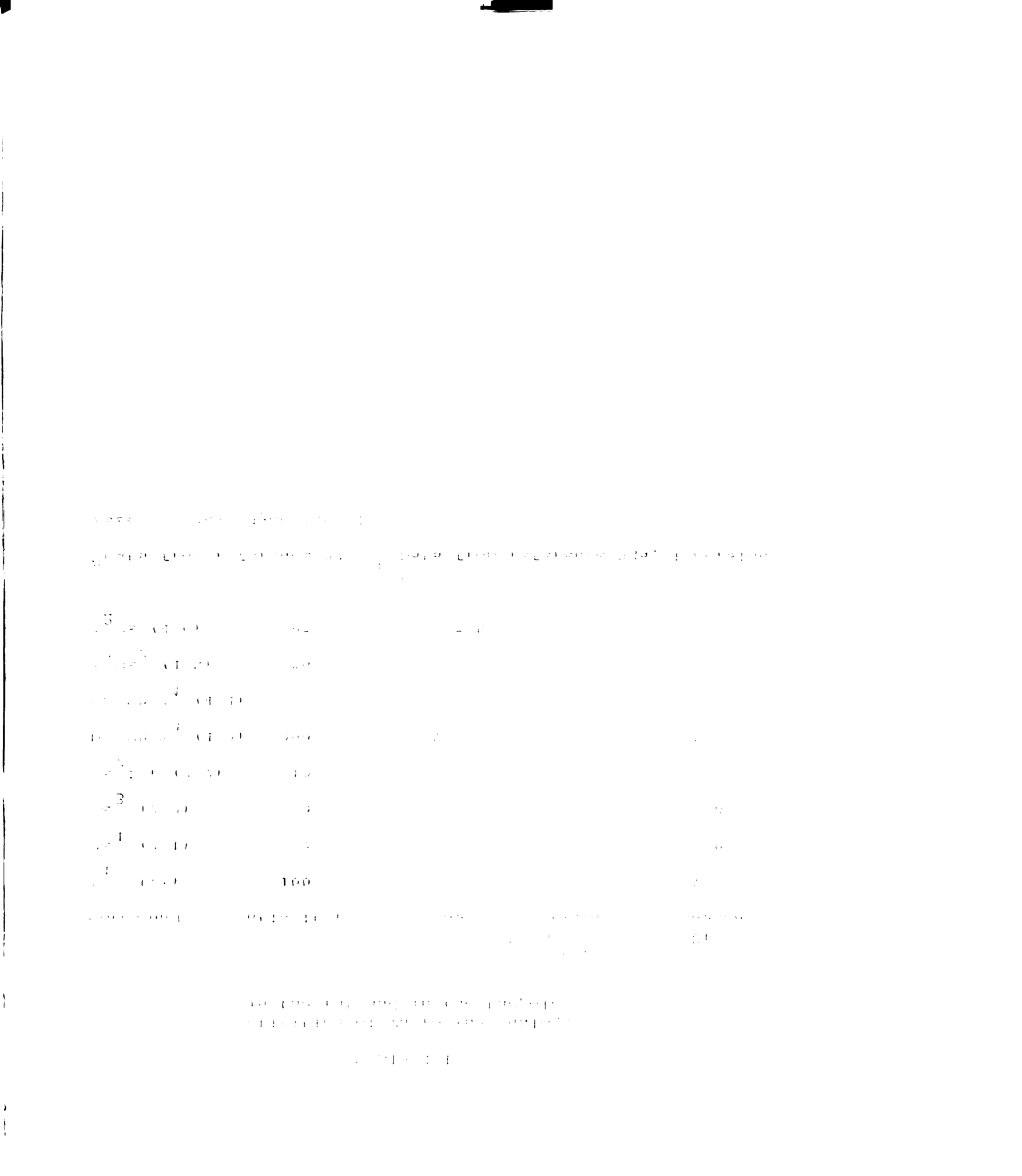
Spangenberg[83] has studied the effects of alkyl thyronines

Table 4-1

Activities of Thyroxine Analogs
in the rat and in the tadpole

	Rat	Tadpole		
Compound	Antigoiter	Tail Decrease	Urea Excretion	Tail Disc Regression
T_4 (1-1)	100	100	100	100
Me_4 (2-1)	2	15[a]	15[a]	0.6[a]
Me_3 (2-3)	3	15[a]	15[a]	0.2[a]
Me_2iPr (2-2)	18	25[a]	20[a]	3[a]
DL-MB-T_3 (4-3)	300	1200[a]	900[a]	500[a]
DL-MB-T_4 (4-4)	-	150[a]	250[a]	-
T_2Me_2 (4-5)	50	140[b]	-	-
T_2Me (4-6)	85	100[c],500[d]	-	-

[a] Data from reference 82. [b] Data from reference 93a, immersion test. [c] Data from 93b, immersion test. [d] Data from reference 93c, injection test.



($L\text{-}Me_2$, $L\text{-}Me_2iPr$ and $L\text{-}Me_3$) on the initiation of strobilation in jellyfish (Aurella aurita). She finds that the alkylated analogs are as effective as $L\text{-}T_4$ and are active down to dose levels of 10^{-7}M. However, she also finds that iodide ion, diiodotyrosine and monoiodotyrosine are also effective at starting the strobilation process; in fact in most instances more effective than thyroxine in both rapid onset and in the percentage of strobilae. She has also found that 3,5-dimethyl-DL-tyrosine is the most effective analog studied thus far, although the sample of dimethyl-tyrosine may have been contaminated with iodide ion, a potent stimulator. However inconclusive the evidence, it appears possible that substituted tyrosines may either be responsible for the "hormonal" action in this invertebrate, or may act as metabolic precursor to the active hormones.

Goldfine, et al.[70c] have studied the effect of a variety of thyroid hormone analogs on the uptake of cycloleucine in the rat thymocyte cell-culture system. Employing an albumin-free test medium they find the biological activities given in Table 4-2, where activity of analogs are compared to the activity of $L\text{-}T_3$ (100%). In most cases their in vitro data closely resembles that found for in vivo systems. For example 3,5-diiodo-3'-isopropyl-L-thyronine is found to be 2 times more active than $L\text{-}T_3$ in the uptake of cycloleucine while in the rat anti-goiter and tadpole metamorphosis assays it is 3 times as effective[3]. The alkyl analog, 3,5-dimethyl-3'-isopropyl-L-thyronine, however is found to be much more potent in their assay (70% the activity of $L\text{-}T_4$ vs. 18% reported for in vivo).

Oppenheimer, et al.[84] have studied the in vitro nuclear

Table 4-2

Effect of thyroid hormone analogs on the uptake of cycloeucine in a rat-thymocyte cell-culture system.[70]

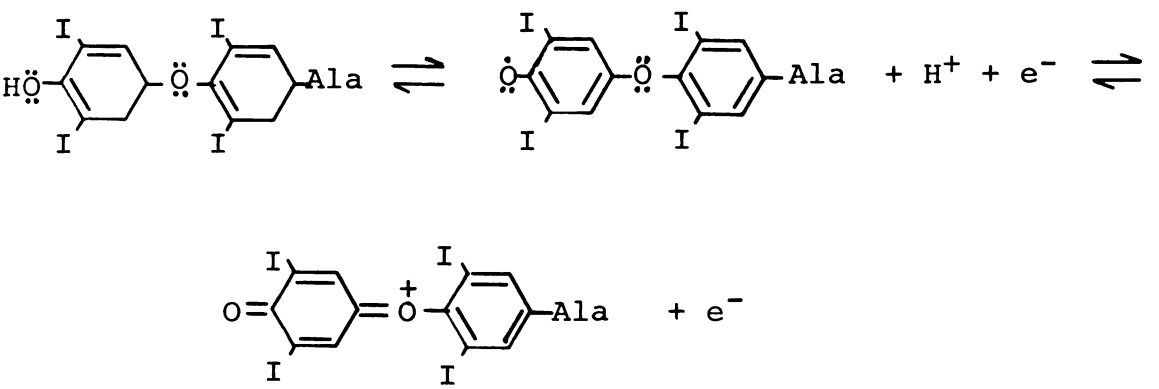
Compound	Activity (% of T_3)
L-T_4 (1-1)	55
L-Thyronine	1
DL-3-Iodothyronine	3
DL-3'-Iodothyronine	5
DL-3',5'-Diiodothyronine	12
DL-3,5-Diiodothyronine	20
DL-3,3'-Diiodothyronine	60
DL-3,3',5'-Triiodothyronine	35
L-T_2Me (4-6)	8
L-T_2Me_2 (4-5)	8
L-3,5-Diiodo-3'-isopropylthyronine	200
L-Me_4 (2-1)	10
L-Me_2iPr (2-2)	60
DL-MB-T_3 (4-3)	75

binding constants with their relative *in vivo* biological activities. Table 4-3 gives the results of this comparison and as can be seen, there is a remarkable similarity. Such close similarity indicates that the nuclear binding site may be the long sought receptor of the thyroid hormones.

DISCUSSION AND CONCLUSIONS

Chapter Two discussed the implications and importance of the biological activities of the alkylated thyronones in relation to postulated functional roles for halogen. Since halogen free analogs have been shown to be active in a variety of test systems, hypotheses giving iodine or other halogens unique properties contributing toward thyroid hormone action cannot be valid.

A long-standing hypothesis by Nieman[85] is that the potential for the phenolic ring of T_4 to undergo reversible oxidation to a quinoid form is related to its hormonal activity. Most analog



studies carried out to date support this hypothesis. For example the activity of a 2'-hydroxy analog[86] compared to the inactivity of a 3'-hydroxy analog[87] was rationalized on the basis that "ortho"-thyroxine (4-1) can undergo the same radical stabilization process as T_4 whereas "meta"-thyroxine (4-2) cannot.

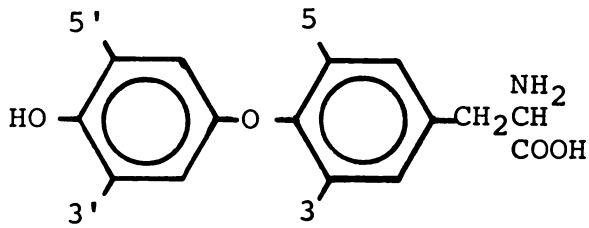
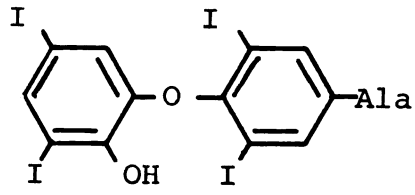


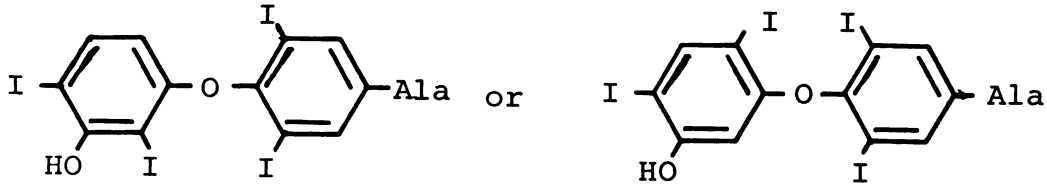
Table 4-3

Comparison of the nuclear *in vitro* displacement activity and *in vivo* biological activity of thyroid hormone analogs.

Compound	Antigoiter	Nuclear Binding
T_4 (1-1)	100	100
T_3 (1-2)	300 -800	800
3-5-Diiodo thyronines		
3'-iPr	1000	1280
3'-tBu	118	120
3'-Me	108	85
3',5'-diMe (4-5)	50	50
Halogen-free		
L-Me_3 (2-3)	3	0.8
L-Me_4 (2-1)	2	0.8
L-Me_2iPr (2-2)	18	5.6
DL-MB-T_3 (4-3)	2000	300
DL-MB-T_4 (4-4)	21	-



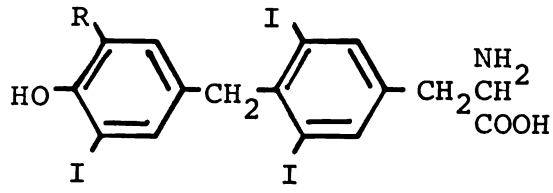
4-1, "ortho"-thyroxine



4-2, "meta"-thyroxine

Acceptance of the hypothesis was deferred, however because of the uncertainty of the substitution pattern in "meta"-thyroxine; the iodines could have been either 2',4'- or 4',6'-. The synthesis of "meta"- and "ortho"- thyroxine has been repeated and infra-red analysis indicated that the iodine atoms were in the 4',6'-positions[88]. In any case the inability of the hydroxy group to undergo the electronic shift necessary for the two-step, two-electron oxidation would be the same for both isomers.

The high activity of the DL-methylene bridged analog of T_3, 3,5-diiodo-4-(4'-hydroxy-3'-iodo)benzyl-DL-phenylalanine (DL-MB-T_3, 4-3) invalidates the functional requirement for a quinoid form, since the oxidation potential for a p-tolyphenol would be much higher than that for a p-phenoxyphenol such as T_4. Indeed, DL-MB-T_3 appears to be equally active as its oxygen-bridged



4-3, DL-MB-T_3, R = H

4-4, DL-MB-T_4, R = I

isostere, DL-T_3, since L-T_3 is about six times as active as L-T_4 in the antigoiter assay[89].

Psychoyos, et al.[90] have assayed the activity of DL-MB-T_3 and DL-MB-T_4 in a variety of in vitro and in vivo tests. They found that DL-MB-T_3 increased mitochondrial glycerophosphate dehydrase activity from various organs and tissues and also caused increased metabolic rates as measured by oxygen consumption in normal rats. DL-MB-T_4 was tested in thyroidectomized rats and found to be active; but the studies made no effort to quantitate the activity with respect to molar ratios of L-T_4. Therefore, no conclusions can be reached relative to activity other than to say that DL-MB-T_3 and DL-MB-T_4 appear to be thyromimetic on peripheral metabolism. Other non-specific tests were conducted, e.g. effects on lipid peroxidation, but these tests tell us nothing regarding thyroid hormone activity.

The tadpole metamorphosis data of Frieden show that DL-MB-T_3 is approximately 10 times more active than L-T_4 and 8-9 times more active than DL-MB-T_4.[22] Oppenheimer, et al.[84] show DL-MB-T_3 has 20 times the activity of L-T_4 on nuclear binding displacemnet and has 100 times the activity of DL-MB-T_4. Goldfine, et al.[70c] on the other hand, shows that DL-MB-T_4 shows half the activity

of DL-MB-T_3.

The hormonal activities of the 3,5,3'-trialkyl-L-thyronines and of 3,5,3',5'-tetramethyl-L-thyronine and of the methylene bridged analogs of T_3, demonstrate that unique electronic or functional characteristics of either iodine or of the ether oxygen do not play primary roles in determining thyroid hormone activity. These results emphasize the steric specificity of thethyromimetic agents, and when combined with the molecular orbital study of Chapter Three, may be used to describe the thyroid hormones and their analogs in terms of structural features.

The active structure of the thyroid hormones appears to consist of an aromatic, lipophilic core sterically constrained by bulky 3,5-substituents (which may be either halogen or methyl groups) into an energetically favored conformation. In this the planes of the two aromatic rings are mutually perpendicular and angled at about 120^0 by a connecting atom or group -O-, -S-, $-CH_2-$) which serves as both a steric and insulating linkage. Specific polar groups, a phenolic hydroxyl and an anionic side chain, are required at opposite ends (4'- and 1- positions) of the central core, which appears to possess a characteristic electron distribution pattern, although further analog studies are necessary to confirm this. Lipophilic 3'-substituents (halogen, alkyl or aryl) enhance the activity of this general structure, which is illustrated in Figure 4-3. These general characteristics have also been used with modification to describe the steriod hormones.

These new structural specifications turn our attention

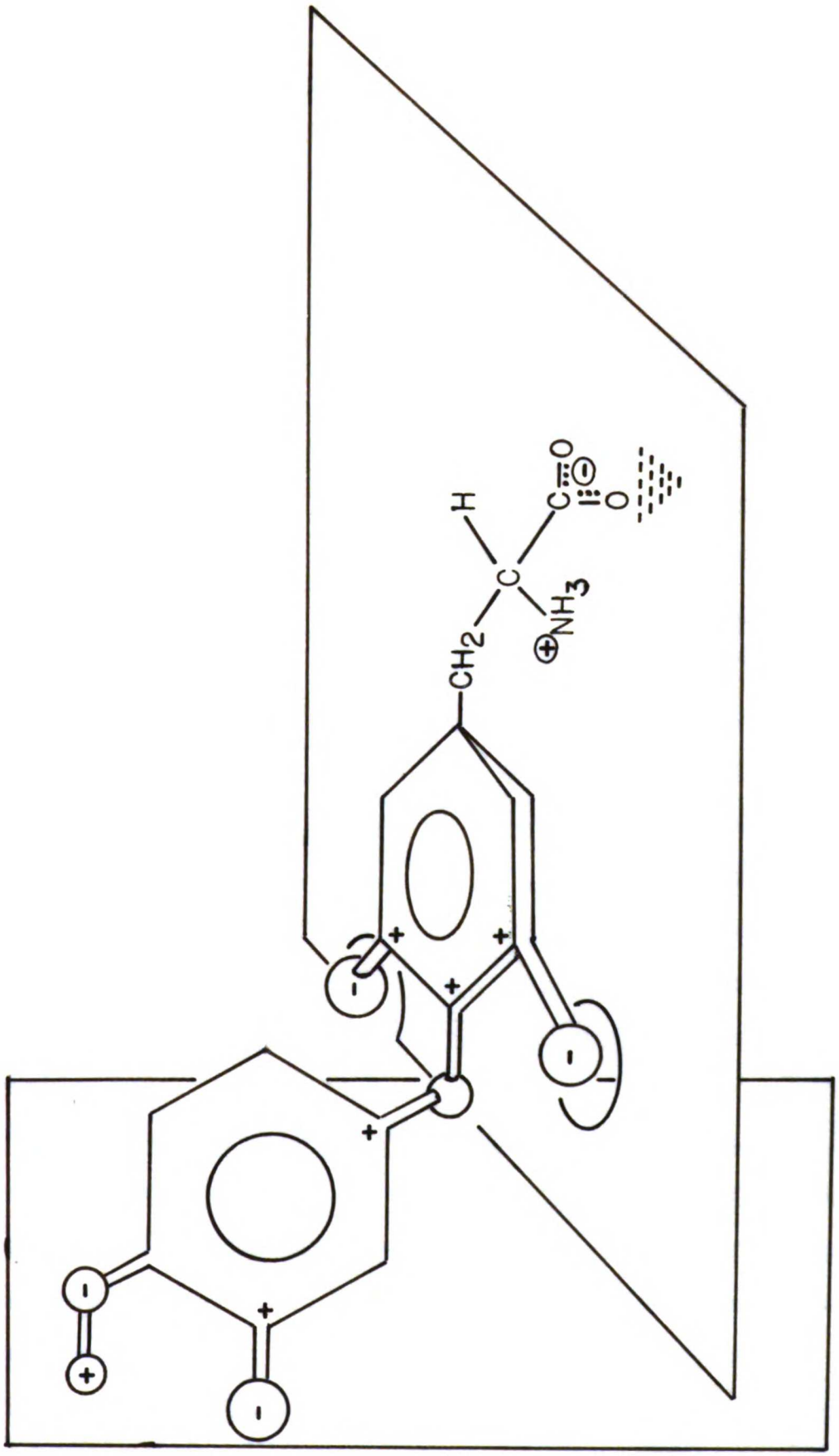


Figure 4-3. Stereoelectronic Features of Active Thyroxine Analogs.

away from the concept of a functional portion of the molecule being involved in the hormonal action. Rather they redirect our attention to the whole molecule, and support the concept that the hormone acts as a structurally specific matrix, inducing in its receptor a new and specific conformation which initiates events leading to the observed hormonal response.

Discovery of specific binding sites in the pituitary gland[78] and in the liver and kidney tissues[77] for T_3 and not T_4, coupled with reports of the metabolic conversion of T_4 to T_3[74, 75, 76] in amounts indicating that 50 - 85% of the potency of T_4 could be attributed to its formation of T_3 have given impetus to the notion that T_3 is <u>the</u> hormone of primary activity. Because the single iodine on the phenolic ring imparts a degree of asymmetry on T_3 not shared by T_4, many investigators have studied the role this asymmetry might play in biological activity. This asymmetry results from the possibilty that two distinct conformations may exist; one in which the 3'-iodine is oriented <u>distally</u>, or away from the alanine-bearing ring, and another in which it is oriented <u>proximally</u>, toward the alanine-bearing ring.

Kier and Hoyland[58], using extended Hückel theory, calculated the rotational barrier for the 3,5,3'-triiodo-, -tribromo- and -trichloro- derivatives of thyronine as well as the unsubstituted molecule. The relative size of the energy barrier is clearly related to the size of the substituted atom. Both the triiodo and tribromo compounds, which are biologically active, were found to possess sufficient internal barriers to rotation to lock the two aromatic rings into a perpendicular conformation.

Furthermore, their calculations imply that the distal and proximal conformations are equal in energy and that it is the size of the rotational barrier which confers a conformational preference to the molecule.

One of the reasons for carrying out our CNDO/2 molecular orbital studies (Chapter Three) was to examine the energy differences between the proximal and distal conformations. Our calculations were in agreement with Kier's and differed from the Camerman's in that they showed very little energy difference between the conformations. We calculated the proximal to be 0.2 kcal/mole more stable than the distal, due, perhaps to a weak attractive interaction between the 3'-iodine and the alanine-bearing ring.

Moreover, we calculated a rotational energy barrier of 17.3 kcal/mole for T_3, using bond distances and bond angles obtained from the x-ray data of Cody and Duax. This indicates that at room temperature and assuming a barrier of 15 kcal/mole there would be rapid interconversion (10^7 times/sec) between the proximal and distal conformations and that, therefore, both conformations would coexist. Kier and Hoyland in their work calculated a barrier of 50 kcal/mole for T_3 and concluded that T_3 was locked into a particular (proximal or distal) conformation (a 50 kcal/mole barrier implies a proximal → distal interconversion rate of about 10^{-51} times/sec or an average lifetime of about 10^{51} sec).

A barrier of approximately 15 kcal/mole is probably closer to reality. This rotational barrier precludes any 'fixed" conformational arrangement since at room temperature rotation

about the C-O bond would be likely. The fact that the PMR of thyronines with 2',6'-protons have a 'normal' aromatic region[24] for the 2',6'-absorption supports the order of magnitude we found for the rotational barrier. If the diphenyl ether were locked into a certain conformation, as in the 2'-methyl compounds, one would observe a very diamagnetically shifted 6'-proton due to the presence of the alanine-bearing ring's π-cloud. The absence of this shifted proton in 2',6'-H compounds, such as T_3 implies an averaging of the 2'-and 6'-proton absorptions and a rotational barrier less than 15 kcal/mole[91].

The evidence leading to the conclusion that T_3 is the thyroid hormone primarily responsible for biological activity may be summarized as follows:

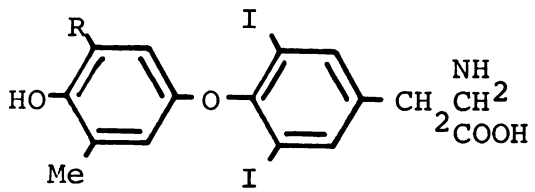
(1) Discovery of specific binding sites for T_3 and not T_4 in all tissues responsive to physiological levels of thyroid hormone but no such sites in tissues of organs or glands irresponsive to the thyroid hormones, e.g. gonads[92];

(2) Evidence supporting sufficient extrathyroidal conversion of T_4 to T_3 to account for 50 to 85% of the activity of T_4; and

(3) The empirical generalization that 3,5,3'-trisubstituted thyronines are more active than their 3,5,3',5'-tetrasubstituted counterparts.

Contrary to this hypothesis we find virtually no difference in activity between the alkylated analogs of T_3 and T_4. The data of Frieden on tadpole metamorphosis[82], Oppenheimer's nuclear displacement results[84] and Goldfine's amino acid uptake studies[70c] parallel our findings. The metabolic removal of an aryl methyl

group, necessary for the conversion of Me_4 to Me_3, has no precedence; thus, both the tri- and tetra-substituted thyronines must possess inherent hormonal activities. This is further substantiated by the high activity shown by both 3,5-diiodo-3'-methyl-L-thyronine (L-T_2Me, 4-6) and 3,5-diiodo-3',5'-dimethyl-L-thyronine (L-T_2Me_2, 4-5) in our goiter prevention assay (85% and 50% respectively) and, previously, in the tadpole metamorphosis assay[93].



4-5, R = Me

4-6, R = H

Also, Oppenheimer[84] finds that the 3'methyl analog has a relative nuclear displacement of 108 while the 3',5'-dimethyl compound shows a value of 50. Goldfine[70c] shows both compounds possess 8% the activity of L-T_3.

However, the observation that T_3 is much more active than T_4 appears to carry over to their methylene-bridged counterparts. Psyshoyos, et al.[90] observed effects with DL-MB-T_4 similar to T_4 using nine times the molar dose levels (DL-MB-T_4= 10% L-T_4). It must be pointed out, however, that this group made no effort to quantitate the activity and this is only an educated estimation. Frieden[82] observed that DL-MB-T_3 is approximately ten times more active than DL-MB-T_4. Oppenheimer's

nuclear binding results which appear to match closely *in vivo* data, indicate that DL-MB-T_3 is 100 times more active than DL-MB-T_4[84]. Goldfine's data[70] show DL-MB-T_3 to be only twice as effective as DL-MB-T_4. Our data show DL-MB-T_3 to be three times more active than T_4. These results indicate a close parallel between the oxygen-bridged and methylene-bridged series with perhaps the methylene-bridged compounds showing a greater preference for the tri-substituted compound i.e. T_3 is three times more active than L-T_4 whereas DL-MB-T_3 appears to be 10 to 100 times more active than DL-MB-T_4.

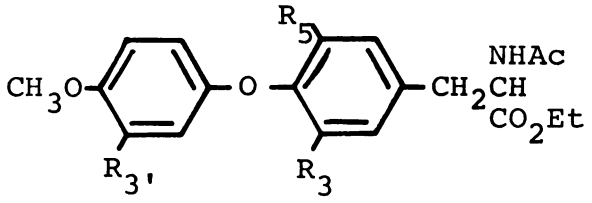
Apparently, in those analogs iodinated in the phenolic ring there is the definite preference of the 3'-monosubstituted compounds over the 3',5'-disubstituted in all assay systems whereas the alkylated systems show little or no difference. One possible explanation for this data is the effect of *ortho*-substitution on the ionization of the phenolic group. It has been repeatedly demonstrated that the 4'-position must contain a hydroxyl group or be so substituted that the compound can metabolically acquire a free hydroxyl group[94]. It is well known that T_4 is more firmly bound to plasma proteins, e.g. TBG, than T_3. This is attributed in large part to the greater acidity of T_4 ($pK'_a = 6.45$) compared to T_3 ($pK'_a = 8.4$)[95]. Hence, at physiological pH T_3 will be considerably less ionized than T_4 and the plasma proteins have a high affinity for ionized species. It is easy to imagine the receptor sites for the thyroid hormones, e.g. the nuclear binding sites, having the reverse requirement, i.e. the necessity for the phenolic group to be in the unionized form or to have an optimum ionization value.

CHAPTER FIVE: PRELIMINARY STUDIES ON PHOTOCHEMICAL AND NITRATION REACTIONS INVOLVING THYROID HORMONE ANALOGS

"Had we but world enough and time,
This coyness, lady, were no crime..."

From "To a Coy Mistress"
Andrew Marvel
18th Century English Poet

This chapter presents some initial efforts to incorporate 3,5-diphenyl and 3'-nitro groups onto the thyronine nucleus as part of the continuing structure-activity program in this laboratory.



5-1, $R_3 = R_5 = I$; $R_{3'} = H$

5-2, $R_3 = R_5 =$ -Phenyl; $R_{3'} = H$

5-3, $R_3 = R_5 = I$; $R_{3'} = -NO_2$

5-4, $R_3 = R_5 = I$; $R_{3'} = -NH_2$

Studies by Wolf and Kharasch[96] indicated that halogenated aryl compounds could be easily converted to their phenyl counterparts

via a photochemical reaction. This appeared to represent a convenient synthetic route to convert the protected 3,5-diiodothyronine (5-1) into protected 3,5-diphenylthyronine (5-2); a substitution which would aid in further characterization of the role of 3,5 substituents in thyroid hormone activity.

Although the effect of nitration on tadpole metamorphosis had been studied[2], there were no subsequent studies on its effect on goiter-prevention or some of the newer assays (Chapter Four). Vallee, et al.[97] found that tetranitromethane (TNM) converted tyrosine and tyrosyl residues to 3-nitrotyrosine in a tris-buffered system. This method was attempted with 3,5-diiodo-L-thyronine (5-1) since the reactivity conferred upon the ortho-position by the phenolic groups of these two amino acids should be similar. Earlier, Johnson and Kohmann[98] described the synthesis of 3-nitrotyrosine using nitric acid. This route was also followed using 5-1.

The conversion of 3-nitrotyrosine to 3-aminotyrosine using sodium hydrosulfite has also been described[99] and we repeated the method with the nitrated thyronine, 5-3, since the 3'-amino analog would have also been an interesting compound to study and also because of its possible conversion to other compounds.

EXPERIMENTAL

The Experimental Section of Chapter Two describes the instrumental methods of analyses and the tlc systems used in preliminary examinations. In addition gradient elution column chromatography was used in some purification procedures. Silica

gel (Baker) and alumina (acid washed, Merck) columns were prepared. Elution was accomplished by starting with benzene solvent migrating to chloroform and ending with ethyl acetate. Preparative tlc was also used [Silica gel, F254 fluorescent 2mm glass plates, E. Merck; EtOAc-$HCCl_3$ (4;1) solvent system].

Photochemical Apparatus and Procedures. All photochemical reactions were carried out in a Rayonet Reactor (The Southern New England Ultraviolet Co., Middletown, Conn.) equipped with a revolving carousel. 2537 Å low pressure mercury lamps were used. The solvents employed in this study were benzene (Eastman, spectrograde, Na dried), acetonitrile (Aldrich, spectrograde, dried over 3A molecular sieve) and 1% pyridine (Mallinckrodt, AR, dried over NaOH) in benzene. Solutions of N-acetyl-3,5-diiodo-4-(4'-methoxy)phenoxy-L-phenylalanine ethyl ester (5-1) were made to 0.02 to 0.03 M in a 100 ml volumetric flask. Aliquots of 12 ml were transferred to each of eight quartz reaction vessels of 15 ml capacity. Vycor 7910 glass surrounded these vessels in the reactor. Prior to irradiation the solutions were purged with nitrogen. Dried solvents would minimize hydrogen replacement reactions by eliminating water, a hydrogen donor. Oxygen was minimized with N_2 purging to prevent possible oxidative reactions. Future studies to determine the effect of oxygen saturation on reaction products should be performed.

Irradiation times varied from 6 to 20 hours. Reactions were monitored by tlc [Eastman Chromatogram, silica gel, 254 F using EtOAc-$HCCl_3$ (4:1)]. Following irradiation, the following steps were employed to separate the inorganic iodides and iodine from organic material.

The benzene solutions were transferred with washing to a 250 ml separatory funnel and washed with 5% $NaHSO_3$ (2 times, 50 ml) and water. Following drying over anhydrous $MgSO_4$, the solution was filtered and reduced in volume to ca. 5 ml. This was submitted to preparative thin layer chromatography (ptlc) or to column chromatography.

The acetonitrile solutions were transferred to a 250 ml round bottomed flask and reduced to dryness in vacuo. The residue was taken up in EtOAc and washed succesively with 5% $NaHSO_3$ and water. This too was reduced in volume after drying and treated to ptlc and column chromatography.

The 1% pyridine in benzene solutions were transferred to a 250 ml separatory funnel and washed with 5% $NaHSO_3$, water, 10% HCl and water and dried over anhydrous $MgSO_4$. Following filtration the solution was reduced in volume under vacuum and treated to ptlc and chromatography.

3,5-Diiodo-3'-nitro-L-thyronine and TNM (5-3). 25 ml of 2 N NaOAc was added to a 100 ml round bottomed flask and the pH adjusted to 8.0 with 5% NaOH. 1 gm of 3,5-diiodo-L-thyronine (5-1) (1.9mM) was added to the buffered solution. EtOH was added to aid dissolution. To this mixture at room temperature was added 5 mM of tetranitromethane (1.0 g, Aldrich Chemicals, #T 2500-3) with stirring. Upon addition of TNM the solution turned red-brown and a suspension developed. The pH was measured and found to be 5.0 and so was readjusted to pH 8.0. At the end of 1 hour the orange suspension was adjusted to pH 5.0 with conc. HCl and refrigerated. Tlc [silica gel, iPrOH-conc NH_4OH (4:1)] showed that all the starting material was reacted

and only one orange spot was present at a position lower than T_2. Following refrigeration tlc shows the presence of two orange spots which were assumed to be the mono- and di- nitrated amino acids. No further characterization was done.

3,5-Diiodo-3'-nitro-L-thyronine (5-3). To 0.5 g of T_2 (0.95 mM) in 3 ml of H_2O in a 25 ml Erlenmeyer flask immersed in an ice bath was added 3 g of conc HNO_3 dropwise. This mixture was stirred for 3 hours giving a yellow solid. 1 g of HNO_3 was added to see if the suspension would dissolve but it did not. Stirring was continued an additional one hour. (4 g of 70% HNO_3 = 91 mM) Tlc shows one yellow spot [silica gel, iPrOH-conc NH_4OH (4;1)] and no starting material. The solution was refrigerated overnite. A portion of the sample was submitted to an isoelectric precipitation (conc NH_4OH followed by the addition of 20% HOAc to pH 5.0) resulting in a red precipitate. Drying yields a red powder, mp 223-24 (dec). Analysis: Calculated (x 1 H_2O) C, 30.4; H, 2.02; N, 4.8; Found C, 30.13; H, 2.02; N, 5.07.

3,5-Diiodo-3'-amino-L-thyronine (5-4). In a 12 ml centrifuge tube 34.5 mg of 3,5-diiodo-3'-nitro-L-thyronine (5-3) from the HNO_3 reaction was dissolved in 2 ml of 5% NaOH. 60 mg of $Na_2S_2O_4$ was added to the solution with shaking. The bright red solution goes from red to brown to yellow within 5 min. Shaking was continued for another 15 minutes and the pH adjusted to 5 with 20% HOAc. The solution was cooled in a refrigerator and centrifuged. The beige residue was dissolved in iPrOH - conc NH_4OH (4:1) and tlc in that system (silica gel) gave a spot differing from the 3'-nitro-T_2, but having a brown smear at the

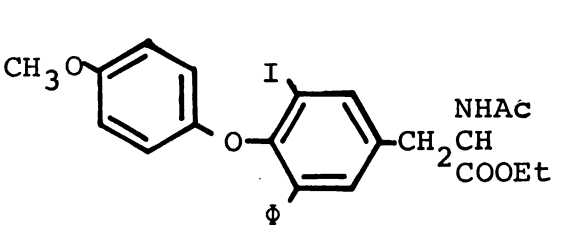
origin.

When 2 N NH_4OH was used as the reaction medium, a dark brown solution resulted and adjustment of the pH to 5 with 20% HOAc gave a dark brown precipitate. The dark precipitate does give a migrating spot not identical with 3'-nitro-T_2, but there was also a great deal more oxidative products left at the origin.

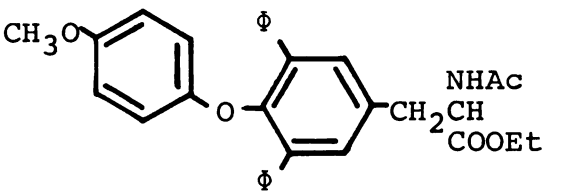
RESULTS AND DISCUSSION

The photochemical reaction of N-acetyl-3,5-diiodo-L-thyronine ethyl ester (5-1) was carried out initially in benzene solvent. The fractions from preparative tlc separation were subjected to pmr analysis. The methoxy region turned out to be an excellent diagnostic test for purity. Each of the three ptlc bands showed from two to three methoxy absorption peaks (δ 3.74, 3.65, 3.90; 5-1 has a methoxy absorption of 3.74 δ). This indicates poor resolution using ptlc. Following ptlc the irradiated material was subjected to column chromatography (alumina) and separated into three fractions. Again pmr showed incomplete separation.

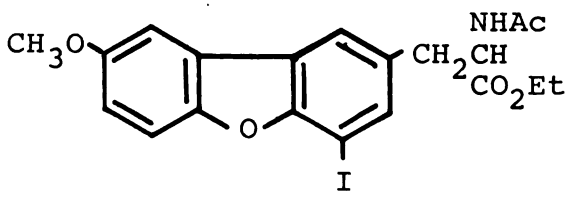
The fractions were then analyzed by CI mass spectrometry. This showed the presence of four [MH^+] peaks 560, 510, 483 and 358 in addition to starting material. The following structures were assigned:



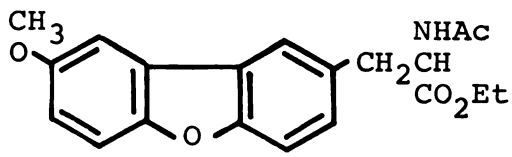
5-5, m/e 560



5-6, m/e 510



5-7, m/e 483



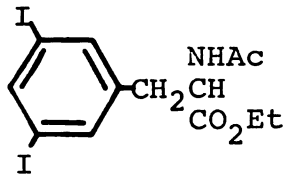
5-8, m/e 358

The cyclization of the protected T_2 (5-1) to the dibenzofuran system excited our interest since this system offered an ideal means to prove or disprove the need for the specific conformation proposed as necessary for biological activity, i.e. two mutually perpendicular aromatic rings. In addition these compounds should also be tested for antagonistic effects since they possess all the other requisites for hormonal activity except the postulated correct information.

Many other groups have reported the photochemical cyclization of halogenated aromatics[100] and the conversion of bridged systems, e.g. diphenylamines and stilbenes, to cyclized systems[101]. The halogenated compounds appear to go through a free-radical pathway[100i] whereas unsubstituted aromatics seem to go through a dihydro-intermediate[102]. Thinking that by eliminating benzene as the reaction solvent we would promote the formation of dibenzofurans, we switched to acetonitrile. Acetonitrile was chosen because 5-1 is insoluble in less polar hydrocarbons while more polar solvents generally have exchangeable hydrogens which would promote the replacement of hydrogen for iodine.

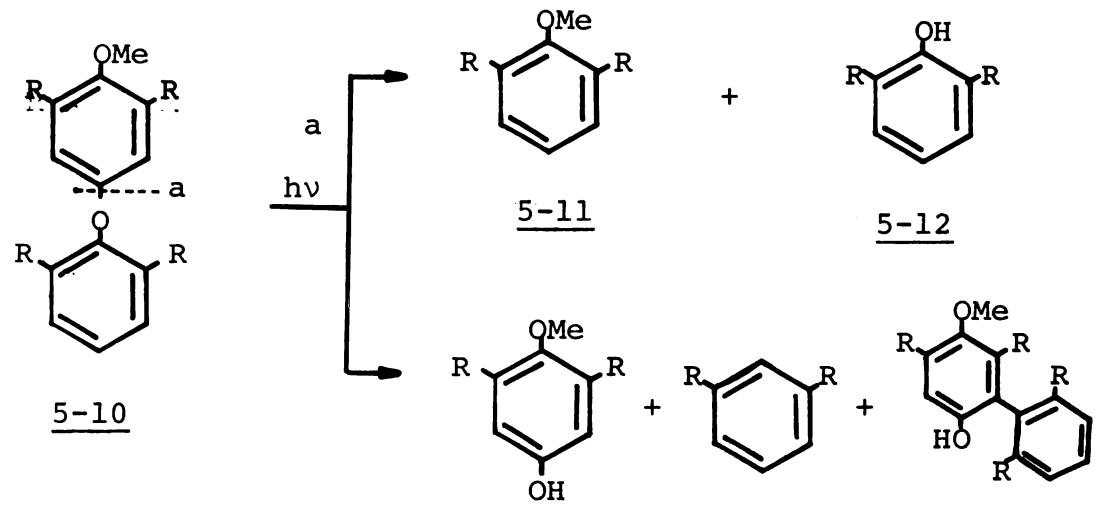
However, upon photolysis in acetonitrile and a preliminary separation using ptlc, analysis by CI mass spectrometry upon

the crude fractions showed that virtually none of the product was the dibenzofuran system. What appeared to be occurring was cleavage of the aromatic ether bond to give as one of the products, 5-9



5-8, m/e 487

A literature search revealed that this type of reaction is not unknown[103]. Hageman and Huymans[103c] report the following reaction scheme:



5-10 5-11 5-12 5-13 5-14 5-15

Ogata, et al.[103b] studied the solvent effect on the photochemical rearrangement of diaryl ethers. They found that 43.5% of their diphenyl ether was converted to rearranged products whereas a benzene solvent gave 23% rearrangement. The tlc profile of 5-1 irradiated in benzene is different than when it is irradiated in Acetonitrile (Figure 5-1)

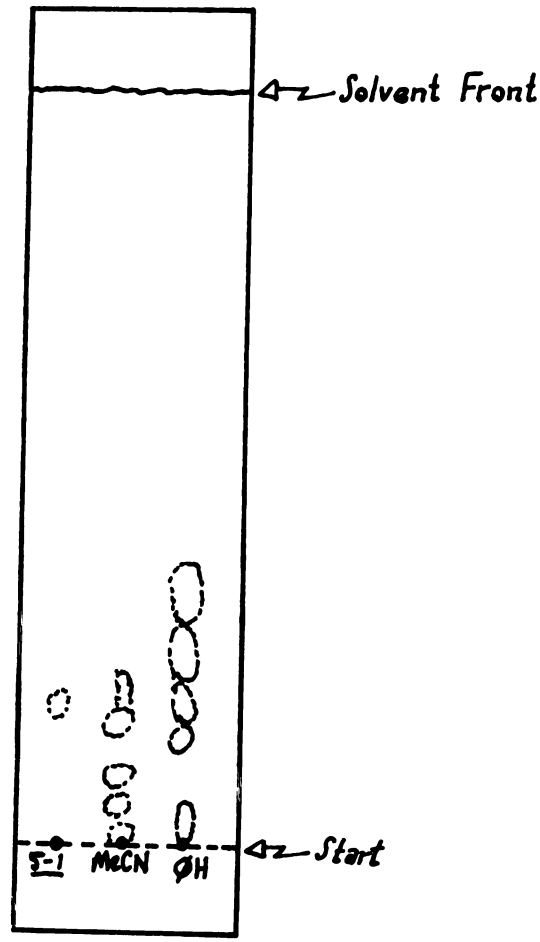


Figure 5-1. Differences between 5-1 irradiated in acetonitrile (MeCN) and benzene (ϕH). [Alumina F254 Eastman Chromatogram, 4:1 EtOAc/hexanes].

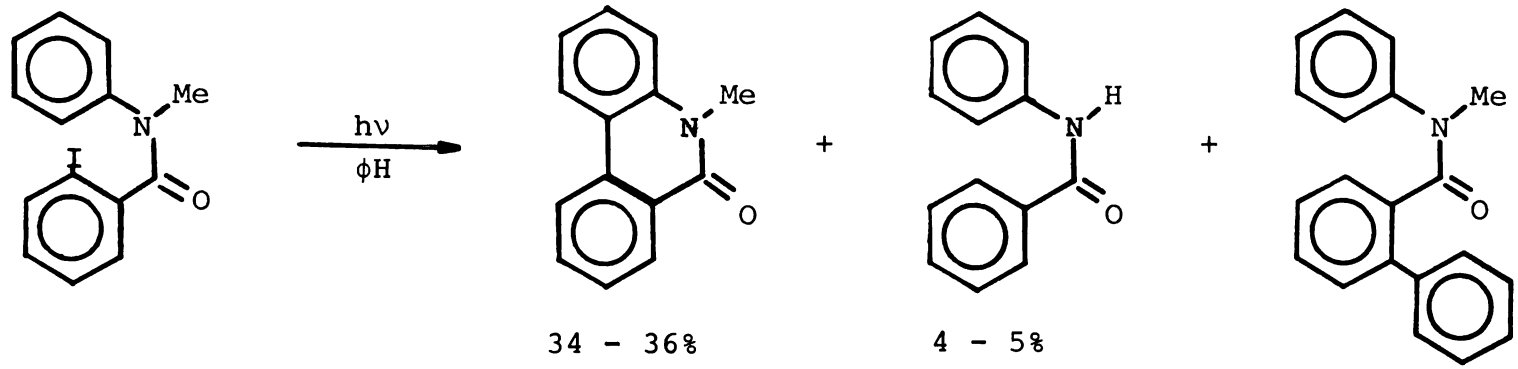
Hey, et al.[100b] observed that small amounts of pyridine prevented the formation of phenylated compounds and spiro dimers while promoting internuclear cyclization of 2-iodo-N-methylbenzanilide to N-methylphenanthridone in benzene. (Figure 5-2)

Using a 1% pyridine in benzene reaction medium we irradiated 5-1. Both alumina and silica gel columns were used to obtain a preliminary separation. Figure 5-3 gives the results of the separation.

Using CI mass spectrometry on the fractions obtained from the 1% pyridine in benzene system we observe [MH^+] peaks for the starting material (5-1) and the iodo-dibenzofuran (5-7) compound. Further studies are necessary to quantitate and further characterize the products from each of the solvent systems before meaningful results can be reported.

As a preliminary study, the nitration reactions also appear to have been successful. However, these also need to be subjected to the normal instrumental methods of analysis and analytical procedures before they become meaningful. Nitration with concentrated HNO_3 is probably the easier of the two and would be the reaction of choice.

The reduction of 5-3 to the 3'-amino compound (5-4) also appears to be facile. Oxidation products can probably be eliminated by performing the reaction under nitrogen atmosphere.



+ spiro-dimers 30 - 36%

Figure 5-2. Photochemical cyclization of 2-iodo-N-methyl-benzamilide.[100b]

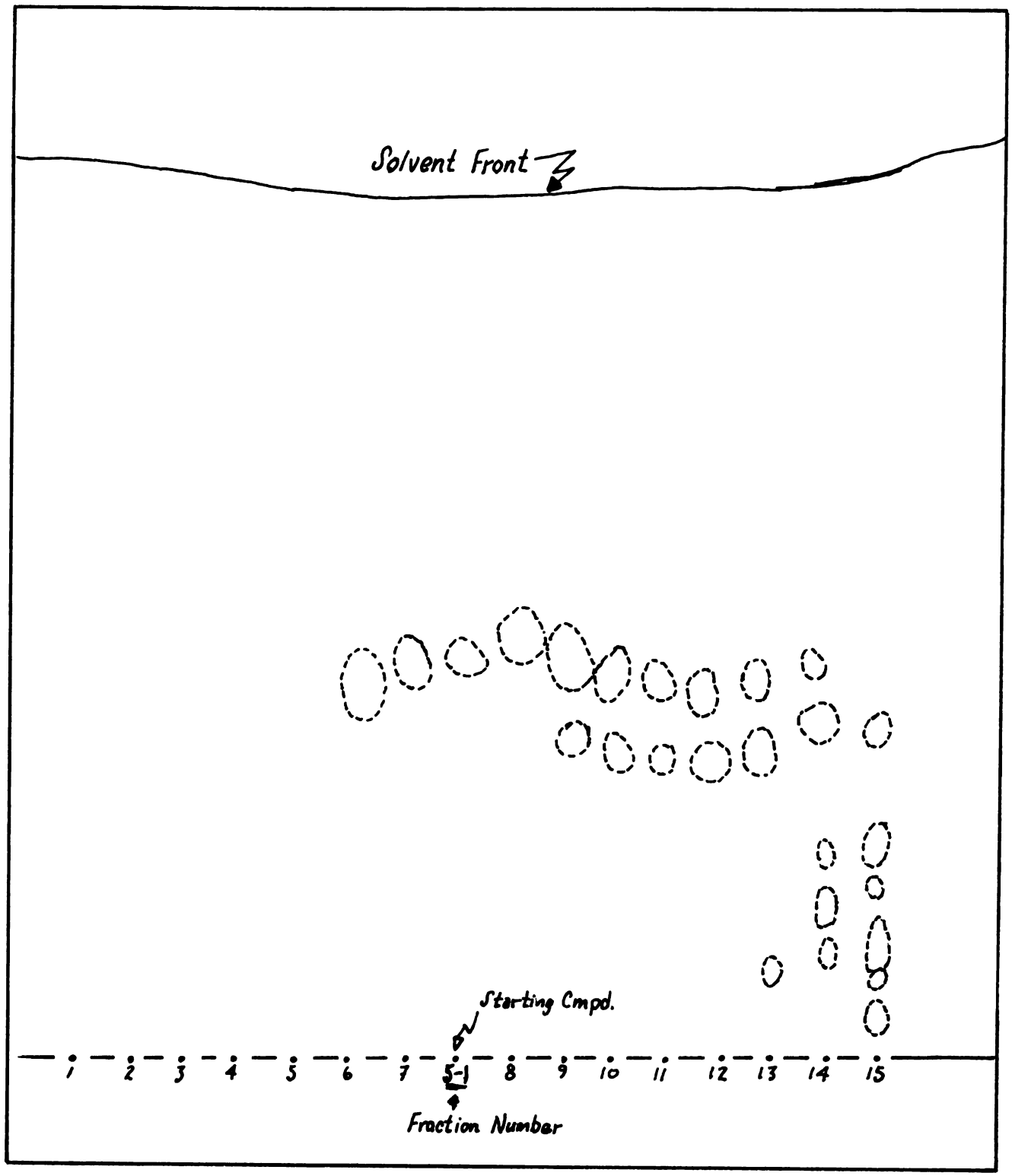


Figure 5-3a. Eluent fractions collected from alumina column separation of 5-1 irradiated in 1% pyridine in benzene. [Silica gel, F254 Eastman Chromatogram; 4:1 EtOAc/hexanes].

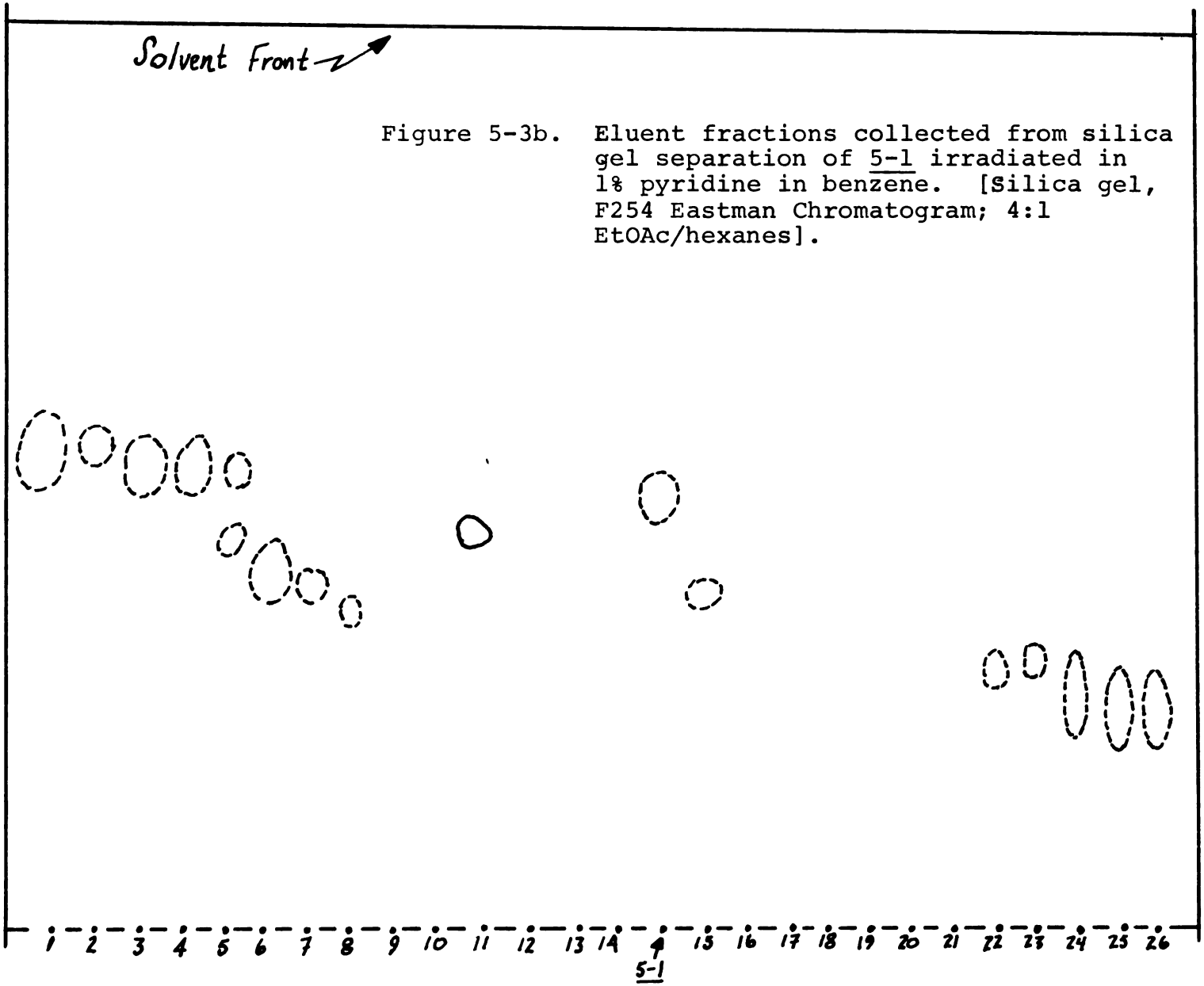


Figure 5-3b. Eluent fractions collected from silica gel separation of 5-1 irradiated in 1% pyridine in benzene. [Silica gel, F254 Eastman Chromatogram; 4:1 EtOAc/hexanes].

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13. Molecules in their lowest energy state have the spin of the paired electrons coupled so that there is no net magnetization, S = 0, and when placed in a magnetic field there are 2S + 1 = 1 energy levels. This is termed a singlet state ($\sum$). In the first excited state the spin of one pair have been uncoupled by reversing the spin on one of them. Since the magnetic quantum nunber for such electrons is $\frac{1}{2}$, S = 1 and 2S + 1 = 3. Thus, when placed in a magnetic field there are three different energy levels and such species are called triplet-state molecules (T). Normally, transitions between $\sum$ and T states (intersystem crossings) are highly improbable, i.e. forbidden, because it requires spin reversal necessitating excitation by UV radiation. Essentially, T-state molecules are highly reactive biradicals which decay to ground state principally by a relatively slow radiative process called phosphorescence.

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Appendix I

Compilation of Spectra

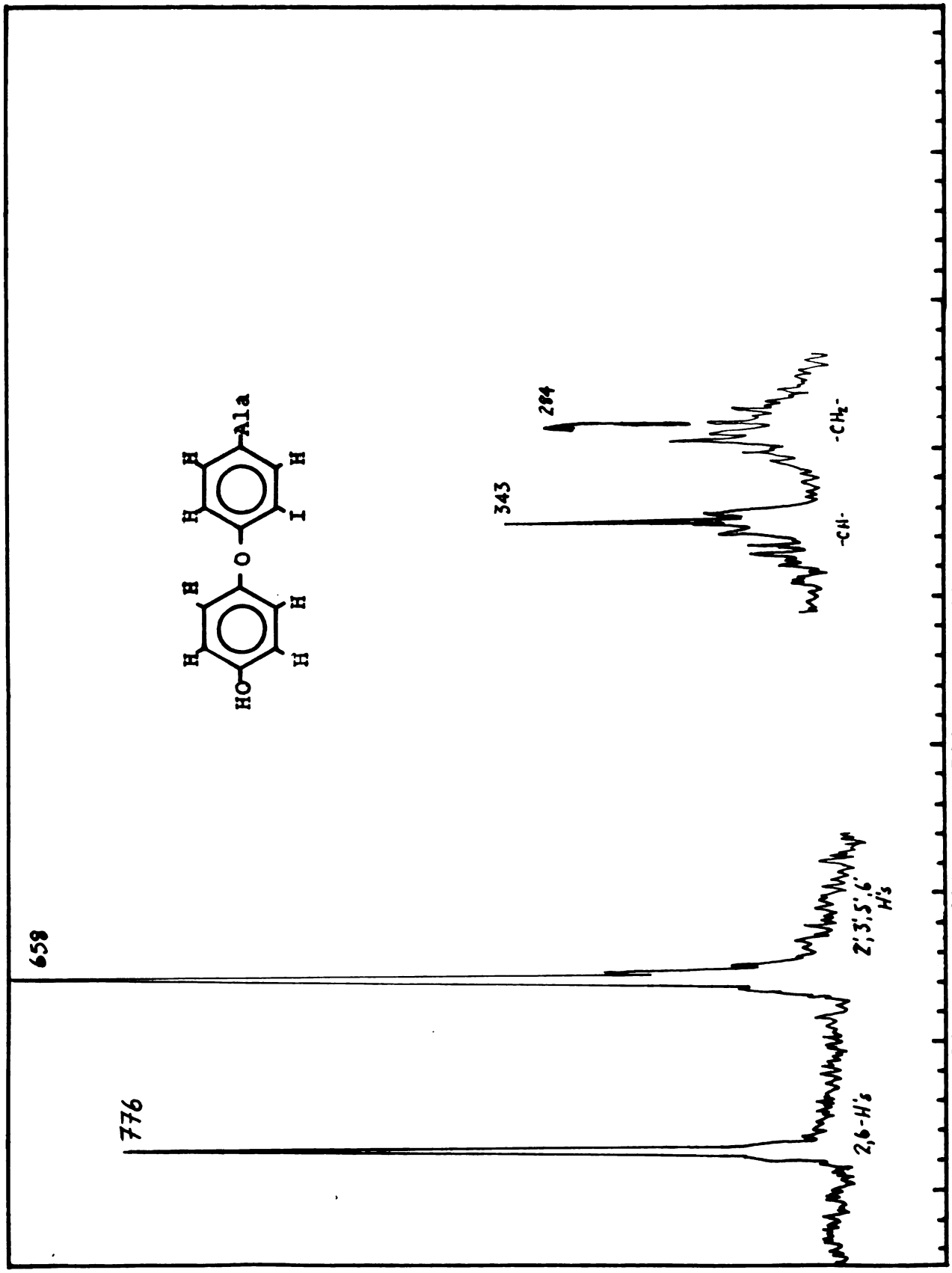


Figure I-1 100 MH_2 PMR spectra of L-T_2; D_2O solvent, DSS reference

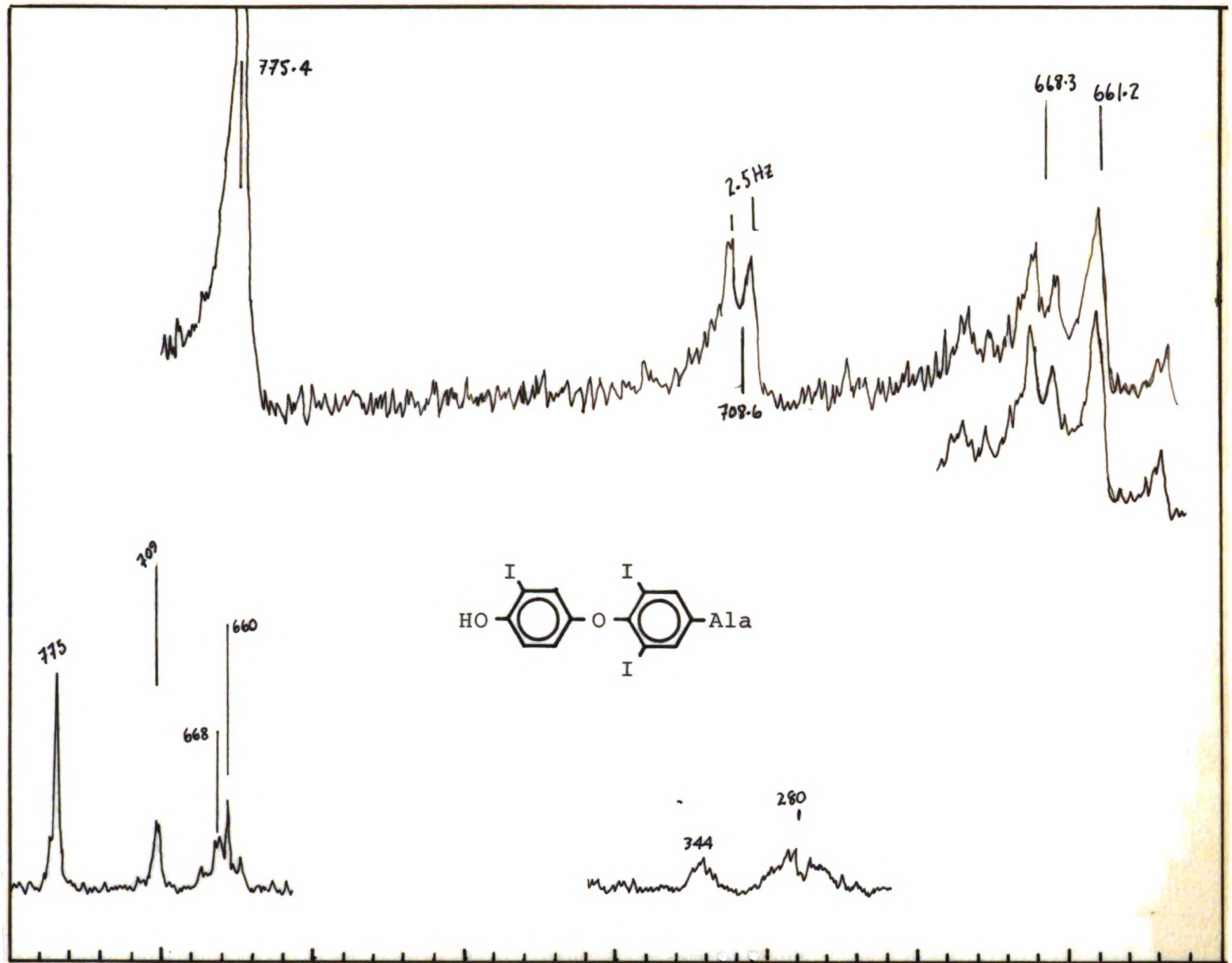


Figure I-2, 100 MHz PMR spectra of L-T_3(1-2), D_2O solvent, DSS reference

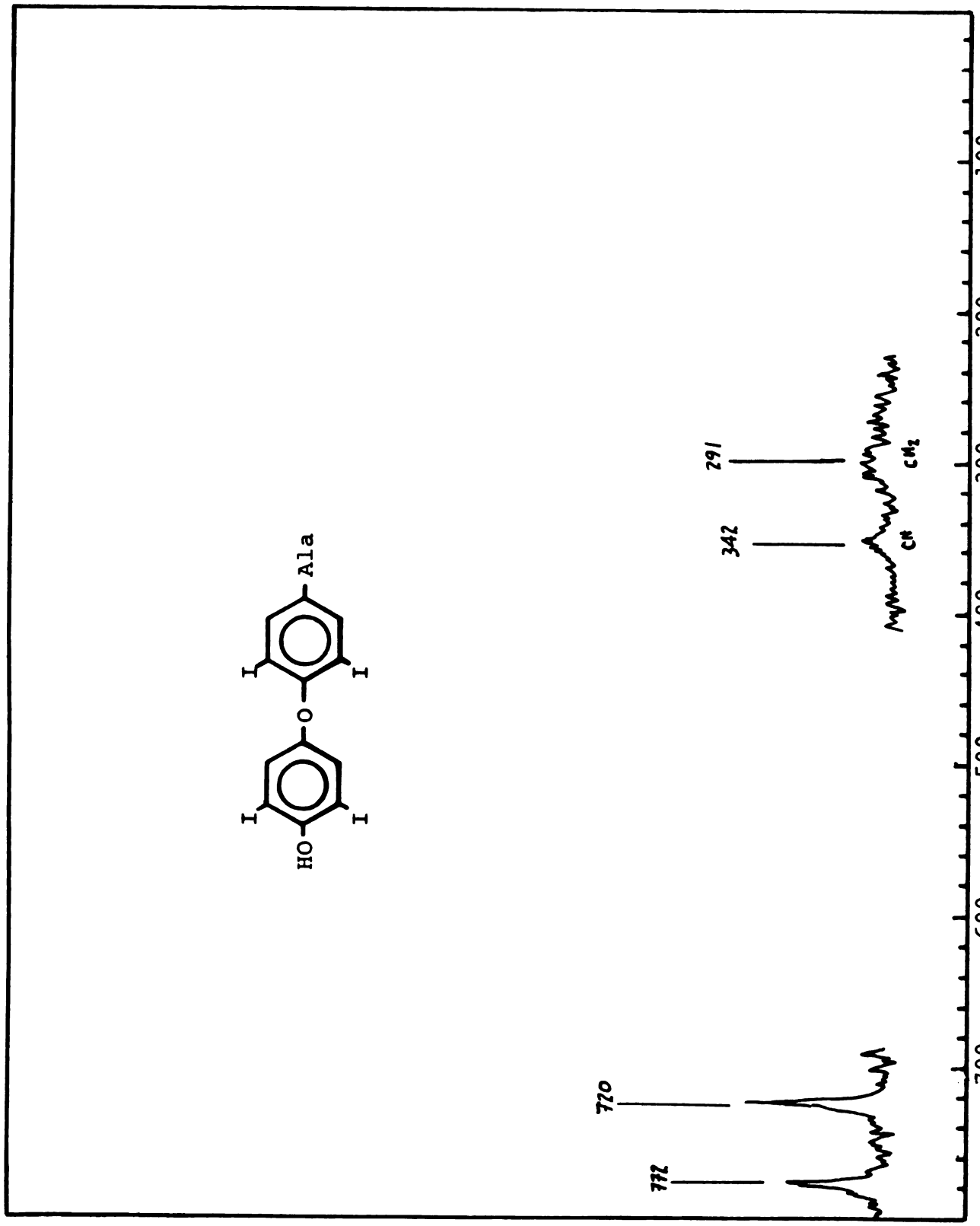


Figure I-3, 100 MHz PMR spectra of L-T_4 (1-1); D_2O solvent, DSS reference.

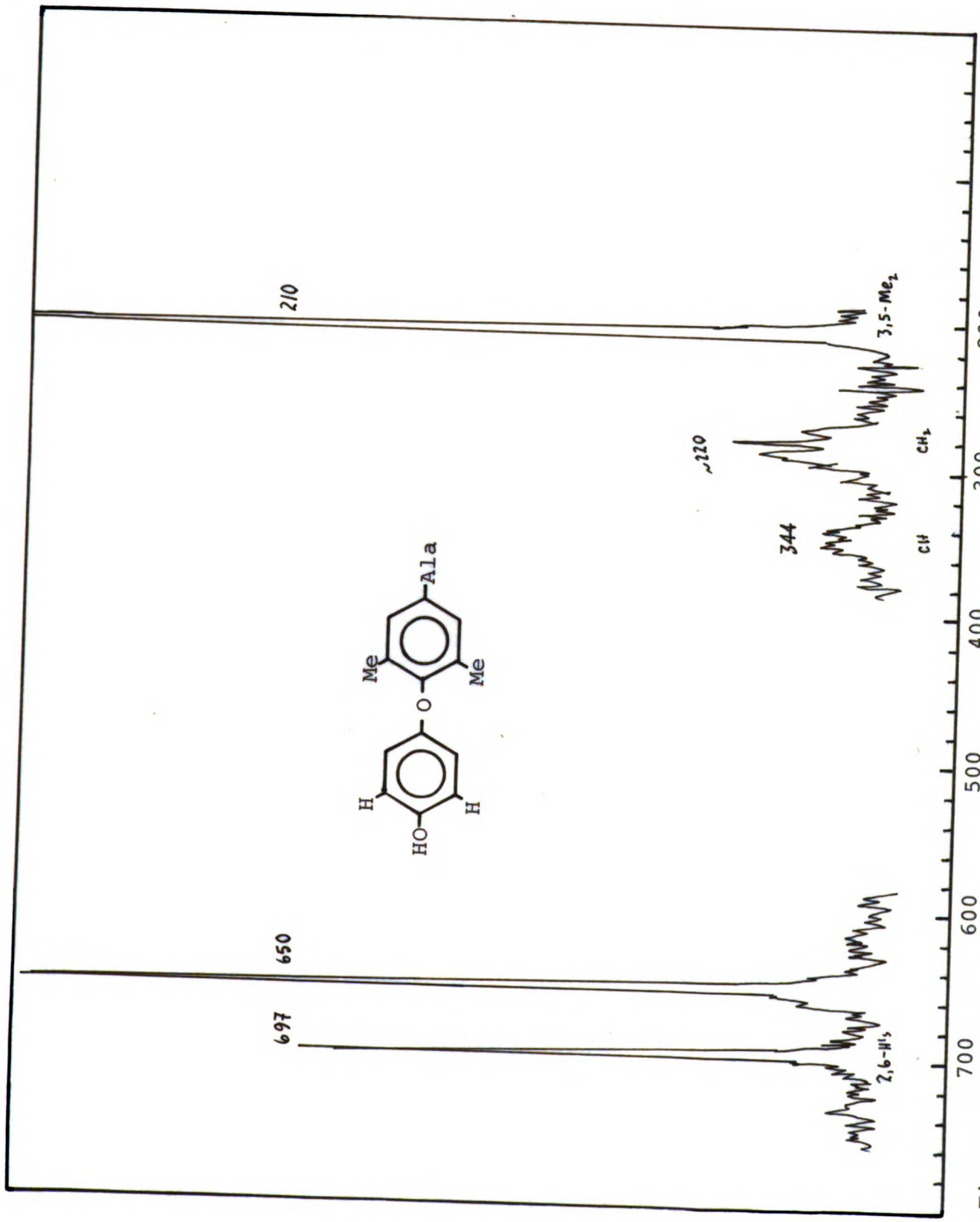


Figure I-4; 100 MHz PMR spectra of L-Me_2; D_2O solvent, NaOD, t-BuOH; DSS ref.

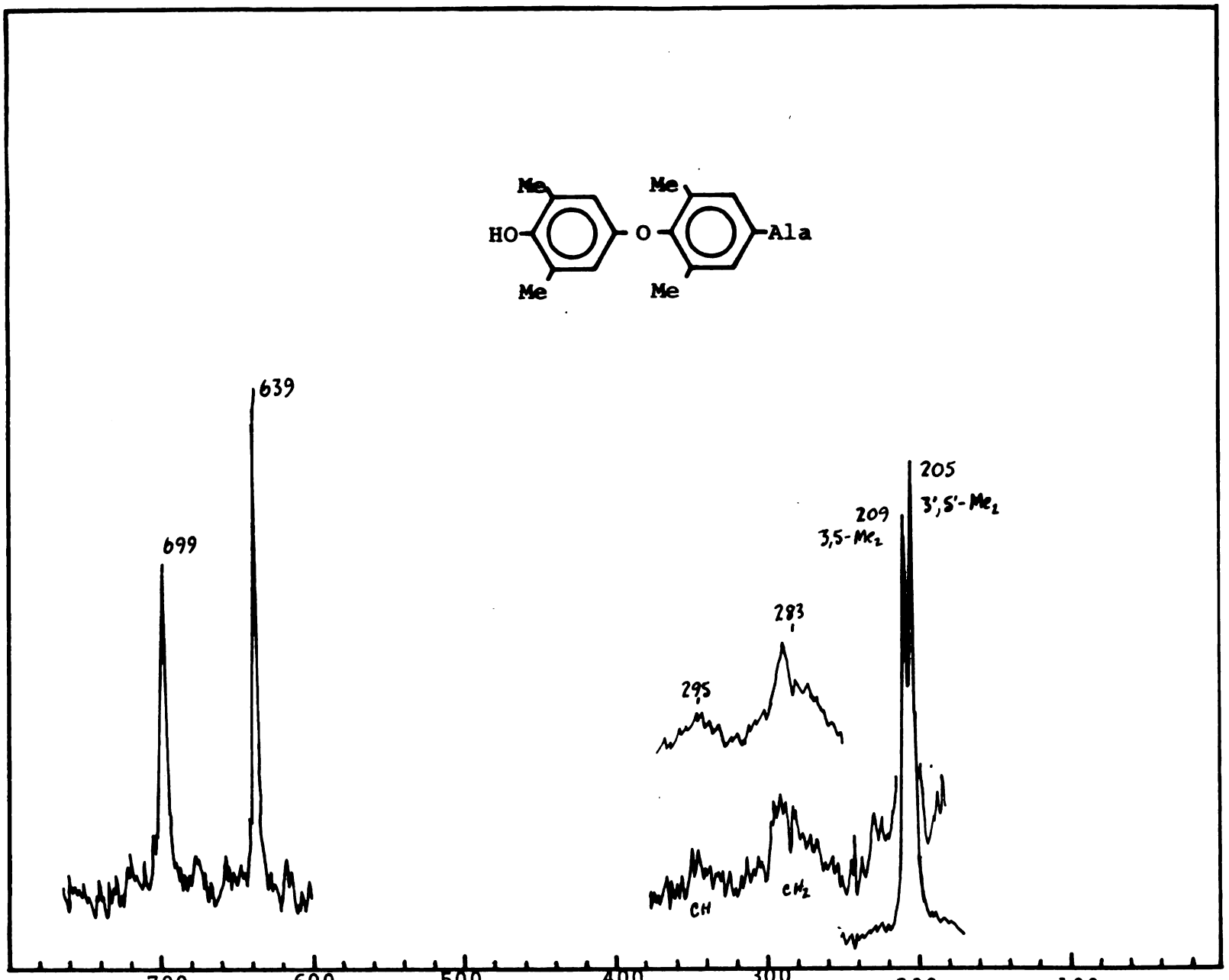


Figure I-5, 100 MHz PMR spectra of L-Me_4 (2-1); D_2O, NaOD, t-BuOH solvent; DSS rf.

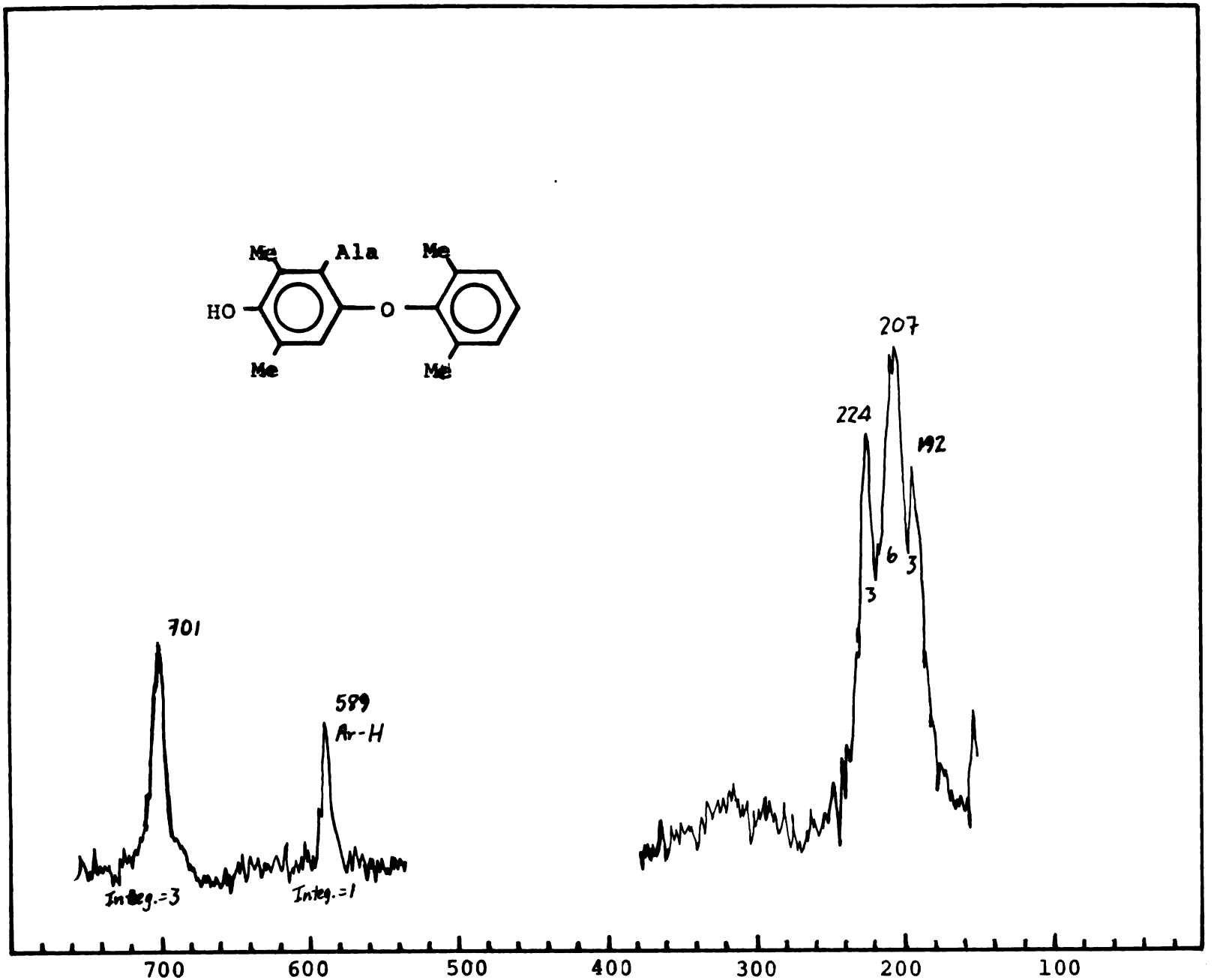


Figure I-6, 100 MHz PMR spectra of i-DL-Me_4 (2-4); D_2O, NaOD, t-BuOH solvent; DSS ref.

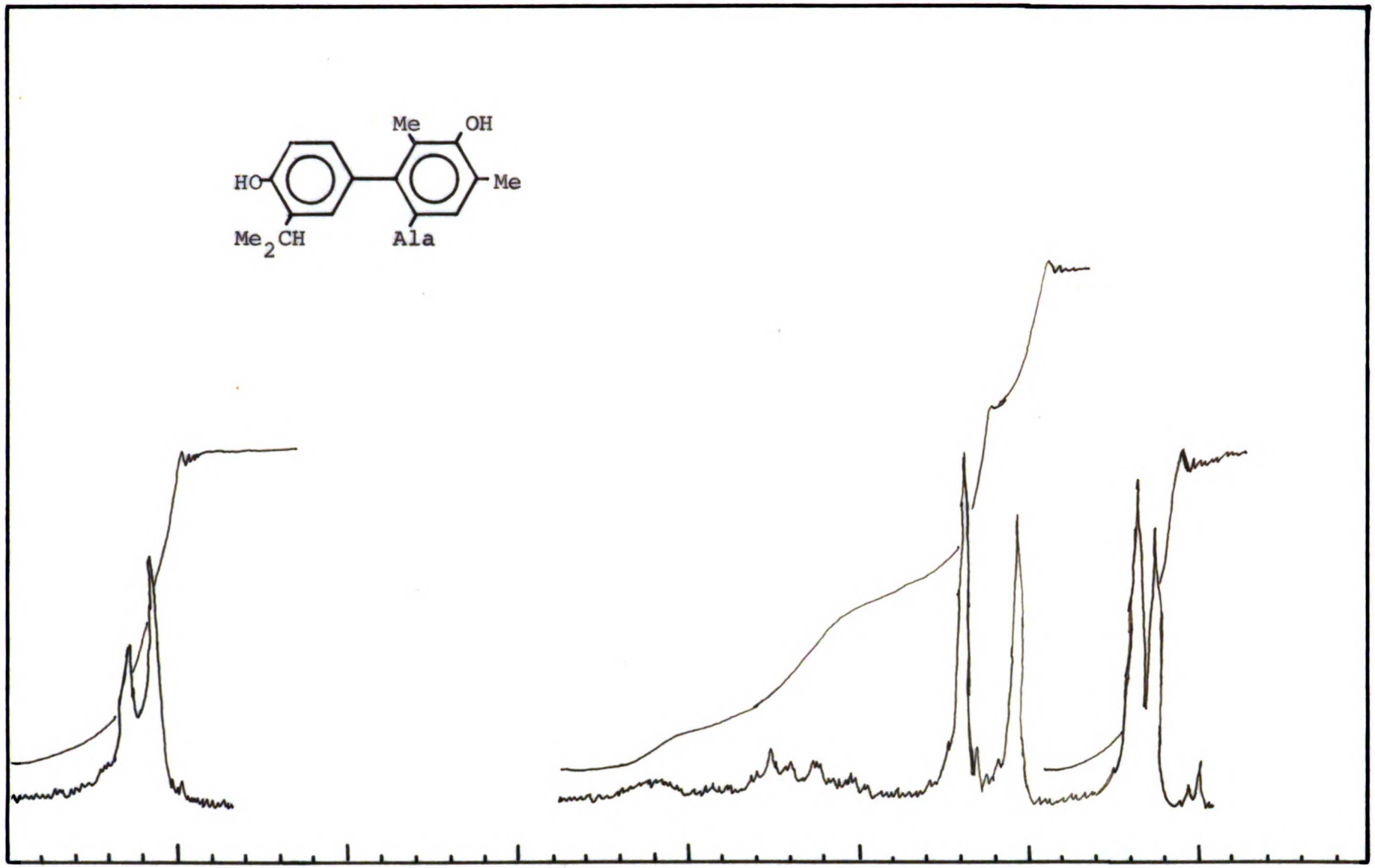


Figure I-7, 60 MHz PMR spectra of i-DL-Me_2iPr (2-15), trifluoroacetic acid solvent, TMS ref.

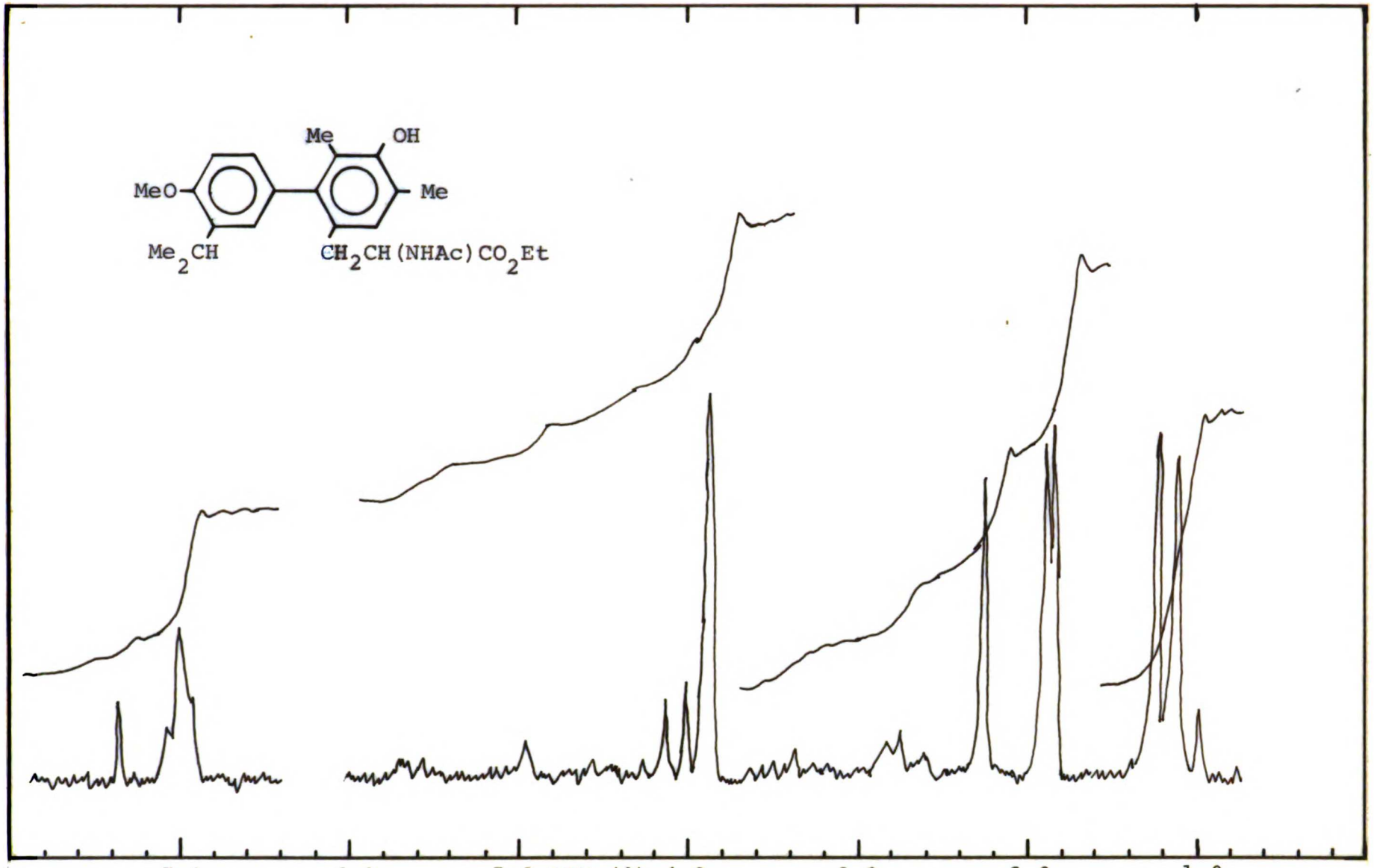


Figure I-8, 60 MHz PMR spectra of the protected amino acid i-DL-Me_2iPr (2-14); deuterated chloroform solvent, TMS reference.

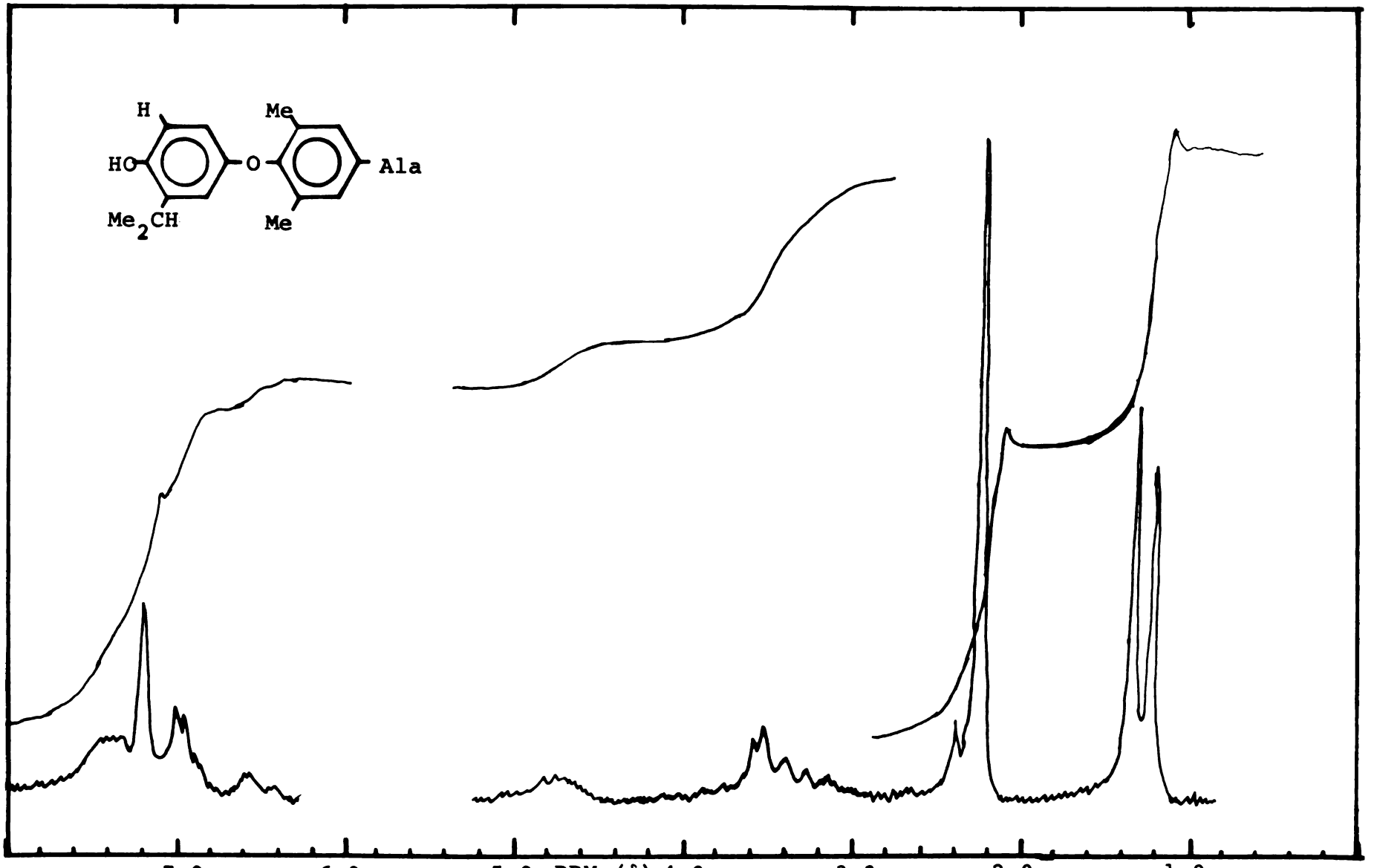


Figure I-9, 60 MHz PMR spectra of L-Me_2iPr (2-2), trifluoroacetic acid solvent, TMS ref.

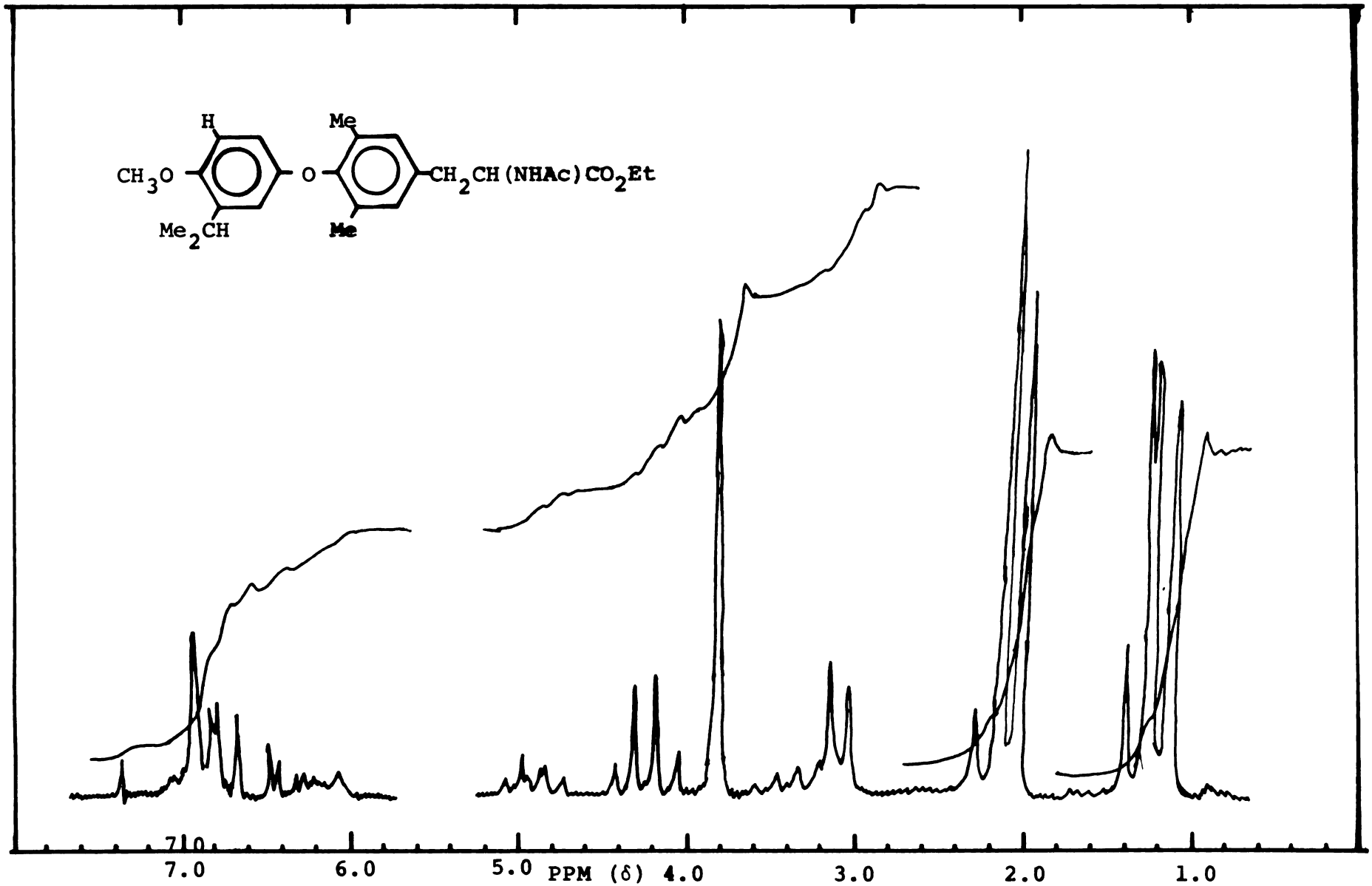


Figure I-10, 60 MHz PMR spectra of the protected aminoacid L-Me_2iPr (2-13); deuterated chloroform, solvent, TMS reference

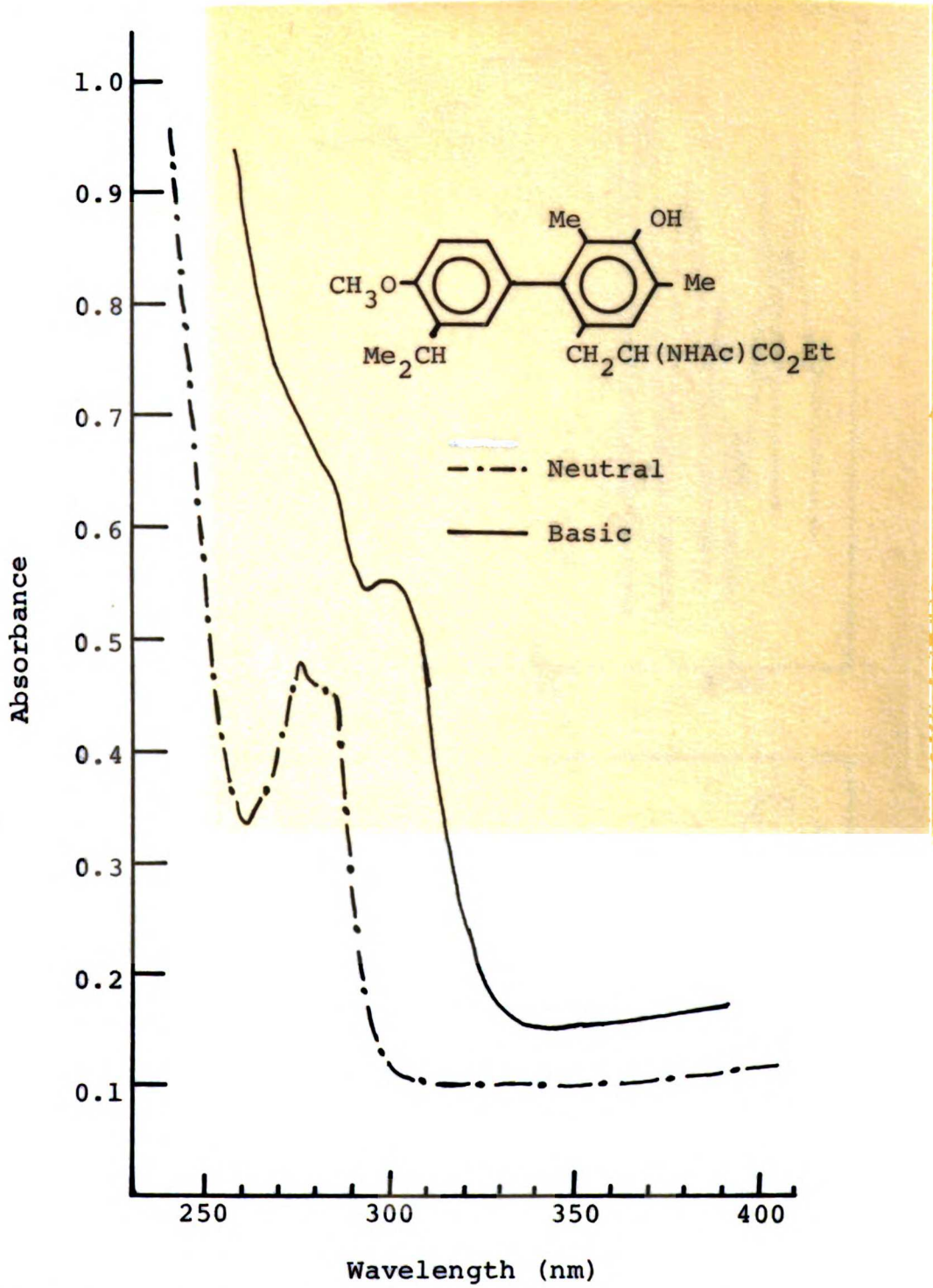


Figure I-11. UV spectra of 2-14 [1 x 10^{-4} M in abs. EtOH = neutral; basic = addition of 1 drop of 15% NaOH]

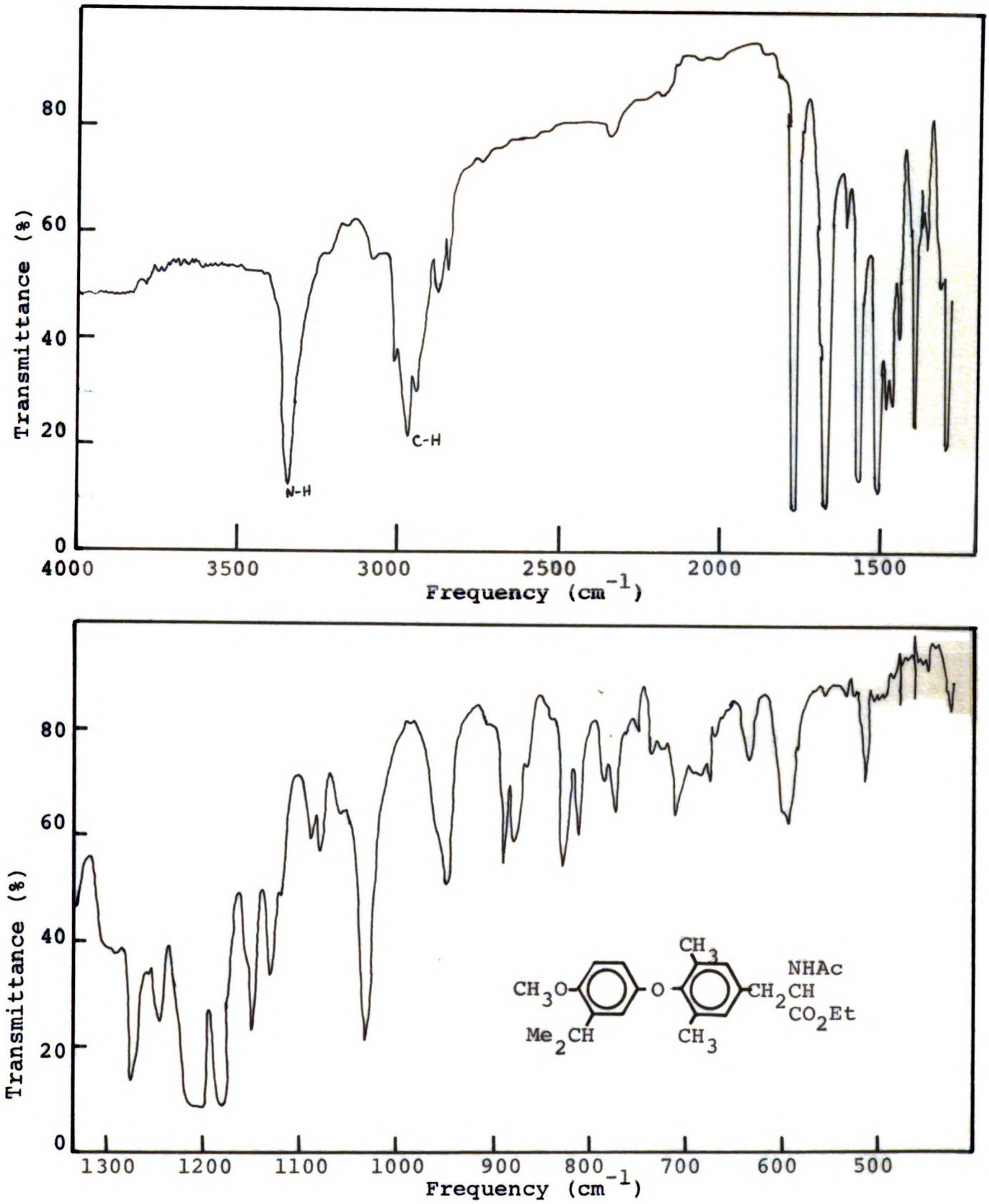
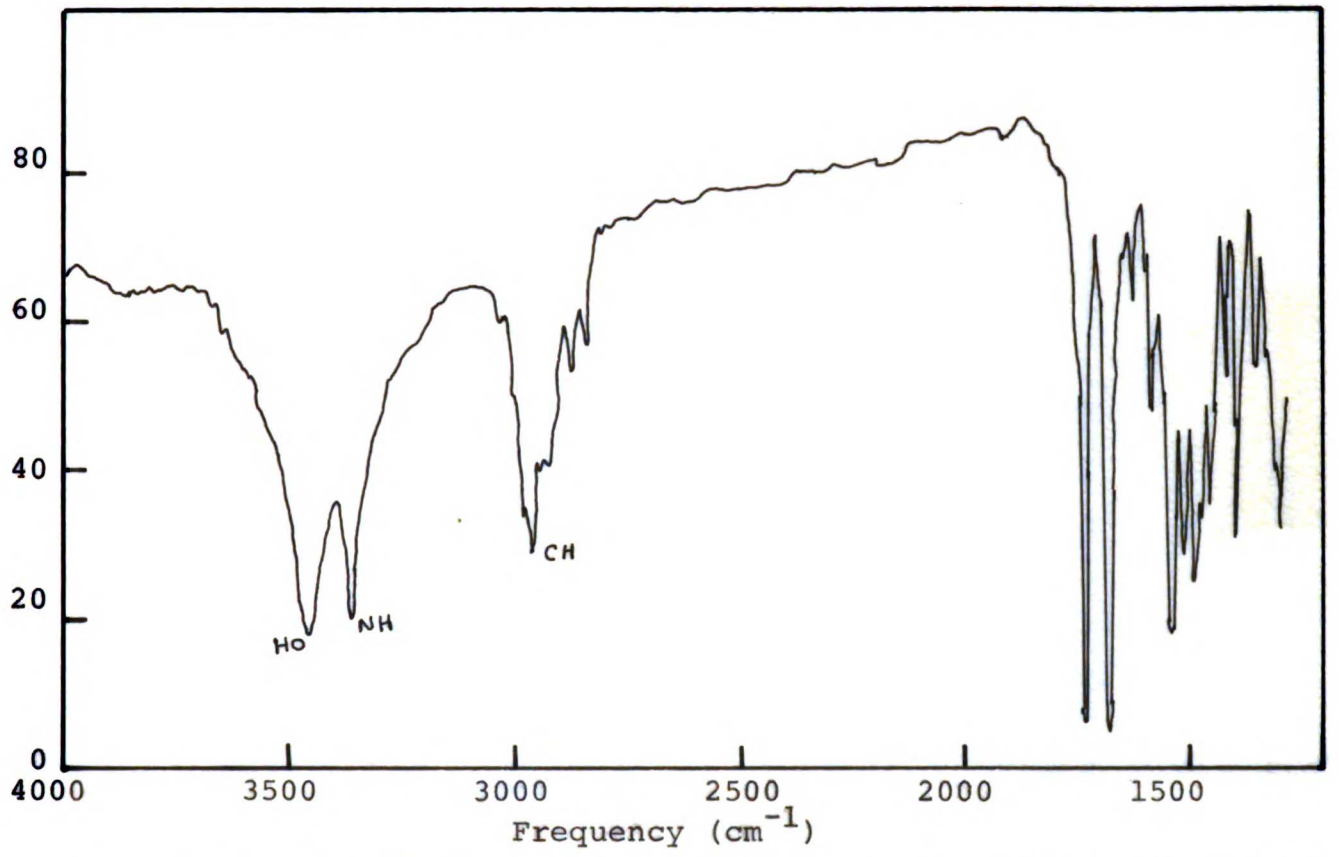


Figure I-12. IR spectra of L-Me$_2$iPr (2-13) KBr pellet.



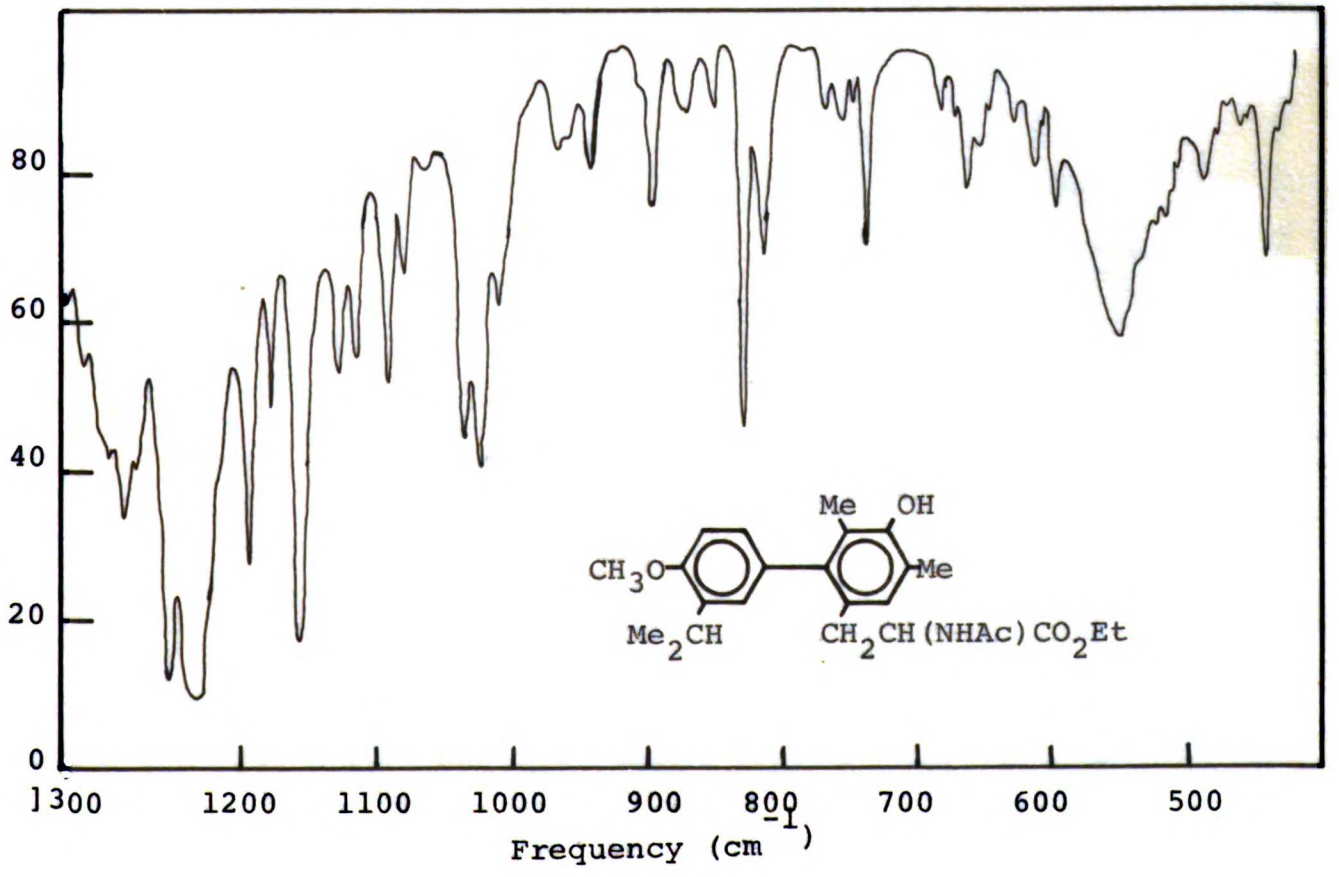


Figure I-13. IR spectrum of 2-14 [KBr Pellet].

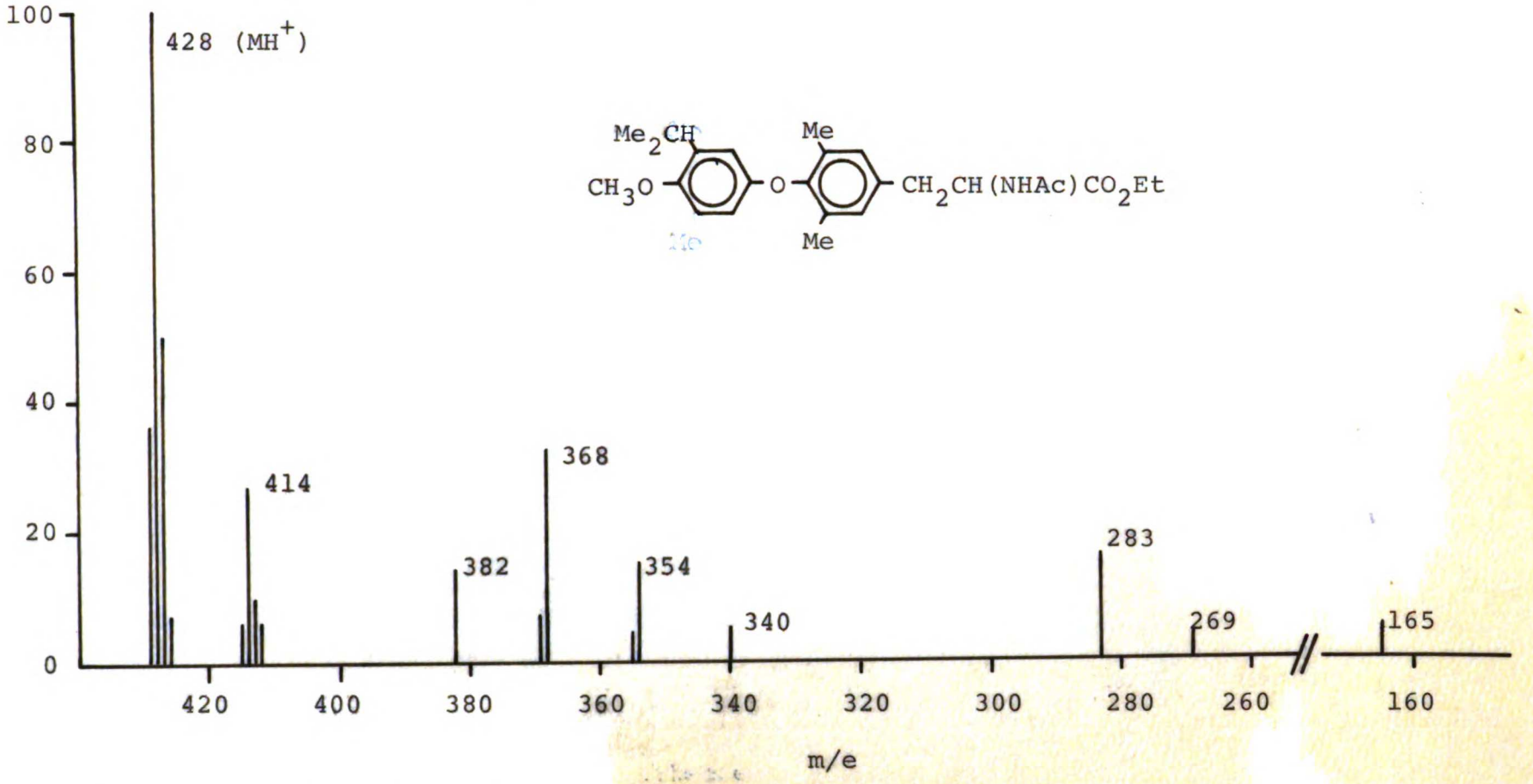


Figure I-14. CIMS spectra of the protected L-Me_2iPr (2-13).

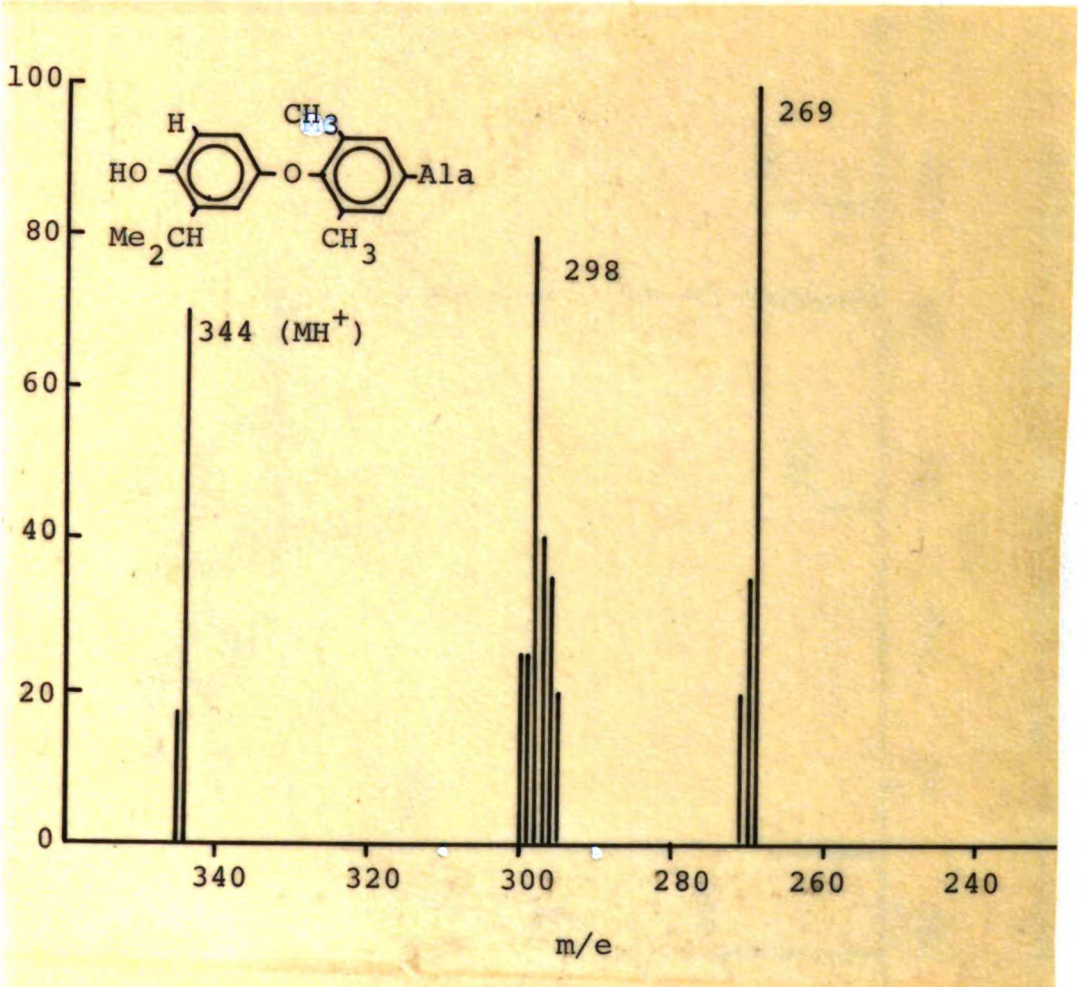


Figure I-15. CIMS spectra of L-Me_2iPr (2-2).

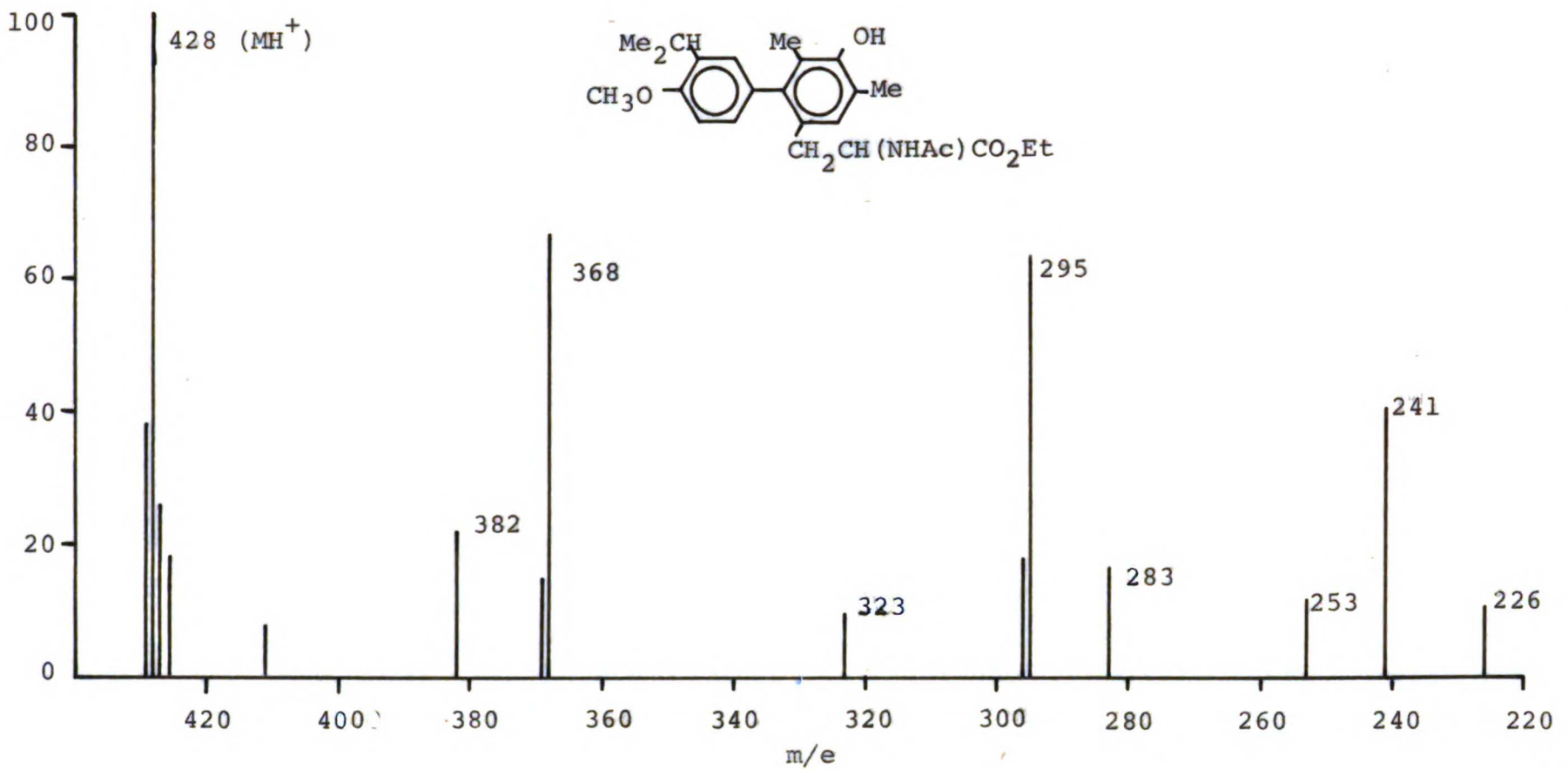


Figure I-16. CIMS spectra of the protected i-DL-Me_2iPr (2-14).

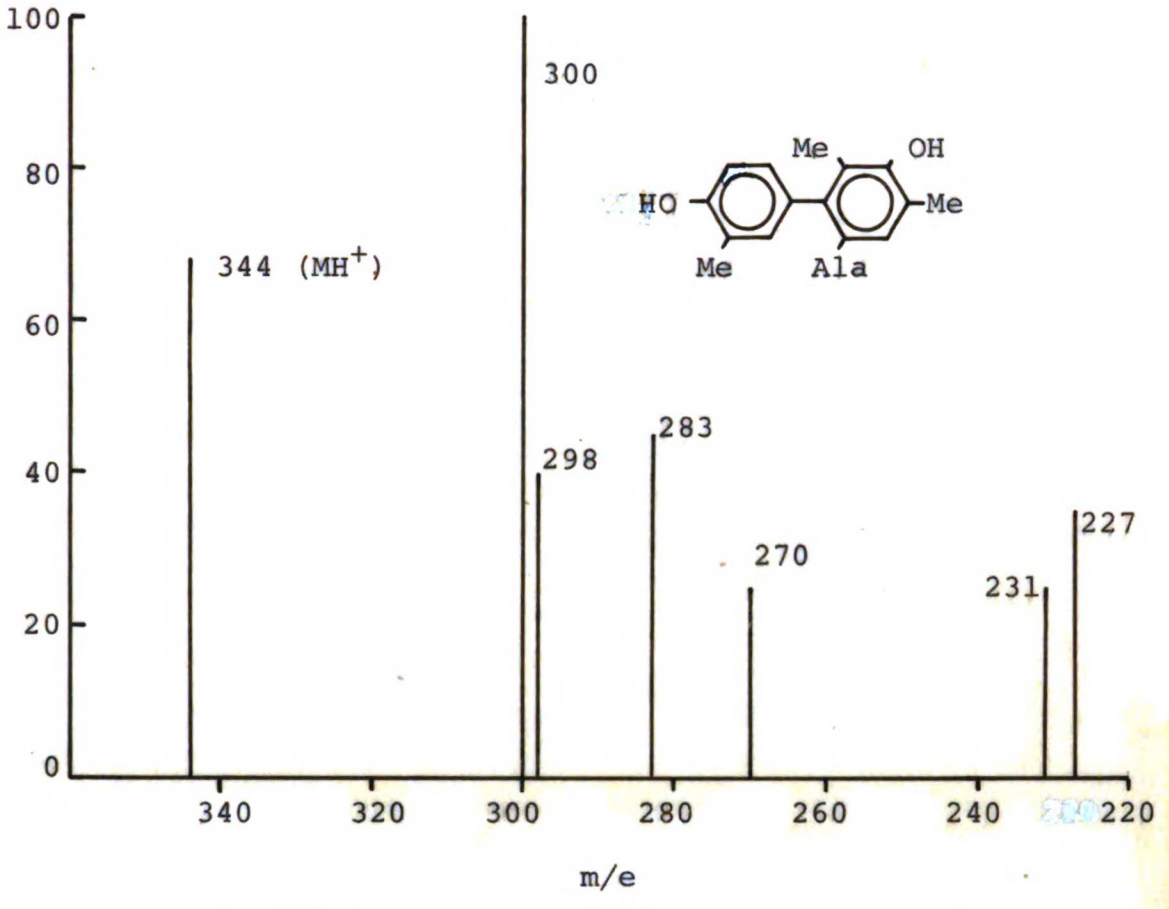


Figure I-17. CIMS spectra of i-DL-Me_2iPr (2-15).

Appendix IIA

Hewlett-Packard Calculator Program for Critical Ratio

Step	Key	Comment	Step	Key	Step	Key
00	clear		21	$x^{\rightarrow}$	42	y ()
1	$x^{\rightarrow}$		2	f	3	c
2	a		3	b	4	$\downarrow$
3	$x^{\rightarrow}$		4	$\uparrow$	5	+
4	b		5	x	6	$y^{\rightarrow}$()
5	$x^{\rightarrow}$		6	c	7	f
6	c		7	x	8	b
7	$x^{\rightarrow}$		8	$y^{\rightarrow}$()	9	$\uparrow$
8	d		9	b	a	e
9	$x^{\rightarrow}$		a	e	b	+
a	e		b	$\uparrow$	c	$\downarrow$
b	$x^{\rightarrow}$		c	x	d	x y
c	f		d	f	50	$\div$
d	STOP	enter x	30	x	1	a
10	$x^{\rightarrow}$		1	$y^{\rightarrow}$()	2	$\uparrow$
1	a		2	e	3	d
2	STOP	enter S_x	3	c	4	-
3	$x^{\rightarrow}$		4	$\uparrow$	5	c
4	b		5	1	6	$\sqrt{x}$
5	STOP	enter n_x-1	6	+	7	ROLL $\uparrow$
6	$x^{\rightarrow}$		7	x y	8	$\sqrt{x}$
7	c		8	$\div$	9	x
8	STOP	enter y	9	f	a	$\downarrow$
9	$x^{\rightarrow}$		a	$\uparrow$	b	$\div$
a	d		b	1	c	$\lvert y \rvert$
b	STOP	enter S_y	c	+	d	$y^{\rightarrow}$()
c	$x^{\rightarrow}$		d	x y	60	a
d	e		40	$\div$	1	clear
20	STOP	enter n_y-1	1	ROLL $\downarrow$	2	a
				+	3	END

Appendix IIB

Rat Antigoiter Assay of Thyroxine Analogs[a]

Assay No. 1 Compound injected	Daily dose per 100 g µg	Molar ratio	Throid Weight per 100 g mg	± sd
Normal Control	-	-	10.10	1.09
Thiouracil	-	-	31.12	8.46
L-T_4[c] (1-1)	1.0	1	17.8[d]	2.31
	2.0	2	7.3[d]	0.36
	4.0	4	6.2[d]	1.43
L-Me_4[e] (2-1)	7.4	10	26.5	4.90
	14.8	20	20.7[f]	3.16
	74	100	22.3	3.68
L-Me_3 (2-3)	7.1	10	23.4	3.30
	14.2	20	16.7[d]	2.57
	71	100	6.1[d]	1.04
L-Me_2I	5	5	22.9	5.30
	20	20	11.3[d]	1.90
DL-MB-T_3 (4-3)	1.48	1	6.5[d]	1.03
	2.96	2	6.2[d]	1.38
	14.8	10	6.5[d]	1.20

Appendix IIB
Rat Antigoiter Assay of Thyroxine Analogs[a]

Assay No. 2 Compound injected	Daily dose per 100 g μg	Molar ratio	Thyroid Weight per 100 g mg	± sd
Normal Control[b]	-	-	9.45	1.38
Thiouracil Control	-	-	28.98	7.30
L-T_4[c] (1-1)	0.5	0.5	25.97	3.72
	1.0	1	15.91[d]	3.70
	2.0	2	7.48[d]	0.81
L-Me_3[g] (2-3)	10.6	30	22.37[f]	2.74
	21.2	60	13.76[d]	7.09
	42.5	120	9.16[d]	3.82
L-Me_4[g] (2-1)	18.5	50	14.95[d]	8.01
	37	100	6.25[d]	1.23
	74	200	6.27[d]	0.64
L-Me_2iPr[g] (2-2)	2.9	7.5	11.95[d]	5.97
	5.8	15.0	6.33[d]	1.02
	11.6	30.0	6.60	0.65
MB-DL-T_3[g] (4-3)	0.185	0.25	21.30[d]	5.62
	0.37	0.50	13.95[d]	3.85
	0.74	1	5.42[d]	1.08

Appendix IIB

Rat Antigoiter Assay of Thyroxine Analogs[a]

Combined Assay Compound injected	Daily dose per 100 g µg	Molar ratio	Thyroid Weight per 100 g mg	± sd
L-T_4 (1-1)	0.5	0.5	26.0	3.72
	1.0	1	15.9[d]	3.70
	2.0	2	7.5[d]	0.81
	4.0	4	6.2[d]	1.43
L-Me_4 (2-1)	7.4	10	26.5	4.90
	14.8	20	20.7[f]	3.16
	18.5	50	15.0[d]	8.01
	37	100	6.25[d]	1.23
	74	200	6.27[d]	0.64
L-Me_3 (2-3)	7.1	10	23.46	3.30
	14.2	20	16.7[d]	2.57
	16.6	30	22.4[f]	2.74
	21.2	60	13.8[d]	7.09
	71	100	6.12[d]	1.03
	85	120	9.16[d]	3.82
L-Me_2iPr (2-2)	2.9	7.5	11.95[d]	5.97
	5.8	15.0	6.35[d]	1.02
	11.6	30.0	6.30[d]	0.65
L-Me_2I	5	5	22.9	3.70
	20	20	11.3[d]	1.90
L-Me_2I_2		40	23.0	3.70
		100	12.6[d]	5.70
L-I_2Me (4-6)		2.5	8.15[d]	3.45

Appendix IIB

Rat Antigoiter Assay of Thyroxine Analogs[a]

Combined Assay Compound injected	Daily dose per 100 g µg	Molar ratio	Thyroid Weight per 100 g mg	± sd
L-I_2Me_2 (4-5)		2.5	12.17[d]	5.12
		5	6.11[d]	0.69
DL-MB-T_3 (4-3)	.37	.25	21.30[d]	5.62
	.74	.50	13.95[d]	3.85
	1.48	1	6.47[d]	1.13
	2.96	2	6.22[d]	1.38
	14.8	10	6.50[d]	1.20

[a]Six rats in each control and experimental group, see E.C. Jorgensen and P. Slade, J. Med. Chem., 5, 729 (1962) for bioassay details, [b]Untreated control group, all other rats received 0.3% thiouracil in their diets. [c]Sodium L-thyroxine pentahydrate. [d]At this dose level thyroid weights were significantly lower ($P > 0.99$) than the thiouracil control and were therefore active dose levels. [e]In dilute aq NaOH, blue-green solution. [f]At this dose level thyroid weights were significantly lower ($P > 0.95$) than the thiouracil control. [g]Colorless solution in EtOH diluted with 0.9% aq NaCl.