TABLE I.—INHIBITION OF GROWTH OF TEST ORGANISMS[a]

Test Compound	*Strep. pyogenes*, β-hemolytic	*P. vulgaris*	*E. coli*	*Staph. aureus*	*Salmonella enteritidis*	*B. cereus* var. *terminalis*
1-Hydroxy-4-aminoanthraquinone	−	−	−	−	±	++
1-Hydroxy-4-dimethylaminoanthraquinone	−	+	+	−	−	−
6,9-Dihydroxybenzo[g]quinoline-5,10-dione	++	−	±	−	±	−
6-Hydroxy-9-chlorobenzo[g]quinoline-5,10-dione	−	−	−	−	+	−
6,9-Dichlorobenzo[g]quinoline-5,10-dione	−	−	−	−	−	−
Bis-(4-chlorophenyl)quinolinate	−	−	−	−	−	−
2-Hydroxybenzo[g]quinoline-5,10-dione-4-carboxylic acid	+	±	−	−	−	−
Tetracycline (40 mcg.)	+	−	++	++	−	+

[a] Radii of zones of inhibition represented by different symbols are: (−) No inhibition. (±) Less than 2 mm. (+) 2–10 mm. (++) Greater than 10 mm.

activity: 6,9-dihydroxybenzo[g]quinoline-5,10-dione(I), 6-hydroxy-9-chlorobenzo[g]quinoline-5,10-dione(II), 6,9-dichlorobenzo[g]quinoline-5,10-dione(III), bis-(4-chlorophenyl) quinolinate (IV), 1-hydroxy-4-dimethylaminoanthraquinone(V).

In addition, two previously reported compounds were prepared and tested: 2-hydroxybenzo[g] quinoline-5,10-dione-4-carboxylic acid(VI) and 1-hydroxy-4-aminoanthraquinone(VII).

While most of the compounds tested showed some degree of inhibition to the growth of some organisms, none were found to approach tetracycline in effectiveness.

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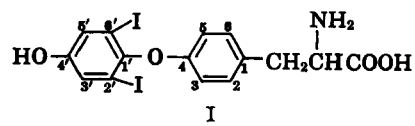
Thyroxine Analogs IX

4′-Deoxy-4′-β-L-alanyl-3,5-diiodo-L-thyronine and Related Stereoisomers as Thyroxine Antagonists

By EUGENE C. JORGENSEN and RICHARD CAVESTRI

On the basis of structural considerations and the activity of 2′,6′-diiodothyronine (I), 4′-deoxy-4′-β-L-alanyl-3,5-diiodo-L-thyronine (VIIb)[1] and a mixture of related stereoisomers have been prepared and tested for activity as thyroxine antagonists in the rat antigoiter assay. The L,L-isomer (VIIb) proved moderately effective as an antagonist at a molar ratio to L-thyroxine of 100:1. The mixed DL,DL-isomers (VIIa) as well as the L,L-isomer (VIIb) were ineffective when tested at a molar ratio to L-thyroxine of 200:1.

STUDIES directed at the preparation of peripheral antagonists to the thyroid hormones have had two principal objectives: (*a*) the development of an agent capable of rapidly reducing the undesirable effects due to high levels of circulating hormone in the various forms of hyperthyroidism, and (*b*) the gaining of a better understanding of the nature of interactions between the thyroid hormones and their biological receptor sites (1). 2′,6′-Diiodo-DL-thyronine (I) was the first compound containing the intact thyronine nucleus characteristic of thyroxine which was shown to possess thyroxine antagonistic properties. Among a number of halogenated thyronines prepared by Niemann and McCasland (2) and tested by Cortell (3),



I

only the 2′,6′-diiodo analog (I) showed thyroxine

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Paper VIII in this series is Reference 17.
[1] An alternate chemical name, 3,5-diiodo-4-(4′-L-phenylalanyloxy)-L-phenylalanine, is used in the *Experimental* section.

antagonistic activity when tested together with thyroxine at a molar ratio of 150:1 in the rat antigoiter assay. This compound has been extensively studied since. Antagonistic activity in the rat antigoiter assay has been confirmed (4) as well as the absence of detectable thyroxine-like activity in the rat and in man (5). Weak activity has been shown by the DL-isomer in antagonizing the thyroxine-induced elevation of oxygen consumption in the mouse (6).

With the exception of 2′,6′-diiodothyronine, all thyronine derivatives thus far studied have required at least one substituent in the 3 or 5 positions of the alanine-bearing ring for the presence of either thyroxine-like or antagonistic activity. For example, thyronine and its 3′-iodo- and 3′,5′-diiodo-derivatives have been shown to possess neither thyroxine-like (7–10) nor thyroxine-antagonistic (11, 12) properties. Thyronine derivatives with a single iodine atom in the alanine-bearing ring, *e.g.*, 3-iodo-, 3,3′-diiodo-, and 3,3′,5′-triiodo-DL-thyronines, have been shown to act as thyroxine antagonists by a variety of biological tests (12). 3,3′-Diiodothyronine (L-isomer (10), DL-isomer (13)), and 3,3′,5′-triiodo-DL-thyronine (14) have also been reported to show weak thyroxine-like activity. Maximal thyroxine-like activity in mammals has been invariably associated with the 3,5-diiodotyrosinyl grouping present in the natural hormones, L-thyroxine and 3,3′,5-triiodo-L-thyronine (7, 15). These consistent structural requirements for 3 or 3,5 substitution in the thyronine nucleus have led to the hypothesis (16) that the ring bearing these substituents may be responsible for primary binding to the biological receptor. Appropriate activating groups in the 3 or 5 positions (such as halogen or methyl (17)) may then function by contributing to the binding forces between the ring bearing these groups and the biological receptor. Bulky 3,5 substituents may also serve by steric effects to orient the two aromatic rings of the thyroid hormones and their analogs, so that the planes of the rings are perpendicular to each other. The suitably oriented phenolic ring with its hydroxyl group conjugated 1,4 to the ether oxygen may then comprise the functional unit of the thyromimetic molecule (16).

A peripheral antagonist may be visualized as one capable of binding to the hormonal receptor site but considerably less effective than the natural hormone in its functional role. Since in all other compounds tested 3 substitution is a requirement for minimal thyroxine-like activity, it seems possible that the iodine-bearing ring of 2′,6′-diiodothyronine may be the portion of the molecule fulfilling the binding role of the 3,5-diiodophenyl ring of thyroxine. Interaction of the 2′,6′-diiodo analog (I) with a hypothetical receptor structure (16) may be pictured as in Fig. 1. If so bound, the alanyl side chain of 2′,6′-diiodothyronine would occupy a position in space normally filled by the 4′-hydroxyl group of thyroxine. Phenyl ethers of 3,5-diiodotyrosine with a methyl group blocking the 4′ position against potential metabolic hydroxylation have shown thyroxine antagonistic properties in the rat antigoiter assay (16, 18). If the normal binding and functional roles of the alanine-bearing and phenolic rings are reversed with 2′,6′-diiodothyronine, the functional requirement for a 4′-hydroxyl group is not met and the alanyl residue acts as a 4′ blocking group. The molecule contains features which would be expected to facilitate transport to the biological receptor site—the alanyl group and a weakly dissociated phenolic group. It also possesses the diiodophenoxy structure for binding to the receptor, but if so bound, it lacks the structural characteristics at the "functional receptor" (Fig. 1) necessary for the functional role of a thyroxine-like compound—a hydroxyl group in 1,4 conjugation to the ether oxygen.

Because of the potential ability for an amino acid side chain to form ionic and hydrogen bonds with functional groups which might occupy positions peripheral to the receptor area, it was considered that the alanyl group could serve both as a blocking group and one providing a more firmly bound drug-receptor complex. To test this concept, a thyroxine analog possessing the 3,5-diiodotyrosinyl inner ring and a 4′-alanyl blocking group in the outer ring was prepared. The L-alanyl side chain in the inner ring was considered the most desirable for transport and binding since L-thyroxine and related

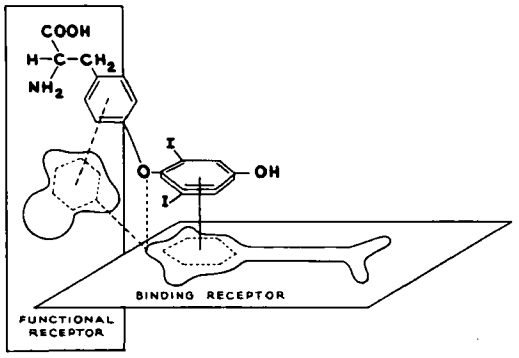


Fig. 1.—Schematic representation of 2′,6′-diiodothyronine interacting with a hypothetical thyroid hormone receptor.

L analogs display strong hormonal activity, while the corresponding D isomers possess relatively little (7). However, the more desirable configuration for the 4′-alanyl group (if such exists) could not be deduced, since activity has been reported only for the DL isomer of 2′,6′-diiodothyronine (3, 4, 6). Therefore, both the DL,DL compound (VIIa), comprising a mixture of all optical forms, and one of its enantiomers, the L,L compound (VIIb), were prepared.

The analogs were synthesized by the Meltzer (19) modification of the method of Chalmers, *et al.* (20). The appropriate DL or L isomer of N-acetyl 3,5-dinitrotyrosine ethyl ester was allowed to react with methanesulfonyl chloride and pyridine to form the N-acetyl 3,5-dinitro-4-pyridinium phenylalanine ethyl ester methanesulfonate (IIa,b). This was not isolated but was allowed to react with either the DL or L isomer of N-acetyl tyrosine ethyl ester (IIIa,b) to form the substituted dinitrodiphenyl ethers (IVa,b). These were converted to the diamines by hydrogenation and were bis-diazotized under anhydrous conditions without isolation and converted to the diiodo compounds (Va,b). Mild alkaline hydrolysis afforded the di-N-acetyl derivative (VI), while acidic conditions yielded the desired bis-amino acids (VIIa,b).

EXPERIMENTAL

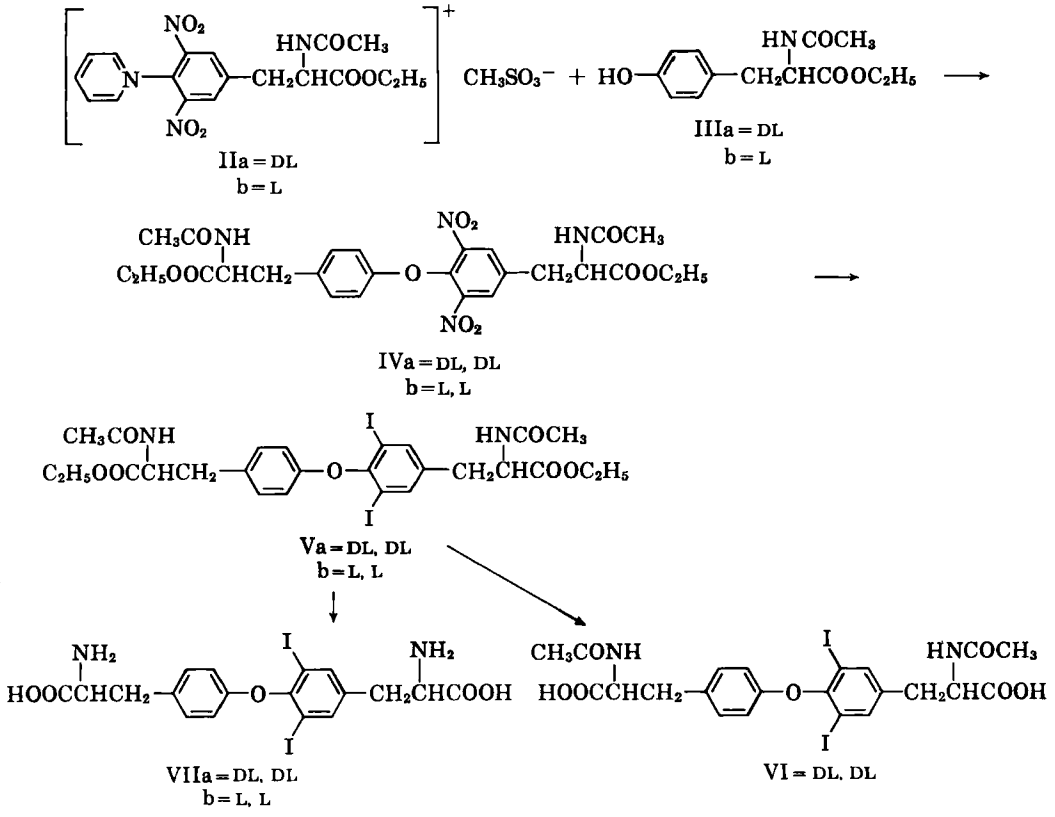
Synthetic

All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured with a Rudolph polarimeter. Microanalyses were carried out by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley.

N-Acetyl 3,5-Dinitro-4-(N-acetyl 4′-DL-phenylalanyloxy ethyl ester)-DL-phenylalanine Ethyl Ester (IVa).—To a solution of N-acetyl 3,5-dinitro-DL-tyrosine ethyl ester (21) (17.0 Gm., 0.05 mole) in dry pyridine (100 ml.), methanesulfonyl chloride (6.3 Gm., 0.055 mole) was added, and the mixture heated under reflux for 2 minutes. N-Acetyl DL-tyrosine ethyl ester (2) (IIIa, 15.9 Gm., 0.063 mole) was added and the solution heated under reflux for 15 minutes. The reaction mixture was poured into cold water (200 ml.) and extracted with benzene and chloroform. The combined extracts were washed successively with 2 *N* hydrochloric acid, water, 0.3 *N* sodium hydroxide, and water. The residue obtained following removal of the solvents under reduced pressure was crystallized from aqueous ethanol yielding 13.7 Gm. (46%), m.p. 171–172°.

Anal.—Calcd. for $C_{26}H_{30}N_4O_{11}$; C, 54.35; H, 5.25. Found: C, 54.30; H, 5.41.

N-Acetyl 3,5-Dinitro-4-(N-acetyl 4′-L-phenylalanyloxy ethyl ester)-L-phenylalanine Ethyl Ester (IVb).—The condensation of N-acetyl 3,5-dinitro-L-tyrosine ethyl ester (20) and N-acetyl L-tyrosine ethyl ester (6) (IIIb) was carried out as described above, except that reflux time was increased from 15



to 30 minutes. Crystallization from 50% ethanol yielded 16.5 Gm. (58%), m.p. 163–164°. $[\alpha]_D^{23}$ −8.0° (*c* 2.00, $CHCl_3$).

Anal.—Calcd. for $C_{26}H_{30}N_4O_{11}$; C, 54.35; H, 5.25. Found: C, 54.52; H, 5.33.

N-Acetyl 3,5-Diiodo-4-(N-acetyl 4′-DL-phenylalanyloxy ethyl ester)-DL-phenylalanine Ethyl Ester (Va).—The dinitro compound (IVa, 10.7 Gm., 0.019 mole) dissolved in acetic acid (200 ml.) was shaken for 45 minutes in the presence of palladium-on-charcoal (10%, 2.0 Gm.) and hydrogen (45 p.s.i. initial pressure). Concentrated sulfuric acid (40 ml.) was added with cooling. The catalyst was removed by filtration through Celite, and the solution of the diamine was added from a pressure-equalized dropping funnel under nitrogen during 1 hour to a well-stirred mixture of nitrosylsulfuric acid maintained at −5°. The nitrosylsulfuric acid was prepared from sodium nitrite (5.52 Gm., 0.08 mole), added in small portions to concentrated sulfuric acid (60 ml.) at 60–70°, then diluted when cool with acetic acid (60 ml.). After the addition was complete, the dark solution of the bis-diazonium compound was stirred for an additional hour at −5°, then poured rapidly into a well-stirred mixture at 5° of iodine (21.4 Gm., 0.084 mole), sodium iodide (30.0 Gm., 0.2 mole), urea (3.12 Gm., 0.052 mole), water (300 ml.), and chloroform (300 ml.). After stirring for 1 hour below 15°, the reaction mixture was allowed to reach room temperature and stirring was continued for an additional hour. The chloroform phase was removed, the aqueous layer was extracted with chloroform, and the combined chloroform extracts were washed successively with water, 10% aqueous sodium bisulfite, water, 1 *M* sodium bicarbonate, and water. The chloroform extract was dried over anhydrous sodium sulfate, and the chloroform removed at reduced pressure. The residue was taken up in acetone, and the oil which precipitated upon addition of an equal volume of ligroin (b.p. 35–60°) crystallized after standing for about 1 month at 0° yielding 4.2 Gm. (35%), m.p. 140–143°.

Anal.—Calcd. for $C_{26}H_{30}I_2N_2O_7$; C, 42.41; H, 4.11. Found: C, 42.89; H, 4.35.

N-Acetyl 3,5-Diiodo-4-(N-acetyl 4′-L-phenylalanyloxy ethyl ester)-L-phenylalanine Ethyl Ester (Vb).—The L,L-dinitro compound (IVb, 3.3 Gm., 0.0058 mole) was treated in the same way as above. The oily residue obtained crystallized readily on scratching and was recrystallized from ethanol yielding 2.4 Gm. (57%), m.p. 197–198°. $[\alpha]_D^{23}$ −6.3° (c 2.00, $CHCl_3$).

Anal.—Calcd. for $C_{26}H_{30}I_2N_2O_7$; C, 42.41; H, 4.11. Found: C, 42.67; H, 4.09.

N-Acetyl 3,5-Diiodo-4-(N-acetyl 4′-DL-phenylalanyloxy)-DL-phenylalanine (VI).—The bis-N-acetyl amino acid ethyl ester (Va, 1.11 Gm., 1.5 mmoles) was dissolved in absolute ethanol (30 ml.), sodium hydroxide (1.12 Gm., 30 mmoles) was added, and the mixture was stirred at room temperature for 2 hours. The solution was diluted with an equal volume of hot water, acidified to pH 3 with 2 *N* hydrochloric acid and allowed to crystallize overnight at 0°. Recrystallization from aqueous acetone yielded 0.27 Gm. (38%), m.p. 143–145°.

Anal.—Calcd. for $C_{22}H_{22}I_2N_2O_7$; C, 38.85; H, 3.26. Found: C, 38.6; H, 3.4.

3,5-Diiodo-4-(4′-DL-phenylalanyloxy)-DL-phenylalanine (VIIa).—The bis-N-acetyl amino acid ethyl ester (Va, 1.0 Gm.) was heated under reflux for 12 hours in glacial acetic acid (10 ml.) and concentrated hydrochloric acid (10 ml.). The mixture was treated with decolorizing carbon and taken to dryness under reduced pressure. The cream-colored residue was dissolved in 15 ml. of hot water with the aid of a few drops of concentrated hydrochloric acid. The hot solution was adjusted to pH 5 with a saturated solution of sodium acetate. After three such isoelectric precipitations, the white amino acid weighed 0.337 Gm. (37%), m.p. approximately 350° dec.

Anal.—Calcd. for $C_{18}H_{18}I_2N_2O_5 \cdot H_2O$; C, 35.21; H, 3.48. Found: C, 35.41; H, 3.57. On a separate sample dried at 65° (2 mm.)—Calcd. for $C_{18}H_{18}I_2N_2O_5$; I, 42.53. Found: I, 42.48.

3,5-Diiodo-4-(4′-L-phenylalanyloxy)-L-phenylalanine Dihydrochloride (VIIb).—The bis-N-acetyl amino acid ethyl ester (Vb, 1.0 Gm.) was heated under reflux for 12 hours in glacial acetic acid (10 ml.) and concentrated hydrochloric acid (10 ml.). The clear solution was taken to dryness at reduced pressure. The solid residue was dissolved by heating in boiling water (15 ml.) and concentrated hydrochloric acid (1 ml.). Cooling yielded the crystalline dihydrochloride, 0.832 Gm. (93%), m.p. 293–295° dec. $[\alpha]_D^{23}$ −1.6° (c 2.00, 1 *N* sodium hydroxide-ethanol (1:2)).

Anal.—Calcd. for $C_{18}H_{18}I_2N_2O_5 \cdot 2HCl$; C, 32.34; H, 2.99; I, 37.81. Found: C, 32.5; H, 2.9; I, 37.7.

Biological

The rat antigoiter assay was based on that of Dempsey and Astwood (22) and Cortell (3), in which thyromimetic activity was estimated by determining the amount of the test compound required to prevent the increase in thyroid weight brought about by the goitrogen, thiouracil. Thyroxine-antagonistic activity was tested by the ability of the test compound to reverse the antigoiter effect of thyroxine when administered concomitantly in thiouracil-fed rats. The assay was carried out as previously described (16). Specific assay conditions and results are collected in Table I.

DISCUSSION

At a molar ratio to L-thyroxine of 100:1, the L,L-isomer (VIIb) produced a 46.5% reversal[2] of the goiter preventing effect of thyroxine (Table I, assay 1). When the data was examined by the student's "t" test, the probability that the thyroid weight produced by thiouracil, thyroxine, and VIIb (14.9 ± 3.0 mg./100 Gm.) was the same as that produced by thiouracil and thyroxine alone (10.3 ± 4.0 mg./100 Gm.) was $P < 0.10$. Thus a moderate degree of antagonism of the antigoitrogenic effect of thyroxine was indicated. This is the same order of effectiveness reported[3] for 2′,6′-DL-diiodothyronine, where a 63% reversal of the effect of DL-thyroxine was observed at a molar ratio of 82:1; a constant reversal of between 43–48% was observed in a duplicate assay at molar ratios of 82:1,

[2] For the method of calculation see Reference 18.

[3] Values for per cent reversal and molar ratios were calculated from the data presented in Reference 4.

TABLE I.—RAT ANTIGOITER ASSAY OF 4′-DEOXY-4′-ALANYL-3,5-DIIODOTHYRONINES

Assay No.	Food	Compound Injected[a]	Daily Dose per 100 Gm., mcg.	Molar Ratio	Thyroid Wt. per 100 Gm. mg.	± s.d.
1[b]	Untreated				6.6	0.8
	TU[c]				20.2	3.9
	TU	Thyroxine	2.0	0.67	14.4	3.5
	TU	Thyroxine	3.0	1.0	10.3	4.0
	TU	Thyroxine	4.5	1.5	6.1	3.0
	TU	Thyroxine plus L,L-isomer (VIIb)	3.0 225	1.0 100	14.9[d]	3.0
2[b]	Untreated				8.2	1.6
	TU[c]				29.5	4.5
	TU	Thyroxine	2.0	0.67	18.2	5.2
	TU	Thyroxine	3.0	1.0	10.7	3.6
	TU	Thyroxine	4.5	1.5	9.1	3.9
	TU	Thyroxine plus L,L-isomer (VIIb)	3.0 450	1.0 200	12.7[e]	3.6
	TU	Thyroxine plus DL,DL-isomers (VIIa)	3.0 400	1.0 200	10.6[f]	2.6

[a] Thyroxine was used as sodium L-thyroxine pentahydrate, kindly provided by Dr. James Kerwin, Smith Kline and French Laboratories. [b] Six rats at each control and dose level. [c] Thiouracil 0.3%. [d] Reversal of thyroxine effect, 46.5% ($P < 0.10$). [e] Reversal of thyroxine effect, 11%; not significant. [f] No reversal of thyroxine effect.

164:1, and 328:1. This difficulty in producing a greater response with increased dose or duplicating the level of significance found earlier was observed in the second assay shown in Table I. At a molar ratio to L-thyroxine of 200:1, the L,L-isomer (VIIb) produced an 11% reversal of thyroxine's antigoitrogenic effect, a value which was not significantly different from the thyroxine-control value. The mixture of DL,DL-isomers (VIIa) had no effect at a molar ratio to L-thyroxine of 200:1 in the same assay.

The results with the L,L-isomer (VIIb) are indicative of a low order of thyroxine antagonistic activity. However, the inability to obtain reproducible and significant results in the rat antigoiter assay makes it undesirable to use this data in support of the proposals put forth regarding the nature of the antagonistic activity demonstrated by 2′,6′-diiodothyronine without further evaluation in other test systems. Since such evaluation will not be possible in the near future, the rationale for the preparation of these compounds and the results obtained to date are presented now.

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