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Synthesis of tritium labelled thiorphan, an enkephalinase inhibitor

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Tritium labelling of the enkephalinase inhibitor, thiorphan, is complicated by the presence of mercapto functional group. Reactions often used in aromatic tritiation, such as halogination and catalytic halogen/tritium displacement, are adversely affected by the presence of the divalent sulfur moeity. By protecting the SH group with *t*-butyl group, the tritiation reaction proceeded smoothly without catalyst poisoning. The mercapto functionality was re-generated with great ease and efficiency using 2-nitrobenzenesulfenyl chloride and dithiothreitol (DTT). [^{3}H]-Thiorphan, thus, obtained had a radiochemical purity of $>$99% by AN-HPLC and a specific activity of 18.42 Ci/mmol. [^{3}H]-Thiorphan showed good stability when stored at 4°C in an aqueous solution containing 10% methanol and 0.2% DTT in the dark.

Keywords: tritium labelled thiorphan; enkephalinase; neutral endopeptidase; NEP; radioligand; tritiation; thiorphan stability

Introduction

Thiorphan is a thiol containing drug, which has been clinically administered as its *S*-acetyl *O*-benzyl prodrug racecadotril (also known as acetorphan) for the treatment of diarrhea.[1,2] Thiorphan shows low nanomolar inhibitory activity against neutral endopeptidase (NEP or enkephalinase), a zinc-metallopeptidase widely distributed in peripheral tissues and in the brain whose biological functions include catabolism of the opioid peptides.[3,4] Enkephalinase inhibitors have received comprehensive studies for their potential therapeutic applications namely in CNS and digestive tract diseases.[5] Tritium labelled thiorphan[6,7] and acetorphan[8] have been used as radioactive probes for characterization of enkephalinase (Figure 1).

There are two types of tritium labelled thiorphan known in the literature, one with the labelling on glycine methylene (**1**) utilizing commercially available [^{3}H]-glycine as tritium source,[6,7] and another labelled on phenyl ring (**2**) through tritium–bromine exchange reaction, which required preparation of the corresponding halogenated precursor.[9] However, the yield and specific activity in both cases were very low, encountering either catalyst poisoning due to the presence of the divalent sulfur group or somewhat lengthy experimental preparation. These problems are the predominant factors that have limited the availability and use of [^{3}H]-thiorphan. Therefore, a new and robust synthesis of [^{3}H]-thiorphan is needed.

Results and discussion

Initial experiments indicated that direct halogenations of thiorphan or acetorphan suffered from *S*-oxidation, forming either disulfide in aromatic iodination with bis(pyridine)iodonium tetrafluoroborate or sulfonic acid in bromination with bromine. In the latter experiment the phenyl group was brominated.

It became clear that bromine had to be introduced prior to generation of free thiol group in order to avoid the *S*-oxidation. Two bromine containing thioesters **8** and **9** were then synthesized (Scheme 1).[10] Methyl 3-(dimethylamino)propionate **3** was coupled individually with 3- and 4-bromobenzyl bromides **4** to form aminoesters **5** in 36 and 32% yield, respectively. The low yields from these reactions were mostly due to competing *N*-benzylation followed by elimination leading to *N*,*N*-dimethylbromobenzylamine by-products. Compounds **5** were further derivatized into the ammonium iodides **6** (85 and 82% yield), which underwent base-catalyzed elimination to afford vinyl acids **7** (85 and 93% yield). Sulfur was introduced by Michael addition of thioacetic acid to give thioesters **8** in 95% and **9** in 87% yield.

Following an analogous literature procedure[9] compound **9** was converted into disulfide **12** through three transformations (Scheme 2). Amide formation under standard peptide coupling conditions afforded **10** in 91% yield. The protecting groups in compound **10** were then removed by treatment with NaOH in methanol to give mercapto acid **11,** which underwent oxidation by iodine to form the disulfide **12** in 81% yield in two steps. Catalytic debromination by deuterium gas for compound **12**, as well as for simpler analog **8**, was examined under various conditions (catalysts: Pd/C, Pd/$BaSO_4$, $Pd(OH)_2$/C, $Pd(OAc)_2$, $PdCl_2$(dppf); reductants: D_2, $NaBD_4$; solvents: THF, dioxane, AcOEt, DMF, EtOH, MeOH, H_2O; bases: TEA, EDTA). Most of the conditions showed either no reaction or undesired side reactions (S–C and S–S cleavage). In the best cases where reductions were conducted in H_2O with 20% $Pd(OH)_2$/C as catalyst (catalyst/substrate $\sim$2:1 or more, by weight) and in the presence of triethylamine, only trace amounts ($<$5%) of the desired product **13** from **12** and up to 20% of

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bromine/deuterium exchange product from **8** were observed along with the starting materials and some S–C and S–S cleavage by-products. Such poor conversions were mostly due to catalysts poisoning caused by divalent sulfur groups in the molecules.

Activity of the sulfur groups in these molecules (SAc in **8** and SS in **12**) needed to be attenuated in order to increase its stability and minimize the observed catalyst poisoning effects. Among SH protecting groups, the more bulky and stable *S-t*-butyl group is considered a better choice for preventing the interaction between sulfur and the active sites of catalyst as well as reducing the chance to release the free SH group or undergo S–C cleavage.

The acetyl group in compound **8** was removed by basic hydrolysis to form mercapto acid **15** (Scheme 3), which was then heated with *t*-butanol in the presence of aq. HCl to form **16** with the desired *S-t*-butyl group[11] in 51% yield in two steps. The coupling reaction between **16** and glycine benzyl ester gave the amide **17** in 79% yield. Compound **17** was used as the precursor for tritium incorporation. It was expected that under palladium-catalyzed tritiation reaction, the bromo and benzyl groups would be reduced simultaneously to afford tritium labelled *S-t*-butyl thiorphan.

Indeed, in a cold run using deuterium gas (Scheme 4), bromine/deuterium exchange and de-benzylation occurred under the optimized catalytic reduction conditions (D_2, $Pd(OH)_2$/C, TEA, H_2O) to afford the D-labelled **18** quantitatively. MS analysis of **18** (D) revealed the deuterium distribution as D_0 30%, D_1 47%, and D_2 22%, respectively. The effectiveness of *S-t*-butyl protection was also observed when compound **16** was subjected to a similar bromine/deuterium exchange conditions affording the reduction product exclusively with D-incorporation as D_0 10%, D_1 60%, D_2 29%. In contrast, when **17** was deuterated to form **18** in ethyl acetate with Pd/C as catalyst, product **18** (D) had a more specific D_1 labelling (D_0 3%, D_1 94%, D_2 3%) even though the reaction was slower and not quantitative (79% yield). The removal of *t*-butyl group in **18** (D) was accomplished by using 2-nitrobenzenesulfenyl chloride,[11] which effectively displaced the *t*-butyl group with nitrobenzenethio group giving the disulfide **19** (D) via a sulfonium ion intermediate.[12] Upon treatment with DTT[13] and triethylamine in methylene chloride, the S–S bond in **19** (D) was easily reduced to thiols yielding deuterium-labelled thiorphan **20** quantitatively.

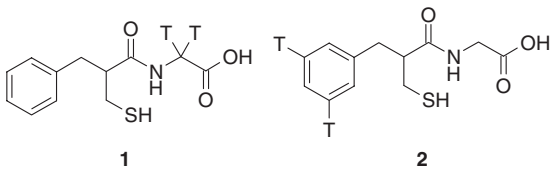
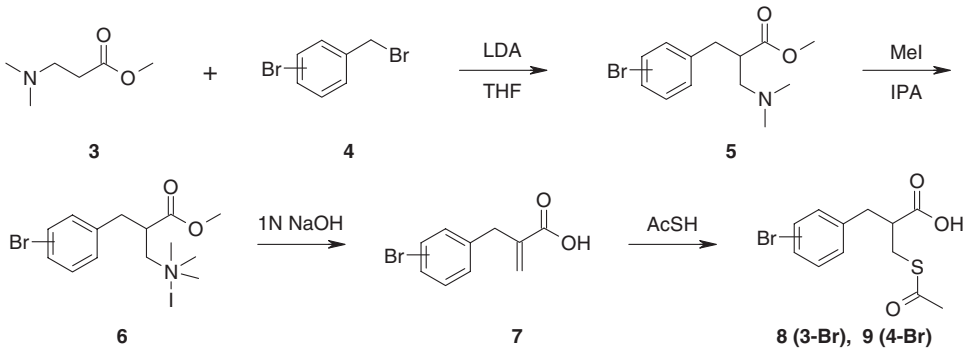
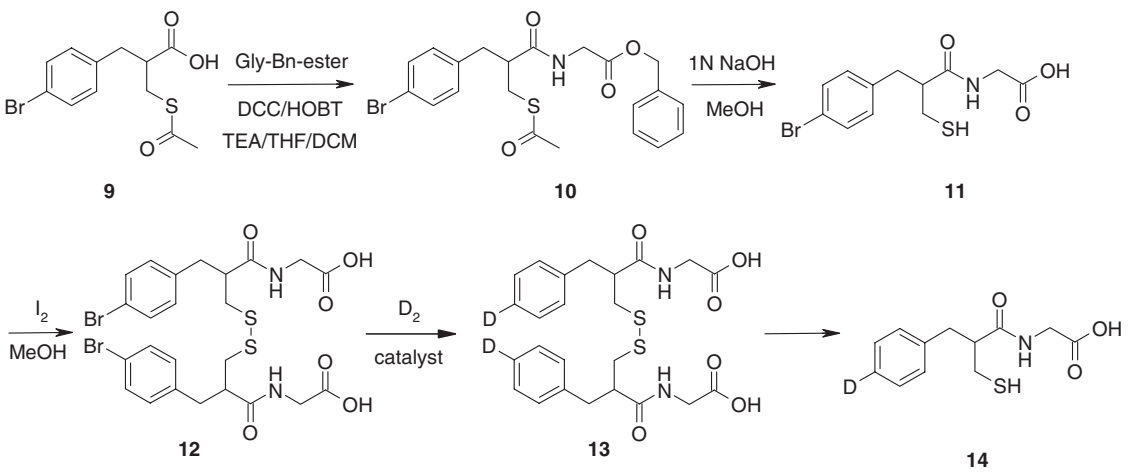


Figure 1. [^{3}H]-Thiorphan in the literature.



Scheme 1. Preparation of 2-(acetylsulfanylmethyl)-3-(3- or 4-bromophenyl) propionic acid **8** and **9**.



Scheme 2. Preparation of brominated disulfide **12** as labelling precursor.

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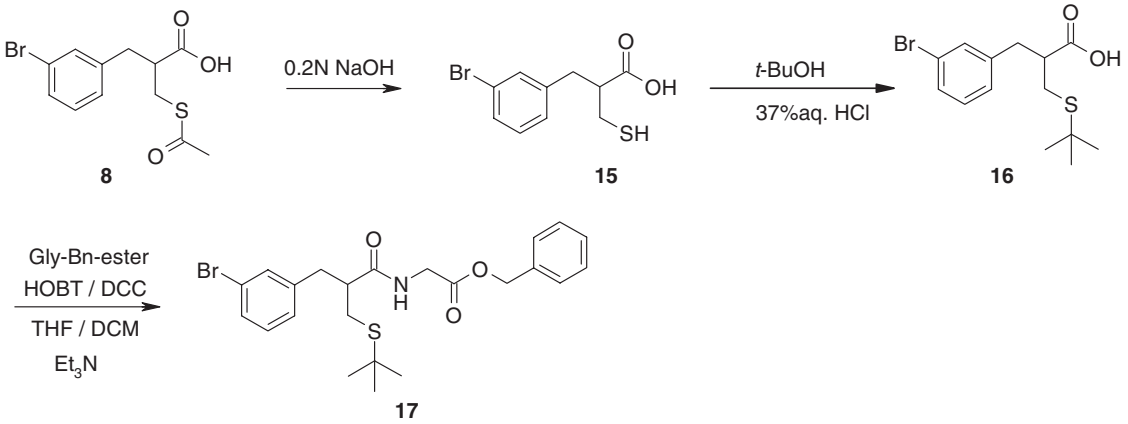
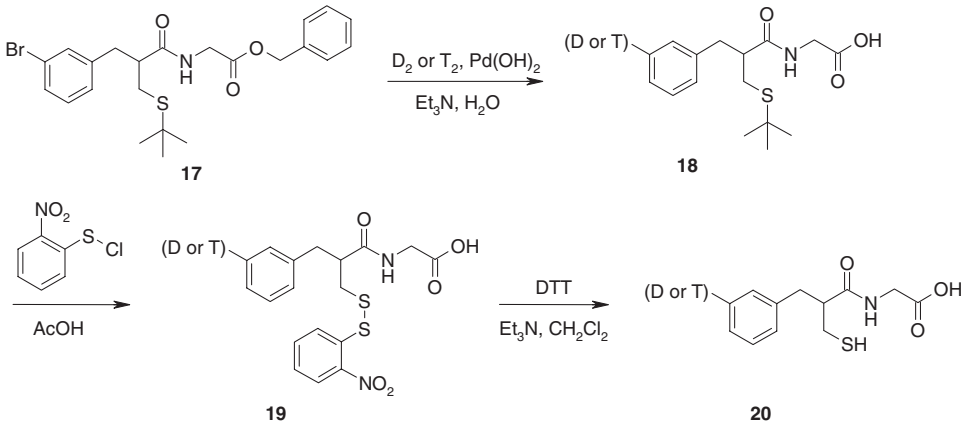


Scheme 3. Preparation of 2-[3-(3-bromophenyl)-2-(*S*-*t*-butylsulfanylmethyl)propionylamino] acetic acid benzyl ester **17** as labelling precursor.



Scheme 4. Preparation of [^{2}H] and [^{3}H]-thiorphan.

It is well known that thiorphan is unstable in solutions and easily undergoes oxidation to form the corresponding disulfide. According to some reports,[14,15] aqueous solutions with slightly acidic pH seem essential for prolonging shelf-life. An initial study indicated that when unlabelled thiorphan was kept as a 0.005% ethanol solution at 4°C under Ar with or without addition of acetic acid (0.01%), its purity degraded to 61 or 64% in 30 days, respectively. The major degradation product was the expected diastereomeric pair of thiorphan disulfides, which showed characteristic 1:1 peaks on HPLC and identical MS profiles. In search of a suitable solvent system to store the synthesized [^{3}H]-thiorphan, stability studies were conducted on non-labeled thiorphan in H_2O and EtOH as the primary solvents and DTT or β-mercaptoethanol (BME) as an antioxidant. Some of the solutions contained a cosolvent (MeOH, H_2O) and/or an acid additive (TFA, HCl, AcOH). The solutions were kept at 4°C under N_2 in the dark and were analyzed by HPLC at 3, 15, 30 and 90 days after initial preparations. Thiorphan was more stable in solutions containing DTT than BME. Acid additives had adverse effects in most cases. Solutions in the water–DTT series showed the highest level of purity retention across all the time points. Among them, the one containing 10% methanol and 0.2% DTT had the least degradation (4% in 90 days). Solutions in the other series showed moderate to severe degradation and thiorphan purity dropped 20–90% in 90 days. In those that had the most degradation, the major impurity was the disulfide arising from cross coupling of thiorphan and anti-oxidant BME. The disulfide of thiorphan itself was consistently less than 1% in all solutions.

The deuterium labelling conditions were then applied for tritiation reaction (Scheme 4). Compound **17** (6 mg) was reduced with 5 Ci of carrier-free tritium gas affording 219 mCi of crude product **18** (T) at 87% radiochemical purity. A portion of the crude **18** (T, 44 mCi) underwent consecutive de-*t*-butylation and S–S bond reduction reaction in one-pot. These two reactions proceeded well at ambient temperature and reached completion within 1 h for de-*t*-butylation and 30 min for DTT-induced S–S reduction. The HPLC radiochemical purities of starting material **18** (T, 87%), the crude products **19** (T, 88%) and **20** (T, 86%) were remarkably consistent, indicating highly specific transformations affording [^{3}H]-thiorphan in 71% isolated yield. From **17** to **20** (T), only one purification (prep-HPLC) was needed. The radiochemical purity was >99%. The specific activity was determined by MS as 18.4 Ci/mmol. [^{3}H]-thiorphan showed good stability when stored at 4°C in an aqueous solution containing 10% methanol and 0.2% DTT in the dark.

Experimental

Proton NMR spectra were recorded on Bruker 300 MHz spectrometers. LC/MS analyses were carried out on a Finnigan LCQ-Advantage ion trap mass spectrometer equipped with an electrospray ionization source. Analytic HPLC was run on a Waters 2695 System with a Waters 2996 Photodiode Array Detector, and preparative HPLC on a Beckman System 32 Karat Gold using a 125 Solvent Module and a 166 detector. Column

chromatography was run on a Teledyne Isco Combiflash Companion System with Thompson prepacked E-Merck silica gel cartridges. Thin layer chromatography used Analtech Silica Gel GF 250 micro plates. UV detection was at 220 or 256 nm and radio detection was on an IN/US System β-Ram (HPLC) and a Bioscan System 200 Imaging Scanner (TLC).

*3-(3- or 4-Bromophenyl)-2-dimethylaminomethylpropionic acid methyl ester (**5**)*

To a 100 ml round bottom flask under N_2 was added anhydrous THF (20 ml) and lithium diisopropylamide (1.8 M in THF/heptane/ethylbenzene, 4 ml, 7.2 mmol) and the mixture was cooled to -30°C with a dry ice/MeOH bath. To this, a solution of methyl 3-(dimethylamino)propionate **3** (0.92 g, 7 mmol) in THF (5 ml) was added dropwise; the initial yellowish solution turned into a yellowish suspension. The mixture was aged at -30°C for 15 min, a solution of 3-bromobenzyl bromide **4** (1.75 g, 7 mmol) in THF (5 ml) was added dropwise causing the suspension to become clear. Stirring continued at $-30 \sim -15^\circ$C for 3 h. The mixture was quenched with saturated NH_4Cl solution to pH $\sim$8, and extracted with ether three times. Organic layers were combined, washed with water and dried over sodium sulfate. The crude product was purified by silica gel flash column chromatography eluting with 0–5% MeOH in CH_2Cl_2 to give the desired 3-bromo **5** as a light yellow oil (0.762 g) in 36% yield. ^{1}H NMR ($CDCl_3$)δ2.24 (s, 6H), 2.28, 2.65(2H, m), 2.83 (m, 3H), 3.62 (s, 3H), 7.13 (m, 2H), 7.36 (s, d, 2H); MS (ES^+) [M+H$^+$]: 300, 302. The 4-bromo isomer of **5** was prepared similarly from 4-bromobenzyl bromide **4** in 32% yield. ^{1}H NMR ($CDCl_3$)δ2.23 (s, 6H), 2.28, 2.64(2H, 2 m), 2.82 (m, 3H), 3.61 (s, 3H), 7.03 (d, J = 8.42 Hz, 2H), 7.39 (d, J = 8.42 Hz, 2H); MS (ES+) [M+H$^+$]: 300, 302. As a by-product, *N,N*-dimethyl-4-bromobenzylamine was isolated in 13% yield. ^{1}H NMR ($CDCl_3$)δ2.22 (s, 6H), 3.37 (s, 2H), 7.18 (d, J = 8.32 Hz, 2H), 7.44 (d, J = 8.36 Hz, 2H); MS (CI) [M+H$^+$]: 214, 216.

*2-(3- or 4-Bromobenzyl)-3-methoxy-*N,N,N*-trimethyl-3-oxo-1-propanaminium iodide (**6**)*

To a 10 ml pear-shaped flask containing 3-(3-bromophenyl)-2-dimethylaminomethylpropionic acid methyl ester **5** (0.762 g, 2.54 mmol) was added isopropanol (5 ml) and iodomethane (480 µl, 1.09 g, 7.68 mmol). The mixture was stirred at ambient temperature overnight. The resulting white suspension was filtered, washed with *i*-PrOH and air-dried to give the 3-bromo ammonium iodide **6**, (0.95 g) in 85% yield. The 4-bromo isomer **6** was prepared similarly from 4-bromo **5** in 82% yield. MS (ES^+) [M$-$I$^-$]: 314, 316. HPLC purity was 95%.

*2-(3- or 4-Bromobenzyl)acrylic acid (**7**)*

To a 10 ml round bottom flask containing 2-(3-bromobenzyl)-3-methoxy-*N,N,N*-trimethyl-3-oxo-1-propanaminium iodide **6** (0.95 g, 2.15 mmol) was added 1 N NaOH (4.3 ml), the mixture was heated at 105°C for 2 h. After cooling to room temperature, it was acidified with 0.5 N HCl to pH $\sim$1, the white precipitate was isolated by filtration, washed with water and air-dried to give 3-bromo **7** (0.44 g) in 85% yield. AN-HPLC purity was 93%. The 4-bromo isomer **7** was prepared similarly from 4-bromo **6** in 93% yield. HPLC purity was 94%. ^{1}H NMR ($CDCl_3$)δ3.58 (s, 2H), 5.61 (s, 1H), 6.38 (s, 1H), 7.08 (d, J = 8.27 Hz, 2H), 7.42 (d, J = 8.35 Hz, 2H); MS (ES^-) [M$-$H$^+$]: 239, 241; [2M+Na$^+-$2H$^+$]: 501, 503, 505.

*2-(Acetylsulfanylmethyl)-3-(3- or 4-bromophenyl)propionic acid (**8** or **9**)*

To a 5 ml pear-shaped flask containing 2-(3-bromobenzyl)acrylic acid **7** (0.44 g, 1.83 mmol) was added thioacetic acid (1 ml, 1.065 g, 13.99 mmol). The mixture was heated at 50°C for 2.5 h and after HPLC confirmation of completion, excess thioacetic acid was removed by rotary evaporation. The residue was co-evaporated with toluene twice and purified with silica gel column chromatography eluting with 0–2% MeOH in CH_2Cl_2 to give **8** as a colorless thick oil (0.55 g) in 95% yield. HPLC purity: 97%. The 4-bromo isomer **9** was prepared similarly from 4-bromo **7** in 87% yield. ^{1}H NMR ($CDCl_3$)δ2.35 (s, 3H), 2.98 (m, 5H), 7.07 (d, J = 8.30 Hz, 2H), 7.43 (d, J = 8.50 Hz, 2H); MS (ES^-) [M$-$$CH_3CO^+$]: 273, 275; [2M+Na$^+-$2H$^+$]: 653, 655, 657.

*2-[3-(4-Bromophenyl)-2-(S-acetylsulfanylmethyl)propionylamino]acetic acid benzyl ester (**10**)*

To a 100 ml round bottom flask containing the thioester **9** (320 mg, 1 mmol) was added THF (10 ml) and the mixture was cooled at 0°C. A solution of glycine benzyl ester hydrochloride (201 mg, 1 mmol) and triethylamine (101 mg, 1 mmol) in CH_2Cl_2 (10 ml) was added dropwise, followed by additions of a solution of HOBT (153 mg, 1 mmol) in THF (7 ml) and a solution of DCC (247 mg, 1.2 mmol) in CH_2Cl_2 (8 ml). The cooling bath was removed and the reaction mixture was stirred at ambient temperature overnight. Both HPLC and TLC analysis confirmed completion of the reaction. The mixture was concentrated and the residue purified by silica gel flash column chromatography eluting with 0–2% ethyl acetate in CH_2Cl_2 to give **10** as a white solid (420 mg) in 91% yield. ^{1}H NMR ($CDCl_3$)δ2.33 (s, 3H), 2.61 (m, 1H), 2.92 (m, 4H), 3.95 (m, 2H), 5.15 (d, J = 2.3 Hz, 2H), 5.84 (m, 1H), 7.05 (d, J = 8.20 Hz, 2H), 7.36 (m, 9H); MS (ES+) [M+H$^+$]: 264, 266; (ES^-) [M$-$$CH_3CO^+$]: 420, 422.

*2-[3-(4-Bromophenyl)-2-(sulfanylmethyl)propionylamino]acetic acid (**11**)*

To a 100 ml round bottom flask containing the benzyl ester **10** (420 mg, 0.9 mmol) was added MeOH (15 ml). The resulting solution was cooled at 0°C and 1 N NaOH solution (2 ml) was added via a syringe pump over 1 h. The mixture was stirred at 0°C for 2.5 h and then at room temperature for 3 h. HPLC analysis showed 75% of desired product **11** and 10% of disulfide **12**. The remainder consisted of partially hydrolyzed products. MS of **11**: (ES^+) [M+H$^+$]: 332, 334. This reaction mixture was used without workup for the preparation of the disulfide **12**.

*2-[3-(4-Bromophenyl)-2-(sulfanylmethyl)propionylamino]acetic acid disulfide (**12**)*

The crude mixture of **11** was cooled to 0°C, a solution of I_2 (127 mg, 0.5 mmol) in MeOH (1.0 ml) was added slowly over 5 min and the resulting mixture was stirred for additional 30 min. A few drops of sat. Na_2SO_3 solution was added to de-colorize the mixture followed by careful addition of 0.2 N HCl to adjust pH to $\sim$2. The mixture was extracted with ethyl acetate twice, organic phases were combined, washed with water, brine and dried over sodium sulfate. After filtration and solvent evaporation, the residue was passed through a short silica gel cartridge eluting with 5–10% MeOH in CH_2Cl_2 to afford 244 mg of **12** in 81% yield from **9**. HPLC purity was $>$90%. MS of **12**: (ES^+) [M+H$^+$]: 661, 663, 665.

*Catalytic hydrodebromination of 2-[3-(4-bromophenyl)-2-(sulfanylmethyl)propionylamino]acetic acid disulfide (**12**)*

A typical reaction is as follows: to a 25 ml two-neck flask were added the substrate **12** (12 mg), water (4 ml), 20% $Pd(OH)_2$ on carbon (24 mg), and triethylamine (20 µl). The mixture was stirred under D_2 (balloon) and monitored by HPLC. Sampling was done at 2, 5 and 16 h. The reaction gave $<5\%$ of the desired product **13**.

*2-Sulfanylmethyl-3-(3-bromophenyl)propionic acid (**15**)*

To a two-neck 25 ml round bottom flask containing the thioester **8** (0.25 g, 0.79 mmol) was added 0.2 N NaOH (5 ml, 1 mmol). The mixture was stirred at ambient temperature for 17 h. Additional base (1.9 N NaOH, 0.7 ml) was added and the mixture was stirred for additional 15 min. HPLC analysis showed completion of reaction. HPLC purity: 81% (14% disulfide), LC–MS was used to identify the products. MS of **15**: (ES^-) $[M-H^+]$: 273, 275. This was used for the next reaction without further purification.

*2-(S-t-butylsulfanylmethyl)-3-(3-bromophenyl)propionic acid (**16**)*

To the crude reaction mixture of **15** under N_2 was injected a mixture of *t*-BuOH (3 ml) and 37% HCl (2.5 ml). It was heated at reflux for 4 h, cooled to room temperature and partitioned between water and CH_2Cl_2. Aqueous phase was re-extracted with CH_2Cl_2, the combined CH_2Cl_2 extracts were washed with brine twice and dried over sodium sulfate. The crude product was purified by silica gel chromatography eluting with 0–5% EtOH in CH_2Cl_2 to give **16** as a colorless thick oil (0.133 g) in 51% yield in two steps. HPLC purity: 95%, MS of **16**: (ES^-) $[M-H^+]$: 329, 331.

*2-[3-(3-Bromophenyl)-2-(S-t-butylsulfanylmethyl)propionylamino]acetic acid benzyl ester (**17**)*

To a 25 ml pear-shaped flask containing acid **16** (0.22 g, 0.665 mmol) and THF (7 ml) at 0°C was dropwise added a solution of glycine benzyl ester hydrochloride (0.134 g, 0.665 mmol) and triethylamine (93 µl, 67.3 mg, 0.665 mmol) in CH_2Cl_2 (7 ml), followed by a dropwise addition of a solution of HOBT (0.102 g, 0.665 mmol) in THF (5 ml) and a solution of 1,3-dicyclohexyl carbodiimide (0.164 g, 0.796 mmol) in CH_2Cl_2 (6 ml). The ice/water cooling bath was removed and the mixture was stirred at ambient temperature overnight. After concentration, the residue was partitioned between water and CH_2Cl_2 and the aqueous layer was extracted twice with CH_2Cl_2. The combined extract was washed with brine, dried over sodium sulfate and purified by silica gel flash column chromatography eluting with 0–50% ether in hexane to give **17** as a colorless thick oil (0.25 g) in 79% yield. HPLC purity: 100%. ^1H NMR ($CDCl_3$)δ1.29 (s, 9H), 2.51 (m, 1H), 2.64 (m, 1H), 2.85 (m, 3H), 4.02 (m, 2H), 5.16 (s, 2H), 5.95 (m, 1H), 7.12 (m, 2H), 7.35 (m, 7H); MS (ES^+) $[M+H^+]$: 478, 480; (ES^-) $[M-H^+]$: 476, 478.

*[2H]- or [^{3}H]-S-t-Butyl thiorphan ([2H]- or [^{3}H]-**18**)*

Procedure for reduction with deuterium gas: to a 10 ml pear-shaped flask containing a magnetic stirrer bar was added the ester **17** (2.5 mg, 0.0052 mmol), 20% $Pd(OH)_2/C$ (7.2 mg), H_2O (1 ml), and triethylamine (10 µl). The air inside the flask was removed by reduced pressure and it was refilled with D_2 gas from a balloon (about 10 ml of open space inside the flask was filled up with D_2 gas). The mixture was stirred for 2 h at ambient temperature. The catalyst was removed by filtration and the solvent was evaporated. The residue was analyzed by HPLC, which showed a clean and complete conversion. MS of [2H]-**18** (ES^+) $[M+H^+]$: 254, 255, 256. The amount of deuterium incorporation was determined by LC/MS as 30% D_0, 47% D_1, 22% D_2 and 3% D_3.

The tritiation was performed by ViTrax Co. based on a slightly modified reaction scale (**17**/20%$Pd(OH)_2/H_2O$/TEA/reaction time = 6 mg : 18 mg : 2.5 ml : 25 µl : 5 h). This resulted in a total activity of 219 mCi of crude product [^{3}H]-**18,** which showed radiochemical purity of $\sim$90% by TLC and a 87% purity by HPLC. Analytical HPLC conditions: Zorbax SB-C8, 4.6 × 150 mm, 5 µm. A: 0.1% TFA in H_2O, B: acetonitrile, gradient from 30 to 90% B 0–20 min; hold 90% B 5 min; post time 10 min; flow rate: 1 ml/min; temp. 30°C, radiodetector. Retention time for [^{3}H]-**18**: 8.28 min.

*[^{3}H]-S-(2-Nitrophenylsulfanyl) thiorphan ([^{3}H]-**19**)*

To a 25 ml pear-shaped flask was added a solution of [^{3}H]-**18** (43.8 mCi) in MeOH (4 ml). Solvent was evaporated and to the residue was added acetic acid (6 ml) followed by addition of a solution of 2-nitrobenzenesulfenyl chloride (3.0 mg) in acetic acid (3 ml). The mixture was stirred for 1 h. AcOH was evaporated at reduced pressure and the residue was used for the next reaction without further purification. Analytical HPLC conditions: Zorbax SB-C8, 4.6 × 150 mm, 5 µm. A: 0.1% TFA in H_2O, B: acetonitrile, gradient from 30 to 90% B 0–20 min; hold 90% B 5 min; post time 10 min; flow rate: 1 ml/min; temp. 30°C, adiodetector. Retention time for [^{3}H]-**19**: 10.20 min.

*[^{3}H]-Thiorphan (**20**)*

To the crude [^{3}H]-**19** were added CH_2Cl_2 (4 ml), DTT (2.9 mg) and triethylamine (10 µl). The mixture was stirred under N_2 for 30 min, concentrated to dryness, re-dissolved in 200 µl of 0.2% DTT in MeOH/H_2O (1:1) and purified by preparative HPLC to give 26.97 mCi [^{3}H]-thiorphan with a radiochemical purity of 99.8% by AN-HPLC. Specific activity was 18.42 Ci/mmol. It was stored as 0.2375 mCi/ml aqueous solution containing 10% MeOH and 0.2% DTT. Analytical HPLC conditions: Zorbax SB-C8, 4.6 × 150 mm, 5 µm. A: 0.1% TFA in H_2O, B: 0.1% TFA in ACN, gradient from 10 to 90% B 0–20 min; hold 90% B 5 min; post time 10 min; flow rate: 1 ml/min; temp. 30°C, radiodetector. Rentention time for [^{3}H]-thiorphan: 9.16 min.

Thiorphan stability in solutions

Four series of 0.01% unlabelled thiorphan solutions were prepared by a combination of a primary solvent and 0.2% of an anti-oxidant (water–DTT, water–BME, ethanol–DTT, and ethanol–BME, respectively) with or without additives such as co-solvent (MeOH, H_2O) or various acids. The five solutions in each of the two aqueous series included (in addition to water–DTT or water–BME) (1) 10% methanol, (2) 40% methanol, (3) 10% methanol+0.005% TFA, (4) 10% methanol+0.005% conc. HCl, (5) 10% methanol+0.01% acetic acid. The five solutions in each of the two ethanol series included (in addition to ethanol–DTT or ethanol–BME) (1) no other additives, (2) 40% water, (3) 0.005% TFA, (4) 0.005% conc. HCl, (5) 0.01% acetic acid. These solutions were stored at 4°C under N_2 in dark, and HPLC samplings were done at 3, 15, 30 and 90 days after initial

preparations. Based on the findings, the labelled [^{3}H]-thiorphan with 99.8% radiochemical purity was stored under optimal conditions (aqueous solution containing 10% methanol and 0.2% DTT at 0.24 mCi/ml, at 4°C under N_2 in dark) and re-analyzed by HPLC after 130 days; only 6% radiochemical degradation was found.

Conclusion

The synthetic methodology that we have developed for [^{3}H]-thiorphan overcomes the difficulties that were encountered in the past due to the presence of SH group in the molecule, which is labile to side reactions and causes severe catalyst poisoning. This method is quite robust in terms of yields and reproducibility based on the trial deuterium incorporation experiments and the final tritiation run. Although it requires re-generation of the SH group after tritiation, the reactions involved can be easily done in one-pot without purification of intermediates and afford [^{3}H]-thiorphan in excellent yield and purity. It is anticipated that a different ^{3}H-labelling position or higher tritium incorporation should be possible by replacing compound **4** with an other mono- or multi-bromobenzyl bromide in the initial stage of the synthesis. [^{3}H]-Thiorphan showed good stability when stored at 4°C in an aqueous solution containing 10% methanol and 0.2% DTT in the dark.

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