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The synthesis of $^{13}C_6$-labeled L-thyronine, 3,5-diiodothyronine, 3,3′,5-triiodothyroacetic acid and 3,3′,5,5′-tetraiodothyroacetic acid

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ABSTRACT

The effects of thyroid hormone metabolites (THMs) other than T3, rT3 and T4 are largely unknown, partially due to the lack of adequate methods. For adequate analysis, internal standards for all THMs are essential, but unfortunately not commercially available. Reported approaches for the synthesis of T0, 3,5-T2, TA3 and TA4 lack sensitivity and/or are not adaptable for $^{13}C_6$-labeled analogues. In this paper, we describe the synthesis of four $^{13}C_6$-labeled THMs, T0-$^{13}C_6$, 3,5-T2-$^{13}C_6$, TA3-$^{13}C_6$, TA4-$^{13}C_6$. Starting with $^{13}C_6$-bromo-benzene, a short and versatile synthesis route was developed in which the formation of the diphenyl ether by a Chan-Lam coupling reaction was fundamental.

1. Introduction

Thyroid hormones (THs) have diverse effects in several important organs such as bone, heart, fat, liver, pituitary, muscle and brain regulating differentiation, growth and metabolism [1]. The thyroid gland is regulated by thyrotropin-releasing hormone (TRH) in the hypothalamus and thyroid-stimulating hormone (TSH) in the pituitary. This pathway is known as the hypothalamic-pituitary-thyroid (HPT) axis. Predominantly the prohormone 3,3′,5,5′-tetraiodothyronine (T4) and approximately 20% of the active metabolite 3,3′,5-triiodothyronine (T3) are produced by the thyroid gland. A negative feedback loop maintains the equilibrium of THs in the bloodstream as increased T4 and T3 in the bloodstream inhibits the secretion of TRH in the hypothalamus. TH metabolism produces 3,5-diiodothyronine (3,5-T2) and L-thyronine (T0) via deiodination or 3,3′,5-triiodothyroacetic acid (TA3) and 3,3′,5,5′-tetraiodothyroacetic acid (TA4) via decarboxylation and oxidative deamination (Fig. 1) [2]. Their role in (patho)physiology during health, disease and comorbidity remains unknown due to the lack of adequate methods to measure these thyroid hormone metabolites (THMs). Potential roles are described for 3,5-T2 in energy metabolism, for TA3 in brain, liver, muscle and heart and for TA4 in heart and pituitary, also elevated levels are observed in Graves' disease patients [3–8]. To study the role of THMs in health, disease and comorbidity, selective and sensitive LC-MS/MS methods measuring multiple THMs in a single analysis are needed. In recent years, several LC-MS/MS methods have been developed to measure single and multiple THMs [5,9–14]. Despite the apparent chemical and physical differences of these THMs, internal standards of other THMs have predominantly been used with the exception of $^{13}C_9$–^{15}N-labeled 3,5-T2 in the most recent studies [11,12,15]. This internal standard is however reported to be contaminated with 3,5-T2 itself [12]. Using the correct internal standard in LC-MS/MS panels is essential, as sample preparations and liquid chromatography and mass spectrometry settings are less optimal. For the synthesis of T0, 3,5-T2, TA3 and TA4 several approaches are previously reported [16–19]. These approaches either lack sensitivity and/or are not adaptable for the synthesis of $^{13}C_6$-labeled analogues. In this paper, we describe the synthesis of four $^{13}C_6$-labeled THM, T0-$^{13}C_6$, 3,5-T2-$^{13}C_6$, TA3-$^{13}C_6$, TA4-$^{13}C_6$, which were used to

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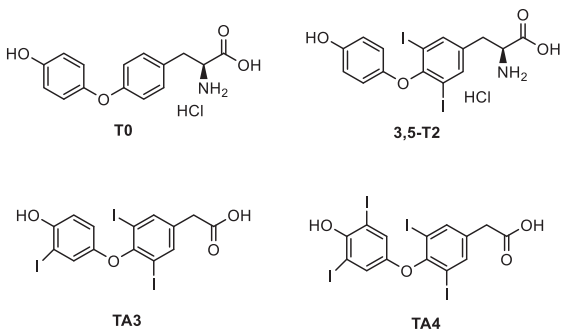


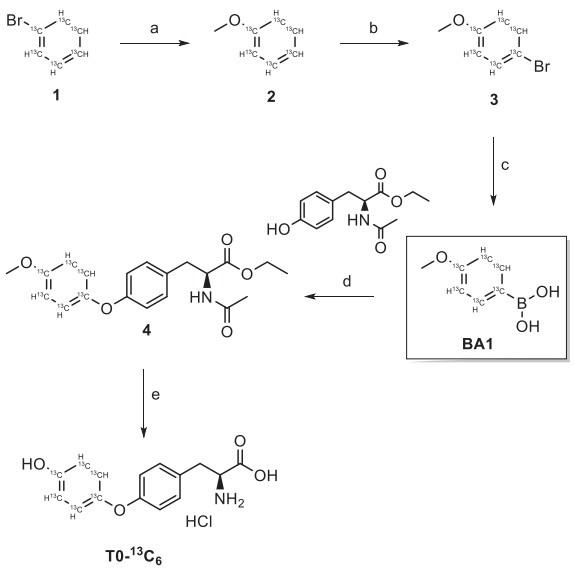
Fig. 1. Chemical structures of thyroid hormone metabolites **T0**, **3,5-T2**, **TA3** and **TA4**.

develop a nine-THM panel with liquid chromatography-tandem mass spectrometry (LC-MS/MS) [14].

2. Results/discussion

We developed a new synthesis route for four $^{13}C_6$-labeled THM analogues starting from readily available $^{13}C_6$-bromo-benzene which was first converted to $^{13}C_6$-anisole **2** using sodium methoxide and copper(I) bromide in DMF under Dean-Stark conditions (Scheme 1) [20]. Subsequently, **2** was selectively brominated using selectfluor and sodium bromide [21]. The boronic acid **BA1** was prepared via lithium exchange with the use of triisopropyl borate. This procedure [22] resulted in a higher yield of **BA1** compared to borylation with trimethoxy borate [23] or Grignard chemistry [24]. The formation of **BA1** was further optimized performing cryogenic quench with Glauber's salt instead of HCl in water to yield **BA1** quantitively.

In the next step, the ether bond was prepared by a Chan-Lam coupling reaction of **BA1** with *N*-Acetyl-L-tyrosine ethyl ester using copper (II) acetate, triethylamine and pyridine in dichloromethane at room temperature [25]. One-pot removal of the protective groups using hydroiodic acid or hydrobromic acid in boiling acetic acid was previously reported for T0-$^{13}C_6$ analogues [26]. Unfortunately, this procedure was not robust and often led to decomposition of the intermediate **4**. Therefore, the protective groups were removed stepwise instead of one-pot removal. First, boron tribromide was used to remove the methoxy ether followed by saponification of the methyl ester with lithium hydroxide and deacetylation of the amide group with hydrochloric acid in boiling acetic acid. With this synthesis route, T0-$^{13}C_6$ was obtained as a HCl salt in a good yield and high purity.



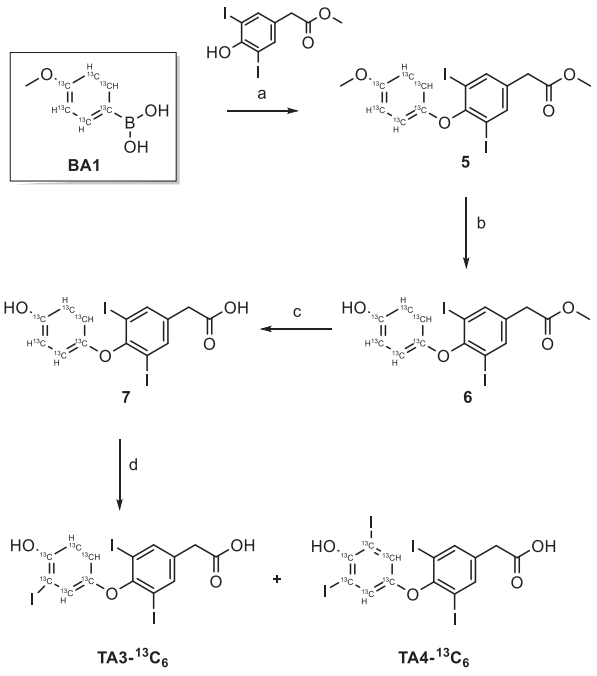
Scheme 1. Synthesis of T0-$^{13}C_6$.

Reagents and conditions: a) NaOMe, Cu(I)Br, anh. DMF, 120 °C, 5 h, 91%, b) selectfluor, NaBr, MeCN, 16 h, room temp., 98%, c) *n*-butyllithium, B(O*i*Pr)$_3$, anh. THF/toluene (1:4), −78 °C, 2 h, Glauber's salt, quant., d) *N*-Acetyl-L-tyrosine ethyl ester monohydrate, Cu(OAc)$_2$, pyridine, powdered molsieves, DCM, 16 h, room temp., 58%. E). i. BBr$_3$, anh. DCM, −78 °C, 1 h, ii. THF, LiOH, 3 h, iii. HCl/AcOH (1/2), reflux temp., 6 h, 87%.

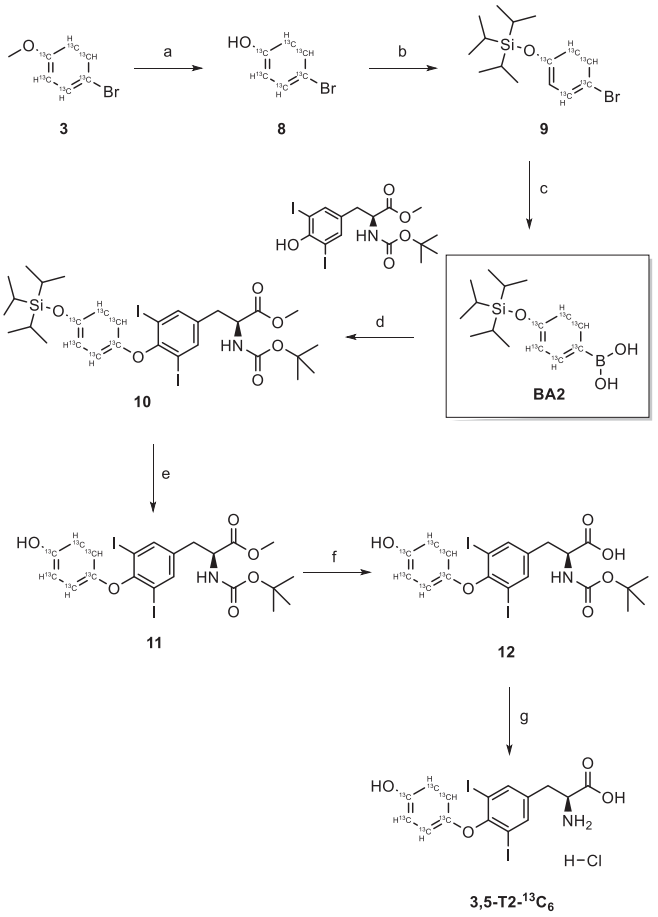
This strategy was also used for the synthesis of TA3-$^{13}C_6$ and TA4-$^{13}C_6$ (Scheme 2). The precursor **5** was obtained via a Chan-Lam coupling reaction of BA1 with commercially available methyl 2-(4-hydroxy-3,5-diiodophenyl)acetate. Direct iodination of **5** using iodide in ammonia to form TA3-$^{13}C_6$ and TA4-$^{13}C_6$ analogues was not successful and led to over iodination and decomposition. Therefore, both protective groups were removed stepwise and intermediate **7** was halogenated using iodide in ammonia. With this synthesis route, a 2:1 mixture of TA3-$^{13}C_6$ and TA4-$^{13}C_6$ was formed. TA3-$^{13}C_6$ and TA4-$^{13}C_6$ were separated by preparative HPLC, which yielded 10% of TA3-$^{13}C_6$ and 30% of TA4-$^{13}C_6$ in a high purity.

Reagents and conditions: a) Cu(OAc)$_2$, pyridine, powdered molsieves, DCM, 16 h, room temp., 57%, b) BBr$_3$, anh. DCM, −78 °C, 79% c) THF, LiOH, 16 h, 82% d) NH$_3$, I$_2$, MeOH, 0 °C, 1 h, 10% TA3-$^{13}C_6$, 30% TA4-$^{13}C_6$.

Metabolite 3,5-T2-$^{13}C_6$ was synthesized via a Chan-Lam coupling reaction using triisopropyl silyl ether protected boronic acid **BA2** and Boc-protected 3,5-diiodo-L-tyrosine methyl ester (Scheme 3). The previously used conditions to remove *O*-methoxy and *N*-acetyl groups in the synthesis of T0-$^{13}C_6$ (Scheme 1) were



Scheme 2. Synthesis of TA3-$^{13}C_6$ and TA4-$^{13}C_6$.



Scheme 3. Synthesis of 3,5-T2-$^{13}C_6$.

considered to be too harsh for 3,5-T2-$^{13}C_6$ and would cause decomposition of the product. To prepare **BA2**, bromoanisole **3** was first deprotected followed by protection as its triisopropyl silyl ether. Next the bromide was converted into its boronic acid by lithium exchange with the use of triisopropyl borate [27]. The Chan-Lam coupling reaction with Boc-3,5-diiodo-L-tyrosine methyl ester and boronic acid **BA2**, gave the diphenyl ether in moderate yield. The silyl ether was cleaved using TBAF in good yield and the ester group hydrolysed using lithium hydroxide. After deprotection of the amine by hydrochloric acid in dioxane, 3,5-T2-$^{13}C_6$ was obtained as HCl salt in a high purity.

3. Conclusion

In this paper, we described a new synthesis route for the preparation and isolation of pure T0-$^{13}C_6$, TA3-$^{13}C_6$, TA4-$^{13}C_6$ and 3,5-T2-$^{13}C_6$. The Chan-Lam coupling reaction can be used to synthesize $^{13}C_6$-labeled thyroid hormone analogues.

Reagents and conditions: a) BBr_3, anh. DCM, −78 °C, 87%, b) TIPSCl, imidazole, 0 °C, DCM, 1 h, 62%, c) *n*-butyllithium, B(O*i*Pr)$_3$, anh. THF, −70 °C, 2 h, 66%, d) Cu(OAc)$_2$, pyridine, powdered molsieves, DCM, 16 h, room temp., 49%, e) TBAF, THF, room temp., 10 min, 76%, f) THF/water (4/1), LiOH, 0 °C, 3 h, 99%, g) HCl, dioxane, 3 h, 54%.

4. Experimental section

NMR spectra were recorded on a Bruker AMX 400 NMR (400 MHz) spectrometer. Signal positions are recorded as chemical shifts (δ) in parts per million (ppm) and referenced to the residual solvent peaks ($CDCl_3$ or DMSO-d_6) and TMS as an internal standard. The multiplicities are reported as follows br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and combination of these. Doublet of multiplets (dm) is used to describe the complex splitting patterns in ^{13}C-labeled compounds, and the reported $^1J_{H,C}$ coupling constant is measured as peak-to-peak distance between the middle of each multiplet group. HRMS spectra were recorded on a LC-MS Q Exactive Focus HRMS spectrometer, which was calibrated with the Pierce calibration solution both in positive and negative mode.

Bromobenzene- [13]C_6 (99 atom% ^{13}C) was purchased from Sigma-Aldrich (Darmstadt, Germany).

4.1. *Anisole-1,2,3,4,5,6−$^{13}C_6$* ***2***

In a one neck-flask equipped with Dean-Stark apparatus, bromobenzene-1,2,3,4,5,6−$^{13}C_6$ (1.9 mL, 18.4 mmol) was dissolved in *N,N*-dimethylformamide (dry) (5 mL). A 5.4 M solution of sodium methoxide in methanol (5.1 mL, 27.6 mmol) was added dropwise with a syringe. The mixture was refluxed at 120 °C for ~1.5 h until ~3 mL of methanol was collected. Then, copper (I) bromide (0.264 g, 1.841 mmol) was added and the reaction was continued for ~2 h. The mixture was diluted with dichloromethane (25 mL) and filtered through a plug of kieselguhr. Subsequently, the filtrate was mixed with water/dichloromethane (1:1; 100 mL) and the layers were separated. Additionally, the dichloromethane layer was washed four times with 50 mL of water, separated and dried with sodium sulphate. After concentration under reduced pressure, the title compound was obtained in 91% yield.

^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.30 (dm, J_{HC} = 132 Hz, 2H); 6.94 (dm, J_{HC} = 168 Hz, 3H); 3.81 (d, J = 4.1 Hz, 3H).

4.2. *1-Bromo-4-methoxybenzene-1,2,3,4,5,6−$^{13}C_6$* ***3***

To a solution of **2** (0.95 g, 5.00 mmol) in anhydrous acetonitrile (20 mL) was added sodium bromide (0.51 g, 5.0 mmol) and selectfluor (1.77 g, 5.0 mmol). The reaction mixture was stirred overnight at room temperature under nitrogen atmosphere. The suspension was diluted with ethyl acetate (50 mL) and filtered through a silicagel plug. The filtrate was concentrated under reduced pressure to yield **3** (1.05 g, 4.90 mmol) in a 98% yield.

^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.38 (dm, $J_{H,C}$ = 156 Hz, 2H); 6.80 (dm, $J_{H,C}$ = 176 Hz, 2H); 3.78 (d, 3H, J = 4.2 Hz).

4.3. *(4-methoxyphenyl-1,2,3,4,5,6-$^{13}C_6$)boronic acid* ***BA1***

In a three neck flask equipped with nitrogen in and outlet, **3** (0.6 g, 3.11 mmol) was dissolved in a mixture of anhydrous tetrahydrofuran (2 mL) and anhydrous toluene (8 mL) and cooled down to −75 °C. Then, a 2.5 M solution of *n*-butyllithium in hexanes (1.37 mL, 3.42 mmol) was added dropwise over the course of 15 min, maintaining the temperature below −70 °C. After stirring for 1 h at −75 °C, triisopropyl borate (0.79 mL, 3.42 mmol) was added dropwise with the temperature not exceeding −60 °C. The reaction was continued for 30 min and quenched with 10 g of Glauber's salt at −50 °C. After 15 min at −50 °C, 50 mL of methanol was added and the mixture was left to warm up to room temperature. Subsequently, the mixture was filtered and concentrated under reduced pressure. The crude material was co-evaporated three times with 20 mL of toluene and three times with a 1:1

solution of dichloromethane and pentane to yield the title compound (490 mg, 3.11 mmol) quantitively as a white solid.

^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 7.80 (dm, $J_{H,C}$ = 164 Hz, 2H); 6.87 (dm, $J_{H,C}$ = 160 Hz, 2H); 3.78 (d, 3H, J = 4.2 Hz).

4.4. Ethyl (S)-2-acetamido-3-(4-(4-methoxyphenoxy-1,2,3,4,5,6−$^{13}C_6$)phenyl)propanoate **4**

BA1 (62.8 mg, 0.398 mmol), ethyl acetyl-L-tyrosinate (50 mg, 0.199 mmol) and copper (II) acetate (108 mg, 0.597 mmol) were mixed in dichloromethane (1 mL) and powdered 4 Å molsieves in 8 mL screw cap reaction vial. Then pyridine (0.161 mL, 1.990 mmol) was added and the mixture was stirred overnight. The crude mixture was concentrated under reduced pressure and purified on silica gel column using 0−20% ethyl acetate in heptane as eluents to yield the title compound (42 mg, 0.116 mmol, 58% yield).

^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.02 (d, J = 8.5 Hz, 2H); 7.00 (dm, J_{HC} = 160 Hz, 2H); 6.90 (dm, J_{HC} = 160 Hz, 2H); 6.85 (d, J = 8.5 Hz, 2H); 5.93 (d, J = 7.6 Hz, 1H); 4.84 (q, J = 7.2 Hz, 1H); 4.16 (q, J = 7.2 Hz, 2H); 3.81 (d, J = 4.2 Hz, 3H); 3.11 (dd, J = 14.0, 5.8 Hz, 1H); 3.06 (dd, J = 14.0, 5.8 Hz, 1H); 2.0 (s, 3H), 1.26 (t, J = 7.1 Hz, 3H).

4.5. (S)-2-Amino-3-(4-(4-hydroxyphenoxy-1,2,3,4,5,6−$^{13}C_6$) phenyl)propanoic acid HCl-salt ***T0-$^{13}C_6$***

To a solution of **4** (45 mg, 0.124 mmol) in anhydrous dichloromethane (10 mL) at −78 °C under nitrogen atmosphere, a 1 M solution of boron tribromide in dichloromethane (0.8 mL, 0.800 mmol) was dropwise added and stirred for 1 h at −78 °C. The mixture was then quenched with methanol and concentrated under reduced pressure. The crude material was dissolved in tetrahydrofuran (10 mL) and lithium hydroxide (1 M) (0.619 mL, 0.619 mmol) was added. The reaction was stirred at room temperature for 3 h. Subsequently, the reaction mixture was diluted with 30 mL ethyl acetate and 30 mL of 1 M HCl solution in water. The layers were separated and the water layer was extracted twice with 50 mL ethyl acetate. The organic layers were dried and concentrated under reduced pressure. The crude mixture was dissolved in a solution of hydrochloric acid (10 mL, 329 mmol) and acetic acid (20 mL, 349 mmol) and refluxed for 6 h. The slightly yellow solution was concentrated under reduced pressure and the obtained solids were triturated with dichloromethane (10 mL). The title compound was obtained as a HCl-salt (30 mg, 0.107 mmol) in 87% yield.

^{1}H NMR (400 MHz, DMSO-d_6) δ: 13.71 (s, 1H); 9.40 (s, 1H); 8.24 (bs, 2H); 7.21 (d, J = 8.5 Hz, 2H); 6.86 (dm, J_{HC} = 161 Hz, 2H); 6.85 (d, J = 8.5 Hz, 2H); 6.78 (dm, J_{HC} = 162 Hz, 2H); 4.13 (t, J = 6.0 Hz, 1H); 3.08 (dd, J = 14.4, 6.1 Hz, 1H); 3.02 (dd, J = 14.5, 6.9 Hz, 1H).

^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 153.9 (td, J = 65.3, 9.0 Hz), 147.8 (td, J = 68.4, 9.0 Hz), 121.7−119.9 (m), 117.1−115.3 (m).

HRMS 280.127,513 ($[M+H]^+$, calculated), 280.12558 ($[M+H]^+$, found), Δ = −6.91 ppm.

4.6. Methyl 2-(3,5-diiodo-4-(4-methoxyphenoxy-1,2,3,4,5,6−$^{13}C_6$) phenyl)acetate ***5***

To **BA1** (200 mg, 1.267 mmol), methyl 2-(4-hydroxy-3,5-diiodophenyl)acetate (265 mg, 0.633 mmol), copper (II) acetate (345 mg, 1.900 mmol), molecular sieves (1 g) in anhydrous dichloromethane (10 mL) was added pyridine (0.102 mL, 1.267 mmol). The brown/green mixture was stirred at room temperature overnight. Subsequently, **BA1** (100 mg, 0.633 mmol) was added and the reaction was stirred for 1 h. The reaction mixture was diluted with dichloromethane (20 mL) and concentrated in vacuo. The product was purified using flash column chromatography with 360% ethyl acetate in heptane as eluents to yield the title compound (192 mg, 0.362 mmol, 57% yield) as a colourless oil.

^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.78 (s, 2H); 6.84 (dm, $J_{H,C}$ = 156 Hz, 2H); 6.72 (dm, $J_{H,C}$ = 156 Hz, 2H); 3.78 (s, 3H), 3.75 (s, 3H); 3.57 (s, 2H).

4.7. Methyl 2-(4-(4-hydroxyphenoxy-1,2,3,4,5,6−$^{13}C_6$)-3,5-diiodophenyl)acetate ***6***

To a solution of **5** (220 mg, 0.394 mmol) in anhydrous dichloromethane (30 mL) at −78 °C under nitrogen atmosphere was added a 1 M solution of boron tribromide in dichloromethane (1 mL, 1.000 mmol) and the mixture was allowed to reach room temperature. After 2.5 h, the mixture was cooled to −78 °C under nitrogen atmosphere and a 1 M solution of boron tribromide in dichloromethane (1 mL, 1.000 mmol) was added dropwise. The cold mixture was quenched with 60 mL of methanol and concentrated under reduced pressure. The crude product was purified on a silica gel column with 0−40% ethyl acetate in heptane as eluents to yield the title compound (170 mg, 0.310 mmol, 79% yield).

^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.77 (s, 2H); 6.76 (dm, $J_{H,C}$ = 161 Hz, 2H); 6.67 (dm, $J_{H,C}$ = 156 Hz, 2H); 4.48 (s, 1H); 3.75 (s, 3H); 3.57 (s, 2H).

4.8. 2-(4-(4-hydroxyphenoxy-1,2,3,4,5,6−$^{13}C_6$)-3,5-diiodophenyl) acetic acid **7**

To a solution of **6** (50 mg, 0.097 mmol) in tetrahydrofuran (10 mL) was added lithium hydroxide (0.6 mL, 0.600 mmol) dropwise during 2 min. The reaction was stirred overnight at room temperature. Additional lithium hydroxide (0.1 mL, 0.100 mmol) was added and reaction was continued until completion (2 h). The mixture was partitioned between ethyl acetate (50 mL) and brine (20 mL). The water layer was extracted two additional times with ethyl acetate (50 mL). The combined organic layers were dried on sodium sulphate and concentrated under reduce pressure. The solids were triturated with dichloromethane and pentane, and dried to yield the title compound (40 mg, 0.080 mmol, 82% yield).

^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 12.22 (bs, 1H); 9.13 (s, 1H); 7.85 (s, 2H); 6.69 (dm, J_{HC} = 156 Hz, 2H); 6.52 (dm, J_{HC} = 156 Hz, 2H); 3.51 (s, 2H).

4.9. 3,3′,5-triiodo thyroacetic acid- [13]C_6 ***TA3-$^{13}C_6$*** and 3,5,3′,5′-tetraiodo thyroacetic acid- [13]C_6 ***TA4-$^{13}C_6$***

To a solution of **7** (30 mg, 0.060 mmol) in methanol (0.6 mL) was added a solution of 32% ammonia in water (0.5 mL, 7.32 mmol) at 0 °C, followed by the dropwise addition of 0.1 M iodine in methanol (0.149 mL, 0.015 mmol). The light yellow mixture was reacted at 0 °C for 1 h, followed by a second portion of 0.1 M iodine in methanol (0.447 mL, 0.045 mmol). After 1 h the reaction mixture was poured out in 10% solution of sodium metabisulfite in water (10 mL) and extracted with ethyl acetate (3 × 10 mL). The combined organic layers were dried on Na_2SO_4 and concentrated in vacuo. The crude mixture was purified by preparative LC-MS and lyophilized to yield pure TA3-$^{13}C_6$ (3.71 mg, 0.006 mmol, 10% yield) and pure TA4-$^{13}C_6$ (13.59 mg, 0.018 mmol, 30% yield).

TA3-$^{13}C_6$

^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 12.54 (bs, 1H); 10.06 (s, 1H); 7.82 (s, 1H); 6.99 (dm, $J_{H,C}$ = 163 Hz, 1H); 6.83 (dm, $J_{H,C}$ = 160 Hz, 1H); 6.58 (dm, $J_{H,C}$ = 164 Hz, 2H); 3.57 (s, 2H).

^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 152.7−151.1 (m), 149.9−148.1 (m), 124.7 (t, J = 68.0 Hz), 117.0−114.2 (m), 85.3−83.6 (m).

HRMS 626.776,342 ([M − H]⁻, calculated), 626.77685 ([M − H]⁻, found), Δ = 2.56 ppm.

TA4-$^{13}C_6$

^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 12.50 (bs, 1H); 9.20 (bs, 1H); 7.84 (s, 2H); 7.06 (dm, $J_{H,C}$ = 165 Hz, 2H); 3.58 (s, 2H).

^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 152.6–148.5 (m), 125.9–124.1 (m), 88.6–86.9 (m).

HRMS 752.672,985 ([M − H]⁻, calculated), 752.67334 ([M − H]⁻, found), Δ = 1.93 ppm.

4.10. 4-Bromophenol- [13]C_6 **8**

To a solution of **3** (2.3 g, 8.34 mmol) in dichloromethane (50 mL) was added boron tribromide (0.868 mL, 9.18 mmol) and left stirring overnight at room temperature. As the reaction was not completed two more portions of a 1 M solution of boron tribromide in dichloromethane were added over a period of 24 h (8.34 mL and 4.17 mL). The mixture was quenched with 100 mL water. The organic layer was evaporated at 35 °C and 300 mbar reduced pressure. The residue was dissolved in diethyl ether (100 mL) and washed with saturated aqueous solution of sodium bicarbonate, brine and dried over sodium sulphate, filtered and concentrated at 35 °C and 300 mbar reduced pressure to afford the title compound (2.46 g, 7.29 mmol, 87% yield) as a brown oil containing 13% of unreacted starting material. The title compound was used as such in step 4.11.

^{1}H NMR (CDCl3, 400 MHz) δ (ppm): 7.31 (dm, $J_{H,C}$ = 166 Hz, 2H); 6.72 (dm, $J_{H,C}$ = 160 Hz, 2H); 6.55–6.45 (m, 1H); 5.90 (s, 1H).

4.11. (4-Bromophenoxy-1,2,3,4,5,6–$^{13}C_6$)triisopropylsilane **9**

Triisopropylsilyl chloride (1.698 mL, 8.01 mmol) was added to a solution of **8** (2.46 g, 7.29 mmol) in dichloromethane (50 mL). The reaction mixture was cooled with an ice bath and imidazole (1.240 g, 18.21 mmol) was added. Subsequently, the reaction was stirred for 30 min at 0 °C and slowly warmed up to room temperature and stirred overnight. The reaction mixture was diluted with diethyl ether and washed two times with 0.5 M hydrochloric solution in water and saturated aqueous sodium bicarbonate and brine. Then, the mixture was dried over sodium sulphate, filtered and concentrated in vacuo. The crude product was purified by flash chromatography with 0–35% solution of ethyl acetate in heptane as eluents to obtain the title compound (1.52 g, 4.53 mmol, 62.2% yield).

^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.30 (dm, $J_{H,C}$ = 160 Hz, 2H); 6.75 (dm, $J_{H,C}$ = 156 Hz, 2H); 1.35–1.15 (m, 3H); 1.11 (s, 9H); 1.09 (s, 9H).

4.12. (4-((triisopropylsilyl)oxy)phenyl-1,2,3,4,5,6–$^{13}C_6$)boronic acid **BA2**

Under nitrogen atmosphere, **9** (1.52 g, 4.53 mmol) was dissolved in tetrahydrofuran (dry) (50 mL). The reaction mixture was cooled to −78 °C and 2.5 M *n*-butyllithium solution in hexanes (2.176 mL, 5.44 mmol) was added. The reaction mixture was stirred for 1 h followed by dropwise addition of triisopropyl borate (1.421 mL, 6.12 mmol). The reaction mixture was stirred for 1 h at −78 °C and was then allowed to warm up to room temperature. After stirring overnight at room temperature the reaction mixture was cooled to 0 °C and quenched with 3 N solution of HCl in water (3 mL) and stirred for 1 h at 0 °C. Then, the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over sodium sulphate, filtered and concentrated in vacuo. The crude product was purified by flash chromatography with 0–60% solution of ethyl acetate in heptane as eluents to yield **BA2** (905 mg, 3.01 mmol, 66.5% yield).

^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 8.08 (dm, $J_{H,C}$ = 163 Hz, 2H); 6.98 (dm, $J_{H,C}$ = 160 Hz, 2H); 1.35–1.20 (m, 3H); 1.14 (s, 9H); 1.12 (s, 9H).

4.13. Methyl (S)-2-((tert-butoxycarbonyl)amino)-3-(3,5-diiodo-4-(4-((triisopropylsilyl)oxy)phenoxy-1,2,3,4,5,6–$^{13}C_6$)phenyl) propanoate **10**

Molecular sieves (4 Å, powdered, 700 mg, 0.555 mmol), Boc-3,5-diiodo-L-tyrosine methyl ester (304 mg, 0.555 mmol), **BA2** (250 mg, 0.833 mmol) and copper (II) acetate (302 mg, 1.665 mmol) were mixed in anhydrous dichloromethane (8 mL). Pyridine (0.090 mL, 1.110 mmol) was added and the reaction mixture was stirred overnight. Additional pyridine (0.045 mL, 0.555 mmol) was added and the reaction was continued at room temperature for 24 h. The reaction mixture was quenched with 0.5 M solution of HCl in water. The aqueous layer was extracted two times with ethyl acetate and the combined organic layers were washed with brine, dried over sodium sulphate, filtered and concentrated in vacuo. The crude product was purified by flash chromatography with 0–50% solution of ethyl acetate in heptane as eluent to yield the title compound (220 mg, 0.274 mmol, 49% yield).

^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.62 (s, 1H); 6.79 (dm, $J_{H,C}$ = 162 Hz, 2H); 6.61 (dm, $J_{H,C}$ = 160 Hz, 2H); 5.09 (d, J = 8.1 Hz, 1H); 4.54 (d, J = 7.2 Hz, 1H); 4.50 (bs, 1H); 3.76 (s, 3H); 3.10 (dd, J = 13.7 Hz, 5.6 Hz, 1H); 2.92 (dd, 13.9 Hz, 6.5 Hz, 1H); 1.45 (s, 9H); 1.15–1.30 (m, 3H); 1.09 (s, 12H); 1.07 (s, 6H).

4.14. Methyl (S)-2-((tert-butoxycarbonyl)amino)-3-(4-(4-hydroxyphenoxy-1,2,3,4,5,6–$^{13}C_6$)-3,5-diiodophenyl)propanoate **11**

To a solution of **10** (220 mg, 0.274 mmol) in anhydrous tetrahydrofuran (5 mL) was dropwise added a 1 M solution of tetra-*n*-butylammonium fluoride in tetrahydrofuran (0.412 mL, 0.412 mmol). After 10 min the reaction mixture was quenched with 0.5 M solution of HCl in water. The reaction mixture was diluted with water and extracted three times with ethyl acetate. The combined organic layers were washed with brine, dried over sodium sulphate, filtered and concentrated in vacuo. The crude product was purified by flash chromatography with 0–60% solution of ethyl acetate in heptane as eluent to yield the title compound (134 mg, 0.208 mmol, 76% yield) as a white solid.

^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.63 (s, 2H); 6.76 (dm, $J_{H,C}$ = 159 Hz, 2H); 6.65 (dm, $J_{H,C}$ = 159 Hz, 2H); 5.09 (d, J = 8.1 Hz, 1H); 4.55 (bs, 1H); 4.50 (bs, 1H); 3.76 (s, 3H); 3.10 (d, J = 13.0 Hz, 1H); 2.90 (dd, 13.6 Hz, 6.6 Hz, 1H); 1.45 (s, 9H).

4.15. (S)-2-((tert-butoxycarbonyl)amino)-3-(4-(4-hydroxyphenoxy-1,2,3,4,5,6–$^{13}C_6$)-3,5-diiodophenyl)propanoic acid **12**

11 (134 mg, 0.208 mmol) was dissolved in a mixture of tetrahydrofuran (4 mL) and water (1 mL) and cooled down with an ice bath. Then, lithium hydroxide monohydrate (43.6 mg, 1.038 mmol) was added and the reaction mixture was conducted for 3 h at 0 °C. Reaction mixture was acidified with 0.5 M solution of HCl in water and extracted two times with ethyl acetate. The combined organic layers were washed with brine, dried over sodium sulphate, filtered and concentrated to yield the title compound (130 mg, 0.206 mmol, 99% yield).

4.16. 3,5-Diiodo-L-thyronine- [13]C_6 HCl-salt **3,5-T2-$^{13}C_6$**

12 (130 mg, 0.206 mmol) was dissolved in 4 M hydrochloric acid

in 1,4-dioxane (2 mL, 57.6 mmol) and reacted for 3 h at room temperature. Then, the reaction mixture was concentrated under reduced pressure and the resulting solids triturated in methyl tert-butyl ether (2 mL), filtered and dried to yield a white solid. This material was dissolved in acetonitrile/water and lyophilized to yield the title compound (63 mg, 0.111 mmol, 53.9% yield) as a white solid.

^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 9.11 (s, 1H); 8.09 (bs, 2H); 7.82 (s, 2H); 6.68 (dm, $J_{H,C} = 161$ Hz, 2H); 6.55 (dm, $J_{H,C} = 156$ Hz, 2H); 4.03 (s, 1H); 3.13 (dd, $J = 14.3$, 5.3 Hz, 1H), 3.02 (dd, $J = 14.3$ Hz, 7.7 Hz, 1H).

^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 153.0–151.1 (m), 149.7–147.9 (m), 116.8–114.8 (m).

HRMS 531.920,800 ([M+H]$^+$, calculated), 531.91812 ([M+H]$^+$, found), $\Delta = 5.04$ ppm.

Declaration of Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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