

Chemical Synthesis of the Organoarsenical Antibiotic Arsinothrinicin

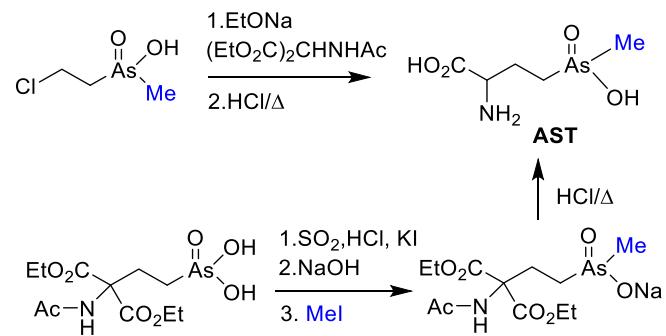
A. Hasan Howlader,^{1,¶} Sazzad H. Suzol,^{1,†} Venkadesh Sarkarai Nadar,² Adriana Emilce Galván,² Aleksandra Nedovic,³ Predrag Cudic,³ Barry P. Rosen,² Masafumi Yoshinaga,^{2,*} and Stanislaw F. Wnuk^{1,*}

¹Department of Chemistry and Biochemistry, Florida International University, Miami, Florida 33199, United States

²Department of Cellular Biology and Pharmacology, Herbert Wertheim College of Medicine, Florida International University, Miami, Florida 33199, United States

³Department of Chemistry and Biochemistry, Charles E. Schmidt College of Science, Florida Atlantic University, Boca Raton, Florida 33431, United States

TOC Graphic



ABSTRACT: We report two routes of chemical synthesis of arsinothrinicin (AST), the novel organoarsenical antibiotic. One is by condensation of the 2-chloroethyl(methyl)arsinic acid with acetamidomalonate, and the second involves reduction of the *N*-acetyl protected derivative of hydroxyarsinothrinicin (AST-OH) and subsequent methylation of trivalent arsenic intermediate with methyl iodide. The enzyme AST *N*-acetyltransferase (ArsN1) was utilized to purify L-AST from racemic AST. This chemical synthesis provides a source of this novel antibiotic for future drug development.

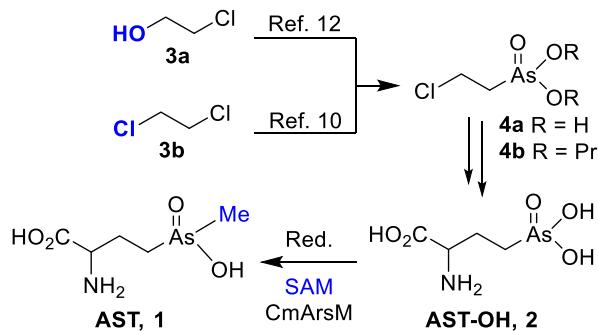
INTRODUCTION

Arsenic has been utilized therapeutically since the Eras of Ancient Greece and China. Numerous arsenic species present in the environment¹ are used in treatment of number of human diseases² and are biotransformed in microbes and animals, including humans.³ Synthesis of organoarsenic compounds is crucial to characterize arsenic species produced by arsenic biotransformations. A variety of synthetic protocols are being developed for novel organoarsenicals.^{4,5} The structures of the new aromatic arsenical metabolites discovered in chicken liver were confirmed by chemical synthesis.⁶

Recently a new arsenic-containing compound, arsinothricin [2-amino-4-(hydroxymethylarsinoyl)butanoic acid, AST (**1**); Scheme 1], was shown to be synthesized by the rice rhizosphere bacterium *Burkholderia gladioli* GSRB05.⁷ A three-gene cluster, *arsQML*, responsible for the AST biosynthesis has been identified recently.⁸ AST, a nonproteogenic amino acid analog of glutamate, is a broad-spectrum antibiotic effective against both Gram-positive and Gram-negative bacteria.⁹ Importantly, AST effectively inhibits growth of major pathogens such as carbapenem-resistant *Enterobacter cloacae*, one of the World Health Organization's global priority pathogens. It can control growth of *Mycobacterium bovis*, a causative agent of both human and animal tuberculosis. The mechanism of action of AST is by inhibition of bacterial glutamine synthetase (GS), an essential enzyme for production of glutamine and for control of ammonia toxicity. AST is chemically similar to the glutamate acylphosphate intermediate in the GS catalytic cycle and binds to the active site of the enzyme, producing inhibition.

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, including some of the most effective commercially available herbicides, pesticides and human drugs. While modest amounts of **1** can be generated using the source organism, drug development requires a reliable source of **1**. For that reason, we recently developed a semi-synthesis of racemic **1** that involves chemical synthesis of the 2-amino-4-(dihydroxyarsonoyl)butanoic acid [hydroxyarsinothricin, AST-OH; (**2**); Scheme 1] precursor; and its enzymatic methylation to AST¹⁰ using the robust thermostable enzyme CmArsM, the As(III) S-adenosylmethionine (SAM) methyltransferase from the acidothermophilic eukaryotic alga *Cyanidioschyzon* sp. 5508.¹¹

Scheme 1. Reported synthesis of **2 (AST-OH)^{10,12} and its enzymatic conversion to **1** (AST)¹⁰**



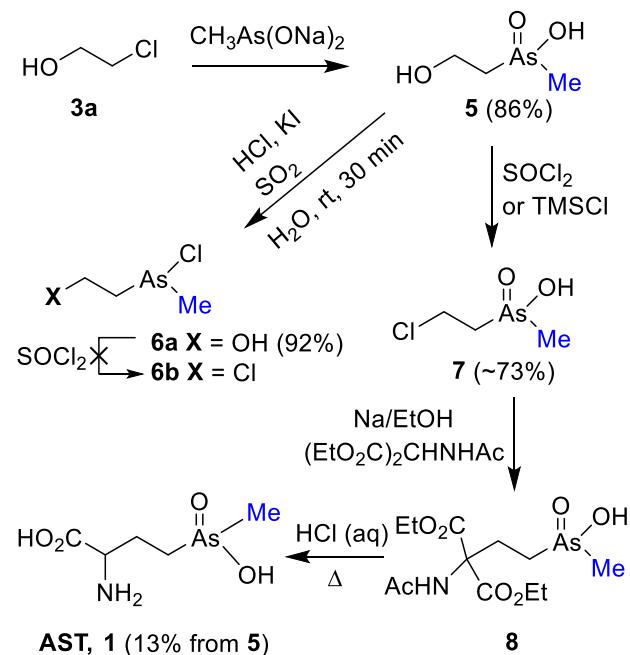
Synthesis of AST-OH from (2-chloroethyl)arsenate ester **4b** that was prepared from 2-chloroethanol **3a** in 5 steps that included elaboration via trivalent arsenic species has been reported (Scheme 1).¹² Coupling of **4b** with sodium salt of diethyl acetamidomalonate followed by deprotection and decarboxylation gave **2**. We reported that condensation of 1,2-dichloroethane **3b** with sodium arsenite provided (2-chloroethyl)arsonic acid **4a** in a single step; which eliminates the necessity of conversion of the pentavalent (2-hydroxyethyl)arsonic acid to trivalent dichloro(2-hydroxyethyl)arsine and challenging displacement of hydroxy group with chloride. We also found that esterification of **4a** to **4b** was not necessary, as subjection of **4a** directly to coupling with acetamidomalonate and deprotection/decarboxylation sequence provided **2** in higher yield.¹⁰ Reduction of **2** and enzymatic methylation of the resulting trivalent arsenicals by CmArsM provided **1**.¹⁰ To reduce effort and complexities associated with synthesis of pure **1** from either bacterial culture medium⁷ or enzymatic buffer¹⁰ and to provide reliable source of larger quantities of **1** for future drug development, we report here two straightforward methods for the synthesis of racemic AST: One by condensation of the 2-chloroethyl(methyl)arsinic acid with acetamidomalonate and the second by methylation of an AST-OH derivative with methyl iodide. Enzymatic separation of racemic AST is also described.

RESULTS AND DISCUSSION

In our first route, pentavalent 2-hydroxyethyl(methyl)arsinic acid **5** was designed as crucial precursor for the synthesis of AST, which in turn could be converted to 2-chloroethyl(methyl)arsinic acid **7** via direct chlorination or a path involving trivalent arsines of type **6b**. Thus, nucleophilic displacement of chloride in 2-chloroethanol **3a** with sodium methylarsonite [$\text{MeAs}(\text{ONa})_2$] provided **5** in 86% yield (Scheme 2). The sodium methylarsonite¹³ was prepared in 97% yield by *in situ* reduction of the sodium salt of methyl arsonate [$\text{MeAs}(\text{O})(\text{OH})\text{ONa}$] with SO_2 gas in the presence of HCl and catalytic amount of KI ¹⁴ followed by hydrolysis of the resulting diiodo(methyl)arsine (MeAsI_2) with aqueous NaOH . Reduction of **5** with $\text{SO}_2/\text{HCl}/\text{KI}$ yielded less polar trivalent chloro(2-hydroxyethyl)(methyl)arsine **6a**, which appears to be susceptible to hydrolysis as it was observed for dichloro(2-hydroxyethyl)arsine.¹⁰ Treatment of crude **6a** with SOCl_2 resulted in vigorous reaction and failed to give **6b**, instead producing dichloro(2-

hydroxyethyl)arsine with loss of the methyl group. Since other approaches for the synthesis of **6b** by halogenation of **6a** were also unsuccessful,¹⁵ we turned our attention to prepare **7** by direct chlorination of **5**.

Scheme 2. Synthesis of 2-chloroethyl(methyl)arsinic acid **7 and its conversion to AST **1****

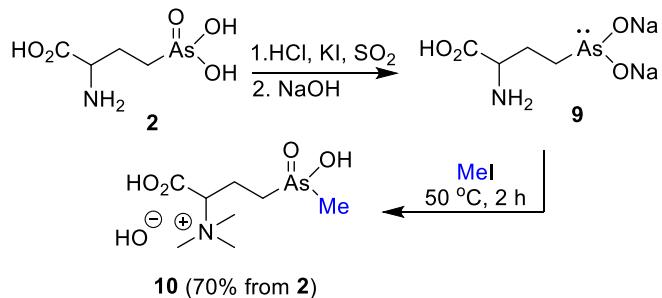


Treatment of the purified and iodide-free sodium salt of **5** with TMSCl in DMSO afforded minor quantities of **7** in addition to unchanged **5** [8:92 mixture based on ^1H NMR and HPLC coupled with ICP-MS (inductively coupled plasma mass spectrometry)]. Subsequent reaction of this mixture with acetamidomalonate in the presence of sodium ethoxide at $70\text{ }^\circ\text{C}$ yielded malonate **8**. Reflux of crude **8** in 6 M HCl effected global deprotection and decarboxylation providing a mixture of products containing 5% of AST as estimated based on ICP-MS and ^1H NMR. However, we were fortunate to find that treatment of sodium salt of **5** with SOCl_2 effected efficient chlorination to provide **7** contaminated by the acidic form of substrate **5** as 80:20 mixture (based on ^1H NMR).¹⁶ Additional purification yielded pure **7** (4%) and a mixture of **7** and **5** (85:15, ~69%). Treatment of the latter mixture with acetamidomalonate followed by deprotection and decarboxylation of the resulting **8** yielded AST¹⁷ (13% from **5**) after purification by Dowex and Sephadex column chromatography.

Building on our enzymatic methylation of AST-OH to AST¹⁰, we chemically methylated reduced $\text{As(III)}\text{-OH}$ **9** with MeI as a source of an electrophilic methyl group. Reduction of **2** with $\text{SO}_2/\text{HCl}/\text{KI}$ (rt/15 min) followed by treatment with 6 M NaOH gave the reduced arsenic salt **9** (Scheme 3). Treatment of the alkaline solution of crude **9** with excess MeI effected methylation at arsenic atom. However, the

reaction also resulted in methylation of the amino group yielding, after purification on cation exchange resin (Dowex^(R) H⁺ form) with NH₄OH, the trimethylammonium salt **10** (70%, from **2**).

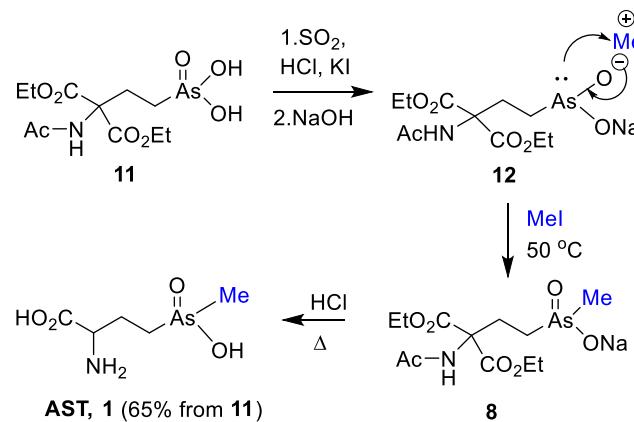
Scheme 3. Attempted synthesis of AST via methylation of AST-OH with methyl iodide



Following encouraging methylation of **9** with MeI, we envisioned that reduction/methylation sequence of the AST-OH derivative bearing the protected amino group would result in an alternative straightforward synthetic route to AST. We selected *N*-acyl protection for the amino group in AST-OH, especially since the original synthesis of AST-OH¹² and our improved protocol¹⁰ required synthesis of the *N*-acetyl protected derivative of type **11**¹⁰ (Scheme 4). Thus, treatment of **4a** with acetamidomalonate as described¹⁰ following purification from the excess of malonate afforded **11**. Reduction of pure **11** with SO₂/HCl and catalytic KI followed by pH adjustment to ~11 with 6 M NaOH gave sodium salt of the trivalent arsenic compound **12**. Subsequent treatment of **12** with MeI (50 °C/4 h) resulted in the methylation at the arsenic atom, providing protected pentavalent AST derivative **8**. Excess MeI and elevated temperature were critical for the optimal yield. The progress of the methylation reaction was monitored by HPLC-ICP-MS. Formation of RAs(O)Me(OMe) byproduct was not observed in agreement with the comparable methylation of trivalent phenylarsenicals⁶ with MeI. Reflux of sodium salt of **8** in 6 M HCl effected global deprotection and decarboxylation providing crude AST. Purification on Dowex (H⁺ form) column with 0.25 M NH₄OH followed by size-exclusion chromatography on Sephadex LH-20 with 70% (v/v) of EtOH/H₂O afforded AST **1** (65%, from **11**) as a racemic mixture. The formation of AST and reduced As(III)T-OH byproduct(s) were also observed, whereas formation of dimethylated product was not detected.

We propose that the methylation of **12** with MeI in basic solution involves S_N2 attack of the nucleophilic arsenic species on the electrophilic methyl iodide with concurrent formation of the arsenic–oxygen double bond, which also oxidized trivalent arsenic to the pentavalent species **8**. The reaction resembles a Michaelis–Arbuzov reaction of trivalent phosphorus esters with alkyl halides to form pentavalent phosphonate esters. Analogous conversion of trivalent to pentavalent organoarsenicals with alkyl halides has been noted.¹⁸

Scheme 4. Synthesis of AST from *N*-acetyl protected AST-OH derivative 11 via reduction and methylation



The antibiotic properties of the chemically synthesized AST **1** (cAST, a mixture of the D/L-enantiomers) were characterized and compared with those of biogenic AST^{7,9} (bAST, the L-enantiomer) and semisynthesized AST¹⁰ (sAST, a mixture of the D/L-enantiomers). Approximately twice as much cAST or sAST was required to inhibit growth (Figure 1) and GS activity (Table 1) of *Escherichia coli* as bAST, consistent with the L-enantiomer of b-AST as the active species.⁹ ArsN1, the bacterial enzyme that confers AST resistance, catalyzes transfer of the acetyl group of acetyl coenzyme A (AcCoA) to the amine group of **1**, generating acetyl-AST (AcAST, **13**; Scheme 5). Purified PpArsN1 (ArsN1 from *Pseudomonas putida* KT2440) nearly completely converted bAST to an arsenic species predicted to be AcAST, while only 50% of racemic cAST or sAST were converted to the putative species and the other half was unmodified (Figure 2), consistent with only the L-enantiomer being the substrate of ArsN1, as predicted from L-AST-bound ArsN1 crystal structures.⁹

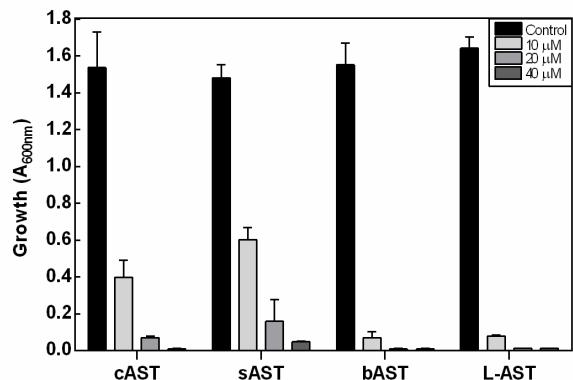


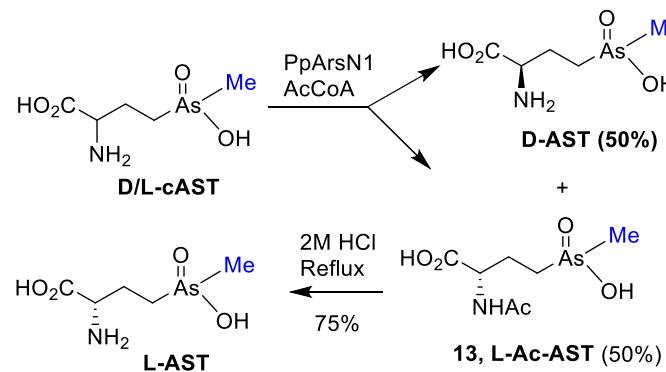
Figure 1. AST inhibits growth of *E. coli*. Cells were cultured in M9 medium in the absence or presence of the indicated concentrations of D/L-cAST, D/L-sAST, L-bAST, L-AST. Growth was estimated from the $A_{600\text{nm}}$ after 16 h. Data are the mean \pm SE ($n = 3$).

Table 1. Inhibition of *E. coli* glutamine synthetase by AST

AST	K_i (μM)
Chemically synthesized (D/L-cAST)	0.75 ± 0.20
Semisynthetic (D/L-sAST)	0.65 ± 0.20
Biogenic (L-bAST)	0.30 ± 0.10

We utilized PpArsN1 to purify L-AST from D/L-cAST. 7 mg of cAST was incubated with purified PpArsN1 and AcCoA overnight, resulting in a mixture of D-AST and L-AcAST **13** (Scheme 5). Purification by size-exclusion chromatography on Sephadex LH-20 afforded **13** (3.0 mg, 36%) and D-AST (2.1 mg, 30%). Reflux of **13** in 2 M HCl effected acetyl deprotection providing L-AST after purification on Sephadex LH-20 column with 70% (v/v) of EtOH/H₂O. Chiral purity of L-AST (ee 87%) has been established by derivatization with Marfey's reagent (see Figure S1 in SI section).¹⁹ The L-AST when treated with PpArsN1/AcCoA was acetylated to **13** proving further its enantiomeric purity (Figure 2). L-AST inhibited growth of *E. coli* as effectively as bAST (Figure 1), additionally supporting the enantiomeric purity.

Scheme 5. Enzymatic acetylation of cAST to L-AcAST **13 and chemical deacetylation of **13** to L-AST**



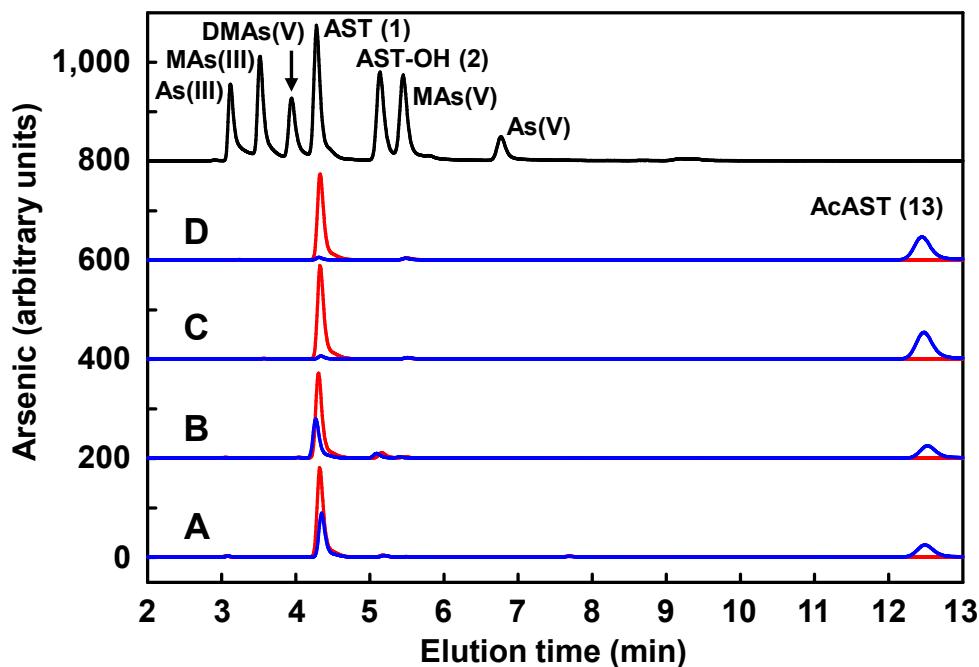


Figure 2. Enzymatic acetylation of **1** to produce **13**. Chemically synthesized D/L-cAST (A), semisynthetic D/L-sAST (B), biogenic L-bAST (C) or L-enantiomeric (D) **1** was incubated in the absence (red lines) or presence (blue lines) of PpArsN1. The arsenic species in the reaction solutions were analyzed by HPLC-ICP-MS. Abbreviations: As(III), arsenite; MAs(III), methylarsenite; DMAs(V), dimethylarsenate; AST (1), arsinothricin; AST-OH (2), hydroxyarsinothricin; MAs(V), methylarsenate; As(V), arsenate; AcAST (13), *N*-acetylarsinothricin.

CONCLUSIONS

In summary, we developed two chemical methods for synthesis of the novel antibiotic arsinothricin. One is by reduction of the *N*-acetyl protected analogue of AST-OH and subsequent methylation of the resulting trivalent arsenic intermediate with methyl iodide, while the second involves condensation of the 2-chloroethyl(methyl)arsinic acid with acetamidomalonate. Enzymatic separation by enantioselective acetylation with PpArsN1 was utilized to purify L-AST from racemic AST. This convenient chemical synthesis can be scaled up to gram quantities to produce AST in sufficient amounts for further drug development.

EXPERIMENTAL SECTION

General Information. ^1H 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 reported in parts per million (ppm) and referenced to

the residual solvent peaks of CDCl_3 (7.26) and D_2O (4.79 ppm) for ^1H NMR and the CDCl_3 (77.16) peaks for ^{13}C NMR spectra, with coupling constant (J) values reported in Hz. HRMS were obtained in TOF (ESI) negative or positive mode. TLC was performed on Merck Kieselgel 60-F₂₅₄, and products were detected by staining with 1% ninhydrin solution. 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-Hydroxyethyl(methyl)arsinic acid (5). (a) Preparation of diiodo(methyl)arsine (MeAsI_2). A solution of KI (41.5 g, 0.252 mol) in H_2O (40 mL) was added into the solution of monosodium salt of the commercially available methylarsonate (MeAs(O)(OH)ONa ; 0.126 mol, 3.15 M/ H_2O , 40 mL). Conc. HCl (30 mL) was slowly added into the mixtures with continuous stirring. Then SO_2 gas was passed into the mixtures for 30 min. The resulting mixture was extracted with CH_2Cl_2 (3 x 70 mL) and dried over anhydrous Na_2SO_4 . The volatiles were evaporated to afford MeAsI_2 (46 g, 97%) as orange liquid.¹⁴ (b) Condensation with 2-chloroethanol. 12 M aqueous NaOH (40 mL, 19.2 g, 0.48 mol) was slowly added into MeAsI_2 (46 g, 0.122 mol) placed in a round bottom flask (0 °C, ice bath) over a 20 min with vigorous stirring. During the addition of NaOH, the yellow color was disappeared resulting in colorless solution. The resulting MeAs(ONa)_2 solution was stirred for 15 min and then 2-chloroethanol (**3a**; 8.2 mL, 9.8 g, 0.122 mol) was slowly added over 10 min. The mixture was allowed to warm to ambient temperature (approximately 1 h) and stirring was continued for 12 h. The mixture was acidified with 6 M HCl to pH~4 and white precipitate was filtered out. The filtrate was evaporated at reduced pressure yielding a white solid, which was suspended in MeOH. The white precipitate was removed by vacuum filtration and the mother liquor was evaporated at reduced pressure to give **5** (19.9 g, 86%) as a white solid: ^1H NMR δ 1.92 (s, 3H), 2.55 (t, J = 6.4 Hz, 2H), 3.97 (t, J = 6.4 Hz, 2H); ^{13}C NMR δ 18.06, 37.30, 55.63; HRMS m/z calcd for $\text{C}_3\text{H}_8\text{AsO}_3$ [M-H]⁺ 166.9694, found 166.9693.

Note: Removal of volatiles from the reaction mixture prior neutralization with HCl and purification of the residue on silica column with 10-30% MeOH/ CH_2Cl_2 provided iodide-free sodium salt of **5**.

*2-Hydroxyethyl(chloro)(methyl)arsine (**6a**).* Conc. HCl (37%, 70 mL) was slowly added into the stirring solution of **5** (15 g, 79 mmol) in H_2O (40 mL) over a 10 min at rt. Next, catalytic amount of KI (200 mg, 1.2 mmol) was added and SO_2 gas was passed into this solution for 30 min. with continuous stirring. The mixtures were extracted with CH_2Cl_2 (3 x 70 mL) and dried over anhydrous Na_2SO_4 . The volatiles were evaporated under reduced pressure to give **6a** (12.3 g, 92%) as yellowish oil: ^1H NMR (CDCl_3) δ 1.72 (brs, 1H), 2.04 (s, 3H), 2.50 (t, J = 6.8 Hz, 2H), 4.01 (t, J = 6.8 Hz, 2H); ^{13}C NMR (CDCl_3) δ 22.0, 35.8, 61.4. Molecular ion for **6a** was not detected in HRMS experiment instead molecular ion for **5** was observed (HRMS: m/z calcd for $\text{C}_3\text{H}_{10}\text{AsO}_3$ [M+H]⁺ 168.9840, found 168.9840).

Note: Treatment (0 °C to rt, 3 h) of the stirred solution of **6a** (12 g, 70.4 mmol) in anhydrous CH₂Cl₂ (60 mL) with SOCl₂ (12.3 mL, 20.1 g, 0.17 mol) followed by removal of volatiles under reduced pressure provided dichloro-(2-hydroxyethyl)arsine (8.7 g, 65%) as brown liquid with the ¹H and ¹³C NMR spectra as reported¹⁰ (HRMS *m/z* calcd for C₂H₄AsOCl₂ [M-H]⁻ 188.8860 found 188.8862) instead of desired chloro(2-chloroethyl)(methyl)arsine **6b**.

2-Chloroethyl(methyl)arsinic acid (7). **Method A.** SOCl₂ (10 mL) was added to an iodide-free sodium salt of **5** (500 mg, 2.63 mmol) in a dry flask at 0 °C (ice-bath) and was stirred at rt for 15 h. The reaction was quenched by adding 20 mL water and extracted with CH₂Cl₂. The aqueous phase was separated and volatiles were evaporated at reduced pressure. The residue was purified by column chromatography (20 → 30% MeOH/ CH₂Cl₂) to give mixture of **7** and **5** as a sticky solid (450 mg; 80:20). Second silica column purification afforded pure **7** (20 mg, 4%) as colorless gummy solid in addition to mixture of **7** and acidic form of **5** as a gummy solid (85:15; 400 mg, ~69% of **7**) to give overall yield for the conversion of **5** to **7** of approximately 73%. Compound **7** had: ¹H NMR δ 2.08 (s, 2H), 2.94 (t, *J* = 6.6 Hz, 1H), 3.98 (t, *J* = 6.8 Hz, 1H); ¹³C NMR δ 17.47, 36.86, 36.97; HRMS *m/z* calcd for C₃H₉AsClO₂ [M+H]⁺ 186.9501, found 186.9500.

Method B. Trimethylsilyl chloride (755 μL, 647 mg, 5.96 mmol) was added to a stirring solution of sodium salt of **5** (500 mg, 2.98 mmol) in DMSO (1 mL) at rt. The resulting mixture was stirred at rt for 14 h. The reaction was quenched by adding 10 mL water and extracted with EtOAc (5 x 10 mL) to remove DMSO. The aqueous phase was separated, and volatiles were evaporated at reduced pressure to give a gummy solid (~400 mg) containing **7** (8%, based on ¹H NMR and HPLC-ICP-MS) and unchanged **5** (92%). HRMS *m/z* calcd for C₃H₉AsClO₂ [M+H]⁺ 186.9501, found 186.9500 for **7** and *m/z* calcd for C₃H₁₀AsO₃ [M+H]⁺ 168.9840, found 168.9840 for **5**.

N-[1-Carboxy-3-(hydroxyl(methyl)arsonyl)propan-1-yl]-N,N,N-trimethylammonium hydroxide (10). (a) Reduction. AST-OH¹⁰ (**2**; 50 mg, 0.22 mmol) was dissolved in the mixture of concentrated HCl and water (1:1, 5 mL). Then catalytic amount of KI (2.2 mg, 0.013 mmol) was added and SO₂ gas was passed into this solution for 15 min at rt. The pH was then adjusted around ~11 with 6 M NaOH (aq) solution under N₂. (b) Methylation. To the solution from *step a*, MeI (1.1 mL) was added and the mixture was stirred at 50 °C for 2 h. After 2 h, the pH of the reaction mixture showed around 6.5. The volatiles were evaporated under reduced pressure and the residue was suspended in methanol. The off-white precipitate was removed by vacuum filtration. Evaporation of volatiles from the filtrate at reduced pressure gave brown solid. The residue was dissolved in 2 mL H₂O and applied to a Dowex 50W X 8 (H⁺ form) column (30 x 1 cm, 10 g) which was washed with 50 mL of H₂O. The product was eluted with a solution of NH₄OH (0.5 M, 50 mL). The appropriate fractions (TLC, R_f 0.70, i-PrOH/H₂O/NH₄OH, 5:2:3; identified by staining with 1%

ninhydrin solution) from the ammonium elution (~20 mL) were evaporated under reduced pressure to afford **10** (41 mg, 70%) as an off-white solid: ^1H NMR δ 1.69 (s, 3H), 1.90-2.06 (m, 2H), 2.09-2.19 (m, 1H), 2.23-2.32 (m, 1H), 3.19 (s, 9H), 3.68 (dd, J = 11.6, 3.6 Hz, 1H); ^{13}C NMR δ 15.84, 19.61, 28.99, 51.95, 78.62, 170.65; HRMS m/z calcd for $\text{C}_8\text{H}_{19}\text{AsNNaO}_4$ [M+H] $^+$ 290.0344, found 290.0346.

Ethyl-2-Acetamido-2-ethoxycarbonyl-4-(hydroxymethylarsinoyl)butanoate (11). Sodium (1.46 g, 63.6 mmol) was added into a dry flask containing 40 mL of anhydrous EtOH and the mixture was stirred at ambient temperature until the sodium dissolved. Then diethyl acetamidomalonate (10.4 g, 47.7 mmol) was added, and the resulting mixture was stirred for 1 h. During this time, the reaction was turned into milky white mixture. To the above mixture solid **4a**¹⁰ (3.0 g, 15.9 mmol) was added and the resulting mixture was stirred at 70 °C in an oil bath for 4 h. Volatiles were evaporated under reduced pressure to give crude **11**¹⁰ as a brownish solid, which was suspended in H_2O and transferred to a separatory funnel. The mixture was extracted with CH_2Cl_2 (6 x 50 mL) to remove excess malonate and aqueous layer was collected. The volatiles were evaporated under reduced pressure and residue was suspended in 30% MeOH in CH_2Cl_2 . The off-white precipitate was removed by vacuum filtration. Evaporation of the volatiles at reduced pressure gave brown solid which was loaded on the silica column. The residual malonate was removed by 5-10% MeOH in CH_2Cl_2 until all the yellow color eluted. Then the arsenic compound was eluted by 30% MeOH. Only the colorless fractions were collected. Volatiles were evaporated under reduced pressure to afford 6.0 g white solid which on ^1H NMR spectra showed approximate 10% malonate impurity. Second silica column purification gave pure **11** (5.2 g, 89%) as white solid: ^1H NMR δ 1.25 (t, J = 7.2 Hz, 6H), 1.99-2.04 (m, 2H), 2.07 (s, 3H), 2.54-2.58 (m, 2H), 4.29 (q, J = 7.2 Hz, 4H); ^{13}C NMR δ 13.73, 22.14, 26.80, 27.88, 64.77, 67.41, 168.97, 174.21; HRMS m/z calcd for $\text{C}_{11}\text{H}_{21}\text{AsNO}_8$ [M+H] $^+$ 370.0478, found 370.0475.

2-Amino-4-(hydroxymethylarsinoyl)butanoic acid (AST, 1). **Procedure A** (from **11**). (a) Reduction. Compound **11** (500 mg, 1.35 mmol) was dissolved in the mixture of concentrated hydrochloric acid and water (1:1, 14 mL). Then catalytic amount of KI (13.5 mg, 0.08 mmol) was added and SO_2 gas was passed into this solution for 15 min at ambient temperature. The pH was then adjusted to around 11 with 6 M NaOH (aq) solution under N_2 to give crude **12** which was directly used in next step. (b) Methylation. To the product from *step a*, MeI (7 mL) was added and the mixture was stirred at 50 °C for 4 h. The reaction progress was monitored by HPLC-ICP-MSA. After 4h, the pH of the reaction mixture showed around 6.7. The volatiles were evaporated under reduced pressure and the residue was suspended in methanol. The off-white precipitate was removed by vacuum filtration. Evaporation of volatiles from the filtrate at reduced pressure gave brown solid crude **8**. (c) Deprotection and decarboxylation. 6 M HCl (20 mL) was added into the crude **8**, and the resulting mixture was refluxed at 120 °C in an oil bath for 3 h. The mixture was

neutralized with 6 M HCl around pH~7 and white precipitate was filtered out. The volatiles were evaporated under reduced pressure and the residue was suspended in methanol. The off-white precipitate was removed by vacuum filtration. Evaporation of volatiles from the filtrate at reduced pressure gave brown solid crude **1**. The residue was dissolved in 15 mL H₂O and applied to a Dowex 50WX8 (H⁺ form) column (30 x 1 cm, 10 g) which was washed with 100 mL of H₂O. The product was eluted with a solution of NH₄OH (0.5 M, 100 mL). The appropriate fractions (TLC, R_f 0.70, i-PrOH/H₂O/NH₄OH, 5:2:3; identified by staining with 1% ninhydrin solution) from the ammonium elution (~40 mL) were evaporated under reduced pressure to afford **1** as an off-white solid with some impurities. The residue was dissolved in 5 mL H₂O and was applied onto a Sephadex LH-20 (GE Healthcare) column with a mobile phase 70% (v/v) EtOH at a flow rate of 1.0 mL/min. Arsenic species in each fraction was analyzed by HPLC-ICP-MS. Fractions containing AST with high purity (>95%) were combined and concentrated by a rotary evaporator to afford **1**^{7,10} (200 mg, 65% from **11**) as an off-white solid. ¹H NMR δ 2.03 (s, 3H), 2.27-2.34 (m, 2H), 2.42-2.48 (m, 1H), 2.52-2.58 (m, 1H), 3.97 (t, J = 6.4 Hz, 1H); ¹³C NMR δ 16.39, 23.34, 29.39, 54.39, 172.93; HRMS m/z calcd for C₅H₁₃AsNO₄ [M+H]⁺ 226.0055, found 226.0055.

Note: Use of pure **11** free of diethyl acetamidomalonate impurities is critical since during deprotection and decarboxylation steps these impurities are converted to glycine that is difficult to separate from the AST product during purification on Dowex.

Procedure B (from **7**). (a) Condensation. Sodium (198 mg, 8.60 mmol) was added into a dry flask containing anhydrous EtOH (5 mL) and the mixture was stirred at rt until the sodium dissolved. Then diethylacetamidomalonate (1.40 g, 6.45 mmol) was added, and the resulting mixture was stirred for 30 min, followed by addition of **7** as a mixture of **7** and **5** (85:15, 400 mg from Method A) dissolved in 2 mL EtOH. The resulting mixture was stirred at 70 °C for 4 h. Volatiles were evaporated under reduced pressure, yielding crude **8**. (b) Deprotection and decarboxylation. Subjection of crude **8** to the same protocol as described for Procedure A (step *c*) gave **1** (72 mg, ~17% from **7**) as an off-white solid: ¹H NMR δ 1.97 (s, 3H), 2.21-2.27 (m, 2H), 2.33-2.41 (m, 1H), 2.43-2.51 (m, 1H), 3.83 (t, J = 6.2 Hz, 1H); ¹³C NMR δ 16.17, 23.53, 29.26, 55.01, 173.73; HRMS m/z calcd for C₅H₁₃AsNO₄ [M+H]⁺ 226.0055, found 226.0055.

Note: Treatment of crude **7** (350 mg, from Method B) with EtONa and diethylacetamidomalonate as described for Procedure B afforded **1** (5% from **5**, based on ¹H NMR and HPLC-ICP-MS). The presence of **1** was confirmed by HPLC-ICP-MS and HRMS: calcd for C₅H₁₁AsNO₄ [M-H]⁻ 223.9909, found 223.9909.

*(S)-2-Acetamido-4-(hydroxymethylarsinoyl)butanoic acid (L-AcAST, **13**).* (a) Enzymatic N-acetylation of AST (**1**). PpArsN1, the AST-selective N-acetyltransferase from *Pseudomonas putida* KT2440, was purified as described previously.⁹ 10 μM of **1** (cAST, sAST bAST, or L-AST) was incubated in a buffer consisting of 20 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetraacetic acid, 0.2 mM acetyl coenzyme A (AcCoA)

at 37 °C for 30 min, with or without 0.2 μ M PpArsN1. The reaction solution was filtered using an Amicon Ultra centrifugal filter with a 3K cutoff membrane (MilliporeSigma), and arsenic species in the filtrate were analyzed by HPLC-ICP-MS. (b) Purification of L-AcAST. A larger amount of D/L-cAST **1** (0.9 mM, 35 mL, 7 mg) was incubated overnight with 1 mM AcCoA and 20 μ M PpArsN1 in a buffer consisting of 20 mM Tris-HCl, pH 7.4 at 37 °C. The reaction solution was filtered using an Amicon Ultra centrifugal filter with a 3K cutoff membrane to remove protein. The filtrate was concentrated to 5 mL by rotary evaporation at reduced pressure and separated by Sephadex LH-20 size-exclusion chromatography gave L-AcAST **13** and D-AST with little impurities. Arsenic species in each fraction was analyzed by HPLC-ICP-MS. Fractions containing putative L-AcAST with high purity (>90%) were combined and concentrated by a rotary evaporation. The concentrated L-AcAST solution was applied again to Sephadex LH-20 size-exclusion chromatography for further purification. Fractions containing purified L-AcAST (>95%) were combined and concentrated to give **13** (3.0 mg, 36%): 1 H NMR δ 1.92 (s, 3H), 19.8 – 2.07 (m, 1H), 2.04 (s, 3H), 2.24 – 2.15 (m, 1H), 2.37 – 2.26 (m, 2H), 4.24 (dd, J = 8.2, 4.6 Hz, 1H); 13 C NMR δ 15.22, 21.89, 24.07, 29.42, 55.01, 173.80, 177.32; HRMS m/z calcd for $C_7H_{15}AsNO_5$ [M+H] $^+$ 268.0161, found 268.0162.

Note: Fractions containing D-AST (>95%) were combined and concentrated to give D-AST (2.1 mg, 30%): HRMS m/z calcd for: $C_5H_{13}AsNO_4$ [M+H] $^+$ 226.0055, found 226.0059.

(S)-2-Amino-4-(hydroxymethylarsinoyl)butanoic acid (L-AST): 2 M HCl (5 mL) was added into **13** (3.0 mg, 0.013 mmol) in round bottom flask, and the resulting mixture was refluxed at 120 °C (oil bath) for 3 h. Volatiles were evaporated at reduced pressure. The residue was dissolved in 2 mL H₂O and separated by Sephadex LH-20 size-exclusion chromatography. Fractions containing L-AST (>95%) were combined and concentrated at reduced pressure to give L-AST (1.9 mg, 75%) as a white solid; 1 H NMR δ 1.73 (s, 3H), 2.08-2.29 (m, 4H), 3.83 (t, J = 5.6 Hz, 1H); 13 C NMR δ 15.66, 23.35, 28.80, 54.70, 173.52; HRMS m/z calcd for $C_5H_{13}AsNO_4$ [M+H] $^+$ 226.0055, found 226.0056.

Derivatization of AST with Marfey's reagent. Sample of racemic AST or L-AST (5 μ mol) is dissolved in 100 μ L of water. 2 mg of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) is dissolved in 200 μ L of acetone (1% solution). Solution of Marfey's reagent is added to the dissolved AST sample followed by 40 μ L of 1 M NaHCO₃. Reaction is set up at 40°C for 1 h after which is quenched by addition of 20 μ L of 2 M HCl. Small aliquot is taken, diluted with DMSO and injected on RP HPLC (column: Vydac Denali C-18, 5 μ m, 120 \AA). Elution method 100% A for 5 min followed by linear gradient of 0-100% B over 40 min where A is 0.1% TFA in H₂O and B is 0.1% TFA in ACN. Eluting products were detected by UV at 340 nm. Results are shown in Figure S1 in SI section.

Arsenic Speciation by HPLC-ICP-MS. Arsenic species were determined by HPLC-ICP-MS using the HPLC retention time of known standards as described previously.¹⁰

Assay of Antibiotic Activity. Single colonies of *E. coli* W3110 were inoculated in M9 medium (47.7 mM Na₂HPO₄, 22 mM KH₂PO₄, 8.6 mM NaCl, 18.7 mM NH₄Cl, 2 mM MgSO₄ and 0.1 mM CaCl₂) supplemented with 0.2% glucose (w/v) and cultured in the absence or presence of the indicated concentrations of chemically synthesized AST (cAST), semisynthetic AST (sAST)¹⁰, biogenic AST (bAST)⁷, and L-AST for 16 h at 37 °C. The A_{600nm} was determined to compare the antibiotic activity of each compound.

Assay of Glutamine Synthetase (GS) Inhibition. Inhibition of GS activity by bAST, sAST, cAST or L-AST was analyzed by a coupled assay using GS from *E. coli* (Millipore-Sigma), as described previously⁹ with minor modifications. Briefly, the GS reaction was initiated by addition of L-glutamate (5, 10, 20 or 50 mM) to the reaction mixture (100 mM Tris-acetate (pH 8.6), 9 mM ATP, 1 mM phosphoenolpyruvate, 60 mM Mg₂Cl, 19 mM KCl, 45 mM NH₄Cl, 0.25 mM NADH, 13-20 units of L-lactic dehydrogenase, 8-14 units of pyruvate kinase and 1 unit of GS) and incubated at 37 °C in the absence or presence of 0.5 or 2.0 μM of bAST, sAST, cAST or L-AST and the decrease in A_{340 nm} was monitored to quantify oxidation of NADH to NAD⁺ using an extinction coefficient 6230 M⁻¹cm⁻¹. Activities were corrected with the values from control assays without enzyme. The inhibition constant (K_i) for each AST was determined based on the apparent K_m of GS using Sigma Plot (Systat Software, Inc., Sun Jose, CA).

ASSOCIATED CONTENT

Supporting Information

Electronic supplementary information (ESI) available: RP-HPLC traces after derivatization of AST with Marfey's reagent and copies of ¹H, ¹³C NMR and HRMS spectra for all new compounds. DOI:

AUTHOR INFORMATION

Corresponding Author

* Email: wnuk@fiu.edu

myoshina@fiu.edu

Present Addresses

¶ Department of Chemistry, Johns Hopkins University, Baltimore, Maryland, USA.

† Department of Chemistry & Biochemistry, Rowan University, Glassboro, New Jersey, USA.

CONFLICT OF INTEREST

There are no conflicts of interest to declare

Author Contributions

AHH and SHS conducted the synthetic experiments. VSN, AEG and MY conducted the HPLC-ICP-MS analysis and biological studies. AN and PC established chiral purity of L-AST. AHH, BPR, MY and SFW designed the experiments and wrote the manuscript.

ACKNOWLEDGMENT

This work was supported by NSF BIO/MCB grant 1817962 to MY and NIH grants GM55425 and GM136211 to BPR. AHH is recipient of the FIU Presidential Fellowship and Dissertation Year Fellowship.

NOTES and REFERENCES

- (1) Foster, S.; Maher, W. *J. Environ. Sci.* **2016**, *49*, 131.
- (2) Chen, B.; Liu, Q.; Popowich, A.; Shen, S.; Yan, X.; Zhang, Q.; Li, X.-F.; Weinfeld, M.; Cullen, W. R.; Le, X. C. *Metallomics* **2015**, *7*, 39.
- (3) Wang, Q. Q.; Thomas, D. J.; Naranmandura, H. *Chem. Res. Toxicol.* **2015**, *28*, 281.
- (4) Tay, W. S.; Pullarkat, S. A. *Chem. Asian J.* **2020**, *15*, 2428.
- (5) Cullen, W. R.; Liu, Q.; Lu, X.; McKnight-Whitford, A.; Peng, H.; Popowich, A.; Yan, X.; Zhang, Q.; Fricke, M.; Sun, H.; Le, X. C. *J. Environ. Sci.* **2016**, *49*, 7.
- (6) Peng, H.; Hu, B.; Liu, Q.; Li, J.; Li, X.-F.; Zhang, H.; Le, X. C. *Angew. Chem. Int. Ed.* **2017**, *56*, 6773.
- (7) Kuramata, M.; Sakakibara, F.; Kataoka, R.; Yamazaki, K.; Baba, K.; Ishizaka, M.; Hiradate, S.; Kamo, T.; Ishikawa, S. *Environ. Chem.* **2016**, *13*, 723.
- (8) Galván, A. E.; Paul, N. P.; Chen, J.; Yoshinaga-Sakurai, K.; Utturkar, S. M.; Rosen, B. P.; Yoshinaga, M.; Tang, X. *Microbiol. Spectr.* **2021**, *9*:e00502.
- (9) Nadar, V. S.; Chen, J.; Dheeman, D. S.; Galván, A. E.; Yoshinaga-Sakurai, K.; Kandavelu, P.; Sankaran, B.; Kuramata, M.; Ishikawa, S.; Rosen, B. P.; Yoshinaga, M. *Commun. Biol.* **2019**, *2*, 131.
- (10) Suzol, S. H.; Hasan Howlader, A.; Galván, A. E.; Radhakrishnan, M.; Wnuk, S. F.; Rosen, B. P.; Yoshinaga, M. *J. Nat. Prod.* **2020**, *83*, 2809.
- (11) Qin, J.; Lehr, C. R.; Yuan, C.; Le, X. C.; McDermott, T. R.; Rosen, B. P. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 5213.
- (12) Adams, S. R.; Sparkes, M. J.; Dixon, H. B. F. *Biochem. J.* **1983**, *213*, 211.
- (13) Reported synthesis of sodium methylarsonite [MeAs(ONa)₂] involves the multistep preparation of chloro(2-chloroethyl)(methyl)arsane from methyl(oxo)arsane which upon treatment with aq. NaOH yields MeAs(ONa)₂: Kuskov, V. K.; Vasil'ev, V. N. *Zh. Obshch. Khim.* **1951**, *21*, 90.
- (14) Spuches, A. M.; Kruszyna, H. G.; Rich, A. M.; Wilcox, D. E. *Inorg. Chem.* **2005**, *44*, 2964.
- (15) Attempted displacement of OH group in trivalent arsine **6a** with NBS or X₂/PPh₃ (X = Br or I) also failed to produce the halogenated products of type **6b** (X = Br or I).

(16) Direct halogenation of **5** with HX (X = Br or I) failed to give halogenated products of type **7**.

(17) Numerous attempts to displace activated hydroxyl group in the protected homoserine with diiodo(methyl)arsine (MeAsI_2) in the presence of aqueous NaOH failed to produce AST **1**, instead yielding dihydro-3-amino-2-(3H)-furanone lactone.

(18) Abalonin, B. E. *Russ. Chem. Rev.* **1991**, *60*, 1346.

(19) Bionda, N.; Cudic, M.; Barisic, L.; Stawikowski, M.; Stawikowska, R.; Binetti, D.; Cudic, P. *Amino Acids* **2012**, *42*, 285.