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Note

Semisynthesis of the Organoarsenical Antibiotic Arsinothricin

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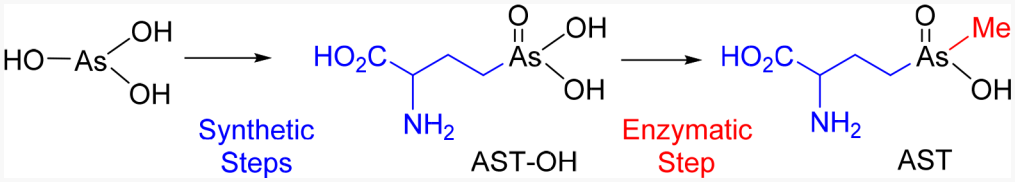

ABSTRACT: Arsinothricin [AST (**1**)], a new broad-spectrum organoarsenical antibiotic, is a nonproteinogenic analogue of glutamate that effectively inhibits glutamine synthetase. We report the chemical synthesis of an intermediate in the pathway to **1**, hydroxyarsinothricin [AST-OH (**2**)], which can be converted to **1** by enzymatic methylation catalyzed by the ArsM As(III) *S*-adenosylmethionine methyltransferase. This is the first report of semisynthesis of **1**, providing a source of this novel antibiotic that will be required for future clinical trials.

The emergence and spread of bacterial resistance have rendered nearly every clinically used antibiotic ineffective. This emphasizes the urgent need for new antibiotics. Infectious diseases such as tuberculosis, the top global infectious disease killer caused by the bacterium *Mycobacterium tuberculosis* (MTB), has become even more difficult to treat due to drug resistance.[1] The World Health Organization (WHO) has declared multi-drug-resistant tuberculosis (MDR TB) a global public health crisis, calling for a pressing need for development of new and innovative antibiotics. In addition to *M. tuberculosis*, the WHO recently issued a global priority pathogen list of antibiotic-resistant bacteria that pose the greatest threat to human health, including the six nosocomial pathogens whose first initials form the acronym ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp.) pathogens.[2]

Arsenic is the most ubiquitous environmental toxic substance and carcinogen, ranking first on the 2019 Environmental Protection Agency's (EPA's) and Agency for Toxic Substances and Disease Registry's (ATSDR's) Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) Substance Priority List http://www.atsdr.cdc.gov/spl/. Most organisms merely try to survive life in the presence of arsenic. In contrast, some members of microbial communities have found ways to use arsenic as weapons against other bacteria in the continual battle for dominance in microbial warfare. Recently a new arsenic-containing compound, arsinothricin [2-amino-4-(hydroxymethylarsinoyl)-butanoic acid, or AST (**1**)], was shown to be synthesized by the rice rhizosphere bacterium *Burkholderia gladioli* GSRB05 (Figure 1).[3] AST (**1**) has broad-spectrum antibiotic activity and is effective against both Gram-positive and Gram-negative

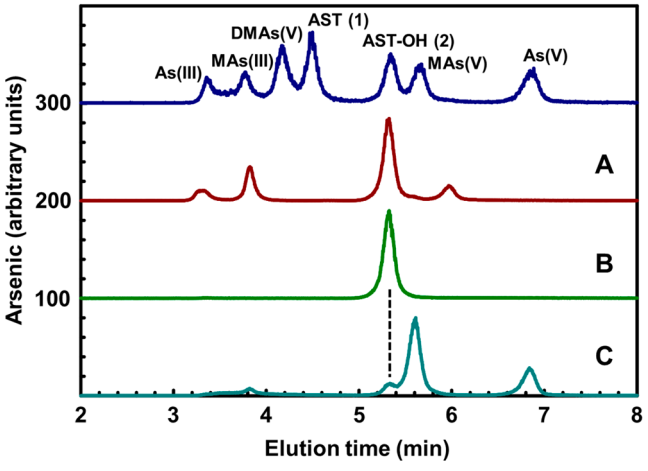

Figure 1. HPLC-ICP-MS analysis of chemically synthesized **2**. See Experimental Section for details. Line A: Crude **2** synthesized by method A. Line B: Purified **2** from method A. Line C: Crude **2** synthesized by method B. Abbreviations: As(III), arsenite; MAs(III), methylarsenite; DMAs(V), dimethylarsenate; AST (**1**), arsinothricin; AST-OH (**2**), hydroxyarsinothricin; MAs(V), methylarsenate; As(V), arsenate.

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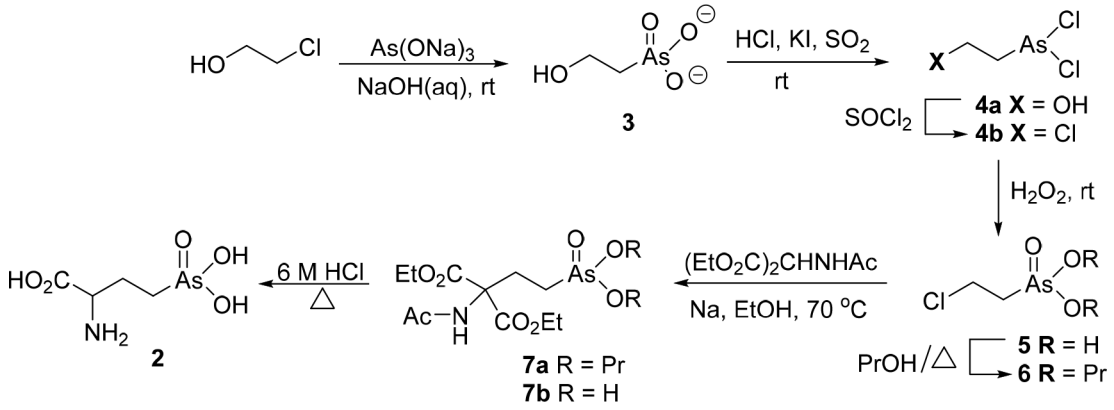
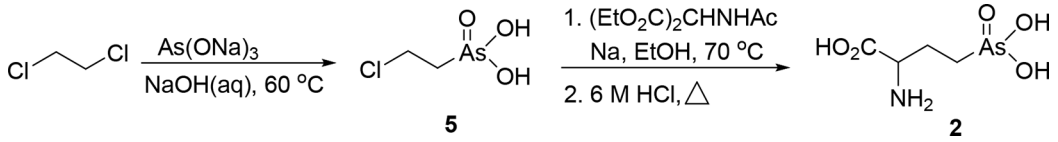
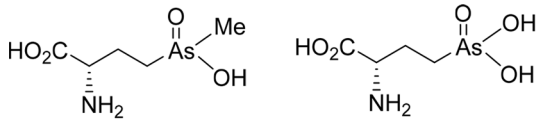
Scheme 1. Synthesis of AST-OH (2) from 2-Chloroethanol (Method A)



Scheme 2. Synthesis of AST-OH (2) from 1,2-Dichloroethane (Method B)



bacteria, including *Mycobacterium bovis* BCG, a causative agent of animal tuberculosis that is closely related to the human pathogen MTB, and carbapenem-resistant *Enterobacter cloacae* (CRE), a WHO priority pathogen.[4] AST is a nonproteinogenic amino acid analogue of glutamate that inhibits glutamine synthetase, a crucial enzyme in the formation of nitrogen compounds, presumably by mimicking the γ-acylphosphoglutamate intermediate in the glutamine synthetase reaction.



We predict that **1** and related arsenic-containing compounds may be the progenitors of a new class of antibiotics. They may prove to be more effective as drugs than chemically related phosphonates, which include some of the most effective commercially available herbicides, pesticides, and human drugs. While modest amounts of **1** can be generated by the source organism, drug development requires a reliable source of the compound. For that reason, we embarked on a synthetic approach to produce **1**. Although we have not yet been successful in the complete synthesis of **1**, we developed a semisynthetic procedure that involves chemical synthesis of the precursor of **1**, 2-amino-4-(dihydroxyarsonoyl)butanoic acid [hydroxyarsinothricin, or AST-OH; (**2**)], which is then enzymatically methylated to **1** using the robust thermostable enzyme CmArsM, the As(III) *S*-adenosylmethionine methyltransferase from the acidothermophilic eukaryotic alga *Cyanidioschyzon* sp. 5508.[5] In 1983 a synthesis of **2** was reported.[6] AST-OH (**2**) is an immediate precursor of 4-arsono-2-hydroxybutanoic acid, which was identified from the crystal structure as a racemic mixture of the D/L-enantiomers.[7] We report here an improved synthesis of **2**. The product of the chemical synthesis can then be methylated enzymatically by CmArsM to produce **1**. This semisynthetic protocol for the synthesis of **1** will provide a reliable source for future development of new and novel antimicrobial agents.

AST-OH (**2**) was prepared from arsonic acid **3** by the reported method with minor modifications (Scheme 1).[6] An excess of hydrochloric acid was required to minimize hydrolysis of **4a**. Also, to prevent decomposition of **5**, the reaction mixture was evaporated under reduced pressure, providing pure **5** (65%) after recrystallization from acetone/ethyl ether. Treatment of crude ester **6** with diethyl acetamidomalonate (3 equiv) in the presence of freshly prepared sodium ethoxide (4 equiv) at 70 °C yielded the crude malonate product **7a**. Heating the reaction mixture is necessary since this reaction at ambient temperature failed to produce **7a**. Reflux of crude **7a** in 6 M HCl effected global deprotection and decarboxylation to yield crude **2**. Purification by cation exchange chromatography on a Dowex 50WX8 (H^+ form) column with triethylammonium acetate (TEAA)/AcOH buffer afforded 2-amino-4-arsonobutanoic acid (**2**) (18%, from **5**).

In a modification of the published route, it was found that esterification of (2-chloroethyl)arsonic acid **5** to **6** was not necessary, as subjection of **5** directly to coupling with diethyl acetamidomalonate followed by deprotection and decarboxylation also provided **2** (56%, method A). HPLC-ICP-MS analysis suggests that the purities of the crude **2** and the purified **2** are approximately 60% and nearly 100%, respectively, with respect to arsenic (Figure 1, lines A and B).

A shorter three-step synthesis of **2** from 1,2-dichloroethane was also developed (Scheme 2). Condensation of 1,2-dichloroethane with basic sodium arsenite afforded (2-chloroethyl)arsonic acid (**5**, 13%) in a single step. Product **5** contained ∼10% vinyl arsonic byproduct(s), as judged by the appearance of the characteristic vinylic peaks from the CH_2═CH group in the ^{1}H NMR spectrum in addition to the 1,2-diarsonoethane adduct detected in HRMS. This approach eliminates (a) the necessity of conversion of the pentavalent (2-hydroxyethyl)arsonic acid to trivalent dichloro(2-hydroxyethyl)arsine with toxic SO_2 gas and (b) the challenging displacement of the hydroxy group with chloride. Coupling of crude **5** with diethyl acetamidomalonate followed by deprotection and decarboxylation of the resulting **7b** also

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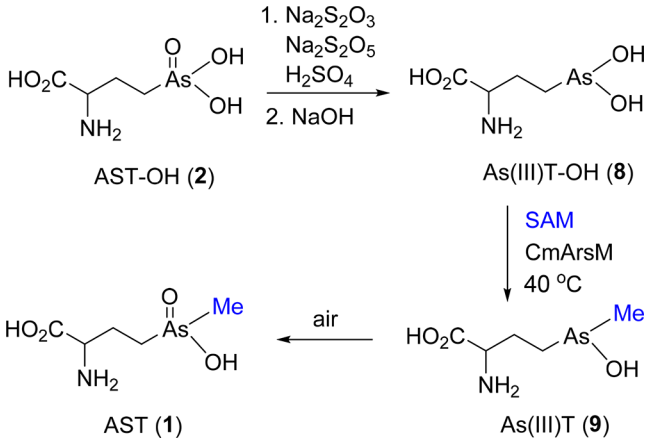
yielded **2** but in lower yield (10%). HPLC-ICP-MS analysis suggests that the purity of crude **2** is roughly 8% with respect to arsenic (Figure 1, line C). However, these reactions can be performed on a fairly large scale from readily accessible starting materials, and the arsenic byproducts can be removed quite efficiently, which makes the low yields less critical.

When exposed to trivalent inorganic arsenite, *B. gladioli* GSRB05 initially produces **2**, followed by gradual biotransformation to **1**, indicating that the final step of AST biosynthesis is methylation of **2** to **1**.[3] Microbial methylation of trivalent arsenicals is catalyzed by the enzyme ArsM, an As(III) *S*-adenosylmethionine (SAM) methyltransferase.[5] AS3MT, the mammalian ortholog of ArsM, was shown to methylate aromatic arsenicals such as 3-nitro-4-hydroxyphenylarsonic acid (roxarsone), producing methyl-roxarsone.[8] AST-OH (**2**) is structurally similar to methylarsenate (MAs(V)) and roxarsone, which suggested that it could be a substrate for enzymatic methylation by ArsM to produce **1**. To examine this possibility, **2** was first chemically reduced to trivalent AST-OH (**8**) with an acidic mixture of $Na_2S_2O_3$, $Na_2S_2O_5$ and H_2SO_4 and then incubated with purified CmArsM enzyme from *Cyanidioschyzon merolae*[10] (Scheme 3). CmArsM catalyzed

Scheme 3. Enzymatic Methylation of 2 to 1



[a]Reaction conditions: Reduction step, 2.5 mM **2** was mixed with 27 mM $Na_2S_2O_3$, 66 mM $Na_2S_2O_5$, and 82 mM H_2SO_4, followed by pH adjusted to 6 with NaOH; methylation and oxidation steps, 100 μM **8** was incubated with 0.75 mM SAM and 10 μM CmArsM at 40 °C overnight.

transfer of the *S*-methyl group of SAM to **8**, largely (>70%) converting it into the trivalent form of AST (**9**), presumably as a mixture of the D/L-enantiomers, which then spontaneously oxidized in air to the final product, AST (**1**) (Figure 2). In the absence of CmArsM, most of **8** reoxidized to **2**. Thus, **1** can be quantitatively produced from chemically synthesized **2** by enzymatic methylation. Sequential purification by cation exchange chromatography on a Dowex 50WX8 (H^+ form) column and size-exclusion chromatography on Sephadex LH-20 afforded **1** (1.2 mg, 29% from **2**). Our semisynthetic method drastically reduces the effort as compared to the purification of **1** from bacterial culture, which requires large-scale culture (5 L) and long culture time (16 days).[3]

In this report we describe a robust method for semisynthesis of the novel antibiotic arsinothricin (**1**). The procedure combines chemical synthesis of the precursor **2** with methylation by the thermostable enzyme CmArsM. Pure

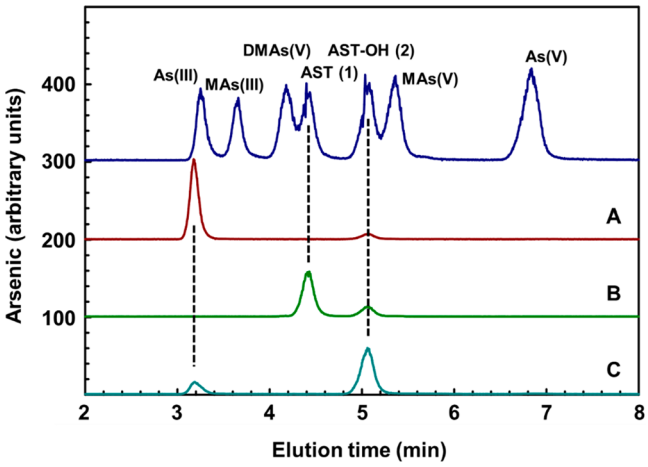


Figure 2. Enzymatic methylation of **2** to produce **1**. Reduced **8** (line A) was incubated in the presence (line B) or absence (line C) of CmArsM, and the arsenic species in the reaction solutions were analyzed by HPLC-ICP-MS, as described in the Experimental Section. Abbreviations: As(III), arsenite; MAs(III), methylarsenite; DMAs(V), dimethylarsenate; AST (**1**), arsinothricin; AST-OH (**2**), hydroxyarsinothricin; MAs(V), methylarsenate; As(V), arsenate.

CmArsM has been produced in substantial amounts for X-ray crystallographic structural analysis. This indicates that both the chemical synthesis and enzymatic methylation can be scaled up for the production of **1** in amounts sufficient for further drug development.

EXPERIMENTAL SECTION

General Experimental Procedures. ^{1}H NMR spectra at 400 MHz and ^{13}C NMR at 100.6 MHz were recorded in D_2O unless otherwise noted. All chemical shift values are referenced to the residual solvent peaks of DMSO-d_6 (2.5 ppm), $CDCl_3$ (7.26), and D_2O (4.79 ppm) for ^{1}H NMR and the DMSO-d_6 (39.52 ppm) or $CDCl_3$ (77.16) peaks for ^{13}C NMR spectra. HRMS data were obtained in TOF (ESI) negative mode. TLC was performed on Merck Kieselgel 60-F_{254}, and products were detected with 254 nm light. Merck Kieselgel 60 (230−400 mesh) was used for column chromatography. All reagents and solvents were purchased from commercial suppliers and used without further purification.

(2-Chloroethyl)arsonic acid (5). Method A: see Supporting Information.

Method B: A 6 M aqueous NaOH (100 mL, 24 g, 0.6 mol) solution was added over 20 min into the cooled (ice bath) suspension of As_2O_3 (20.1 g, 0.1 mol) in H_2O (50 mL) in a round-bottom flask with continuous stirring. Then 1,2-dichloroethane (16 mL, 20 g, 0.2 mol) was slowly added into the resulting homogeneous mixture. The reaction mixture was stirred at 60 °C for 48 h with continuous stirring. The mixture was then concentrated to 100 mL at reduced pressure, and the pH of the solution was adjusted to ∼2.0 with 4 M HCl. The off-white precipitate was removed by vacuum filtration. Evaporation of volatiles from the filtrate at reduced pressure gave 30 g of a white solid, which was suspended in isopropyl alcohol. The white precipitate was removed by vacuum filtration. Evaporation of volatiles from the filtrate at reduced pressure afforded crude **5** (3.0 g, 12.8%) as a transparent gummy solid containing ∼10% of the vinylarsonic acid: ^{1}H NMR (600 MHz, D_2O) δ 4.06 (t, J = 6.4 Hz, 2H), 2.88 (t, J = 6.4 Hz, 2H); 6.48 (d, J = 18.4 Hz, 0.1H), 6.56 (t, J = 11.6 Hz, 0.1H), 6.65 (dd, J = 18.4, 11.6 Hz, 0.1H); HRMS m/z 186.9151 $[M - H]^-$ (calcd for $C_2H_6AsO_4$, 186.9149).

2-Amino-4-arsonobutanoic acid (AST-OH, 2). Via direct concentration of **5** (step a): Sodium (244 mg, 10.6 mmol) was added into a dry flask containing 5 mL of anhydrous EtOH, and the mixture was stirred at ambient temperature until the sodium

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dissolved. Then diethylacetamidomalonate (1.74 g, 8.0 mmol) was added, and the resulting mixture was stirred for 5 min, followed by addition of a freshly prepared solution of **5** (500 mg, 2.65 mmol; from method A) dissolved in 3 mL of EtOH. The resulting mixture was stirred at 70 °C in an oil bath for 4 h. Volatiles were evaporated under reduced pressure, yielding crude **7b** as a brownish solid, which was directly used in next step. Step b: 6 M HCl (10 mL) was added into the crude **7b**, and the resulting mixture was refluxed at 120 °C in an oil bath for 3 h. Volatiles were evaporated, and the residue was dissolved in 20 mL of H_2O. The solution was applied to a Dowex 50WX8 (H^+ form) column (30 × 1 cm, 10 g), which was washed with 50 mL of H_2O. The product was eluted with a solution of NH_4OH (0.5 M, 100 mL). Fractions from the ammonium elution (~100 mL) were evaporated under reduced pressure, and the residue was dissolved in 40 mL of H_2O. The diluted solution was passed through a (Dowex 50WX8 H^+ form) column (30 × 1 cm, 12 g) equilibrated with a weakly acidic TEAA buffer solution (acetic acid 30 mM and triethylamine 15 mM). Compound **2** (TLC, R_f 0.35, *i*-PrOH/H_2O/NH_4OH, 5:2:3; identified by staining with 1% ninhydrin solution) eluted with the same buffer followed by glycine byproduct (TLC, R_f 0.55). The appropriate fractions were evaporated and coevaporated (3×) with a mixture of EtOH/H_2O (1:1, 12 mL) to afford **2**[6] (337 mg, 56% from **5**) as a white solid: ^{1}H NMR (D_2O) δ 2.13−2.28 (m, 4H), 3.81 (t, J = 5.2 Hz, 1H); ^{13}C NMR (D_2O) δ 23.85, 28.83, 54.63, 173.41; HRMS m/z 225.9704 $[M - H]^-$ (calcd for $C_4H_9AsNO_5$, 225.9702).

Subjection of **5** (800 mg, 4.25 mmol; from method B) to the same protocol as described above also gave **2** (95 mg, 10% from **5**): HRMS m/z 225.9704 $[M - H]^-$ (calcd for $C_4H_9AsNO_5$, 225.9702).

Enzymatic Methylation of AST-OH (2) to form AST (1). AST-OH (**2**) was methylated by the enzyme CmArsM. CmArsM was expressed and purified as described previously.[10] Briefly, cells of *E. coli* BL21(DE3) pET28-arsM7B were grown at 37 °C in LB medium supplemented with 25 μg/mL kanamycin to an absorbance of 0.5 at 600 nm, at which time 0.3 mM isopropyl β-D-1-thiogalactopyranoside was added to induce expression of CmArsM. The cells were grown for another 4 h, harvested by centrifugation (5 000 *g*) at 4 °C for 20 min, washed once with buffer A (50 mM morpholinopropane-1-sulfonic acid (MOPS), pH 7.5, containing 20% (wt/vol) glycerol, 0.5 M NaCl, 20 mM imidazole, and 10 mM 2-mercaptoethanol), and suspended in 5 mL of buffer A per g of wet cells. The cells were lysed by a single pass through a French pressure cell at 20 000 psi, and 2.5 μL per g wet cell of the protease inhibitor diisopropyl fluorophosphate was added immediately. Membranes and unbroken cells were removed by centrifugation at 150 000 *g* for 1 h, and the supernatant solution was loaded at a flow rate of 0.5 mL/min onto a Ni(II)-NTA column preequilibrated with buffer A. The column was then washed with 150 mL of buffer A, followed by elution with 60 mL of buffer A with a concentration gradient of imidazole from 0 to 0.2 M. CmArsM was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing CmArsM were concentrated by centrifugation using a 10-kDa-cutoff Amicon Ultrafilter (MilliporeSigma). Protein concentrations were estimated by the method of Bradford using BSA (MilliporeSigma) as a standard. Chemically synthesized AST-OH was reduced to trivalent As(III)T-OH (**8**) as described previously.[9] Briefly, 27 mM $Na_2S_2O_3$, 66 mM $Na_2S_2O_5$, and 82 mM H_2SO_4 were added to and mixed well with 2.5 mM **2** (1.2 mL, 0.7 mg) (method A), on a one-by-one basis, to reduce it to **8**, immediately followed by adjustment of the pH to 6 with NaOH. HPLC-ICP-MS analysis suggests that roughly 80% of **2** was converted to **8** (Figure 2, line A). Compound **8** (100 μM, 25 mL, 0.5 mg) was methylated by incubation with 0.75 mM *S*-adenosylmethionine and 10 μM CmArsM in a buffer consisting of 50 mM MOPS, 0.15 M KCl, 3 mM tris(2-carboxyethyl)phosphine (TCEP), and 1 mM cysteine, pH 7.0, at 40 °C overnight in aerobic conditions with shaking at 180 rpm. HPLC-ICP-MS analysis suggests that roughly 70% of **2** was converted to **1** (Figure 2, line B). Another five batches of reaction were further carried out. In total, 3 mg of **8** (100 μM, 150 mL) was used. The reaction solution was filtered using an Amicon Ultra centrifugal filter with a 3K cutoff membrane (MilliporeSigma) to remove protein. The filtrate was concentrated to 10 mL by rotary evaporator at reduced pressure and applied to a Dowex 50WX8 (H^+ form) column (30 × 1 cm, 10 g), which was washed with 100 mL of H_2O to remove most of the inorganic salts. The product was eluted with a solution of NH_4OH (0.5 M, 100 mL). The appropriate fractions (TLC, R_f 0.70, i-PrOH/H_2O/NH_4OH, 5:2:3; identified by staining with 1% ninhydrin solution) from the ammonium elution (~100 mL) were evaporated under reduced pressure. The residue was dissolved in 5 mL of H_2O and applied to size-exclusion chromatography with a glass Econo-Column (25 mm i.d. × 950 mm) packed with Sephadex LH-20 (GE Healthcare) with a mobile phase 70% (v/v) EtOH at a flow rate of 1.0 mL/min. The arsenic species in each fraction was analyzed by HPLC-ICP-MS. Fractions containing AST with high purity (>90%) were combined and concentrated by a rotary evaporator. The concentrated AST solution was applied again to Sephadex LH-20 size-exclusion chromatography for further purification. Fractions containing AST with high purity (>95%) were combined and concentrated by a rotary evaporator, affording **1** (1.2 mg, overall 29% from **2**) as an off-white solid: ^{1}H NMR (D_2O) δ 1.96 (s, 3H), 2.21−2.28 (m, 2H), 2.32−2.47 (m, 2H), 3.84 (t, J = 6.0 Hz, 1H); ^{13}C NMR (D_2O) δ 15.49, 22.90, 28.59, 54.38, 173.09; HRMS m/z 226.0054 $[M + H]^+$ (calcd for $C_5H_{13}AsNO_4$, 226.0055).

Arsenic Speciation by HPLC-ICP-MS. Arsenic species, including trivalent and pentavalent forms of **2** and **1**, were analyzed by high-pressure liquid chromatography (HPLC) (Series 2000; PerkinElmer, Waltham, MA, USA) coupled to inductively coupled plasma mass spectrometry (ICP-MS) (ELAN DRC-e; PerkinElmer), as described previously,[3,5] with minor modifications. Briefly, arsenic species in samples were separated by HPLC on a BioBasic 18 LC column (250 mm × 4.6 mm, 5 μm, 300 Å) (Thermo Fisher Scientific) using a mobile phase consisting of 3 mM malonic acid and 5% MeOH (v/v) (pH 5.6 adjusted with tetrabutylammonium hydroxide) with a flow rate of 1 mL min^{-1} at 25 °C. Arsenic was monitored by ICP-MS. Arsenic species were determined from the HPLC retention time of known standards (Figures 1 and 2).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c00522.

Copies of ^{1}H NMR, ^{13}C NMR, and HRMS spectra for compounds (PDF)

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Author Contributions

⊥S.H.S. and A.H.H. contributed equally.

Notes

The authors declare no competing financial interest.

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