Synthesis of a phosphonate-linked aminoglycoside-coenzyme A bisubstrate and use in mechanistic studies of an enzyme involved in aminoglycoside resistance

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Abstract

Aminoglycoside *N*-6'-acetyltransferases (AAC(6')s) are important determinants of antibiotic resistance. A good mechanistic understanding of these enzymes is essential to overcome aminoglycoside resistance. We have previously reported the synthesis of amide-linked and sulfonamide-linked aminoglycoside-coenzyme A conjugates which were useful mechanistic and structural probes of AAC(6')s. We report here the synthesis of a phosphonate-linked aminoglycoside-coenzyme A variant, which is expected to be a superior mimic of the tetrahedral intermediate proposed for catalysis by AAC(6')s. This synthetic target is especially challenging for a number of reasons including the presence of multiple functional groups, the water solubility of both starting materials, and incompatibility of P(III) chemistry with water. We have overcome these challenges by adding the expensive coenzyme A in the last step via an elegant Michael-type addition onto a vinylphosphonate in water. Overall, a single protection step was needed. The decreased inhibitory potency of this bisubstrate compared to that of the amide-linked analog suggests that *Enterococcus faecium* AAC(6')-Ii may not stabilize the proposed tetrahedral intermediate, and may act mainly via proximity catalysis.

Keywords

Coenzyme A; enzyme inhibitors; Michael addition; neamine; pharmaceutical chemistry

Introduction

Aminoglycosides are useful antibiotics that target the 16S ribosomal RNA A site of bacteria. Aminoglycoside resistance is however spreading rapidly. It is mainly caused by the expression of drug-modifying enzymes such as nucleotidyltransferases (ANTs), phosphoryltransferases (APHs) and acetyltransferases (AACs). Among them, aminoglycoside *N*-6'-acetyltransferases (AAC(6')s) are some of the most clinically relevant. AAC(6')s selectively transfer an acetyl group from acetyl coenzyme A (AcCoA) to the 6'-

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Supporting Information Available: General experimental methods as well as 1D and 2D NMR spectra of the final product and selected intermediates are provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

NH₂ of most aminoglycosides. It is proposed that a tetrahedral intermediate is formed during the acetyl transfer by these enzymes (Figure 1). The acetylated aminoglycosides display a greatly reduced antibacterial activity. We have reported the use of amide-linked^[1] and sulfonamide-linked^[2] aminoglycoside-CoA bisubstrates as structural and mechanistic probes for these enzymes (e.g. see **1** and **2**, Figure 1). Most of these conjugates were nanomolar inhibitors of AAC(6')-Ii and successfully crystallized with the enzyme.^[1] They were also useful mechanistic probes of *Salmonella enterica* AAC(6')-Iy.^[3] To our surprise, the bisubstrate with a sulfonamide linker (**2**) was 40 fold less potent than the amide-linked analog (**1b**). These results support the earlier suggestion by Wright and co-workers that AAC(6')-Ii may catalyze the acetyltransfer reaction mainly via proximity effects.^[4,5] Alternatively, sulfonamides may not be good mimics of the enzymatic intermediate.

Indeed, very few examples of the use of sulfonamides as tetrahedral intermediate mimetics have been reported.^[6–9] Phosphoryl groups, on the other hand, have been extensively used for this purpose.^[10–21] Aminoglycoside-CoA bisubstrates that mimic the tetrahedral intermediate better than amide-linked bisubstrates represent useful probes to clarify the mechanism of AAC(6')s. We therefore embarked on the syntheses of bisubstrates **3a-c**, which pose a number of challenges: 1) both starting materials, aminoglycosides and CoA, display multiple reactive functional groups; 2) the starting materials are only soluble in aqueous solutions, while the more efficient P(III) chemistry is not compatible with such conditions; 3) the functional groups of CoA cannot withstand the typical conditions employed for protection/deprotection of aminoglycosides; and finally 4) CoA is expensive and only available in small amounts. We report here a 5 steps synthesis of the phosphonate-linked neamine-CoA conjugate **3c**.

Results and Discussion

Targets **3a-b** were designed based on our previously reported structure-activity relationships for amide-linked bisubstrates.^[1,22] Compared to the proposed enzymatic tetrahedral intermediate (Figure 1), **3a-c** have extra carbons between N-6' of the aminoglycoside and S of CoA. As suggested by our previous results with the bisubstrate inhibitors **1a-d**, these extra atoms are not expected to compromise inhibition, and n = 2 may even strengthen it as was the case for **1b**.^[1]

The key intermediate in the synthesis of bisubstrate **3a** was methyl iodomethylphosphonyl chloride (**4**) (Scheme 1A), which may be derived from dimethyl iodomethylphosphonate **8c**. α -Hydroxymethylphosphonate **7** was used in numerous attempts to prepare the corresponding α -halomethylphosphonates **8a-c** without success (Scheme 1B). Conventional halogenation procedures led to complex mixtures, even with very mild chlorination reagents such as trichlorocyanuric acid.^[23] Alternatively, attempts to convert chloromethyl dimethylphosphonate (**9**) into iodomethyl dimethylphosphonate via the Finkelstein reaction yielded the demethylation product **10** (Scheme 1C).

As an alternative route, the Michaelis-Arbuzov reaction of trimethylphosphite and diiodomethane was attempted but proceeded with an extremely low yield due to the decomposition of diiodomethane and the rearrangement of trimethylphosphite to dimethyl

methylphosphonate.^[24–26] To optimize this reaction, various conditions and catalysts were tested. The addition of copper powder to slow down the decomposition of diiodomethane, reaction at a lower temperature (130°C instead of the literature reported 160°C)^[24] and for a shorter reaction time (3 h instead of 12 h) afforded iodomethyl dimethylphosphonate (**8c**) in ca. 30% yield (Scheme 2). A typical two-step chlorination procedure^[15,27] was adopted to prepare phosphonyl chloride **4**. A number of nucleophilic reagents such as NaOH/MeOH,^[15,16] LiN₃/DMF^[28], TMSBr/Py,^[29] *tert*-butylamine,^[27] thiophenol/TEA,^[30] and NaI/acetone^[31,32] were reported to cleave dimethyl phosphonates to the mono-phosphonic acids. We found that *tert*-butylamine worked the best to convert **8c** to **11**. The phosphonic acid **11** was next converted to phosphonyl chloride **4** in good yield using oxalyl chloride. Treatment of 1,3,2'-tri-*N*-(*tert*-butoxycarbonyl)neamine **5**^[2] with phosphonyl chloride **4** in the presence of base afforded the *N*-6'-phosphoramidate intermediate **12**, which yielded **13** after removal of the Boc protecting groups. The iodomethylphosphoramidate **13** was reacted with CoA (shown as CoA-SH in Scheme 2) under various conditions, none of which yielded the desired product; instead the demethylated product **14** was observed. It was reasoned that steric hindrance may prevent approach by the thiol. To avoid this problem, we embarked on the synthesis of **3b**. It was envisaged that Michael addition of CoA onto vinylphosphonate **18** (Scheme 3A) would likely be a reasonable route.

To investigate the feasibility of this route, model reactions were carried out using commercial dimethyl vinylphosphonate and *N*-acetylcysteamine (as a surrogate for CoA) (Scheme 3B). Surprisingly, the addition was complete within one hour at RT in both organic and aqueous solutions.

Thus, the synthesis of bisubstrate **3b** was attempted as shown in Scheme 4. Mono demethylation of commercially available dimethyl vinylphosphonate yielded phosphonic acid **19**,^[33] which was converted to phosphonyl chloride **16**. Treatment of tri-*N*-(*tert*-butoxycarbonyl)neamine (**5**) with phosphonyl chloride **16** in the presence of base afforded the *N*-6'-phosphoramidate intermediate **20**, which yielded phosphoramidate **17** after deprotection. The Michael addition of CoA onto vinylphosphoramidate **17** was not successful under the conditions tested (Scheme 4), and mostly demethylated product was observed. Alternatively, radical reactions were also tested but did not produce the desired product either.

This was unexpected considering the high efficiency of the model reaction (Scheme 3C), and made us realize that the model reaction may not have been representative because the P-N bond was replaced with a P-O bond. This prompted us to attempt the synthesis of **3c** (Figure 1), in which neamine is replaced with paromamine to better mimic the model reaction. We reasoned that the more electronegative O (vs. N) would make vinyl phosphonate **23** (Scheme 5) a better Michael acceptor than **17** (Scheme 4). Moreover, this modification was not expected to decrease inhibition because both *N*-6'-OH and *N*-6'-NH₂ containing aminoglycosides bind AAC(6') tightly, with the former being inhibitors and the latter being substrates. Thus, after methanolysis of paromomycin to paromamine,^[34] reaction with Boc anhydride provided 1,3,2'-tri-*N*-(*tert*-butoxycarbonyl) paromamine **21**, which was reacted with **16** to yield **22**. Removal of the Boc protecting groups generated **23**, which was reacted with CoA under basic aqueous solution to produce the desired bisubstrate **3c** as two

diastereoisomers (~1:1) in 31% total yield (non-optimized) after HPLC purification. The regioselectivity of the phosphorylation is demonstrated by a ^{31}P - ^1H HMBC correlation spectrum, in which the 6'-CH₂ is correlated to the linker phosphorus. The high cost of CoA (and the low activity, see below) prevented us from making enough material to allow separate studies of the diastereoisomers.

The affinity of bisubstrates **3c** for AAC(6')-II was measured both using the standard enzyme assay^[22] ($K_i = 2.0 \pm 0.9 \mu\text{M}$) and isothermal calorimetry ($K_d = 0.81 \pm 0.04 \mu\text{M}$). To our surprise, the expected better mimetic of the tetrahedral intermediate, **3c**, was a much weaker inhibitor than the corresponding amide-linked bisubstrate (**1b**, $K_i = 0.04 \pm 0.02 \mu\text{M}$). Even if one diastereoisomer was more potent than the other, based on the K_i of the diastereomeric mixture, it is unlikely to compete with **1b**. The extra atoms between the aminoglycoside and CoA compared to the tetrahedral intermediate cannot explain the reduced activity of inhibitor **3c** because the latter has a linker (phosphonate) of the same length as that of **1b** (amide-linked), yet **1b** is a low nanomolar inhibitor, the most potent AAC(6')-II inhibitor reported to date.^[1] Moreover, the crystal structure of AAC(6')-II in complex with **1b** clearly shows that the extra methylene groups reach out into a groove without affecting the relative positions of the aminoglycoside and CoA.^[1] Based on enzyme kinetic studies, Wright and coworkers^[5] have suggested that this enzyme may not stabilize the tetrahedral intermediate and only provide catalysis via a proximity effect. Our results are in good agreement with this suggestion. Indeed, the reduction in affinity observed when the amide linker (as in **1b**) is replaced with a phosphonate such as **3c**, may result from repulsion of the phosphonate negative charge within the enzyme catalytic site. Examination of the various crystal structures reported for this enzyme are also in agreement with this proposition as no residues able to stabilize the intermediate have been identified.^[1,35,36] In summary, we have reported the first synthesis of a phosphonate-linked aminoglycoside-CoA derivative (**3c**) via an elegant Michael-type addition of the CoA thiol onto a vinylphosphonate in water. Our route includes only one protection step and the expensive CoA is added in the last step.

Experimental Section

Preparation of bisubstrate **3c**

Crude product **23** (55 mg, ca. 0.06 mmol) was dissolved in a mixture of water (5 mL) and acetone (1 mL), CoA (sodium salt, 15 mg, 0.02 mmol) was directly added to this solution and followed by the addition of DIPEA (0.25 mL). The mixture was sonicated for 10 min and stirred for 16 h at 4°C. The solution was then poured into water (30 mL) and washed with EtOAc (10 mL $\times$ 2). The aqueous layer was separated and freeze-dried to produce a yellowish powder, which was further purified by reverse phase HPLC to yield the bisubstrate **3c** as a pair of diastereoisomers (6 mg, 31% yield). HPLC separation was achieved on an Agilent Zorbax SB-C8 column (4.6 $\times$ 250 mm, 5 μ). Samples were eluted at a flow rate of 3 mL/min, using the linear gradients shown in Table 1 below. The two diastereoisomers have retention time at 13.30 and 13.75 min, respectively. The purity of the bisubstrate was evaluated by HPLC with the same equipment and column but using isocratic eluent with a ratio of phase A/phase B of either 85/15 (method A) or 64/40 (method B). The purity of **3c** used in the enzymatic inhibition assays was > 95%. ^1H NMR (D₂O, 500 MHz) δ 8.55 (s,

1H), 8.31 (s, 1H), 6.08 (d, $J = 4.0$, 1H), 5.64 (br s, 1H), 4.49 (bs, 1H), 4.24 (bs, 2H), 4.16 (bs, 2H), 3.90 – 3.20 (m, 21H), 2.66 (m, 2H), 2.54 (t, $J = 6.5$ Hz, 2H), 2.34 (m, 3H), 2.17 (m, 2H), 1.79 (q, $J = 12.5$, 1H), 0.78 (bs, 3H), 0.70 (bs, 3H); **^{13}C NMR** (D_2O , 400 MHz by HMQC and HMBC): δ 174.7, 174.4 (C=O of CoA), 150.2, 148.7, 144.9, 142.7, 118.5 (aromatic carbons of adenine), 96.7, 87.5, 83.3, 79.3, 74.9, 74.3, 73.8, 72.2, 71.8, 71.6, 68.6, 68.5, 65.1, 64.7, 53.8, 53.7, 53.2, 51.3, 39.9, 38.2, 35.4, 35.3, 30.2, 27.6, 24.8, 23.4, 23.3, 20.8, 18.4; **^{31}P NMR** (D_2O , 81 MHz) δ 35.7, 35.6 (diastereoisomers); **ESI-MS** for $\text{C}_{36}\text{H}_{66}\text{N}_{10}\text{O}_{25}\text{P}_4\text{S}$ $[\text{M}+\text{H}]^+$ calcd. 1195.29, found 1195.09; **HR-MS** for $\text{C}_{36}\text{H}_{66}\text{N}_{10}\text{O}_{25}\text{P}_4\text{S}$ $[\text{M}+\text{H}]^+$ calcd. 1195.28956, found 1195.29446.

Paromamine [34]

Paromomycin sulfate (5 g, 7.0 mmol) was dissolved in MeOH (100 mL), and conc. HCl (12 M, 4 mL) was added. The mixture was refluxed for 6 h, and concentrated to a volume of 20 mL. Ether (50 mL) was added and the product was allowed to crystallize at ca. 8°C for 2 h. Filtration was used to collect the paromamine hydrogen chloride salt as white crystals (3.65 g, yield: 84%). **^1H NMR** (D_2O , 400 MHz) δ 5.52 (d, $J = 4.0$, 1H), 3.82-3.69 (m, 4H), 3.62 (dd, $J = 6.8$, 5.2, 1H), 3.55 (t, $J = 8.8$, 1H), 3.46 (m, 2H), 3.40-3.32 (m, 2H), 3.21 (m, 1H), 2.38 (td, $J = 12.0$, 4.0, 1H); 1.75 (q, $J = 12.0$, 1H); **^{13}C NMR** (D_2O , 75 MHz) δ 97.6 ($\text{C}1'$), 80.6 ($\text{C}5'$), 74.7 ($\text{C}4'$), 74.3 ($\text{C}3'$), 73.6 ($\text{C}5$), 69.6 ($\text{C}6$), 68.8 ($\text{C}4$), 60.3 ($\text{C}6'$), 54.1 ($\text{C}2'$), 49.4 ($\text{C}1$), 48.9 ($\text{C}3$), 28.2 ($\text{C}2$); **ESI-MS** for $\text{C}_{12}\text{H}_{25}\text{N}_3\text{O}_7$ $[\text{M}+\text{H}]^+$ calcd. 323.2, found 323.2.

Methyl chloro iodomethylphosphonate (4)

Compound **11** (0.33 g, 1.4 mmol) was dissolved in DMF (2.5 μL , 0.03 mmol) and dry DCM (10 mL). Oxalyl chloride (0.15 mL, 2 mmol) was added dropwise. The solution was stirred at 0°C for 20 min, warmed to RT, and stirred for one hour. The reaction mixture was concentrated to give phosphonochloridate **4** as a yellowish oil, which was used immediately in a reaction with 1,3,2'-tri-*N*-(*tert*-butoxycarbonyl)neamine. **^1H NMR** (CDCl_3 , 300 MHz): δ 3.96 (d, $J = 10.8$, 3H), 3.46 (d, $J = 8.8$, 2H); **^{13}C NMR** (CDCl_3 , 75 MHz) δ 54.7 (d, $J = 5.8$), -9.3 (d, $J = 155$); **^{31}P NMR** (CDCl_3 , 81 MHz): δ 34.0; **ESI-MS** for $\text{C}_2\text{H}_5\text{ClIO}_2\text{P}$ $[\text{M}+\text{H}]^+$ calcd. 254.9, found 254.9.

Dimethyl hydroxymethylphosphonate (7)

This compound was prepared using a previously reported procedure.^[15] Yield: 76%. **^1H NMR** (CDCl_3 , 200 MHz): δ 4.89 (bs, 1H), 3.96 (d, $J = 5.8$, 2H), 3.82 (d, $J = 10.6$, 6H); **^{13}C NMR** (CDCl_3 , 75 MHz): δ 54.3 (d, $J = 5.6$), 62.0 (d, $J = 155$); **^{31}P NMR** (CDCl_3 , 81 MHz): δ 27.9; **ESI-MS** for $\text{C}_3\text{H}_9\text{O}_4\text{P}$ $[\text{M}+\text{H}]^+$ calcd. 141.1, found 141.1.

Dimethyl iodomethylphosphonate (8c)

Diiodomethane (16 g, 60 mmol) and copper beads (1 g, diameter < 0.5 mm) were mixed and heated to 130°C. Trimethyl phosphite (7.7 g, 60 mmol) was added through an automatic syringe pump at speed of 5 mL/h. The mixture was refluxed for 3 h. After distillation of the by-product dimethyl methylphosphonate (house vacuum at about 70°C), the remaining yellowish liquid afforded the desired compound in low yield (32%, purity > 95% by NMR).

¹H NMR (CDCl₃, 300 MHz) δ 3.70 (d, J = 11.0, 6H), 2.95 (d, J = 10.2, 2H); **¹³C NMR** (CDCl₃, 75 MHz) δ 54.1 (d, J = 5.8), -15.8 (d, J = 155); **³¹P NMR** (CDCl₃, 81 MHz): δ 23.9; **HRMS** for C₃H₈IO₃P [M+H]⁺ calcd. 250.9334, found 250.9329.

Dimethyl chloromethylphosphonate (9)

This compound was prepared as previously reported.^[16] Yield: 62%. **¹H NMR** (CDCl₃, 200 MHz) δ 3.71 (d, J = 10.8, 6H), 3.46 (d, J = 10.6, 2H); **¹³C NMR** (CDCl₃, 75 MHz) δ 53.9 (d, J = 5.6), 32.7 (d, J = 156); **³¹P NMR** (CDCl₃, 81 MHz): δ 22.2; **ESI-MS** for C₃H₈³⁵ClO₄P calcd. 158.0, found 158.0.

Methyl iodomethylphosphonate (11)

Compound **8c** (2.50 g, 1 mmol) was dissolved in *tert*-butylamine (20 mL) and the resulting solution was refluxed for 16 h. The reaction mixture was concentrated (10 mL) and filtered to collect the crystalline methyl iodomethylphosphonate *tert*-butylamine salt (3.13 g, quantitative yield). This salt (2.0 g, 6.5 mmol) was dissolved in chloroform (60 mL) and Dowex 8–100 resin (10 g, 200–400 mesh, wet) was added. After 30 min of shaking, the resin was removed by filtration, and the filtrate was dried over Na₂SO₄. After evaporation of the solvent, the desired compound was obtained as a yellowish oil (1.1 g, 72%). **¹H NMR** (CDCl₃, 200 MHz) δ 11.60 (bs, 1H), 3.81 (d, J = 10.8, 3H), 3.00 (d, J = 8.8, 2H); **³¹P NMR** (CDCl₃, 81 MHz) δ 24.1; **ESI-MS** for C₂H₆IO₃P [M+H]⁺ calcd. 236.9, found 236.9.

1,3,2'-Tri-*N*-(*tert*-butoxycarbonyl)-6'-*N*-(methyl iodomethyl phosphonyl) neamine (12)

Compound **5** (125 mg, 0.2 mmol) and diisopropylethylamine (50 μ L, 0.25 mmol) were dissolved in anhydrous DMF (20 mL). The mixture was cooled to 0°C, and methyl iodomethylphosphonate chloride (**4**, 50 mg, 0.2 mmol) was added dropwise. The reaction mixture was stirred for one hour at 0°C and another two hours at RT. Reaction completion was monitored by TLC, R_f = 0.40 (tailing, CHCl₃/MeOH/NH₄OH, 4/1/0.1) and **ESI-MS** for C₂₉H₅₄IN₄O₁₄P [M+Na]⁺ calcd. 863.2, found 863.2. The reaction mixture was evaporated to dryness to give a white powder. This solid was triturated with water (20 mL) to remove the ammonium salt. The remaining solid material was dried to afford the crude product as a white powder (138 mg, 82%). **³¹P NMR** (DMSO-*d*₆, 81 MHz) 28.3 and 28.9 (diastereoisomers). Attempted purification on silica gel led to decomposition. Compound **12** was therefore used as a crude sample in the next step.

6'-*N*-(Methyl iodomethyl phosphonyl) neamine (13)

Crude **12** (80 mg, ca. 0.06 mmol) was mixed with DCM (10 mL) containing 1 drop of anisole. To the mixture, TFA (2 mL) was added and the mixture became homogeneous immediately. The reaction was complete after 1 h at RT as monitored by TLC and ESI-MS. Evaporation of the volatiles afforded a white salt, which was dissolved in water (20 mL) and washed with chloroform (3 $\times$ 5 mL). The aqueous phase was lyophilized to afford the product as a white powder (48 mg, ca. 60%). **³¹P NMR** (D₂O, 81 MHz) δ 26.4, 27.2. **ESI-MS** for C₁₄H₃₀IN₄O₈P [M+H]⁺ calcd. 541.1, found 541.0. This compound is also prone to decomposition on silica gel; it was therefore directly used in the next step.

Methyl chloro vinylphosphonate (16)

Compound **19** (250 mg, 2 mmol) and dry DMF (25 μ L) were mixed in dry DCM (15 mL). The mixture was cooled to 0°C, to which oxalyl chloride (0.26 mL, 3 mmol) was added dropwise. The reaction mixture was stirred for 30 min and then for another hour at RT. All the volatiles were evaporated under vacuum (45°C, 15 mmHg) to yield the desired product as yellowish oil (0.32 g). This crude product was directly used in the next reaction. ¹H NMR (CDCl₃, 200 MHz) δ 6.80-6.22 (m, 3H), 3.95 (d, J = 10.8, 3H); ³¹P NMR (CDCl₃, 81 MHz) δ 29.9.

6'-N-(Methyl vinylphosphonyl) neamine (17)

Crude **20** (75 mg) was mixed with DCM (10 mL) containing 1 drop of anisole. To the mixture, TFA (2 mL) was added and the mixture became homogeneous immediately. The reaction was completed after 1 h at RT as monitored by TLC and ESI-MS. Evaporation of the volatiles afforded a white salt, which was dissolved in water (20 mL) and washed with chloroform (3 $\times$ 5 mL). The aqueous phase was lyophilized to afford the crude product as a white powder (36 mg, ca. 34%). ³¹P NMR (D₂O, 81 MHz) δ 24.2, 24.8. ESI-MS for C₁₅H₃₁N₄O₈P [M+H]⁺ calcd. 427.2, found 427.2. Attempts to purify this compound on silica gel led to decomposition, and therefore the crude product was used in the reaction with CoA.

Dimethyl 2-((2-acetamidoethyl)sulfanyl)ethylphosphonate (18)

Dimethylvinylphosphonate (68 mg, 0.5 mmol) and *N*-acetylcysteamine (60 mg, 0.5 mmol) were dissolved in a mixture of acetone-d₆/D₂O (1 mL, 1/1). The reaction was monitored by NMR. There was no reaction before addition of TEA. The reaction was, however, complete within 2 h after addition of TEA (100 μ L, 0.7 mmol). The mixture was purified on silica gel (R_f: 0.43, CHCl₃/MeOH, 10/1) to yield the desired product (89 mg, 70%). ¹H NMR (CDCl₃, 200 MHz) δ 6.28 (bs, 1H), 3.60 (d, J = 10.6, 6H), 3.31 (q, J = 6.4, 2H), 2.90 (t, J = 6.8, 2H), 2.85 (d, J = 6.4, 2H), 1.95 (s, 3H), 1.80 (m, 2H); ¹³C NMR (CDCl₃, 75 MHz) δ 176.0, 54.2 (d, J = 5.2), 40.5, 33.2, 30.6, 29.2 (d, J = 10.2), 22.5; ³¹P NMR (CDCl₃, 81 MHz) δ 34.1; ESI-MS for C₈H₁₈NO₄PS [M+H]⁺ calcd. 256.1, found 256.1.

Methyl vinylphosphonate (19) [33]

Dimethyl vinylphosphonate (4.0 g, 29.4 mmol) was dissolved in *tert*-butylamine (20 mL) and refluxed for 16 h at 50°C. The reaction mixture was concentrated (ca. 10 mL) and filtered to collect the methyl vinylphosphonate *tert*-butylaminium salt as a white crystal (3.2 g, yield: 56%). ¹H NMR (CDCl₃, 200 MHz) δ 8.59 (bs, 3H), 6.20-5.60 (m, 3H), 3.52 (d, J = 10.8, 3H); ³¹P NMR (CDCl₃, 81 MHz) δ 13.8. This salt (1.95 g, 10 mmol) was dissolved in chloroform (60 mL) and Dowex 8–100 resin (8 g, 200–400 mesh, wet) was added. The heterogeneous mixture was shaken for 30 min. The resin was removed by filtration, the filtrate was dried over Na₂SO₄ and solvents were evaporated under vacuum to give the desired compound as a yellowish oil (0.81 g, 66%). ¹H NMR (CDCl₃, 200 MHz) δ 11.99 (bs, 1H), 6.30-5.98 (m, 3H), 3.71 (d, J = 10.8, 3H); ¹³C NMR (CDCl₃, 75 MHz) δ 127.0, 124.5, 52.3 (d, J = 5.8); ³¹P NMR (CDCl₃, 81 MHz) δ 21.1; ESI-MS for C₂H₆IO₃P[M+H]⁺ calcd. 236.9, found 236.9.

1,3,2'-Tri-*N*-(*tert*-butoxycarbonyl)-6'-*N*-(methyl vinylphosphonyl) neamine (20)

Compound **5** (125 mg, 0.2 mmol) and diisopropylethylamine (50 μ L, 0.25 mmol) were dissolved in anhydrous DMF (20 mL). The mixture was cooled to 0°C, and methyl vinylphosphonyl chloride (**16**, 28 mg, 0.2 mmol) was added. The reaction mixture was stirred for one hour at 0°C and for another two hours at RT. Reaction completion was monitored by TLC, R_f = 0.38 (CHCl₃/MeOH/NH₄OH, 4/1/0.1) and **ESI-MS** for C₃₀H₅₅N₄O₁₄P [M+Na]⁺ calcd. 749.3, found 749.2. ³¹P NMR (DMSO-*d*₆, 81 MHz) δ 25.1 and 25.7.

1,3,2'-Tri-*N*-(*tert*-butoxycarbonyl) paromamine (21)

Paromomycin hydrogen chloride salt (0.86 g, 2 mmol) and NaOH (0.60 g, 15 mmol) were dissolved in MeOH (80 mL). The mixture was stirred for a few minutes to yield an homogeneous solution. Boc anhydride (2.0 g, 9.2 mmol) was added in three batches. The mixture was stirred for 16 h and then evaporated. The residue was triturated with petroleum ether (60 mL $\times$ 3) and washed with water (50 mL $\times$ 2), to yield the desired product (0.93 g, yield: 75%). ¹H NMR (DMSO-*d*₆, 400 MHz) δ 6.63 (d, *J* = 7.5, 1H), 6.46 (bs, 1H), 6.30 (d, *J* = 7.5, 1H), 4.97 (bs, 1H), 4.82 (bs, 1H), 4.73 (bs, 1H), 4.65 (bs, 1H), 4.58 (bs, 1H), 4.18 (bs, 1H), 3.63-3.52 (m, 3H), 3.35-3.04 (m, 8H), 1.75 (bs, 1H), 1.37 (s, 27H), 1.18 (q, *J* = 10.5, 1H); ¹³C NMR (DMSO-*d*₆, 75 MHz) δ 156.5, 155.9, 155.6, 99.8, 82.6, 78.4, 78.2, 77.7, 74.4, 73.2, 71.8, 70.6, 61.0, 56.3, 51.4, 50.3, 36.0, 29.0, 28.8; **ESI-MS** for C₂₇H₄₉N₃O₁₃ [M+H]⁺ calcd. 624.3, found 624.3.

1,3,2'-Tri-*N*-(*tert*-butoxycarbonyl)-6'-*O*-(methyl vinyl phosphonyl) paromamine (22)

Into a flame-dried flask, compound **21** (0.32 g, 0.5 mmol) was dissolved in anhydrous DMF (20 mL), and activated 4 Å molecular sieves (3 g) were added. The mixture was allowed to stir for 2 h and then transferred by cannula into another dry flask. A freshly prepared solution of compound **19** (67 mg, 0.48 mmol) in DCM (10 mL) was added dropwise to the above solution at 0°C. The reaction was complete after 3 h as revealed by TLC (R_f : 0.42, CHCl₃/MeOH/NH₄OH, 5/1/0.1). Evaporation of the solvent under vacuum produced the crude product as a white solid powder. **ESI-MS** gave the correct mass as for C₃₀H₅₄N₃O₁₅ P [M+Na]⁺ calcd. 750.3, found 750.2. Purification using silica column was not possible due to decomposition, thus the crude product was used directly in the next step.

6'-*O*-(Methyl vinyl phosphonyl) paromamine (23)

Crude compound **22** (0.16 g) was dissolved in DCM (5 mL) along with 3 drops of anisole. TFA (2 mL) was added to the mixture which became homogeneous immediately. The mixture was stirred for 1 h at RT and evaporation of the solvent yielded the crude product **23** (200 mg, yield: 100%). ³¹P-NMR (D₂O, 81 MHz): δ 23.9, 23.8; **ESI-MS** for C₁₅H₃₀N₃O₉P [M+H]⁺ calcd. 428.3, found 428.1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Science and Engineering Research Council of Canada (NSERC) and by the Canadian Institute of Health Research (CIHR). F.G. was supported by a scholarship award from the Chemical Biology Strategic Training Initiative of CIHR. The authors are grateful to G. D. Wright at McMaster University for sharing his AAC(6')-II expression plasmid.

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Figure 1.

Examples of previously reported AAC(6') inhibitors (**1** and **2**); targets envisaged here (**3**); and proposed enzymatic tetrahedral intermediate.

Scheme 1.

First synthetic plan for the preparation of bisubstrate **3a**. Reagents and conditions: i) NBS, PPh₃; ii) PBr₃, CCl₄; iii) I₂, PPh₃; d) trichlorocyanuric acid, DMF.

Scheme 2.

Second synthetic plan for the preparation of bisubstrate **3a**. Compound **5** is 1,3,2'-tri-*N*-(*tert*-butoxycarbonyl)neamine² (see Scheme 3). Conditions tested separately: i) TEA/H₂CO₃ buffer, pH ~ 8.4; ii) H₂O/acetone, DIPEA; iii) H₂O/acetone, TEA; iv) H₂O, 0.1 N NaOH

Scheme 3.

Synthetic plan for the preparation of bisubstrate **3b**.

Scheme 4.

Attempted synthesis of bisubstrate **3b**. Conditions tested separately: i) TEA H_2CO_3 buffer, pH ~ 8.4; ii) H_2O /acetone, TEA; iii) H_2O /acetone, DIPEA; iv) H_2O , 0.1 M NaOH; v) H_2O /CAN, AIBN, 50°C .

Scheme 5.

Synthesis of bisubstrate **3c**. The yield for the last step is non-optimized.

Table 1

Gradient profile for HPLC purification

Time (min)	% A (0.05% TFA ^a in H ₂ O)	% B (0.04% TFA in ACN)
0	99	1
20	60	40
30	99	1