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THE SYNTHESIS OF $^{13}\text{C}_9$ - ^{15}N -LABELED 3,5-DIIODOTHYRONINE AND THYROXINE

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Abstract

Thyroid hormones undergo extensive metabolism to regulate hormone activity. A labeled thyroid hormone would be useful to track hormone metabolism through various pathways. While radiolabeled thyroid hormones have been synthesized and used for in vivo studies, a stable isotope labeled form of thyroid hormone is required for studying thyroid hormone metabolism by LC-MS/MS, an analytical technique that has certain advantages without the complications of radioactivity. Here we report the synthesis of $^{13}\text{C}_9$ - ^{15}N -T₂ and $^{13}\text{C}_9$ - ^{15}N -T₄, two labeled thyroid hormone derivatives suitable for in vivo LC-MS/MS studies.

Graphical abstract

Keywords

$^{13}\text{C}_9$ - ^{15}N -T₂; $^{13}\text{C}_9$ - ^{15}N -T₄; thyroid hormone; thyroxine

INTRODUCTION

Thyroid hormones (THs) are important endocrine signaling molecules that regulate a variety of physiological functions, including body temperature, cardiac function, metabolism, and mental status. THs are synthesized and secreted by the thyroid gland predominantly as 3,3', 5,5'-tetraiodothyronine (T₄, thyroxine), which undergoes extrathyroidal deiodination to 3,3',5-triiodothyronine (T₃). T₃ is largely considered to be the active form of the hormone

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and exerts its effects by binding to TH nuclear receptors and regulating transcription of TH-responsive genes. In addition to the commonly known transcriptional actions of TH, there are rapid, nontranscriptionally mediated effects of TH that remain less well understood, suggesting the existence of additional biologically active TH metabolites.^[1,2]

T₁AM is an endogenous compound present in serum and various tissues of rats, hamsters, and humans.^[3–6] Acute administration of T₁AM *in vivo* results in induction of a torpor-like state characterized by hypothermia, bradycardia, a shift in respiratory quotient from carbohydrate to lipid utilization, hyperglycemia, and hypoinsulinemia.^[3,5,7] In an *ex vivo* perfused rat heart, T₁AM decreases cardiac output.^[3,8] These effects tend to be opposite those normally attributed to T₃ and suggest that T₁AM may also play a role in TH signaling by modulating the effects of T₃.

Structural similarities between T₄ and T₁AM (Fig. 1) have led to speculation that T₁AM is a deiodinated and decarboxylated derivative of TH, but this relationship has not been directly investigated. To test this metabolic question, it is necessary to definitively trace the fate of a suspected precursor, which can be accomplished through incorporation of a label in T₄. Several methods for analyzing T₁AM by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) have been reported.^[4,9,10] This technique is ideal for analysis of isotopically labeled compounds, because any metabolites arising from a labeled precursor would contain a mass signature distinct from endogenous compounds. T₄, as the predominant form of TH produced endogenously, is the first candidate to test as a suspected precursor to T₁AM. Based on *in vitro* data showing that T₃ is not a substrate for outer ring deiodination, it remains unclear if 3,5-diiodothyronine (3,5-T₂), another endogenous TH, arises from extrathyroidal metabolism of T₄ or some alternate biosynthetic pathway.^[10] This indicates a second potential candidate for a biosynthetic precursor to T₁AM. Herein we describe the novel syntheses of ¹³C₉-¹⁵N-3,5-T₂ and ¹³C₉-¹⁵N-L-thyroxine (¹³C₉-¹⁵N-T₄) that can be used to study TH metabolism by liquid chromatography–tandem mass spectroscopy (LC-MS/MS).

RESULTS AND DISCUSSION

Synthesis of ¹³C₉-¹⁵N-3,5-T₂

The synthesis of ¹³C₉-¹⁵N-L-T₂ was carried out in parallel with unlabeled T₂ according to the route shown in Scheme 1. Commercially available ¹³C₉-¹⁵N-L-tyrosine **1** was N-Boc and O-methyl ester protected and bis-iodinated with N-iodosuccinimide in dichloromethane (DCM), a modification of the method of Bovonsombat et al., to give ¹³C₉-¹⁵N-3,5-diiodo-L-tyrosine **3** in 59% yield over the three steps.^[11,12] 4-(Triisopropyl)silyloxyphenyl boronic acid was synthesized as previously described and coupled to **3** via a copper(II)-mediated biaryl ether formation.^[13,14] Deprotection with tetrabutylammonium fluoride (TBAF) gave N-boc-3,5-diiodo-¹³C₉-¹⁵N-L-thyronine-OMe **4** in 30% yield over two steps. Sequential deprotection with lithium hydroxide to cleave the methyl ester and hydrochloric acid to cleave the t-Boc gave ¹³C₉-¹⁵N-3,5-T₂ **5**, in 28% yield as the trifluoroacetic acid (TFA) salt after purification by preparative high-performance liquid chromatography (HPLC).^[15] Because of the difficulty in interpreting ¹H NMR spectra as a result of ¹³C coupling effects, labeled intermediates and ¹³C₉-¹⁵N-3,5-T₂ were characterized by high-resolution mass

spectrometry (HRMS) and chromatographic comparison to unlabeled standards, which were synthesized in parallel to $^{13}\text{C}_9$ - ^{15}N -labeled compounds.

Synthesis of $^{13}\text{C}_9$ - ^{15}N -L- T_4

The synthesis of $^{13}\text{C}_9$ - ^{15}N -L- T_4 was carried out in parallel with unlabeled T_4 according to the route shown in Scheme 2. Biaryl ether **4** was bis-iodinated on the outer ring with iodine monochloride and butylamine to give N-Boc- $^{13}\text{C}_9$ - ^{15}N - T_4 -OMe **6** in 37% yield.^[13] Sequential deprotection of **6** as described previously produced $^{13}\text{C}_9$ - ^{15}N - T_4 **7**, which was purified by preparative HPLC as the TFA salt in 23% yield from **6**. As described for $^{13}\text{C}_9$ - ^{15}N -3,5- T_2 , synthetic intermediates and $^{13}\text{C}_9$ - ^{15}N -L- T_4 were characterized by HRMS and chromatographic comparison to unlabeled standards, which were synthesized in parallel.

^{125}I , ^{131}I , and ^{14}C labeled THs have been used to study TH metabolism and pharmacokinetics but the use of radiolabels in metabolism studies is limited to tracing the fate of the radioactivity and does not provide information on metabolic pathways that do not involve radiolabeled portions of the hormones.^[16–25] In addition, the use of outer ring labeled ^{125}I - T_4 , the most commonly employed labeled form of T_4 , could not be used to follow T_4 metabolites that are extrathyroidally deiodinated in the outer ring, such as 3,5- T_2 and T_1AM . The incorporation of nonradioactive isotopes such as ^{13}C or ^{15}N into T_4 and other THs allows for more in-depth investigations of alternate pathways of TH metabolism by LC-MS/MS. The syntheses of unlabeled and of ^{13}C -labeled T_4 and T_3 have been reported, but here we report an alternate synthetic route to produce a novel labeled T_4 containing ^{15}N and ^{13}C .^[14,26–29] Although the synthesis of ^{125}I -3,5- T_2 has been reported, to our knowledge this is the first reported synthesis of a stable isotope labeled T_2 .^[30]

EXPERIMENTAL

All chemicals used for synthesis were purchased from Aldrich, Sigma-Aldrich, Fluka, Acros or TCI. Anhydrous solvents were obtained from an in-house solvent distillation system. Intermediates were purified by flash chromatography (HPFC) on a Biotage SP1 purification system with a fixed wavelength ultraviolet detector (Biotage, Charlotte, NC). Syntheses of unlabeled and ^{13}C - ^{15}N labeled compounds were carried out in parallel. The synthetic scheme was prospected using unlabeled material, which also served as standards to match ^{13}C - ^{15}N -labeled compounds by thin-layer chromatography (TLC). NMR spectra for ^{13}C -labeled compounds were difficult to interpret due to ^{13}C J-couplings. For this reason, ^{13}C labeled compounds were characterized by high-resolution mass spectrometry (HRMS) only. ^1H NMR were taken for unlabeled compounds only. ^1H NMR spectra were taken on a Bruker 400 (400 MHz), and spectra were processed using ACD/NMR Processor Academic Edition software. HRMS and MS/MS fragmentation (contained in the Supplemental Figures, available online) using electrospray ionization (ESI) in positive polarity was performed at the Mass Spectrometry Laboratory at the University of Illinois at Urbana–Champaign. HRMS are reported for all novel compounds, and MS/MS fragmentation for $^{13}\text{C}_9$ - ^{15}N -labeled compounds are available in the Supplementary Material, available online.

Boc-OMe-L-tyrosine

Concentrated hydrochloric acid (2.7 ml) was added dropwise to a stirring solution of L-tyrosine (0.500 g, 2.76 mmol) in 2,2-dimethoxypropane (33 ml). The reaction was stirred at room temperature for 24 h, and the crude reaction mixture was used in the next step.

NaHCO₃ (7.5 ml, 1.1 M in water) was added to crude OMe-L-tyrosine and Boc₂O (0.620 g, 2.85 mmol) in THF (12 ml). The reaction was stirred at room temperature for 24 h. The reaction was diluted with ether (20 ml), and the aqueous layer was extracted twice with ether. The combined organic layers were washed with 0.5 M HCl and brine and dried over MgSO₄. The crude material was purified by HPFC (10–80% ethyl acetate / hexanes over 10 column volumes) to give Boc-OMe-L-tyrosine (0.787 g, 2.66 mmol, 96.5% yield). ¹H NMR (400 MHz, chloroform-d) δ ppm 1.41 (s, 9 H) 2.86–3.21 (m, 2 H), 3.75 (s, 3 H), 4.47–4.63 (m, 1 H), 5.13 (br. s., 1 H), 5.48 (br. s., 1 H), 7.10 (s, 2 H), 7.64 (s, 2 H).

Boc-OMe-¹³C₉-¹⁵N-L-tyrosine **2** was synthesized using the same procedure with ¹³C₉-¹⁵N-L-tyrosine (0.500 g, 2.62 mmol) as starting material, in 87.2% yield (0.702 g, 2.30 mmol). HRMS (ESI+) for C⁶¹³C₉H²²¹⁵NO₅ [M + H] calculated 306.1770, found 306.1776. MS/MS fragmentation spectrum is available in Supplemental Fig. S1, available online.

3,5-Diiodo-Boc-OMe-L-tyrosine

N-Iodosuccinimide (1.2 g, 5.32 mmol) was added to a stirring solution of Boc-OMe-L-tyrosine (0.787 g, 2.66 mmol) in DCM (17.7 ml) at 0 °C. The reaction was monitored by TLC (40% ethyl acetate / hexanes) for consumption of starting material, which occurred in ~40 min. A solution of 10% Na₂S₂O₃ was added dropwise, and the reaction mixture was diluted with water and extracted three times with ethyl acetate. The ethyl acetate layers were combined and dried over MgSO₄. The crude material was purified by HPFC (10–80% ethyl acetate / hexanes over 10 column volumes) to give 3,5-diiodo-Boc-OMe-L-tyrosine as a white solid (0.908 g, 1.66 mmol, 67.5% yield). ¹H NMR (400 MHz, chloroform-d) δ ppm 1.45 (s, 9 H), 2.96 (s, 2 H), 3.68–3.79 (m, 3 H), 4.49 (d, J = 7.07 Hz, 1 H), 5.03 (d, J = 7.58 Hz, 1 H), 5.70 (s, 1 H), 7.44 (s, 2 H).

3,5-Diiodo-Boc-OMe-¹³C₉-¹⁵N-L-tyrosine **3** was synthesized following the same procedure from Boc-OMe-¹³C₉-¹⁵N-L-tyrosine (0.702 g, 2.30 mmol) starting material, in 67.5% yield (0.864 g, 1.55 mmol). HRMS (ESI+) for C⁶¹³C₉H₁₉I²¹⁵NNaO₅ [M + Na] calculated 579.9523, found 579.9528. MS/MS fragmentation spectrum is available in Supplemental Fig. S2, available online.

3,5-Diiodo-Boc-OMe-L-thyronine

Molecular sieves 4 Å were added to a round-bottom flask that was then flame dried and flushed with dry air. 4-(Triisopropyl)silyloxyphenyl boronic acid (0.612 g, 2.08 mmol), DCM (6.3 ml), diisopropylethylamine (4.15 mmol), and pyridine (4.15 mmol) were added under argon. Dry copper(II) acetate (0.151 g, 0.83 mmol) was added, and the reaction mixture was stirred under argon for 10 min. 3,5-Diiodo-Boc-OMe-L-tyrosine (0.452 g, 0.83 mmol) was added in DCM (2 ml) over 5 min. The reaction was stirred at room temperature under dry air for 48 h. The reaction was diluted with ether; filtered through silica/celite;

sequentially washed with HCl, H₂O, and brine, and dried over MgSO₄. The crude reaction material was taken into the next reaction.

Crude biaryl ether was dissolved in THF and cooled to 0 °C. Tetra-N-butyl-ammonium fluoride (3.15 mmol) was added dropwise over 5 min. The reaction was stirred at room temperature for 15 min. The reaction was diluted with ethyl acetate and washed with HCl. The aqueous layer was washed with ethyl acetate, and the combined organic layers were washed sequentially with H₂O and brine and dried over MgSO₄. The crude product was purified by HPFC (10–80% ethyl acetate / hexanes over 10 column volumes) to yield 3,5-diiodo-Boc-OMe-¹³C₉-¹⁵N-L-thyronine as a solid (0.196 g, 0.31 mmol, 37.0% yield over two steps). ¹H NMR (400 MHz, chloroform-d) δ ppm 1.45 (s, 9 H), 2.83–3.19 (m, 2 H), 3.66–3.88 (m, 3 H), 4.54 (br. s., 1 H), 4.69 (br. s., 1 H), 5.10 (br. s., 1 H), 6.62–6.92 (m, 4 H), 7.63 (s, 2 H). HRMS (ESI+) for C₂₁H₂₃I₂NNaO₆ [M + Na] calculated 661.9512; found 661.9512.

3,5-Diiodo-Boc-OMe-¹³C₉-¹⁵N-L-thyronine **4** was synthesized following the same procedure from 3,5-diiodo-Boc-OMe-¹³C₉-¹⁵N-L-tyrosine (0.547 g, 0.98 mmol) starting material, in 29.9% yield (0.190 g, 0.29 mmol). HRMS (ESI+) for C¹²¹³C₉H₂₄I²¹⁵NO₆ [M + H] calculated 649.9965; found 649.9966. MS/MS fragmentation spectrum is available in Supplemental Fig. S3, available online.

3,5-Diiodo-¹³C₉-¹⁵N-L-thyronine (¹³C₉-¹⁵N-T₂) **5**

To cleave the methyl ester, lithium hydroxide (0.39 mmol, 100 μl of 3.9 M in H₂O) was added to a stirred solution of 3,5-diiodo-Boc-OMe-¹³C₉-¹⁵N-L-thyronine (0.051 g, 0.078 mmol) in methanol (300 μl) at 4 °C. The reaction was stirred at 4 °C for 90 min. Solvent was removed under argon, and the crude reaction product was dried in a modified Abderhalden drying apparatus for use in the next step.

The dried crude reaction product from the methyl ester deprotection was dissolved in 4 M HCl in dioxane (300 μl). The reaction was stirred under argon at room temperature overnight. The reaction mixture was diluted with 500 μl of 1:1 water/acetonitrile and purified by preparatory HPLC using a Rainin HPXL solvent delivery system and a Prostar PDA detector (Varian Inc., Palo Alto, CA). Reaction product was injected onto a Varian Dynamax Microsorb C18 21.4 × 250 mm column and monitored at wavelength 254 nm. Mobile phases were A (water + 0.1% TFA) and B (acetonitrile + 0.1% TFA) with gradient conditions as follows: 5% B 0–5 min, 5–95% B 5–50 min, 95% B 50–65 min, 95–5% B 65–75 min, and 5% B 75–90 min. Commercially available 3,5-T₂ was used to determine the retention time of the product, 31.5 min. HRMS (ESI+) for C⁶¹³C₉H₁₄I²¹⁵NO₄ [M + H] calculated 535.9284; found 535.9291. MS/MS fragmentation spectrum is available in Supplemental Fig. S4, available online.

3,3',5,5'-Tetraiodo-N-Boc-OMe-L-thyronine

Iodine monochloride in DCM (0.26 mmol, 260 μl) was added dropwise to a stirring solution of 3,5-diiodo-Boc-OMe-L-thyronine (0.072 g, 0.11 mmol) and butylamine (0.64 mmol, 64 μl) in 4:1 DCM/DMF (3.7 ml) at 0 °C. The reaction was stirred at 0 °C for 20 min. The

reaction was diluted with ethyl acetate; washed sequentially with HCl, 10% sodium thiosulfate, H₂O, and brine; and dried over MgSO₄. The crude product was purified by HPFC (10–80% ethyl acetate / hexanes over 10 column volumes) to yield 3,3',5,5'-tetraiodo-N-Boc-OMe-L-thyronine as a brown solid (0.022 g, 0.025 mmol, 22.9% yield). ¹H NMR (400 MHz, chloroform-d) δ ppm 1.41 (s, 9 H), 2.86–3.21 (m, 2 H), 3.75 (s, 3 H), 4.47–4.63 (m, 1 H), 5.13 (br. s., 1 H), 5.48 (br. s., 1 H), 7.10 (s, 2 H), 7.64 (s, 2 H). HRMS (ESI+) for C₂₁H₂₁I₄NNaO₆ [M + Na] calculated 913.7445; found 913.7437.

3,3',5,5'-Tetraiodo-N-Boc-OMe-¹³C₉-¹⁵N-L-thyronine **6** was synthesized following the same procedure using 3,5-diiodo-Boc-OMe-¹³C₉-¹⁵N-L-thyronine (0.150 g, 0.23 mmol) as starting material, in 36.9% yield (0.076 g, 0.085 mmol). HRMS (ESI+) for C ¹³C₉H₂₁I₄¹⁵NNaO₆ [M + Na] calculated 923.7718; found 923.7715. MS/MS fragmentation spectrum is available in Supplemental Fig. S5, available online.

3,3',5,5'-Tetraiodo-¹³C₉-¹⁵N-L-thyronine (¹³C₉-¹⁵N-T₄) **7**

To cleave the methyl ester, lithium hydroxide (0.43 mmol, 110 μl of 3.9 M in H₂O) was added to a stirred solution of 3,3',5,5'-tetraiodo-Boc-OMe-¹³C₉-¹⁵N-L-thyronine (0.076 g, 0.085 mmol) in methanol (330 μl) at 4 °C. The reaction was stirred at 4 °C for 90 min. Solvent was removed under argon and the crude reaction product was dried in a modified Abderhalden drying apparatus for use in the next step.

The dried crude reaction product from the methyl ester deprotection was dissolved in 4 M HCl in dioxane (320 μl). The reaction was stirred under argon at room temperature overnight. To the reaction mixture, 500 μl of 1:1 water/acetonitrile was added, and the solution was filtered through a 0.22-μm filter prior to purification by preparatory HPLC as described previously. Commercially available T₄ was used to determine the retention time of the product, 37.7 min. HRMS (ESI+) for C₆¹³C₉H₁₁I₄¹⁵NO₄ [M + H] calculated 787.7217; found 787.7218. MS/MS fragmentation spectrum is available in Supplemental Fig. S6, available online.

Supplementary Material

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Figure 1.
Structures of T₄, 3,5-T₂, and T₁AM.

Scheme 1.

Reagents and conditions: (a), HCl, 2,2-dimethoxypropane; (b), BOC₂O, NaHCO₃, THF/H₂O; (c), NIS, DCM; (d), 4-(triisopropyl)silyloxyphenyl boronic acid, Cu(OAc)₂, DIPEA, pyridine, DCM; (e), TBAF, THF; (f), LiOH, MeOH/H₂O; and (g), HCl, dioxane. An asterisk (*) indicates ¹³C or ¹⁵N.

Scheme 2.

Reagents and conditions: (a), ICl, BuNH₂, 4:1 DCM/DMF; (b), LiOH, MeOH/H₂O; and (c), HCl, dioxane. An asterisk (*) indicates ¹³C or ¹⁵N.