



Published in final edited form as:

J Labelled Comp Radiopharm. 2011 November ; 54(13): 795–798. doi:10.1002/jlcr.1933.

Synthesis of carbon-14, carbon-13 and deuterium labeled forms of thioacetamide and thioacetamide S-oxide

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Abstract

Thioacetamide (TA) is a model hepatotoxin that undergoes metabolic activation via two successive *S*-oxidations. The ultimate toxic metabolite thioacetamide *S,S*-dioxide, or its tautomer acetimidoyl sulfinic acid $\text{CH}_3\text{C}(\text{NH})\text{SO}_2\text{H}$, then acylates lysine side chains on cellular proteins leading to cellular dysfunction or death. To identify individual target proteins, quantitate the extent of their modification and elucidate the structural details of their modification we required both radio-labeled and stable-labeled forms of TA and its intermediate metabolite thioacetamide *S*-oxide (TASO). The latter is stable when purified but can be difficult to isolate. Considering currently available isotopic precursors we devised and report here methods for the synthesis and isolation of TA and TASO labeled with C-14, C-13 and/or deuterium. The methods are straightforward, utilize readily available precursors and are amenable to small scale.

Keywords

acetate; thioacetamide; thioacetamide S-oxide; deuterium exchange; pyrolysis

Introduction

Thioacetamide (TA, **1**), a prototype thiono-sulfur ($>\text{C}=\text{S}$) compound, has been used industrially in the leather, textile, and paper industries, as an accelerator in the vulcanization of buna rubber, as a stabilizer in motor fuels and as a replacement for hydrogen sulfide in qualitative analysis.¹ TA was also once used as a fungicide on oranges but it was reported that a single dose of this toxin in animals can produce centrilobular necrosis, and that chronic administration leads to liver cirrhosis and even hepatocarcinoma.² More recently thioacetamide has been used to generate useful animal models of hepatic fibrosis,³ hepatic cirrhosis⁴ and human cholangiocarcinoma.⁵ Thioacetamide^{6, 7} and its congener thiobenzamide (TB, PhCSNH_2)^{8, 9} are also widely used to model acute hepatotoxicity. The cytotoxicity of both thioamides is believed to result from a two-step bioactivation sequence leading first to a thioamide *S*-oxide such as TASO (**2**) and then to a reactive *S,S*-dioxide such as TASO_2 (**3**) (Scheme 1).^{10, 11} The iminosulfinic acid tautomers of TASO_2 and TBSO_2 are highly reactive acylating agents toward lysine residues in proteins and may initiate necrosis by covalently binding to liver macromolecules.^{8, 12}

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Whereas numerous specific cellular protein targets have been identified for thiobenzamide,⁸ and a number of other metabolically activated pro-toxins,¹³ no specific cellular targets for TA metabolites have thus far been identified. Detection of proteins adducted by reactive metabolites commonly depends on treating animals or cells with isotopically labeled protoxins, followed by 2D gel electrophoresis and identification of targets by mass spectrometric analysis. Herein, we report simple and efficient methods for the synthesis of TA and TASO from sodium acetate labeled with either ¹⁴C or ¹³C and deuterium.

Experimental

[1-¹⁴C]-Acetic acid, sodium salt (59.6 mCi/mmol and radiochemical purity >98 %) was obtained from Moravex Biochemical (California, USA). [1,2-¹³C]-acetic acid-d₃, sodium salt was obtained from Cambridge Isotopes (Massachusetts, USA). All other chemicals and solvents were obtained from Sigma Aldrich. Hydrogen peroxide was titrated iodometrically before use. ¹H and ¹³C NMR spectra were recorded on Bruker AV III 500 MHz spectrometer and spectra were referenced to the residual NMR solvent peak. Electrospray ionization spectra were acquired on a LCT Premier time of flight mass spectrometer. GC/MS analyses were conducted with an Agilent 6890N GC interfaced with a quadrupole mass analyzer using an injector temperature of 240 °C and an initial column temperature of 50 °C for 1 min followed by a gradient of 50–300 °C over 13 min. Ionization was by electron impact at 70eV. Thin-layer chromatography was performed on Silica Gel GF plates (2.5 × 10 cm, 0.25 mm) with fluorescence detection under UV light at 254 nm for TA and TASO, or by spraying the plate with 0.05% Rhodamine 6G in 95% ethanol to detect acetamide under UV or ambient light. After eluting with 10% methanol in ethyl acetate (v/v), the R_f values for TA, acetamide and TASO were 0.80, 0.55 and 0.35, respectively. Column chromatography was performed using silica gel (63–200 particle size). Radioactivity was measured using a Packard Tri-Carb liquid scintillation counter.

Thioacetamide S-oxide (TASO, **2**)

Thioacetamide, **1** (0.050 g, 0.666 mmol) was dissolved in 1 mL methanol in a 50 mL screw-capped Corex tube. The solution was cooled to 0 °C and hydrogen peroxide (30% w/w, 10.9 M by iodometric titration, 0.046 mL, 0.501 mmol) was added. After stirring for 2 h at 0 °C, the solvent was removed (rotovap, bath temperature 37 °C). The residue was dissolved in a small quantity of methanol and transferred to a column packed with 20 g silica gel in ethyl acetate. Elution was started with MeOH/ethyl acetate (v/v = 1:9) and compound **2** was eluted with MeOH/ethyl acetate (v/v = 1:4). Removal of the solvent provided **2** as a crystalline white solid (0.054 g, 0.593 mmol, 89% yield). ¹H NMR (DMSO-d₆): δ 1.94 (s, 3H), 8.19 (s, 1H), 9.01 (s, 1H). ¹³C NMR: δ 13.83 (–CH₃), 189.97 (>C=S). TOF MS calcd for C₂H₅OSN [M + H]⁺ 92.0170, found 92.0159. The purified material was quite stable for several months when stored as a crystalline solid in a freezer (ca. –20 °C), as judged by TLC and by retention of its well defined colorless crystalline appearance as tiny plates and pincushions.

[1-¹⁴C]-Thioacetamide (7)

[1-¹⁴C]-Acetic acid, sodium salt (**4**, 0.017 g, 0.126 mmol, 7.5 mCi) was dissolved in 1 mL methanol and transferred to a 12 mL screw-capped culture tube and solid ammonium acetate (**5**, 0.087 g, 1.134 mmol) was added. After dissolution the homogeneous mixture was evaporated to dryness (rotovap). The tube was capped tightly and placed in a six inch length of galvanized iron pipe closed at both ends with threaded pipe caps. The bottom 1.5 inches of the upright assembly was immersed in a sand bath heated electrically to ca. 240 °C overnight (ca. 24 hr) (Scheme 3). After cooling to room temperature the water formed during pyrolysis was removed using a rotovap yielding crude acetamide (**6**, 0.074 g, 1.254 mmol, quantitative yield) as a highly viscous pale yellow liquid. Tetrahydrofuran (THF, 4 mL) and Lawesson's reagent (0.510 g, 1.254 mmol) were added directly to the crude product and the mixture was stirred at room temperature for 2 h. With no work-up, the reaction mixture was transferred to a column packed with 20 g silica gel in ethyl acetate/hexane (1:9 v/v); thioacetamide (**7**) was eluted using ethyl acetate/hexane (1:2 v/v). Removal of the solvent provided **7** as crystalline white solid (0.014 g, 0.187 mmol, specific activity 5.65 mCi/mmol). Radiochemical purity as assessed by TLC was ca. 98%.

[1-¹⁴C]-Thioacetamide S-oxide (8)

Compound **7**, (0.004 g, 0.054 mmol, 5.65 mCi/mmol) was combined with ordinary thioacetamide (0.008 g, 0.110 mmol) in 2 mL methanol in a culture tube containing a magnetic stirring bar (Scheme 2). The reaction mixture was cooled to 0 °C, H₂O₂ (10.9 M by iodometric titration, 0.012 mL, 0.130 mmol) was added and the reaction was stirred at 0 °C for 2 h. After removal of solvent (rotovap, bath temperature 37 °C), the residue was re-dissolved in a small volume of methanol and transferred to a column of silica gel (20 g) packed in ethyl acetate. Elution began with MeOH/ethyl acetate (1:9 v/v) and compound **8** was eluted with MeOH/ethyl acetate (1:4 v/v). Removal of the solvent provided **8** as a crystalline white solid (0.012 g, 0.132 mmol, 1.90 mCi/mmol)

[1,2-¹³C]-Acetic acid-d₄ (10)

A 50 mL pear-shaped flask (19/22 joint) was charged with 1.0 mL of D₃PO₄ (prepared by adding 2 vol of D₂O to 85% H₃PO₄ and then removing excess water using a rotary evaporator at ca. 70 °C for a total of three cycles of D-exchange). The use of D₃PO₄ rather than H₃PO₄ was precautionary to prevent loss of deuterium by exchange. [1,2-¹³C]-acetic acid-d₃, sodium salt trihydrate (**9**, 0.508 g, 3.603 mmol) was then added as a solid powder on top of the D₃PO₄, the flask was rotated several turns to mix and spread out the viscous mass on the walls. It was immediately affixed to a 135° bend connecting tube leading to a vacuum distillation adapter that terminated in a 50 mL round bottom receiving flask. After cooling the receiver flask to -78 °C (Dry Ice/acetone) the system was evacuated to ca. 1 torr and the distillation flask was heated gently with a hair dryer until the acetic acid that was generated all collected in the receiver as a frozen white solid. Compound **10** was thus obtained as colorless liquid at room temperature (0.258 g, 3.91 mmol). Identity, purity and complete retention of deuterium was confirmed by GC/MS analysis of a tiny aliquot dissolved in methanol to wash out the single easily exchangeable proton of the acid group.

[1,2-¹³C, 2,2,2-²H₃]-Thioacetamide (14)

Compound **10** (0.240 g, 3.636 mmol) was cooled to $-78\text{ }^{\circ}\text{C}$ in a 50 mL round bottom receiver flask, anhydrous ammonia (2 M solution in ethanol, 2.2 mL, 4.4 mmol) was added, and the reaction mixture was allowed to warm to room temperature. Successive portions of the solution were transferred to a 16×100 mm screw-cap culture tube and the solvent was removed by rotary evaporation. Once all the material was pooled and evaporated, methanol-*O*-d (1.5 mL) was added to the residue (**11**) and then removed by rotary evaporation to wash out all exchangeable hydrogens; this step was repeated one more time. Final evaporation of the solvent afforded **12** as colorless viscous liquid (0.200 g, 2.326 mmol). The tube was then capped and placed in a metal pipe and heated at $240\text{ }^{\circ}\text{C}$ for 24 h as described for **7** above. After cooling, the water formed during pyrolysis was removed (rotovap) to give **13** as colorless viscous liquid (0.040 g, 0.625 mmol). Complete retention of deuterium was confirmed by GC/MS analysis of a tiny aliquot dissolved in methanol to wash out the two exchangeable protons of the amide group. Compound **13** (0.035 g, 0.547 mmol) was dissolved in 2 mL THF in a culture tube with a magnetic stirring bar. Lawesson's reagent (0.221 g, 0.547 mmol) was added and the reaction mixture was stirred at room temperature for 2 h. Solvent was then removed (rotovap), the crude product was redissolved in small amount of ethyl acetate, and the solution was transferred to a column packed with 20 g silica gel in ethyl acetate/hexane (1:9 v/v). Compound **14** was eluted with ethyl acetate/hexane (1:2 v/v). Removal of the solvent provided **14** as crystalline white solid (0.012 g, 0.150 mmol). GC-MS examination of a methanol solution of **14** showed the expected molecular ion at 80 (vs. 75 for unlabeled TA) and indicated complete retention of deuterium.

[1,2-¹³C, 2,2,2-²H₃]-Thioacetamide S-oxide (15)

Compound **14** (0.010 g, 0.125 mmol) was dissolved in 1 mL methanol and oxidized with standardized hydrogen peroxide solution, and isolated by chromatography as described for **2** above. Removal of solvent provided **15** as tiny white crystals (0.006 g, 0.062 mmol). TOF MS calcd for ¹³C₂H₂D₃OSN [M + H]⁺ 97.0425, found 97.0439.

Results and Discussion

Thioacetamide S-oxide is a stable compound when pure, but its synthesis and isolation are complicated by its extreme polarity and high water solubility. It was first synthesized by Walter who treated TA with aqueous hydrogen peroxide saturated with ammonium sulfate.¹⁴ Later Porter and Neal prepared TASO on a 40 g scale by oxidizing TA with hydrogen peroxide in DMF and isolating the product by precipitation with carbon disulfide.¹⁵ They also prepared [1-¹⁴C]-TASO by oxidizing [1-¹⁴C]-TA (obtained commercially) with excess hydrogen peroxide in acetone and isolating the product by low temperature crystallization.

As a precursor for the synthesis of isotopically labeled TASO, labeled TA has been prepared by addition of H₂S to acetonitrile and by thionation of acetamide (Scheme 2). Antoni et al.¹⁶ prepared ²H-, ¹³C-, and ¹⁵N-variants of acetonitrile by reacting CH₃I with KCN in glycerol (94–98% yield) and converting the latter to TA using Ph₂PS₂H in toluene (55% yield after sublimation). Porter et al.¹¹ synthesized [³H]-TA by treating [³H]-MeCN with

(EtO)₂PS₂H in 40–70% overall yield. Benincori et al.¹⁷ prepared [¹³C₂]-TA at 5% enrichment in 30% overall yield by refluxing an mixture of [¹³C₂]-acetic acid with urea and thionating the resulting acetamide product using P₂S₅ and K₂S in toluene.

For the identification of hepatocyte proteins adducted by TA metabolites we first needed [¹⁴C]-TA to quantitate the overall extent of protein adduction and to locate adducted protein spots on 2D gels by phosphorimaging. To facilitate the mass spectral analysis of tryptic peptides derived from the radioactive spots, we also wanted a stable isotope signature having a mass shift of at least 5 AMU^{8, 18} in the TA and its metabolites. Considering the potential isotopic precursors currently available commercially, we chose to begin with sodium [1-¹⁴C]-acetate and sodium [1,2-¹³C₂-2,2,2-²H₃]-acetate (Schemes 3 and 4, respectively).

Our syntheses of [¹⁴C]-TA was predicated on an early report by Hofmann¹⁹ indicating that acetamide could be prepared simply by pyrolyzing ammonium acetate. We therefore combined sodium [1-¹⁴C]-acetate at high specific activity with an excess of ammonium acetate in methanol (to ensure isotopic mixing and to achieve the desired final specific activity), evaporated the solvent, and pyrolyzed the resulting material overnight in a sealed screw-cap culture tube (protected inside a metal pipe). The acetamide product, formed in essentially quantitative yield, was thionated with Lawesson's reagent in THF to give [¹⁴C]-thioacetamide, and the latter was oxidized to [¹⁴C]-TASO in ca. 30% overall yield. We discovered that it was quite critical to control the amount of H₂O₂ added to just under 1.0 mol-equivalent in order to prevent overoxidation of the TA, and to avoid having un-oxidized TA as an impurity in the material intended for metabolic studies. When stored as a crystalline solid at –20 °C, TASO was found to be stable for several months.

We expected the synthesis of [1,2-¹³C₂-2,2,2-²H₃]-TA to proceed almost as easily, but were surprised by the ease with which the deuterium atoms could be lost during the pyrolysis step, apparently by exchange with the amide NH₂ protons. Therefore a slightly more elaborate route was devised (Scheme 4). Sodium [1,2-¹³C₂-2,2,2-²H₃]-acetate (**9**) was converted to acetic acid-d₄ using 85% phosphoric acid (i.e., H₃PO₄•H₂O) that had been deuterated by several cycles of adding and evaporating D₂O to give (D₃PO₄•D₂O). This completely avoided the partial loss of deuterium that occurred when non-deuterated 85% H₃PO₄ was used. The labeled acetic acid (**10**) was isolated by bulb-to-bulb transfer under vacuum in essentially quantitative yield and then neutralized with anhydrous ammonia in ethanol to give ammonium acetate **11** with full deuterium retention as verified by mass spectrometry. Since pyrolysis of this material consistently lead to the loss of most of the deuterium from the methyl group, we surmised that the protons of the ammonium group of salt **11** were the source of hydrogens. Prior exchange of these with methanol-*O-d* afforded labeled ammonium acetate **12**, pyrolysis of which produced acetamide **13** with no loss of deuterium from the methyl group. The latter was then thionated with Lawesson's reagent and carefully oxidized to TASO using 1.0 eq. of hydrogen peroxide.

Supplementary Material

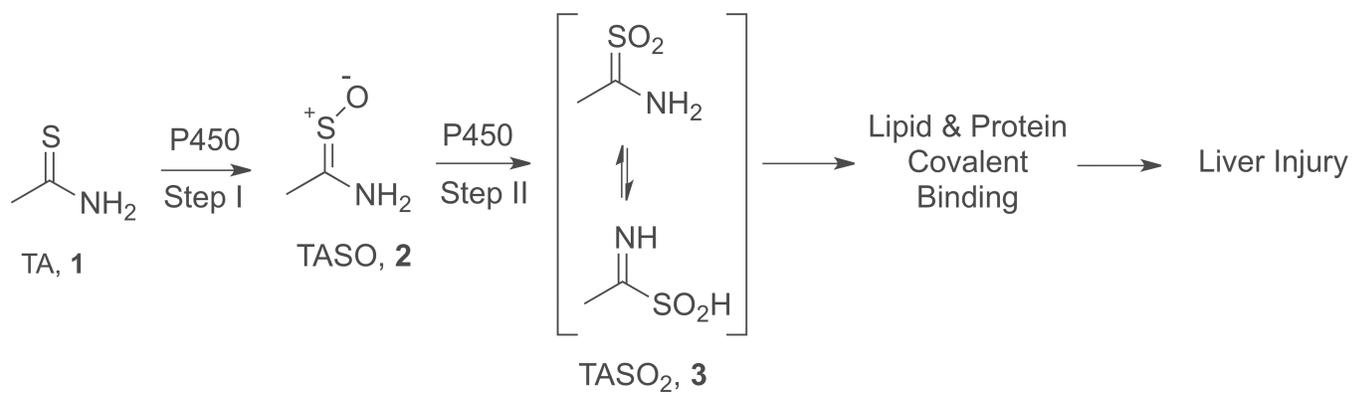
Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

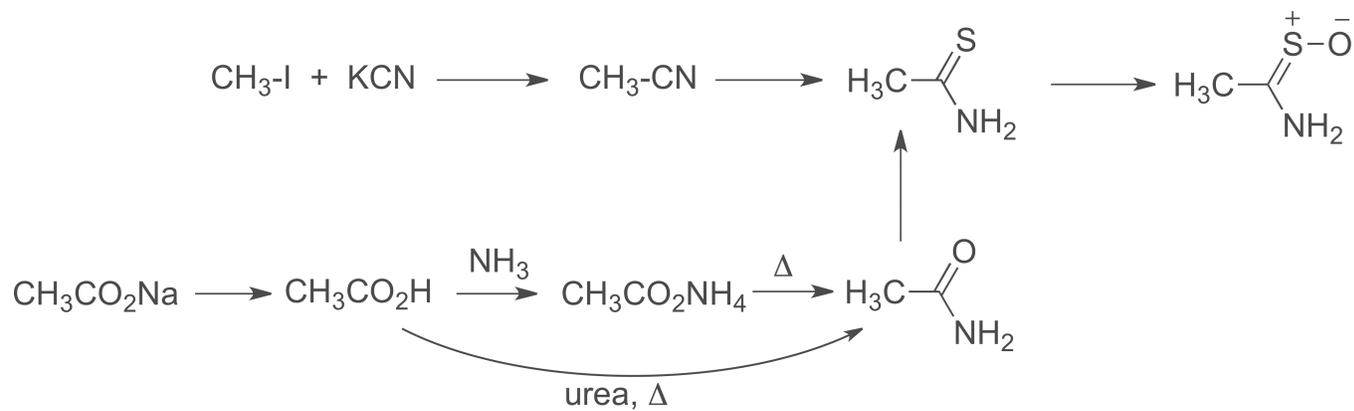
Funding from the National Institutes of Health (GM-21784) is gratefully acknowledged.

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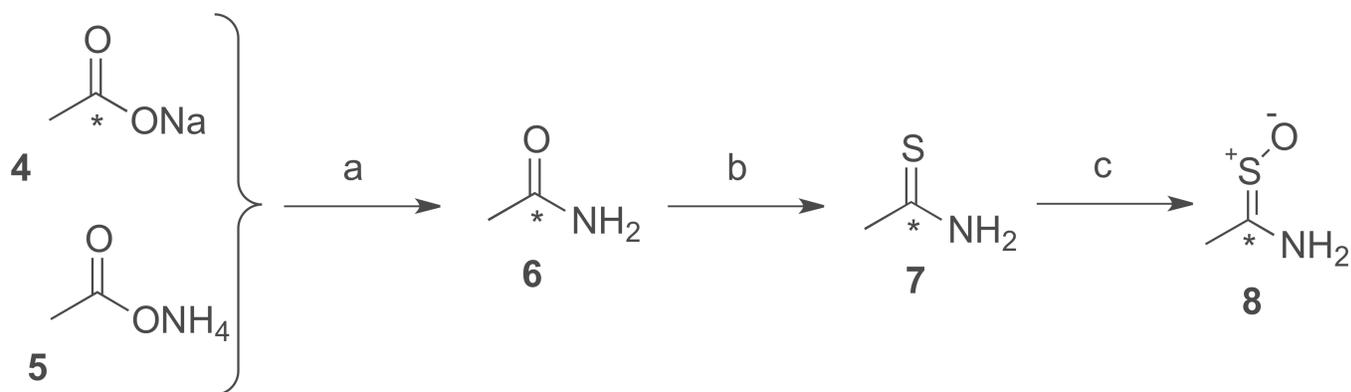
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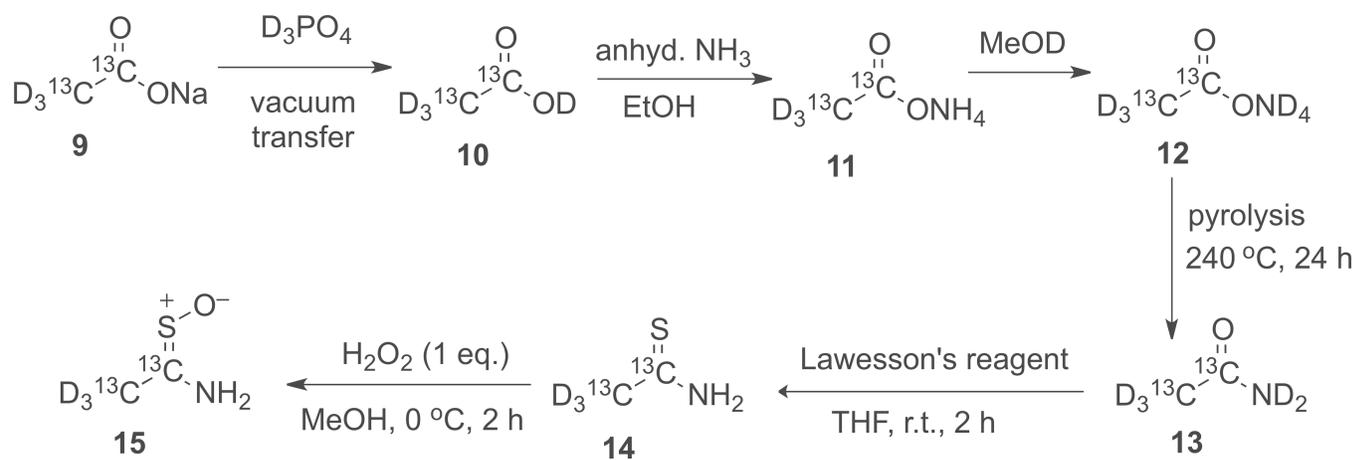
Scheme 1.
Metabolic activation of thioamides.

**Scheme 2.**

Synthetic routes to isotopically-labeled thioacetamide and thioacetamide S-oxide.

**Scheme 3.**

Synthesis of [1-¹⁴C]-thioacetamide S-oxide (a) pyrolysis in sealed screw-cap tube, 240 °C, 24 h; (b) Lawesson' reagent, THF, r.t. 2h; (c) 0.95 eq. H₂O₂, MeOH, 0 °C, 2h.



Scheme 4.
Synthesis of stable labeled TASO