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Synthesis and aminoacyl-tRNA synthetase inhibitory activity of aspartyl adenylate analogs

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Abstract—Three nonhydrolyzable aspartyl adenylate analogs have been prepared and tested as inhibitors of *E. coli* aspartyl-tRNA synthetase. 5′-*O*-[*N*-(L-Aspartyl)sulfamoyl]adenosine is a potent competitive inhibitor (K_i = 15 nM) whereas L-aspartol adenylate is a weaker inhibitor (K_i = 45 μM) with respect to aspartic acid. The corresponding ketomethylphosphonate (a novel isosteric replacement) is also a strong inhibitor (K_i = 123 nM).

1. Introduction

Aminoacyl-tRNA synthetases (aaRSs) are essential enzymes involved in protein biosynthesis in all living organisms. They catalyze the esterification of a particular tRNA with its cognate amino acid in a two-step process.[1,2] In the first step, the amino acid (aa) is recognized by the enzyme and reacts with ATP to form an enzyme-bound mixed anhydride (aaAMP, aminoacyl adenylate) with displacement of pyrophosphate (PP_i). In this intermediate, the high-energy anhydride bond activates the carboxyl group of the amino acid. In the second step, the activated amino acid is transferred to the 3′-terminal adenosine of the corresponding tRNA to form aminoacyl-tRNA (aa-tRNA) and adenosine monophosphate (AMP). This esterification step is a nucleophilic attack by the 2′ or 3′ ribose hydroxyl group of the A76 of the tRNA on the activated carboxyl group of the aminoacyl adenylate.

$$\mathrm{aa} + \mathrm{ATP} + \mathrm{aaRS} \overset{\mathrm{Mg^{2+}}}{\leftrightarrows} \mathrm{aaRS \cdot aaAMP} + \mathrm{PP_i}$$

$$\mathrm{aaRS \cdot aaAMP} + \mathrm{tRNA} \leftrightarrows \mathrm{aa\text{-}tRNA} + \mathrm{AMP} + \mathrm{aaRS}$$

Aminoacyl-tRNA synthetases are classified into two main groups of 10 enzymes each on the basis of common structural and mechanistic features.[3] Aspartyl-tRNA synthetase is a member of the class II group. As aaRSs have been subjected to significant evolutionary divergence, selective inhibition of bacterial enzymes is a valuable strategy for the production of antibiotics.[4–9] Pseudomonic acid, a natural product isolated from *Pseudomonas fluorescens*, is a potent and selective inhibitor of bacterial isoleucyl-tRNA synthetases, and plays an important clinical role.[9,10]

Potent synthetic inhibitors of aaRSs are typically stable analogues of aminoacyl adenylates (aaAMP). The stability is achieved by replacement of the labile mixed anhydride function by nonhydrolyzable bioisosteres (Scheme 1).

Several aminoalkyl adenylates[11–17] or aminoacyl-sulfamoyladenosines[11,13,18–24] have been synthesized and shown to be potent inhibitors of aaRSs. The ketomethylphosphonate (β-ketophosphonate) function can also serve as a stable surrogate for the reactive acylphosphate subunit of aminoacyl adenylates in the design of aaRS inhibitors. The synthesis of β-ketophosphonate analogs of some aminoacyl adenylates has been reported[25,26] but their inhibitory activity on corresponding aaRSs was not determined.

Keywords: Aspartyl-tRNA synthetase; Inhibitors; Aspartyl adenylate; Analogs.
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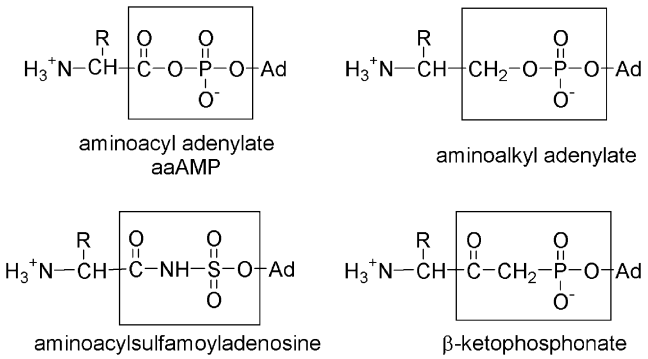


Scheme 1.

With the aim of obtaining inhibitors of AspRS, we have synthesized aminoalkyl adenylate, sulfamoyl, and β-ketophosphonate analogues of aspartyl adenylate, and tested their properties in the aminoacylation reaction of $tRNA^{Asp}$ catalyzed by *Escherichia coli* AspRS.

2. Results and discussion

2.1. Synthesis of L-aspartol adenylate 5

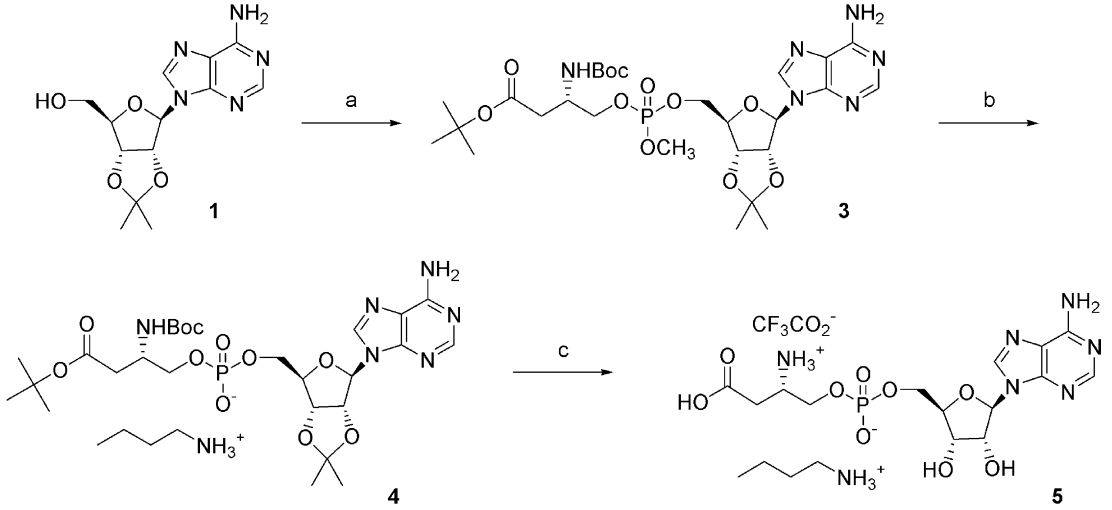
The phosphoramidite-phosphite triester approach was used for the condensation between commercially available 2′,3′-isopropylideneadenosine **1** and known L-aspartol derivative **2**[27] (Scheme 2). Compound **1** was first phosphorylated with *N*,*N*-diisopropylmethylphosphonamidic chloride in the presence of *N*,*N*-diisopropylethylamine in dry dichloromethane. The intermediate phosphoramidite was coupled with aspartol **2** in dry tetrahydrofuran using imidazolium triflate as an activating agent and then the phosphite triester was oxidized to phosphate triester **3** with iodine in tetrahydrofuran–water. In phosphate triester **3**, the phosphorus is a center of chirality and this compound was obtained as a mixture of diastereoisomers. The nonequivalence (chemical shift difference) of some groups was observed in ^{1}H, ^{13}C, and ^{31}P NMR spectra. This phosphotriester was deprotected by sequential treatment with *n*-butylamine in methanol to give phosphodiester **4** and then with wet trifluoroacetic acid (hydrolysis of *tert*-butyl ester, *N*-Boc, and isopropylidene acetal groups) to provide aspartol adenylate **5**.

2.2. Synthesis of 5′-*O*-[*N*-(L-aspartyl)sulfamoyl]adenosine 9

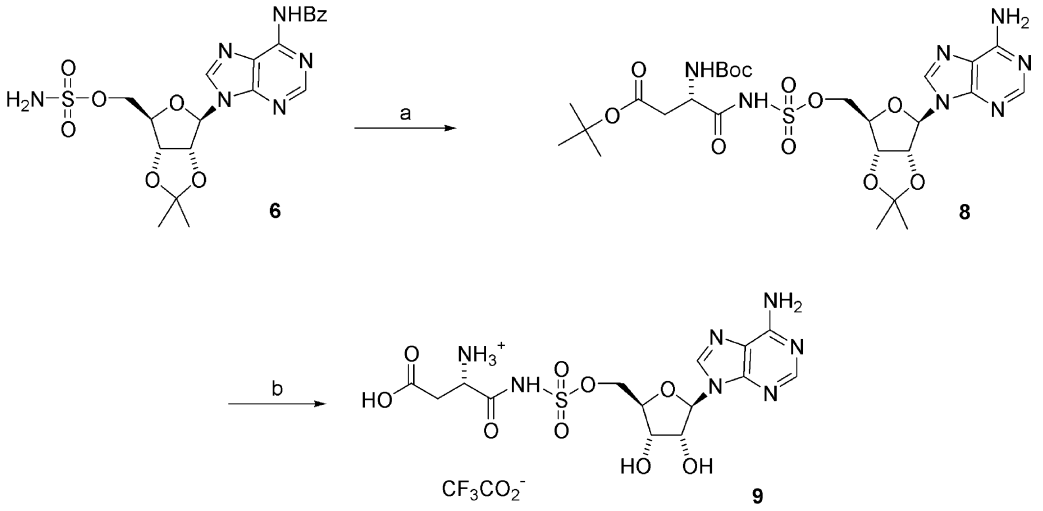
5′-*O*-Sulfamoyladenosine derivative **6** was prepared by reaction of N^6-benzoyl-2′,3′-*O*-isopropylidene-adenosine[28] with sulfamoyl chloride.[29,30] Commercially available *N*-*t*-Boc-L-aspartic acid γ-*tert*-butyl ester **7** was coupled with sulfamoyladenosine derivative **6** in the presence of dicyclohexylcarbodiimide (DCC) and 1 equiv of dimethylaminopyridine (DMAP) in CH_2Cl_2[31] (Scheme 3). Treatment of the crude product with *n*-butylamine in methanol removed the *N*-benzoyl moiety and provided **8**. This deprotection step enhanced the polarity of the compound thus facilitating its purification from the dicyclohexylurea by-product of the coupling reaction. Treatment of **8** with wet trifluoroacetic acid facilitated the removal of the three remaining protecting groups (*N*-Boc, isopropylidene, *tert*-butyl ester) to afford compound **9**. This compound is moisture sensitive and slowly decomposes in the solid state but is stable for several months in a neutral buffer solution (HEPES sodium salt).

2.3. Synthesis of β-ketophosphonate 15

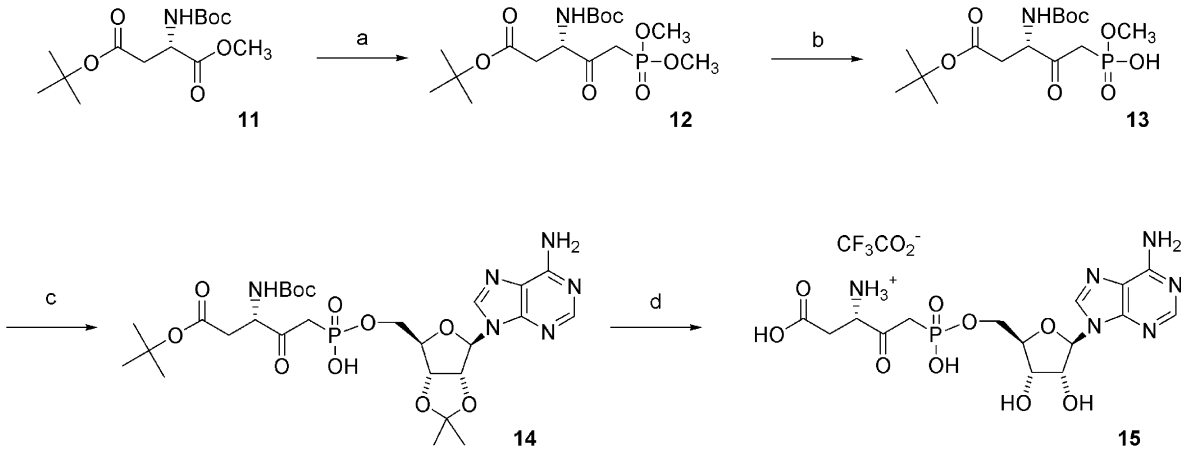
Aspartic methyl ester **11** was prepared by reaction of cesium salt of aspartic acid derivative **7** with iodomethane in DMF.[32] Reaction of ester **11** with the lithiated dimethyl methylphosphonate (generated by addition of *n*-butyllithium to commercially available dimethyl methylphosphonate) in THF at −78 °C afforded β-keto-phosphonate **12** (Scheme 4). Monomethyl phosphonic acid **13** was obtained by selective demethylation of **12** by *tert*-butylamine.[33] Nucleophilic displacement of the



Scheme 2. Reagents and conditions: (a) *i*-Pr_2NEt, $(i\text{-Pr})_2NP(OCH_3)Cl$, CH_2Cl_2, rt, 30 min; (ii) imidazolium triflate, *t*-BuO_2C–CH_2–$CH(NHBoc)CH_2OH$ (**2**), CH_3CN, rt, 4 h 30 min; (iii) I_2, H_2O/THF (1:4); (c) *n*-$BuNH_2$/MeOH (1:1), rt, 48 h; (d) TFA/H_2O (4:1), rt, 15 min.



Scheme 3. Reagents and conditions: (a) (i) *t*-$BuO_2C–CH_2–CH(NHBoc)CO_2H$ (**7**), DCC, DMAP, CH_2Cl_2, rt 2 h; (ii) *n*-$BuNH_2$/MeOH (1:1), rt, 3 h; (b) TFA/H_2O (4:1), rt, 15 min.



Scheme 4. Reagents and conditions: (a) $LiCH_2P(O)(OCH_3)_2$ (**10**), THF, −78 °C, 2 h 30 min; (b) (i) *t*-$BuNH_2$, reflux, 24 h; (ii) HCl; (c) **1**, TsCl, py, rt, 14 days; (d) TFA/H_2O (4:1), rt, 15 min.

5′-tosylate of 2′,3′-*O*-isopropylideneadenosine **1** (generated in situ by reaction of **1** with tosyl chloride) by the pyridinium salt of **13** in pyridine gave **14** after 2 weeks at room temperature. Such a long reaction time resulted in the simultaneous deprotection of the phosphonate moiety. Finally, treatment of **14** with wet trifluoroacetic acid provided β-ketophosphonate **15**.

2.4. Inhibition of *E. coli* aspartyl-tRNA synthetase (AspRS)

Among these three analogs of aspartyl adenylate, 5′-*O*-[*N*-(L-aspartyl)sulfamoyl]adenosine **9** is by far the most efficient inhibitor of AspRS activity (Fig. 1). The kinetic results presented in Figure 2A show that aspartyl-sulfamoyl-adenosine is a competitive inhibitor of *E. coli* AspRS with respect to aspartate. Indeed, the reciprocal plot curves obtained in the absence of inhibitor and in the presence of two concentrations of this inhibitor intersect on the vertical axis ($1/v$). Analysis of these data (Fig. 2B) gives a K_{ic} value of 15 nM aspartyl-sulfamoyl-adenosine for AspRS with respect to aspartate. This value is consistent with the estimated K_i of 8 nM obtained from the data shown in Figure 1 with the assumption that the three analogs of aspartyl-AMP tested here are competitive inhibitors of AspRS with respect to aspartate. This assumption is based on the fact that all amino- acyl-AMP analogs that inhibit the corresponding aaRS are competitive with respect to the amino acid substrate.[5] Estimated K_i values of β-ketophosphonate **15** and aspartol adenylate **5** for *E. coli* AspRS are 123 nM and 45 μM, respectively (Fig. 1). In class I aaRSs, the activated carbonyl group of the aminoacyl adenylate (aaAMP) intermediate is exposed to the solvent and has minimal interaction with the protein. In class II aaRSs such as AspRS, the activated carbonyl of aaAMP is involved into a network of hydrogen bonds with the protein and the adjacent amino group[11]. This fact may explain why aspartol adenylate **5** lacking a carbonyl is a much weaker inhibitor ($K_i = 45\,\mu M$) than the two other inhibitors **9** and **15** ($K_i = 123$ and 15 nM, respectively) bearing a carbonyl group. The comparison of the K_i values of these aspartyl-AMP analogs for *E. coli* AspRS with those to be obtained for a

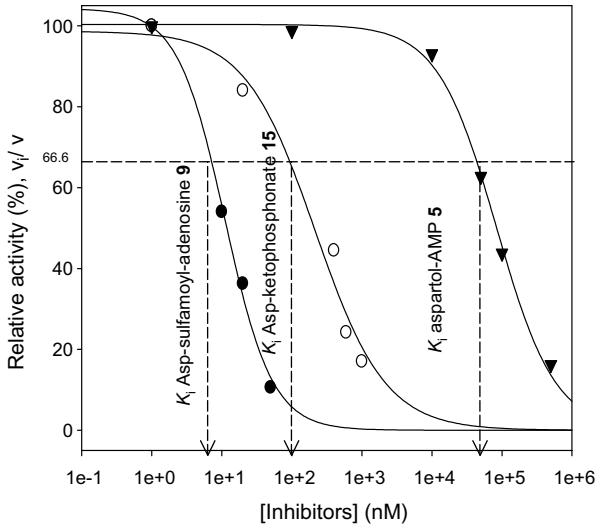


Figure 1. Estimation of the inhibition constants of aspartyl-sulfamoyl-adenosine **9**, aspartyl-phosphonate-adenosine **15**, and aspartol adenylate **5** for *E. coli* aspartyl-tRNA synthetase (AspRS). The relative aminoacylation activities of *E. coli* AspRS were measured at 37 °C in the presence of 90 μM aspartate ([Asp] = K_m^{Asp}), of saturating ATP and tRNA concentrations (2 mM and 50 μM, respectively) and of either aspartyl-sulfamoyl-adenosine **9** (dark circle), or aspartyl-phosphonate-adenosine **15** (empty circle), or aspartol adenylate **5** (triangle). The AspRS concentration was 0.5 nM. Assuming that AspRS inhibition by these analogues of aspartyl-AMP is competitive with respect to aspartate, the ratio of the initial rates v_i of this reaction in the presence of various concentrations of an inhibitor, to its rate v in the absence of inhibitor, was used to estimate the inhibition constant K_{ic} as follows: $v = V_{max}S/(S + K_m)$, $v_i = V_{max}S/(S+K_m[1 + I/K_{ic}])$, where S and I are the substrate and inhibitor concentrations, respectively. Hence $v_i/v = (S+K_m)/(S+K_m[1+I/K_{ic}])$. When $S = K_m$ and $I = K_{ic}$, $v_i/v = 0.66$.

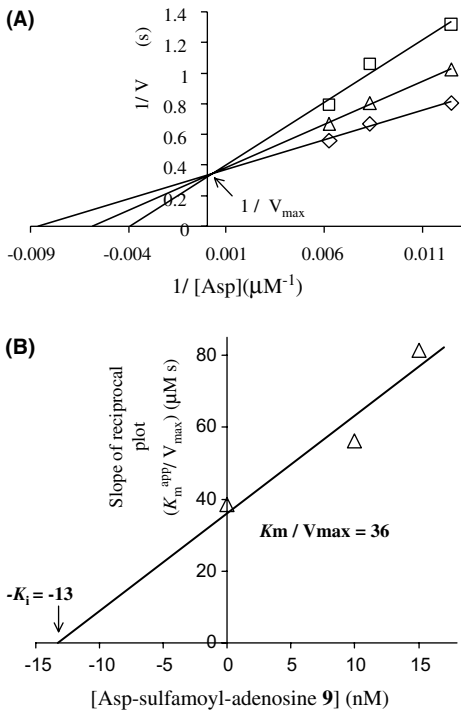


Figure 2. Aspartylsulfamoyladenosine **9** is a competitive inhibitor of *E. coli* AspRS with respect to aspartate. (A) Lineweaver–Burk plot of the kinetics of aspartyl-tRNA formation in the presence of various concentrations of aspartate (160, 120, 80 μM) and saturating concentrations of ATP (2 mM) and unfractionated *E. coli* tRNA (50 μM), in the absence of inhibitor (◊), and for fixed aspartyl-sulfamoyl-adenosine **9** concentrations: of 10 nM (triangle) and 15 nM (squares). (B) Determination of the K_{ic} value of aspartyl-sulfamoyl-adenosine **9** of *E. coli* AspRS with respect to aspartate, using replots of data from the reciprocal plot shown in A, versus the inhibitor concentration.[35]

mammalian AspRS will be useful for the design of an inhibitor specific to bacterial AspRSs. To our knowledge, these are the first compounds to inhibit AspRS. The 3D-structures of complexes between these inhibitors and AspRS could provide mechanistic information complementary to that reported by Cavarelli et al.[34] for yeast AspRS using the structures of various complexes with ATP and analogs.

3. Experimental

3.1. General

Chemical reagents were purchased from Aldrich-Sigma Chemical Company. Infrared spectra were recorded on a Bomem MB-100 spectrometer. Optical rotations were measured using a JASCO DIP-360 digital polarimeter (c as g of compound per 100 mL). Flash column chromatography was carried out using 40–63 μm (230–400 mesh) silica gel. NMR spectra were recorded on a Varian Inova AS400 spectrometer (400 MHz).

3.2. Phosphotriester 3

To a solution of commercially available 2′,3′-*O*-isopropylideneadenosine **1** (603 mg, 1.96 mmol) and diisopropylethylamine (0.85 mL, 4.90 mmol) in dry dichloromethane (30 mL) was added *N*,*N*-diisopropylmethylphosphonamidic chloride (0.38 mL, 1.96 mmol) and the solution was stirred for 30 min. The solution was washed with brine, dried ($MgSO_4$), and evaporated. The crude phosphoramidate was dissolved in dry acetonitrile (30 mL) and treated with (*S*)-*tert*-butyl-3-(*tert*-butoxycarbonylamino)-4-hydroxybutanoate **2** (450 mg, 1.63 mmol), followed by imidazolium triflate (428 mg, 1.96 mmol) at room temperature for 5 h. Then a solution of iodine (400 mg) in THF/H_2O (15 mL/15 mL) was added until no discoloration was observed. The mixture was diluted with ethyl acetate, and the organic layer was washed with aqueous sodium thiosulfate, 1 M HCl, saturated sodium bicarbonate, brine, dried ($MgSO_4$), and evaporated. The crude product was purified by flash chromatography (AcOEt to 15% MeOH/AcOEt) to give **3** (452 mg, 42%) as a white solid: mp 88–91 °C; $[\alpha]_D^{20}$ −14.1 (*c* 1.025, MeOH); IR (KBr) 3406, 2982, 1718, 1270 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 1.40–1.43 (m, 21H), 1.61 (s, 3H), 2.28–2.39 (m, 1H), 2.43–2.51 (m, 1H), 3.68 (d, J = 10.9 Hz, 3H), 3.93–3.98 (m, 2H), 4.10–4.18 (m, 1H), 4.21–4.30 (m, 2H), 4.43–4.48 (m, 1H), 5.15 (dd, J = 6.0 and 3.0 Hz, 1H), 5.49 (d, J = 6.0 Hz, 1H), 6.24 (d, J = 1.9 Hz, 1H), 8.23 (s, 1H), 8.27 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 24.3, 26.2, 27.1, 27.5, 36.9, 48.0, 54.2, 67.3, 68.9 (d, J_{C-P} = 6.1 Hz), 79.2, 80.9, 81.6, 84.1, 85.4 (d,

J_{C-P} = 7.6 Hz), 90.5 (d, J_{C-P} = 7.7 Hz), 114.5, 119.4, 140.5, 148.9, 152.9, 156.2, 156.4, 170.3; ^{31}P NMR (162 MHz, CD_3OD) δ 4.02, 4.08; HRMS (FAB) calcd for $C_{27}H_{44}N_6O_{11}P$ $(M+H)^+$ 659.2806, found 659.2791.

3.3. Phosphodiester 4

A solution of compound **3** (200 mg, 0.304 mmol) in methanol/*n*-butylamine (10 mL/10 mL) was stirred at room temperature for 48 h. The solvents were evaporated and the residue was purified by reversed phase chromatography (25% MeOH/H_2O to 35% MeOH/H_2O) to give **4** (196 mg, 90%) as a white solid: mp 160 °C (dec); $[\alpha]_D^{18}$ −42.9 (*c* 0.76, MeOH); IR (KBr) 3357, 2929, 1713, 1051 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 0.92 (t, *J* = 7.2 Hz, 3H), 1.35–1.38 (m, 23H), 1.45–1.52 (m, 2H), 1.58 (s, 3H), 2.31 (dd, *J* = 15.2 and 8.6 Hz, 1H), 2.49 (dd, *J* = 15.2 and 5.4 Hz, 1H), 2.69 (t, *J* = 7,2 Hz, 2H), 3.69–3.74 (m, 1H), 3.80–3.84 (m, 1H), 3.99–4.02 (m, 3H), 4.45 (br s, 1H), 5.07–5.11 (m, 1H), 5.33 (dd, *J* = 6.2 and 3.6 Hz, 1H), 6.18 (d, *J* = 3.6 Hz, 1H), 8.18 (s, 1H), 8.43 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 13.9, 20.7, 25.7, 27.6, 28.4, 28.8, 30.7, 38.7, 40.6, 66.7 (d, J_{C-P} = 5.4 Hz), 68.0 (d, J_{C-P} = 5.6 Hz), 80.2, 81.9, 83.4, 85.8, 86.6 (d, J_{C-P} = 8.5 Hz), 91.8, 115.3, 120.3, 141.4, 150.6, 154.1, 157.4, 157.5, 173.2; ^{31}P NMR (162 MHz, CD_3OD) δ 3.39; LRMS (ESI) 645 $(M+H)^+$.

3.4. Aspartol adenylate 5

A solution of compound **4** (92 mg, 0.129 mmol) in trifluoroacetic acid/water (4 mL/1 mL) was stirred at room temperature for 20 min. The solvents were evaporated under reduced pressure. The colorless oil was co-evaporated with water and then MeOH/Et_2O to give **5** (79 mg, 98%) as a white solid: mp 93 °C (dec); $[\alpha]_D^{20}$ −21.4 (*c* 0.51, DMSO); IR (KBr) 3386, 3099, 1684, 1204 cm^{-1}; ^{1}H NMR (400 MHz, D_2O) δ 0.73 (t, *J* = 7.6 Hz, 3H), 1.17–1.22 (m, 2H), 1.41 (quint., *J* = 7.6 Hz, 2H), 2.52 (d, *J* = 6.8 Hz, 2H), 2.79 (t, *J* = 7.6 Hz, 2H), 3.61–3.67 (m, 1H), 3.75–3.81 (m, 1H), 3.85–3.90 (m, 1H), 3.93–4.04 (m, 2H), 4.19 (br s, 1H), 4.34 (t, *J* = 4.8 Hz, 1H), 4.56–4.59 (m, 1H), 6.00 (d, *J* = 4.8 Hz, 1H), 8.23 (s, 1H), 8.39 (s, 1H); ^{13}C NMR (100 MHz, D_2O) δ 12.8, 19.1, 28.8, 33.5, 39.3, 48.4 (d, J_{C-P} = 8.4 Hz), 64.7 (d, J_{C-P} = 4.6 Hz), 64.9 (d, J_{C-P} = 5.3Hz), 70.1, 74.4, 83.7 (d, J_{C-P} = 8.4 Hz), 88.1, 116.4 (q, J_{C-F} = 290.0 Hz), 118.7, 142.0, 146.0, 148.5, 150.9, 163.1 (q, J_{C-F} = 35.0 Hz), 174.1; ^{31}P NMR (162 MHz, D_2O) δ 2.69; LRMS (ESI) 449.1 $(M+H)^+$.

3.5. 2′,3′-Isopropylidene-5′-*O*-[*N*(β-*tert*-butyl-*N*-Boc-L-aspartyl)sulfamoyl]adenosine 8

N-Boc-L-aspartic acid β-*tert* -butyl ester **7** (377 mg, 1.30 mmol) was added to a solution of sulfonamide adenosine **6** (640 mg, 1.30 mmol), DCC (269 mg, 1.30 mmol), and DMAP (159 mg, 1.30 mmol) in dry dichloromethane (20 mL), and the mixture was stirred at room temperature for 2 h. The reaction mixture was diluted with ethyl acetate (150 mL), washed with sat. aqueous $NaHCO_3$, water, brine, dried ($MgSO_4$), and evaporated. The crude product was dissolved in MeOH/*n*-butylamine (10 mL/10 mL) and stirred at room temperature for 3 h. The solvents were evaporated and the crude product was purified by flash chromatography (EtOAc to 10% MeOH/EtOAc) to give **8** (610 mg, 71%) as a white solid: mp 155 °C (dec); $[\alpha]_D^{20}$ −37.9 (*c* 1.01, MeOH); IR (KBr) 3393, 2981, 1712, 1152 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 1.34–1.38 (m, 21H), 1.58 (s, 3H), 2.57 (dd, *J* = 15.6 and 7.6 Hz, 1H), 2.72 (dd, *J* = 15.6 and 4.8 Hz, 1H), 4.20 (d, *J* = 4.0 Hz, 2H), 4.25–4.29 (m, 1H), 4.49–4.52 (m, 1H), 5.07 (dd, *J* = 6.0 and 2.0 Hz, 1H), 5.32 (dd, *J* = 6.0 and 3.2 Hz, 1H), 6.20 (d, *J* = 3.2 Hz, 1H), 8.18 (s, 1H), 8.42 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 24.4, 26.6, 27.2, 27.6, 38.9, 53.8, 68.6, 79.2, 80.9, 82.0, 84.4, 84.5, 90.6, 114.1, 118.9, 140.3, 149.2, 152.8, 156.1, 156.4, 171.1, 178.5; HRMS (FAB) calcd for $C_{26}H_{39}N_7O_{11}S$ $(M+H)^+$ 657.2428, found 657.4784.

3.6. Aspartylsulfamoyladenosine 9

A solution of compound **8** (50 mg, 0.076 mmol) in trifluoroacetic acid/water (4 mL/1 mL) was stirred at room temperature for 15 min. The solvents were evaporated then co-evaporated with MeOH/Et_2O to give **9** (40 mg, 95%) as a white solid: mp 105 °C (dec); $[\alpha]_D^{20}$ −15.5 (*c* 0.525, DMF); IR (KBr) 3434, 1684, 1196, 1135 cm^{-1}; ^{1}H NMR (400 MHz, D_2O) δ 3.06 (d, *J* = 5.5 Hz, 2H), 4.18 (t, *J* = 5.5 Hz, 1H), 4.42 (br s, 3H), 4.49–4.51 (m, 1H), 4.74 (t, *J* = 4.9 Hz, 1H), 6.16 (d, *J* = 4.9 Hz, 1H), 8.43 (s, 1H), 8.51 (s, 1H); ^{13}C NMR (100 MHz, D_2O) δ 34.5, 51.5, 68.9, 70.1, 74.4, 82.5, 88.6, 116.3 (q, J_{C-F} = 290.0 Hz, TFA), 118.7, 142.6, 144.7, 148.4, 149.9, 163.8 (q, J_{C-F} = 35.5 Hz, TFA), 172.8, 173.1; LRMS (ESI) 462.1 $(M+H)^+$.

3.7. Dimethyl β-ketophosphonate 12

To a solution of dimethyl methylphosphonate (0.71 mL, 6.59 mmol) in anhydrous THF (20 mL) cooled to −78 °C was added *n*-butyllithium (1.6 M in hexane, 4.12 mL, 6.59 mmol) and the mixture was stirred at −78 °C for 30 min. A solution of **11** (1.00 g, 3.296 mmol) in anhydrous THF (3 mL) was added to the lithiated phosphonate **10** and the mixture was stirred at −78 °C for 2.5 h. The reaction was quenched with saturated aqueous NH_4Cl (20 mL) and THF was evaporated under reduced pressure. The product was extracted with ethyl acetate and the organic phase was washed with saturated aqueous $NaHCO_3$ and brine, dried ($MgSO_4$), and the solvent was evaporated. The crude product was purified by flash chromatography (70% hexane/30% EtOAc to pure EtOAc) to give **12** (0.873 g, 67%, 84% after starting material recovery) as a white solid: mp 78–80 °C; $[\alpha]_D^{20}$ −43.6 (*c* 1.085, $CHCl_3$); IR (KBr) 2981, 1712 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.39 (s, 9H), 1.42 (s, 9H), 2.69 (dd, *J* = 16.8 and 4.8 Hz, 1H), 2.75 (dd, *J* = 16.8 and 5.6 Hz, 1H), 3.15–3.30 (m, 2H), 3.74 (d, *J* = 1.8 Hz, 3H), 3.77 (d, *J* = 1.8 Hz, 3H), 4.47–4.52 (m, 1H), 5.73 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 27.9, 28.2, 36.5, 37.1 (d, J_{C-P} = 130.5 Hz), 53.0 (d, J_{C-P} = 6.5 Hz), 56.9, 80.2, 81.4, 155.3, 170.3, 200.0 (d, J_{C-P} = 6.4 Hz); ^{31}P NMR (162 MHz,

$CDCl_3$) δ 23.1; HRMS (CI) calcd for $C_{16}H_{31}NO_8P$ $(M+H)^+$ 396.1787, found 396.1776.

3.8. Methyl β-ketophosphonate 13

A solution of **12** (600 mg, 1.52 mmol) in *tert*-butylamine (20 mL) was heated to reflux for 48 h. The solvent was evaporated and the crude product was dissolved in CH_2Cl_2 (20 mL), and then treated with 4 M HCl in dioxane (0.379 mL, 1.52 mmol) for 5 min. The organic layer was diluted with ethyl acetate (150 mL), washed with brine, dried ($MgSO_4$), and evaporated. The crude product was purified by flash chromatography (EtOAc to 35% MeOH/EtOAc) to give **13** (532 mg, 92%) as a white solid: mp 83–85 °C; $[\alpha]_D^{20}$ −0.43 (*c* 1.025, MeOH); IR (KBr) 2934, 1705 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 1.42 (br s, 18H), 2.56 (dd, J = 16.0 and 7.8 Hz, 1H), 2.79 (dd, J = 16.0 and 4.8 Hz, 1H), 2.98–3.15 (m, 2H), 3.57 (d, J = 10.8 Hz, 3H), 4.63–4.66 (m, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 27.2, 27.6, 36.3, 38.0–39.5 (C–D coupling, H–D exchange with MeOD), 51.5 (d, J_{C-P} = 5.4 Hz), 56.9, 79.9, 81.1, 156.4, 170.9, 204.3; ^{31}P NMR (162 MHz, CD_3OD) δ 18.4; LRMS (ESI) 404.1 $(M+Na)^+$.

3.9. Phosphonate 14

A solution of phosphonate **13** (140 mg, 0.367 mmol), *p*-toluenesulfonyl chloride (49 mg, 0.257 mmol), and 2′,3′-*O*-isopropylideneadenosine **1** (74 mg, 0.241 mmol) in dry pyridine was stirred at room temperature for 2 weeks. The reaction was quenched with saturated aqueous $NaHCO_3$ (0.2 mL), diluted with ethyl acetate (100 mL), and washed with brine. The aqueous phase was acidified to pH 5 with acetic acid and extracted with ethyl acetate. The organic phases were combines, dried ($MgSO_4$), and evaporated. The crude product was purified by flash chromatography (5% MeOH/EtOAc to 35% MeOH/EtOAc) to give **14** (47 mg, 30%) as a white solid: mp 117–119 °C; $[\alpha]_D^{20}$ −26.5 (*c* 1.5, MeOH); IR (KBr) 3408, 2983, 2933, 1718 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 1.36–1.43 (m, 21H), 1.58 (s, 3H), 2.48–2.55 (m, 1H), 2.75–2.81 (m, 1H), 2.90–3.10 (m, 2H), 4.04 (br s, 2H), 4.45 (br s, 1H), 4.52–4.54 (m, 1H), 5.10–5.12 (m, 1H), 5.28–5.32 (m, 1H), 6.18 (d, J = 2.0 Hz, 1H), 8.18 (s, 1H), 8.44 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 25.6, 27.5, 28.4, 28.8, 37.6, 38.0–40.0 (C–D coupling, H–D exchange with MeOD), 58.4, 65.9, 80.9, 82.2, 83.3, 85.8, 86.7 (d, J_{C-P} = 7.2 Hz), 91.9, 115.4, 120.3, 141.6, 150.6, 154.1, 157.4, 157.5, 172.2, 205.0 (d, J_{C-P} = 3.5 Hz); ^{31}P NMR (162 MHz, CD_3OD) δ 17.0; HRMS (FAB) calcd for $C_{27}H_{42}N_6O_{11}P$ $(M+H)^+$ 657.2649, found 657.2666.

3.10. β-Ketophosphonate 15

A solution of compound **14** (30 mg, 45.7 μmol) in trifluoroacetic acid/water (4 mL/1 mL) was stirred at room temperature for 20 min. The solvents were evaporated under reduced pressure. The colorless oil was co-evaporated with MeOH/Et_2O to give **15** (25.2 mg, 96%) as a white hygroscopic solid: $[\alpha]_D^{20}$ −10.3 (*c* 1.2, H_2O); IR (KBr) 3434, 2927, 1684, 1206 cm^{-1}; ^1H NMR (400 MHz, D_2O) δ 3.05–3.40 (m, 4H), 4.13–4.24 (m, 2H), 4.34–4.40 (m, 1H), 4.47–4.50 (m, 1H), 4.52–4.60 (m, 1H), 4.73–4.79 (m, 1H), 6.17 (d, J = 4.48 Hz, 1H), 8.43 (s, 1H), 8.60 (s, 1H); ^{13}C NMR (100 MHz, D_2O) δ 35.9, 40.1–44.0 (C–D coupling, H–D exchange with D_2O), 58.7, 66.9, 72.9, 77.3, 86.7 (d, J_{C-P} = 7.7 Hz), 91.1, 119.2 (q, J_{C-F} = 290.0 Hz, TFA), 121.6, 145.4, 147.5, 151.2, 152.3, 166.2 (q, J_{C-F} = 35.0 Hz), 175.5, 202.7; ^{31}P NMR (162 MHz, D_2O) δ 15.9; LRMS (ESI) 461.1 $(M+H)^+$.

3.11. Purification of aspartyl-tRNA synthetase (AspRS) from *E. coli*

E. coli AspRS carrying a C-terminal poly-histidine tag was overproduced in *E. coli* BLR (DE3) (Novagen, Madison, WI) and then purified to homogeneity by affinity chromatography on a nickel-containing resin (NiNTA), as previously described.[36]

3.12. Enzyme assays, and kinetic constant measurements

E. coli AspRS activity was determined in the aminoacylation reaction in the presence of 100 mM Na Hepes pH 7.5, 10 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol, 2 mM ATP, 90 μM [^{14}C]aspartate, and 50 μM unfractionated tRNA from *E. coli*. To determine the initial rate of this reaction, the amount of [^{14}C]aspartyl-tRNA formed in 40 μL aliquots after various incubation times at 37 °C was measured as previously described.[37]

For the determination of the K_i value of aspartyl-sulfamoyl-adenosine with respect to aspartate for *E. coli* AspRS, we first measured the K_m^{app} values for aspartate from Lineweaver–Burk plots of initial rates of aminoacylation at various concentrations of aspartate, under fixed and saturating concentrations of ATP and tRNA, in the presence of various fixed concentrations of inhibitor.

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