

and shaking mechanically for 10 min. A 15-ml aliquot of hexane was removed, the hexane extraction was repeated twice more, and the hexane fractions were combined and reduced to dryness with nitrogen. Samples were taken up in 0.3 ml of hexane containing compound 3 as an internal standard, and the samples were used as described above.

A Microtek gas chromatograph equipped with a  $^{63}\text{Ni}$  electron-capture detector was used for analysis. A 3-ft glass column packed with 3% OV-225 on 100–120 mesh Gas Chrom Q was used. Nitrogen was used as carrier gas at 60 cc/min with 8 cc/min purge. Column temperature was 260° for compounds 1 and 3 and a temperature of 230 °C was used for compound 2 determinations. Inlet temperature was 295 °C and detector was 310 °C. The area under the peaks was calculated by xeroxing the chromatograms, cutting them out, and weighing them.

Retention times were 1 = 7 min; 3 = 9.5 min; and 2 = 7.25 min. Compound 3 was used as an internal standard for 1 and 1 was used for 3. 9-Chloro-1-methyl-7-phenylpyrimido[1,2-*a*][1,4]-benzodiazepin-3(5*H*)-one was the internal standard for compound 2 determinations.

One unit of compound 3 gave a response equivalent to 1.81 units of compound 1, and one unit of 9-chloro-1-methyl-7-phenylpyrimido[1,2-*a*][1,4]benzodiazepin-3(5*H*)-one gave a response equivalent to 1.92 units of 2. Results were not corrected for percent recovery.

**B. Gas Chromatograph–Mass Spectrograph Studies.** Essentially the same procedure was used for GC–MS studies of both compounds 5 and 4. Twenty-four male Sprague–Dawley rats, 200–225 g, were used in each study. Rats were injected with 30 mg/kg of the appropriate drug via the ip route as a suspension in 0.5 ml of sterile vehicle no. 122. After the drug was administered (30 min), the rats were anesthetized with halothane and 4–5 ml of blood was removed via heart puncture. The blood from two similarly treated rats was placed in a 100-ml glass-stoppered flask containing 50 ml of benzene and 4 ml of 0.1 M Tris buffer at pH 8.0. Flasks were mechanically shaken for 10 min and centrifuged, and the benzene layer was removed and combined with other samples. The benzene extraction was repeated a second time and combined with the first extraction. The total benzene extracts, approximately 900 ml from the 12 samples, were reduced to approximately 100 ml using a Rotovapor. The benzene solution was transferred to a separatory funnel and washed with 25 ml of 0.1 M Tris buffer, pH 8.0. The benzene layer was then reduced to dryness; the residue was dissolved in 50 ml of acetone, transferred to a clean flask, and reduced to dryness. This residue was dissolved in 50 ml of acetonitrile, transferred to a clean flask, and reduced to dryness. The final residue was taken up in 25 ml of benzene and placed on a 30 × 1 cm glass column containing

silica gel (70–325 mesh) to a height of 4.5 cm. The effluent was collected and the column was washed with 5 ml of benzene. The materials were then eluted from the column with acetone and 5-ml fractions were collected and saved. The entire procedure was monitored for compounds 1, 5, or 4 by using gas chromatographic methods described earlier. Compound 1 was found in acetone fractions 4–7, 5 in fractions 7–10, and 4 in fractions 7–10. The respective fractions were combined and reduced to dryness with a stream of  $\text{N}_2$ . In the compound 5 study, the final sample was dissolved in 0.15 ml of acetonitrile, and in the compound 4 study, 0.1 ml of acetone was used as the final solvent.

Analysis on the LKB was carried out using a 4-ft glass column packed with 3% OV-17 on Gas Chrom Q, with carrier gas flow rate at approximately 20 ml/min. Temperatures were: column, 255 °C in the compound 5 study and 245 °C in the 4 study; flask heater, 290 °C; ion source, 250 °C; separator, 280 °C; and electron energy of 70 eV. In the compound 5 study, the retention time of 1 at 255 °C was 20.6 min and for 5 it was 11 min. In the compound 4 study, the retention time of 1 at 245 °C was 33 min and for 4 it was 62.4 min. Mass spectra were obtained for authentic samples of 1, 5, and 4 and for the materials extracted from rat blood which had retention times comparable to those of the authentic compounds.

**C. Pharmacology Methods.** Antimetrazole activity was determined as previously described.<sup>3</sup>

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## Notes

### Synthesis of 3-Iodo-L-thyronine and Its Iodinated Derivatives

Paul Block, Jr.

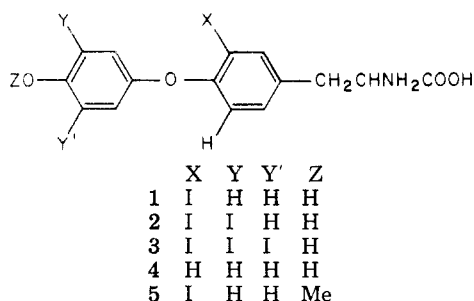
Department of Chemistry, University of Toledo, Toledo, Ohio 43606. Received November 3, 1975

3-Iodo-L-thyronine has been synthesized from iodo-L-tyrosine by coupling with an anisylidonium salt. Further iodination yields the 3,3'-diiodo and the 3,3',5'-triiodo derivatives. Chromatographic systems for separating these compounds and establishing their purity are described.

Interest in 3-iodo-L-thyronine (1) and its 3'-iodo (2) and 3,5'-diiodo (3) derivatives has recently been stimulated by the finding that appreciable amounts of these metabolites of the thyroid hormones may be formed in the developing fetus. Fetal metabolism of thyroxine in the human, implied from analysis of amniotic fluid, differs from that during extrauterine life in the predominance of inner ring

deiodination to produce high levels of 3.<sup>1</sup> Within a few hours of birth, serum levels of 3,5,3'-triiodothyronine rise rapidly from very low levels to those found in adults, accompanied by a more gradual fall in levels of 3.<sup>2</sup> The early appearance of 3 in the readily accessible amniotic fluid provides the first means for early detection and potential treatment of congenital or drug-induced hypo-

thyroidism in the developing fetus.<sup>3</sup> This development



was made possible by the availability of [<sup>125</sup>I]-3,3',5'-triiodothyronine of high specific activity for use in radioimmunoassay. The key intermediate for conventional or radiosynthesis of its further iodinated derivatives is 1. Radioiodination of 1 would provide labeled 2 and 3 of specific activities higher than could be obtained by exchange reactions.<sup>4</sup> Studies of the rate of formation of 2 during fetal development<sup>5</sup> require pure reference compound, as well as labeled compound of high specific activity. This paper describes a new synthesis of 1, free of the potential for contamination by 3,5-diiodo-L-thyronine, which was inherent in previous methods. Conditions are described for the iodination of 1 and for a chromatographic procedure for the isolation of 2.

3-Iodo-DL-thyronine was first synthesized by Roche,<sup>6</sup> who partially deiodinated commercially available 3,5-diiodo-DL-thyronine and separated the products by fractional crystallization. The method was subsequently refined by Varcoe and Warburton, who prepared the compound in the optically active state.<sup>7</sup> Although the method is simple, it is hard to purify the products. Any trace of 3,5-L-thyronine that might remain would, on reiodination, give rise to thyroxine and to the even more potent 3,5,3'-triiodo-L-thyronine, thus vitiating biological results. It would therefore seem preferable to employ a series of reactions in which 3,5-diiodo-L-thyronine has played no part.

A second approach whereby the alanine side chain was elaborated after the diphenyl ether linkage had been formed was employed by Burger and co-workers<sup>8a</sup> who utilized the series of reactions of Harington's original synthesis of thyroxine.<sup>8b</sup> A simplification of this approach was introduced by Roche<sup>9</sup> and subsequently pursued by others.<sup>10,11</sup> These methods lead only to DL compounds.

In the present work the iodonium salt method first suggested by Hillmann<sup>12</sup> is extended to protected 3-iodo-L-tyrosine. Use of variations of this method for synthesis of chloro-L-thyronines<sup>11</sup> has already been described.

Monoiodination of 1 leads to 2 and a lesser amount of 3, a compound also of current interest. Diiodination of 1 would yield 3 in a pure state, but unless a radioiodinated product of high specific activity is sought, this compound can be more simply synthesized in a one-step reaction from commercially available starting materials by Cahnmann's adaptation<sup>13</sup> of the method of Meltzer.<sup>14</sup>

The earlier work with iodonium coupling to monochlorinated tyrosine<sup>11</sup> employed conditions that required the more soluble iodonium chloride<sup>15</sup> rather than the bromide or iodide. It has now been found that the iodonium fluoborate reacts equally well under the same conditions but can be more simply purified because of the water solubility of silver fluoborate.

It has previously been indicated that the ethyl ester of *N*-acetylmonoiodo-L-tyrosine had not been crystallized.<sup>11</sup> In the present work the methyl ester was prepared and converted to the amide, which was readily purified. The

intermediate methyl ester was obtained as a solid, but its recrystallization in good yield was not satisfactory.

## Experimental Section

Melting points (corrected) were determined in a Hershberg apparatus. Microanalyses were done by Huffman Laboratories, Wheatridge, Colo. Optical rotations were measured on a Rudolph polarimeter, Model 26202. Concentrations were calculated on the basis of the anhydrous compounds. Analyses are indicated only by the symbols of the elements and are within 0.3% of the theoretical value. Solvent A<sup>16</sup> was 3-methyl-1-butanol-6 N NH<sub>4</sub>OH, used with Whatman No. 1. Solvent B<sup>17</sup> was chloroform-methanol-concentrated NH<sub>4</sub>OH (20:10:1), used with Quantum Industries Type Q5F and preparative plates Type PLQF, thickness 1 mm containing a preadsorbent area for convenience in sample application. Both types contained the same silica formulation and a phosphor. Solvent C<sup>18</sup> was ethyl acetate-2-propanol-25% NH<sub>4</sub>OH (55:35:20), used with Q5F.

**Di(*p*-anisyl)iodonium Salts.** The method of Beringer,<sup>19</sup> a useful alternative to that of Plati,<sup>20</sup> lent itself readily to a larger scale than described. Thus the iodine trifluoroacetate resulting from 30 g (0.236 g-atom) of iodine was taken up in 160 ml of acetic anhydride and to this solution was added a solution of anisole (52 g, 0.49 mol) in 80 ml of acetic anhydride and 32 ml of trifluoroacetic acid, following the time and temperatures recommended by Beringer. After removing the solvents the residue was dissolved in 350 ml of methanol and treated with a solution of potassium metabisulfite, followed by one of 40 g of sodium iodide in 400 ml of water. Refrigeration yielded 89 g (80%) of crude product. This material, ground if necessary, was suspended in six parts of water and stirred for 2 h with 1 equiv of silver sulfate.<sup>15</sup> Charcoal was added, and the solids were filtered off and washed with water. A slight excess of fluoboric acid was added and the precipitate collected after refrigeration: recovery 80–85%; mp 185–186°. For analysis, recrystallization from 20% ethanol was performed (5 ml/g). Anal. (C<sub>14</sub>H<sub>14</sub>BF<sub>4</sub>I) I.

***N*-Acetyl-3-iodo-L-tyrosine Amide.** *N*-Acetyl-3-iodo-L-tyrosine monohydrate<sup>11</sup> (18.4 g) was refluxed for 16 h in a solution of 15 ml of methanol and 100 ml of dichloroethane containing 1.5 g of toluenesulfonic acid. After cooling, water was added and the acid neutralized with sodium acetate solution. The separated dichloroethane solution was washed once with water, dried, and evaporated under vacuum. Two additions of methanol were reevaporated and the dry residue was treated with concentrated NH<sub>4</sub>OH (180 ml). The solution was left at room temperature in a closed flask for 20 h. After filtration it was evaporated under vacuum to a small volume and the solid collected: crude yield, 15 g (86%). Recrystallization from 50% acetic acid gave 11 g: mp 219–221°; [α]<sub>D</sub><sup>25</sup> +3.1° (c 1.5, DMF). Anal. (C<sub>11</sub>H<sub>13</sub>IN<sub>2</sub>O<sub>3</sub>) C, H, I.

***N*-Acetyl-3-iodo-4-(4-methoxyphenoxy)-L-phenylalanine Amide.** A sodium ethoxide solution containing the equivalent of 0.46 g of sodium was evaporated to dryness and the residue dissolved in 60 ml of DMF. *N*-Acetyl-3-iodo-L-tyrosine amide (7 g, 0.02 mol) was added and dissolved by gentle heating. To this solution 10.3 g (0.024 mol) of di(4-methoxyphenyl)iodonium fluoborate (or 9 g of chloride) was added and the reaction was maintained at 50–55° for 14 h with stirring. The filtered solution was evaporated under vacuum to a small volume, diluted with water, made slightly basic with sodium hydroxide, and refrigerated. The solid product was thoroughly dried, ground, and extracted twice with hexane to remove iodoanisole. There remained 9.6 g of crude product which was suspended in water (75 ml) and dissolved at the boiling point by addition of acetic acid. A second crystallization gave 5.8 g (64%): mp 185–187°. The analytical sample had mp 189–190°; [α]<sub>D</sub><sup>20</sup> -5.0° (c 1.5, DMF). Anal. (C<sub>18</sub>H<sub>19</sub>IN<sub>2</sub>O<sub>4</sub>) C, H, I, N.

**3-Iodo-L-thyronine (1).** The above amide (4 g) was hydrolyzed by boiling for 4.5 h in 60 ml of acetic acid and 10 ml of constant boiling HBr. The solvents were removed under vacuum, 120 ml of water was added, and the solution was neutralized to pH 5.5. The crude amino acid weighed 3.45 g (94%). It was purified by conversion to the hydrochloride to eliminate thyronine (4) and reprecipitated as the free amino acid following the conditions previously described:<sup>7</sup> overall yield, 65%. Chromatography showed a trace of a fast-running spot of the *O*-methyl derivative

**Table I.** Chromatographic Mobility of Some Iodinated Thyronines

Compd	Solvent		
	A	B	C
1	0.42	0.24	0.32
2		0.22	0.22
3		0.14	0.20
4	0.19	0.21	0.29
5	0.73	0.34	0.375

5 which was removed during the preparative TLC after iodination. If the product was not to undergo a chromatographic separation, the small amount of 5 could be removed by increasing the volume of HBr to 15.5 ml and the time of hydrolysis to 5.5 h. A second recrystallization of the hydrochloride from 2 N HCl before conversion to the free amino acid was required to eliminate the larger amount of 4 formed: overall yield, 55%. Purity was checked by paper chromatography (see Table I) (solvent A) (ascending, 18°): mp 253°;  $[\alpha]^{21D} +24.8^\circ$  [c 1, 1 N HCl-EtOH (1:1)] (lit.<sup>7</sup> +24.9°). Anal. (C<sub>15</sub>H<sub>14</sub>INO<sub>4</sub>·H<sub>2</sub>O) I.

**3,3'-Diiodo-L-thyronine (2).** 1 (0.834 mg) in 40 ml of concentrated NH<sub>4</sub>OH was treated dropwise with 1 N iodine solution (4.8 ml, 20% excess). After standing 1 h the solvent was removed under vacuum, adding 1-propanol if necessary to control foaming. The residue was redissolved in water by a few drops of ammonia and separated from an insoluble brown material. On addition of acetic acid to the filtrate, the amino acids precipitated as an off-white solid: 930–990 mg (80–85%). Batches (90–100 mg) were dissolved in ethanol–2 N ammonia (3:1) about 1 ml, and the solutions were streaked onto 20 × 20 cm preparative layer plates and developed in solvent B. They were dried and redeveloped several times until sufficient separation between the two heavy stripes was achieved, as determined by minimum exposure to uv light. A faint stripe of highest *R<sub>f</sub>*, identified as 5, was discarded. The middle stripe held 2; the stripe of lowest *R<sub>f</sub>* contained the triiodothyronine (3). The silica was eluted with ethanolic ammonia and filtered and the filtrate evaporated under vacuum. The dried products were recovered either by adding water and filtering or by dissolving in water by addition of a few drops of ammonia, transferring to a centrifuge tube, precipitating with acetic acid, washing the precipitate repeatedly with water, and lyophilizing. About 50% of the theoretical amount of 2 was obtained (mp 196°), whereas the less soluble 3 recovery averaged 75% (mp 204°). Using analytical plates with solvent B gave a single spot, but since 1 was not separated from the other compounds, solvent C was used. This also produced a single spot. 2 also gave a single peak by gas chromatography after silylation. The technique was that of Cahnmann:<sup>21</sup> column, 2 ft by 2.4 mm i.d. OV-1 (1%) on Supelcoport; oven programmed for 7.5°/min rise; nitrogen carrier (44 ml/min); starting temperature 178°. Under these conditions, compounds 4, 1, 2, and 3 emerged at 194, 210, 233, and 248°,

respectively:  $[\alpha]^{20D} +19.6^\circ$  [c 1, 1 N HCl-EtOH (1:1)] (lit.<sup>7</sup> +18.8°). Anal. (C<sub>15</sub>H<sub>13</sub>I<sub>2</sub>NO<sub>4</sub>·2H<sub>2</sub>O) I. Anal. (C<sub>15</sub>H<sub>12</sub>I<sub>3</sub>NO<sub>4</sub>·H<sub>2</sub>O) I.

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## Antifungal Activity of 4-Substituted Crotonic Acid Esters

Herman Gershon,\* Larry Shanks, and Daniel E. Gawiak

Boyce Thompson Institute for Plant Research, Yonkers, New York 10701. Received January 26, 1976

Twenty-three 4-substituted crotonic acid esters were tested for antifungal activity against *Candida albicans*, *Aspergillus niger*, *Mucor mucedo*, and *Trichophyton mentagrophytes*. For the analogues of the methyl ester containing substituents in the 4 position, the following order of fungitoxicity was observed: I > Br > Cl > CH<sub>3</sub>S > CH<sub>3</sub>O > F = H. Of the homologues of the esters of the 4-iodo and 4-bromo compounds which included methyl, ethyl, *n*-propyl, *n*-butyl, *n*-pentyl, and *n*-hexyl, ethyl 4-iodocrotonate was most toxic to the four fungi at pH 7.0 in the presence of 10% beef serum (*C. albicans*, 18 µg/ml, *A. niger*, 40 µg/ml, *M. mucedo*, 5 µg/ml, *T. mentagrophytes*, 4 µg/ml). It is believed that the mechanism of fungitoxicity is due, in part, to a nucleophilic reaction involving SH-containing compounds. This is based on the correlation of fungitoxicity with the order of leaving groups in the nucleophilic reaction and the protection against the toxicity of the test compounds to the fungi by cysteine and glutathione.

As medicine becomes more complex, iatrogenic diseases follow.<sup>1</sup> In recent years, this has been manifested by an

increase in the incidence of fungal infections.<sup>2</sup> Frequently, the attacking fungi are opportunistic invaders. They cause