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Reaction Intermediate Analogues as Bisubstrate Inhibitors of Pantothenate Synthetase

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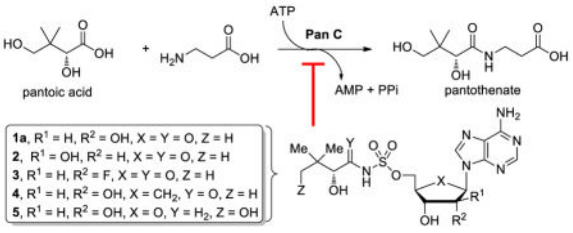
Abstract

The biosynthesis of pantothenate, the core of coenzyme A (CoA), has been considered an attractive target for the development of antimicrobial agents since this pathway is essential in prokaryotes, but absent in mammals. Pantothenate synthetase, encoded by the gene *panC*, catalyzes the final condensation of pantoic acid with β–alanine to afford pantothenate via an intermediate pantoyl adenylate. We describe the synthesis and biochemical characterization of five PanC inhibitors that mimic the intermediate pantoyl adenylate. These inhibitors are competitive inhibitors with respect to pantoic acid and possess submicromolar to micromolar inhibition constants. The observed SAR is rationalized through molecular docking studies based on the reported co-crystal structure of **1a** with PanC. Finally, whole cell activity is assessed against wild-type *Mtb* as well as a PanC knockdown strain where PanC is depleted to less than 5% of wild-type levels.

Graphical abstract



Keywords

pantothenate synthetase; tuberculosis; bisubstrate inhibitor; adenylation; coenzyme A

1. Introduction

Tuberculosis (TB) caused by members of the *Mycobacterium tuberculosis* (*Mtb*) complex is an ancient scourge that remains the leading source of morbidity and mortality today with an estimated nine million new cases and 1.4 million deaths in 2011, primarily in the developing world.[1] The recommended therapy by the World Health Organization (WHO) for drug sensitive TB is known as directly observed treatment, short course (DOTS) and requires 6 months of treatment with isoniazid, rifampin, ethambutol, and pyrazinamide.[2] This extremely long duration of treatment is likely due to the ability of *Mtb* to switch its metabolism to a nonreplicating state,[3] the heterogeneous nature of the bacterial subpopulations residing in different lesions types,[3] and the lack of drug penetration into the site of infection.[4] In order to combat this global health threat, new drugs are needed to shorten the treatment duration and for drug resistant strains including multidrug-resistant (MDR) TB and extensively drug resistant (XDR) TB.[5, 6]

Pantothenate, also known as vitamin B5 is a precursor to coenzyme A (CoA), an essential cofactor required in central and intermediary metabolism where it serves as an acyl group carrier and carbonyl activating group.[7, 8] Bioinformatics analysis has identified the *de novo* biosynthetic pathway to pantothenate as an attractive target for the development of antimicrobial agents since this pathway is absent in mammals, but essential in prokaryotes.[9–11] Biosynthesis of pantothenate is accomplished by four enzymes encoded by the genes *panB*, *pancC*, *panD*, and *panE*. PanB and PanE are responsible for the synthesis of pantoic acid, while PanD, an aspartate α-decarboxylase, produces β-alanine. PanC (Rv3602c, pantothenate synthetase) then catalyzes the condensation of pantoic acid with β-alanine through a two-step adenylation-ligation reaction.[12] In the adenylation half-reaction, ATP and pantoic acid react to form a pantoyl-adenylate intermediate (Fig. 1A). Following the release of pyrophosphate, β-alanine binds and PanC catalyzes its ligation with the activated carbonyl of the pantoyl-adenylate to afford pantothenate. The detailed kinetic characterization of PanC from *Mtb* shows it uses a bi-uni-uni-bi ping pong kinetic mechanism with sequential ordered binding of ATP followed by pantoic acid and sequential ordered release of pantothenate followed by AMP (Fig. 1B).[12] The apparent K_M values for pantoic acid, ATP, and β-alanine are 0.13, 2.6, and 0.8 mM, respectively. PanC is a 33 kDa protein that exists as a homodimer in solution. The active site is located in the N-terminal domain (residues 1–186) while the C-terminal domain (residues 187–309) covers the active site.[13] Substrates must diffuse through a gate comprising residues 75–88, which are flexible and disordered, but becomes ordered upon formation of the pantoyl adenylate. The structures PanC from *Mtb* in complex with substrates, intermediates, and products have been solved providing a step-by-step view of the PanC reaction.[13, 14]

Inhibitors of PanC have been identified by high-throughput screening,[15–17] fragment-based approaches,[18–20] dynamic combinatorial chemistry,[21] and through the rationale design of analogues of the pantoyl-adenylate intermediate.[22, 23] The pantoyl-adenylate intermediate mimic **1**, which is epimeric at the C-2 position of the pantoyl fragment, reported by Ciulli and co-workers is the most potent inhibitor yet reported with a K_i of 0.22 μM with respect to ATP as the varied substrate at saturating concentrations of pantoic acid and β-alanine using a coupled biochemical assay that measures formation of AMP.[23] In this paper, we report the

design, synthesis, and biochemical evaluation of five inhibitors with the C-2 stereocenter retaining the same configuration (i.e. *R*) as the reaction intermediate **1a** (Fig. 2). To prevent the intramolecular lactonization (Fig. 1C), the terminal C-4 hydroxyl of the pantoyl moiety was removed in **1a–4** while the pantoyl carbonyl group was deleted in **5**. All compounds were also evaluated against wild-type *Mtb* and a PanC depleted *Mtb* strain.

2. Results and Discussion

2.1. Chemistry

Synthesis of diastereomerically pure **1a** was achieved starting from commercially available (*R*)-2-amino-3,3-dimethylbutanoic acid **6** that was first converted to the corresponding α-hydroxy acid **7** with retention of configuration via an intermediate α-lactone (Scheme 1).[24] Protection of the resultant hydroxyl group as a TBS ether provided **8**, which was coupled with *N*-hydroxysuccinimide (NHS) using DCC to afford the common reaction intermediate **9**. The pantoyl-adenylate analogue **1a** was then prepared by acylation of 2′,3′-isopropylidene-5′-*O*-(sulfamoyl)adenosine **10**[25] with **9** employing Cs_2CO_3 in DMF followed by concomitant deprotection of the TBS and acetonide groups using aqueous trifluoroacetic acid (TFA).[26]

Analogue **2** that is epimeric at the C-2′ position was synthesized in five steps from vidarabine **11** (Scheme 2). Persilylation with excess TBSCl afforded **12** and regioselective deprotection of the primary 5′-OTBS was accomplished under carefully controlled conditions with TFA in 2:1 H_2O–THF to furnish **13**. Sulfamoylation with freshly prepared sulfamoyl chloride[27] in dioxane yielded **14**, which was coupled to **9** and deprotected with 80% aqueous TFA to provide the arabinosyl nucleoside derivative **2**.

The 2′-fluoroadenosine analogue **3**[28] was also prepared starting from vidarabine **11** through regioselective protection of the less sterically encumbered 3′ and 5′-hydroxyl groups to yield **15** (Scheme 3). Fluorination of **15** with diethylaminosulfur trifluoride (DAST) afforded **16**.[29] After selective removal of the 5′-OTBS employing TFA in 2:1 H_2O–THF, intermediate **17** was converted to sulfamate **18**. The ^1H NMR data of **17** and **18** agreed with reported analytical data,[30] indicating that the C-2′ configuration of **16** was *R*. Acylation of **18** with **9** followed by deprotection of the TBS with 80% aqueous TFA provided **3**.

Carbocyclic analogue **4** was prepared from the reported aristeromycin nucleoside analouge **19**[31] by acylation with **9** followed by deprotection with 80% aqueous TFA (Scheme 4).

The synthesis of sulfamide **5** began from commercially available (D)-(–)-pantolactone **20** that was first reduced to triol **21** with $LiAlH_4$ as described by Burkart et al., then protected as the 1,3-benzylidene acetal **22**.[32] Functional group interconversion of primary alcohol **22** to amine **25** was performed by sequential tosylation, azide displacement, and catalytic reduction. Sulfamoylation of **25** with 4-nitrophenyl chlorosulfate afforded **26**, which was coupled to nucleoside **27**[33] followed by global deprotection of the acetonide and benzylidene acetal with 80% aqueous TFA to furnish **5**.[34]

2.2. PanC Inhibition Studies

Pantothenate synthetase (PanC) from *Mtb* was subcloned from BAC-Rv222 (kindly provided by the Institut Pasteur) into pET28b and expressed in *E. coli* BL21 (DE3) as described in Materials and Methods to provide an N-terminal His-tagged protein with kinetic parameters commensurate with the native enzyme.[12] Kinetic studies to evaluate enzyme inhibition of each compound toward PanC were performed under initial velocity conditions using a continuous coupled assay that measures production of pyrophosphate (see Materials and Methods).[35, 36]

Since compounds **1a–5** are bisubstrate inhibitors, designed to bind both the pantoic acid and ATP binding pockets, we evaluated inhibition with respect to pantoic at fixed non-saturating concentrations of ATP and saturating concentrations of the third substrate β-alanine. Representative inhibition data for compound **1a** are shown in Figure 3. The double-reciprocal plots of initial velocity versus pantoic acid concentration at different inhibitor concentrations of **1a** display a pattern of intersecting lines that converge at the y-axis, indicating that the molecule act as a competitive inhibitor towards pantoic acid, in which K_m values are increased, but there is no effect on V_{max}.[37] The data were fitted to Eq. 1 for competitive inhibition yielding a K_i value of 0.27 ± 0.04 μM.

Inhibitors **2–5** were similarly evaluated and shown to possess identical modalities of inhibition with respect to pantoic acid (see Table 1 for inhibition constants). Inhibitor **1a** containing an acyl-sulfamate linkage and the natural ribosyl subunit is the most potent (K_i = 0.27 μM) in the series while inhibitor **5** containing a sulfamide linkage is the least potent (K_i = 3.47 μM), an approximately 13-fold difference in affinity, demonstrating that the linker carbonyl is more important than the terminal hydroxyl group of the pantoyl fragment. Replacement of the ribofuranosyl ring oxygen with a methylene in carbocyclic analogue **3** and substitution of the 2′-hydroxyl with a fluoro group in analogue **4** are reasonably well tolerated resulting in a modest 2–3 fold loss in affinities. Inversion of the 2′-alcohol in analogue **2** results in more pronounced 4–6 fold loss in potency.

2.3. Computational Modeling

The key interactions between the intermediate analogue (Pan-AMP, Fig. 1A) and PanC involve the secondary hydroxyl and the carbonyl groups of the pantoyl fragment, which form hydrogen bonds with protein residues Gln72 and Thr39, respectively. In the adenylate moiety, the two hydroxyls of ribose form hydrogen bonds with Asp161, Gly158 and Gln 164, while the two nitrogen atoms at the adenine moiety interact with Val187 and Met195. To understand the binding selectivity of our designed PanC inhibitors, we modeled **2–5** into PanC binding site using the reported X-ray structure of the complex of **1** with PanC (Fig. 4A and 4B).[17] The removal of the carbonyl group in **5** results in a loss of critical hydrogen bond interaction with Gln 72 and explains the nearly 13-fold loss of affinity relative to **1a**. For inhibitor **3**, the 2′-fluorine atom maintained hydrogen bond with Asp161. Replacement of the oxygen atom by carbon in inhibitor **4** does not change the compound overall binding mode with the protein and **4** displayed only a slight loss in binding affinity (K_i = 287 nM) compared to **1a**.

2.4. Evaluation against whole cell *M. tuberculosis*

Pantoyl-adenylate analogues **1a–5** were evaluated against whole cell *Mtb* H37RvMA in 7H9 liquid medium; however, none of the compounds displayed any growth inhibition up to 250 μM. Notably, no whole cell activity against wild-type *Mtb* has yet been observed or disclosed for any previously described PanC inhibitor.[15–23] Mizrahi and co-workers recently reported on the preparation of a *Mtb panC* conditional mutant that expresses less than 5% wild-type PanC levels.[38] Depletion of PanC renders this mutant hypersensitive to target-specific inhibitors. In order to provide evidence that **1a–5** possess some target-based activity, the compounds were screened against this panC-depleted strain, but observed no activity was observed with up to 2 mM **1a–5**. Although discouraging, it is important to note that the enzyme potency of these bisubstrate inhibitors is relatively modest compared to related bisubstrate inhibitors for other adenylating enzymes in *Mtb*.[39]

3. Conclusion

We have reported the synthesis as well as the biochemical and biological characterization of five PanC inhibitors as potential antitubercular agents. These compounds structurally mimic the pantoyl-adenylate reaction intermediate by bioisosteric replacement of the labile acyl-phosphate moiety with either acyl-sulfamate or sulfamide functional groups. We demonstrated that structural variation of the glycosyl domain of these inhibitors is well tolerated and the hydrogen bond interactions between the inhibitors and the active site residues Gln72 and Gln164 are critical for high binding affinity. Surprisingly, despite their close mimicry of the pantoyl-adenylate, none of the compounds were exceptionally potent inhibitors with K_i ranging from ~0.3–3 μM. By contrast, related adenylate inhibitors of the enzymes MbtA and BirA in *Mtb* possess picomolar dissociation constants.[40, 41] Ciulli reported the binding of **1** (as a mixture of C-2 epimers) to PanC by isothermal titration calorimetry is accompanied by a large unfavorable entropic term ($-T\Delta S$ = +8.8 kcal/mol),[23] which may reflect the energetic penalty associated with the disorder-to-order transition of the gate residues (amino acids 75–88) that occurs upon ligand binding.[13] Thus, despite an impressive favorable enthalpy of binding PanC (ΔH = −18.2 kcal/mol),[23] the overall potency of **1a** is limited by the large unfavorable entropy that is likely to restrict the potency of all active-site directed inhibitors of this enzyme. Since the inhibitors have limited potency, we used an *Mtb* strain wherein PanC is depleted to less than 5% of the wild-type levels in order to provide evidence that the compounds target PanC. However, none of the compounds demonstrated any activity against this knock-down strain suggesting the compounds failed to accumlate intracelluarly. This could be caused by poor cell penetration or active efflux. Alternatively, PanC may not be particularly vulnerable to inhibition and thus may not represent an ideal target for development of new antitubercular agents.

4. Materials and Methods

4.1 General Procedures

All chemicals (reagent grade) used were purchased from Sigma–Aldrich (USA) and Sinopharm Chemical Reagent Co. Ltd. (China). ^{1}H NMR spectra were measured on Varian Unity Inova 300 or 400 MHz NMR Spectrometers at 25 °C and referenced to TMS.

Chemical shifts are reported in ppm using the residual solvent line as internal standard. Splitting patterns are designed as s, singlet; d, doublet; t, triplet; m, multiplet. HRMS spectra were acquired on an Agilent Technologies 6220 Accurate-Mass TOF LC/MS. Analytical thin-layer chromatography (TLC) was performed on the glass-backed silica gel sheets (silica gel 60 Å GF254). All compounds were detected using UV light (254 or 365 nm). Analytical HPLC was conducted on SHIMADZU LC-20AD. Prior to K_i measurement, all compounds were determined to be >95% pure by HPLC based on the peak area percentage.

4.1.1. (*R*)-2-Hydroxy-3,3-dimethylbutanoic acid (7)—To a solution of **6** (1.0 g, 7.62 mmol, 1.0 equiv) in 0.5 M H_2SO_4 at 0 °C was added a solution of $NaNO_2$ (3.15 g, 45.6 mmol, 6.0 equiv) in H_2O (10 mL) dropwise over 30 min. After addition was complete the solution was warmed to 23 °C and stirred overnight (~16 h). The reaction mixture was diluted with H_2O (30 mL), extracted with diethyl ether (3 × 30 mL) and the combined organic layers were dried (Na_2SO_4), and concentrated to afford **7** (806 mg, 81%) as a colorless oil, which was used directly in next reaction. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.90 (s, 1H), 1.02 (s, 9H); MS (ESI–) calcd for $C_6H_{11}O_3$ $[M–H]^-$ 131.1, found 131.4.

4.1.2. (*R*)-2-(*tert*-Butyldimethylsilyl)oxy-3,3-dimethylbutanoic acid (8)—To a solution of **7** (2.06 g, 15.6 mmol, 1.0 equiv) in DMF (15 mL) were added imidazole (5.09 g, 74.8 mmol, 4.8 equiv) and *tert*-butylchlorodimethylsilane (5.64 g, 37.4 mmol, 2.4 equiv). The mixture was stirred overnight at 23 °C then extracted with 1:1 ethyl acetate–petroleum ether (3 × 30 mL). The organic layer was washed successively with a 10% aqueous citric acid solution, H_2O, saturated aqueous $NaHCO_3$, and brine, then dried (Na_2SO_4), and concentrated. The residue was dissolved in methanol (100 mL), then an aqeuous 0.8 M K_2CO_3 solution (50 mL) was added. After 4 h, 10% citric acid aqueous solution was added to adjust pH to 4. The mixture was extracted with ethyl acetate (3 × 30 mL) and the combined organic layers were dried (Na_2SO_4) and concentrated. The residue was purified by flash chromatography (10:1 petroleum ether–ethyl acetate) to afford the title compound (3.1 g, 81%) as a colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.64 (br s, 1H), 3.83 (s, 1H), 0.97 (s, 9H), 0.93 (s, 9H), 0.08 (s, 3H), 0.06 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.6, 80.0, 35.4, 26.0, 25.8, 18.3, −5.2 (2 C); MS (ESI–) calcd for $C_{12}H_{25}O_3Si$ $[M–H]^-$ 245.2, found 245.2.

4.1.3. (*R*)-2,5-Dioxopyrrolidin-1-yl-2-(*tert*-butyldimethylsilyl)oxy-3,3-dimethylbutanoate (9)—To a solution of **8** (1.99 g, 8.1 mmol, 1.0 equiv) in DME (50 mL) at 0 °C, *N*-hydroxysuccinimide (1.87 g, 16.2 mmol, 2.0 equiv) and DCC (2.34 g, 11.3 mmol, 1.4 equiv) were added and the mixture was stirred overnight at 23 °C. The mixture was filtered through a short pad of Celite washing with ethyl acetate and the filtrate was concentrated. The residue was purified by flash chromatography (1:1 petroleum ether–CH_2Cl_2) to afford the title compound (1.60 g, 60%) as a colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.09 (s, 1H), 2.81 (br s, 4H), 1.05 (s, 9H), 0.92 (s, 9H), 0.12 (s, 3H), 0.09 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.1, 168.1, 78.5, 36.0, 25.7 (3C), 18.2, −5.2, −5.5; MS (ESI–) calcd for $C_{16}H_{29}NO_5Si$ $[M]^-$ 343.2, found 342.8.

4.1.4. 5′-*O*-[*N*-((*R*)-2-Hydroxy-3,3-dimethylbutanoyl)sulfamoyl]adenosine (1a)—To a solution of **10**[25] (100 mg, 0.26 mmol, 1.0 equiv) in DMF (1.0 mL) was added **9** (179 mg, 0.52 mmol, 2.0 equiv) and Cs_2CO_3 (169 mg, 0.52 mmol, 2.0 equiv) at 23 °C. After stirring 24 h at 23 °C, the solution was concentrated in vacuo. Purification by flash chromatography (500:6:1.5 CH_2Cl_2–MeOH–Et_3N) afforded 5′-*O*-{*N*-[(*R*)-2-(*tert*-butyldimethylsilyl)oxy-3,3-dimethylbutanoyl]sulfamoyl}-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (72 mg) as a yellow solid. This compound was dissolved in 80% aqueous TFA (2 mL). After stirring 48 h at 8 °C, the solution was concentrated in vacuo. Purification by flash chromatography (10:1 CH_2Cl_2–MeOH) afforded the title compound (8 mg, 7% yield from compound **10**) as a white solid: ^{1}H NMR (400 MHz, CD_3OD) δ 8.57 (s, 1H), 8.33 (s, 1H), 6.38 (s, 1H), 5.05–5.00 (m, 1H), 4.79–4.77 (m, 1H), 4.74–4.70 (m, 1H), 4.40–4.37 (m, 1H), 4.14–4.10 (m, 1H), 3.62 (s, 1H), 0.99 (s, 9H); ^{13}C NMR (100 MHz, CD_3OD) δ 179.0, 158.7, 150.3, 140.9, 140.6, 121.2, 95.3, 85.2, 80.3, 77.2, 71.7, 59.7, 36.1, 26.5; HRMS (ESI–) calcd for $C_{16}H_{23}N_6O_8S$ $[M–H]^-$ 459.1304, found 459.1310.

4.1.5. 2′,3′,5′-*O*-tri(*tert*-Butyldimethylsilyl)vidarabine (12)—To a solution of vidarabine **11** (200 mg, 0.748 mmol, 1 equiv) in DMF (1.5 mL) were added imidazole (306 mg, 4.49 mmol, 6.0 equiv) and TBSCl (677 mg, 4.49 mmol, 6.0 equiv) and the reaction was stirred overnight at 23 °C The reaction mixture was partitioned between EtOAc and H_2O and the organic layer was dried ($NaSO_4$), and concentrated. Purification by flash chromatography (80:1 CH_2Cl_2–MeOH) afforded the title compound (319 mg, 70%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.36 (s, 1H), 8.03 (s, 1H), 6.48 (d, J = 3.6 Hz, 1H), 5.59 (s, 2H), 4.34–4.32 (m, 1H), 4.19–4.16 (m, 1H), 4.03–3.98 (m, 1H), 3.88–3.84 (m, 2H), 0.94 (s, 9H), 0.92 (s, 9H), 0.72 (s, 9H), 0.15 (s, 6H), 0.09 (s, 3H), 0.08 (s, 3H), −0.08 (s, 3H), −0.45 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.5, 153.0, 149.6, 140.8, 119.3, 86.5, 85.9, 78.3, 77.6, 63.0, 26.1, 25.9, 25.7, 18.5, 18.1, 17.9, −4.4, −5.2, −5.5; MS (ESI+) calcd for $C_{28}H_{56}N_5O_4Si_3[M+H]^+$ 610.4, found 610.0.

4.1.6. 2′,3′-*O*-di(*tert*-Butyldimethylsilyl)vidarabine (13)—To a solution of **12** (50 mg, 0.082 mmol) in THF (0.5 mL) was added 35% aqueous TFA (2 mL). The reaction mixture was stirred for 2 h at 23 °C., then quenched with saturated aqueous $NaHCO_3$ and extracted with EtOAc. The combined organic layers were dried ($NaSO_4$) and concentrated. Purification by flash chromatography (80:1 CH_2Cl_2–MeOH) afforded the title compound (33 mg, 81%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.30 (s, 1H), 8.05 (s, 1H), 6.44 (d, J = 4.0 Hz, 1H), 5.83 (s, 2H), 4.38–4.34 (m, 1H), 4.31–4.27 (m, 1H), 4.13 (s, 1H), 4.08–4.04 (m, 1H), 3.92–3.88 (m, 2H), 0.94 (s, 9H), 0.67 (s, 9H), 0.16 (s, 6H), −0.06 (s, 3H), −0.41 (s, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 156.8, 153.6, 149.7, 141.9, 119.5, 88.0, 87.1, 79.1, 78.4, 62.6, 26.2, 26.0, 18.6, 18.3, −4.2, −4.4, −4.8, −5.4; MS (ESI+) calcd for $C_{22}H_{42}N_5O_4Si_2$ $[M+H]^+$ 496.3, found 496.0.

4.1.7. 2′,3′-*O*-di(*tert*-Butyldimethylsilyl)-5′-*O*-(sulfamoyl)vidarabine (14)—To a solution of **13** (100 mg, 0.2 mmol, 1.0 equiv) in 1,4-dioxane (5 mL) was added NaH (60% w/w in mineral oil, 60 mg, 1.5 mmol, 7.5 equiv). The reaction mixture was stirred for 1 h at 23 °C. Next, NH_2SO_2Cl[27] (58 mg, 0.5 mmol, 2.5 equiv) was added and the reaction was stirred for 24 h, then quenched by the slow addition of 5:1 CH_2Cl_2–MeOH (10 mL). The

reaction mixture was filtered through silica gel and the filtrate was concentrated. Purification by flash chromatography (30:1 CH_2Cl_2–MeOH) afforded the title compound (70 mg, 60%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (s, 2H), 6.48 (d, *J* = 3.2 Hz, 1H), 4.48 (dd, *J* = 10.4, 7.2 Hz, 1H), 4.40–4.24 (m, 4H), 0.98 (s, 9H), 0.72 (s, 9H), 0.22 (s, 6H), −0.02 (s, 3H), −0.43 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 155.9, 152.7, 149.1, 139.8, 118.3, 84.2, 81.9, 77.3, 76.5, 68.1, 25.7, 25.4, 17.6, 17.3, −4.6, −4.7, −5.4, −5.8; MS (ESI+) calcd for $C_{22}H_{43}N_6O_6SSi_2$ $[M+H]^+$ 575.2, found 574.8.

4.1.8. 5′-*O*-[*N*-((*R*)-2-Hydroxy-3,3-dimethylbutanoyl)sulfamoyl]vidarabine (2)— To a solution of **14** (420 mg, 0.70 mmol, 1.0 equiv) in DMF (10 mL) was added **9** (497 mg, 1.40 mmol, 2.0 equiv) and Cs_2CO_3 (476 mg, 1.40 mmol, 2.0 equiv). The reaction was stirred for 24 h then concentrated in vacuo. The residue was purified by flash chromatography (500:6:1.5 CH_2Cl_2–MeOH–Et_3N) to afford 2′,3′-*O*-di(*tert*-butyldimethylsilyl)-5′-*O*-{*N*-[(*R*)-2-(*tert*-butyldimethylsilyloxy)-3,3-dimethylbutanoyl]sulfamoyl}vidarabine triethylammonium salt (80 mg) as a yellow solid. This intermediate was dissolved in 80% aqueous TFA (2 mL). After stirring 60 h at 23 °C, the solution was concentrated in vacuo. Purification by flash chromatography (10:1 CH_2Cl_2–MeOH) afforded the title compound (10 mg, 3% yield from compound **14**) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.58 (s, 1H), 8.19 (s, 1H), 6.38 (d, *J* = 5.2 Hz, 1H), 5.01 (d, *J* = 13.6 Hz, 1H), 4.74 (d, *J* = 2.4 Hz, 1H), 4.68 (dd, *J* = 16.0, 2.4 Hz, 1H), 4.52 (t, *J* = 5.2 Hz, 1H), 3.95 (t, *J* = 5.2 Hz, 1H), 3.64 (s, 1H), 1.00 (s, 9H); ^{13}C NMR (100 MHz, CD_3OD) δ 179.6, 158.7, 150.1, 142.2, 141.5, 121.3, 91.8, 85.7, 81.3, 80.2, 76.9, 59.8, 36.2, 26.5; HRMS (ESI–) calcd for $C_{16}H_{23}N_6O_8S$ $[M–H]^-$ 459.1304, found 459.1318.

4.1.9. 3′,5′-*O*-di(*tert*-Butyldimethylsilyl)vidarabine (15)—To a solution of vidarabine **11** (2.0 g, 7.5 mmol, 1.0 equiv) in DMF (40 mL) were added triethylamine (5.2 mL, 37 mmol, 5.0 equiv) and TBSCl (2.82 g, 18.7 mmol, 2.5 equiv). The reaction mixture was stirred for 16 h at 23 °C then partitioned between EtOAc and H_2O. The organic layer was dried ($NaSO_4$), and concentrated. Purification by flash chromatography (80:1 CH_2Cl_2–MeOH) afforded the title compound (2.4 g, 65%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.35 (s, 1H), 8.27 (s, 1H), 6.33 (d, *J* = 2.4 Hz, 1H), 5.65 (s, 2H), 4.88 (d, *J* = 10.0 Hz, 1H), 4.37–4.34 (m, 1H), 4.14 (d, *J* = 10.0 Hz, 1H), 4.07 (s, 1H), 3.96 (d, *J* = 11.2 Hz, 1H), 3.81 (d, *J* = 11.2 Hz, 1H), 0.93 (s, 9H), 0.93 (s, 9H), 0.15 (s, 6H), 0.14 (s, 6H); MS (ESI+) calcd for $C_{22}H_{42}N_5O_4Si_2$ $[M+H]^+$ 496.3, found 496.4.

4.1.10. 3′,5′-*O*-di(*tert*-Butyldimethylsilyl)-2′-deoxy-2′-fluoroadenosine (16)—To a solution of **15** (100 mg, 0.20 mmol, 1.0 equiv) and pyridine (0.20 mL, 10 mmol, 10 equiv) in CH_2Cl_2 (3 mL) at 23 °C was added diethylaminosulfur trifluoride (DAST) (50.1 μL, 1.0 mmol, 5.0 equiv). The reaction mixture was stirred for 6 h at 23 °C then quenched with 5% aqueous $NaHCO_3$ (15 mL) and extracted with CH_2Cl_2 (3 × 30 mL). The combined organic layers were dried ($NaSO_4$) and concentrated. Purification by flash chromatography (50:1 CH_2Cl_2–MeOH) afforded the title compound (20 mg, 31%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 (s, 1H), 8.16 (s, 1H), 6.25 (dd, *J* = 15.6, 1.6 Hz, 1H), 6.05 (s, 2H), 5.32 (dd, *J* = 52.8, 1.6 Hz, 1H), 4.73–4.64 (m, 1H), 4.16–4.12 (m, 1H), 4.02 (dd, *J* = 12.0, 2.4 Hz, 1H), 3.79 (dd, *J* = 12.0, 2.4 Hz, 1H), 0.93 (s, 9H), 0.89 (s, 9H), 0.14 (s, 3H), 0.13 (s, 3H),

0.08 (s, 3H), 0.06 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.8, 153.3, 149.4, 139.3, 120.1, 93.0 (d, *J* = 191.3 Hz), 87.0 (d, *J* = 33.0 Hz), 84.0, 69.4 (d, *J* = 15.8 Hz), 61.3, 26.0, 25.8, 18.5, 18.2, −4.6, −4.9, −5.3, −5.4; MS (ESI+) calcd for $C_{22}H_{41}FN_5O_3Si_2$ $[M+H]^+$ 498.3, found 498.3.

4.1.11. 3′-*O*-(*tert*-Butyldimethylsilyl)-2′-deoxy-2′-fluoroadenosine (17)—To a solution of **16** (100 mg, 0.20 mmol) in THF (0.5 mL) was added 35% aqueous 7 TFA (2 mL) at 23 °C. The solution was stirred for 20 min, then quenched with saturated aqueous $NaHCO_3$ solution. The mixture was extracted with EtOAc (3 × 10 mL) and the combined extracts were dried ($NaSO_4$), and concentrated. Purification by flash chromatography (50:1 CH_2Cl_2–MeOH) afforded the title compound (71 mg, 92%) as a white solid: ^{1}H NMR (400 MHz, CD_3OD) δ 8.37 (s, 1H), 8.19 (s, 1H), 6.27 (dd, *J* = 15.2, 4.0 Hz, 1H), 5.51 (dt, *J* = 52.8, 4.0 Hz, 1H), 4.79–4.72 (m, 1H), 4.15 (d, *J* = 2.0 Hz, 1H), 3.90 (d, *J* = 12.4 Hz, 1H), 3.71 (dd, *J* = 12.8, 2.4 Hz, 1H), 0.96 (s, 9H), 0.18 (s, 3H), 0.17 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 156.2, 152.6, 148.8, 139.6, 119.2, 92.3 (d, *J* = 189.0 Hz), 85.7 (d, *J* = 32.3 Hz), 84.7, 70.0 (d, *J* = 15.0 Hz), 60.3, 25.6, 17.9, −4.9, −5.1; MS (ESI+) calcd for $C_{16}H_{27}FN_5O_3Si$ $[M+H]^+$ 384.2, found 384.2.

4.1.12. 3′-*O*-(*tert*-Butyldimethylsilyl)-2′-deoxy-2′-fluoro-5′-*O*-(sulfamoyl)adenosine (18)—To a solution of **17** (100 mg, 0.26 mmol, 1.0 equiv) in 1,4-dioxane (5 mL) was added NaH (60 dispersion w/w in mineral oil, 32 mg, 0.78 mmol, 3.0 equiv) at 23 °C. After 1h, NH_2SO_2Cl[27] (75 mg, 0.65 mmol, 2.5 equiv) was added and the solution was stirred for 16 h at 23 °C. The reaction was quenched by the slow addition of 5:1 CH_2Cl_2–MeOH (10 mL) then the mixture was filtered through silica gel and the filtrate was concentrated. Purification by flash chromatography (30:1 CH_2Cl_2–MeOH) afforded the title compound (72 mg, 60%) as a white solid: ^{1}H NMR (300 MHz, CD_3OD) δ 8.26 (s, 1H), 8.21 (s, 1H), 6.31 (dd, *J* = 17.1, 2.7 Hz, 1H), 5.53 (ddd, *J* = 52.5, 4.5, 2.7 Hz, 1H), 4.94–4.91 (m, 1H), 4.46–4.40 (m, 1H), 4.32–4.26 (m, 2H), 0.97 (s, 9H), 0.21 (s, 3H), 0.19 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 156.2, 152.8, 148.8, 139.7, 119.1, 92.2 (d, *J* = 187.5 Hz), 86.1 (d, *J* = 33.8 Hz), 80.3, 70.2 (d, *J* = 15.0 Hz), 67.7, 25.6, 17.8, −4.9, −5.1; MS (ESI+) calcd for $C_{16}H_{28}FN_6O_5SSi$ $[M+H]^+$ 463.2, found 463.2.

4.1.13. 2′-Deoxy-2′-fluoro-5′-*O*-[*N*-((*R*)-2-Hydroxy-3,3-dimethylbutanoyl)sulfamoyl] adenosine (3)—To a solution of **18** (140 mg, 0.30 mmol, 1.0 equiv) in DMF (8 mL) was added **9** (196 mg, 0.60 mmol, 2.0 equiv) and Cs_2CO_3 (206 mg, 0.60 mmol, 2.0 equiv) at 23 °C. The reaction mixture was stirred for 20 h at 23 °C, then concentrated in vacuo. The residue was purified by flash chromatography (500:6:1.5 CH_2Cl_2–MeOH–Et_3N) to afford 3′-*O*(*tert*-butyldimethylsilyl)-5′-*O*-{*N*-[(*R*)-2-(*tert*-butyldimethylsilyloxy)-3,3-dimethylbutanoyl] sulfamoyl}-2′-deoxy-2′-fluoroadenosine triethylammonium salt (80 mg) as a yellow solid. The compound was dissolved in 80% aqueous TFA (1 mL) and the solution was stirred at 20 °C for 72 h. The solvent was removed in vacuo. Purification by flash chromatography (10:1 CH_2Cl_2–MeOH) afforded the title compound (5.0 mg, 4% yield from **18**) as a white solid: ^{1}H NMR (400 MHz, CD_3OD) δ 8.56 (s, 1H), 8.30 (s, 1H), 6.68 (d, *J* = 6.8 Hz, 1H), 4.97 (dd, *J* = 51.6, 4.4 Hz, 1H), 4.86–4.82 (m, 2H), 4.76 (dd, *J* = 14.0, 2.8 Hz, 1H), 4.50 (dt, *J* = 18.8, 4.4 Hz, 1H), 3.63 (s, 1H),

0.98 (s, 9H); ^{13}C NMR (100 MHz, CD_3OD) δ 179.1, 158.7, 150.4, 141.0, 140.5, 121.3, 94.3 (d, *J* = 192.0 Hz), 92.4 (d, *J* = 31.0 Hz), 84.8, 80.2 (d, *J* = 7.0 Hz), 71.5 (d, *J* = 16.0 Hz), 59.5, 36.2, 26.5; HRMS (ESI–) calcd for $C_{16}H_{22}FN_6O_7S$ $[M–H]^-$ 461.1260, found 461.1280.

4.1.14. 5′-*O*-[*N*-((*R*)-2-Hydroxy-3,3-dimethylbutanoyl)sulfamoyl]aristeromycin (4)—To a solution of **19**[31] (89 mg, 0.19 mmol, 1.0 equiv) in DMF (5 mL) was added **9** (128 mg, 0.38 mmol, 2.0 equiv) and Cs_2CO_3 (122 mg, 0.38 mmol, 2.0 equiv). The reaction mixture was stirred for 20 h at 23 °C then concentrated in vacuo. The residue was purified by flash chromatography (500:8:1.5 CH_2Cl_2–MeOH–Et_3N) to afford 5′-*O*-{*N*-[(*R*)-2-(*tert*-butyldimethylsilyl)oxy-3,3-dimethylbutanoyl]sulfamoyl}-2′,3′-*O*-isopropylidenearisteromycin triethylammonium salt (50 mg) as a yellow solid. The compound was dissolved in 80% aqueous TFA (1.0 mL) and the solution was stirred at 8 °C for 48 h. The solvent was removed in vacuo. Purification by flash chromatography (10:1 CH_2Cl_2–MeOH) afforded the title compound (6 mg, 7% yield from compound **19**) as a white solid: ^{1}H NMR (400 MHz, CD_3OD) δ 8.56 (s, 1H), 8.27 (s, 1H), 5.04 (d, *J* = 4.8 Hz, 1H), 4.93 (dd, *J* = 13.6, 3.6 Hz, 1H), 4.55 (dd, *J* = 13.6, 2.8 Hz, 1H), 4.11–4.07 (m, 1H), 4.02 (d, *J* = 4.8 Hz, 1H), 3.69 (s, 1H), 3.05–2.96 (m, 1H), 2.80 (d, *J* = 10.0 Hz, 1H), 2.04 (d, *J* = 14.4 Hz, 1H), 1.00 (s, 9H); ^{13}C NMR (100 MHz, CD_3OD) δ 179.1, 158.6, 150.5, 143.5, 141.2, 121.7, 80.3, 77.5, 74.4, 67.4, 60.6, 44.0, 36.1, 33.5, 26.5; HRMS (ESI–) calcd for $C_{17}H_{25}N_6O_7S$ $[M–H]^-$ 457.1511, found 457.1510.

4.1.15. (4*R*)-5,5-Dimethyl-4-hydroxymethyl-2-phenyl-1,3-dioxane (22)—To a mixture of **21**[32] (371 mg, 2.77 mmol, 1.0 equiv) and benzaldehyde dimethyl acetal (548 mg, 3.04 mmol, 1.1 equiv) in CH_2Cl_2 (300 mL) was added camphorsulfonic acid (50 mg, 0.28 mmol, 0.1 equiv) at 23 °C. The reaction mixture was refluxed for 24 h, then saturated $NaHCO_3$ aqueous solution was added to adjust the pH to 7. The mixture was extracted with EtOAc, and the combined organic extracts were washed with saturated aqueous NaCl, dried ($NaSO_4$), and concentrated. Purification by flash chromatography (6:1 petroleum ether–EtOAc) afforded the title compound (500 mg, 85%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52 (d, *J* = 6.8 Hz, 2H), 7.43–7.33 (m, 3H), 5.51 (s, 1H), 3.69–3.60 (m, 5H), 1.14 (s, 3H), 0.84 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.4, 129.2, 128.4, 126.4, 102.2, 86.0, 79.0, 61.6, 31.7, 21.5, 19.3; MS (ESI+) calcd for $C_{13}H_{19}O_3$ $[M+H]^+$ 223.1, found 223.0.

4.1.16. (4*R*)-5,5-Dimethyl-2-phenyl-4-[(trifluoromethanesulfonyl)oxy]methyl-1,3-dioxane (23)—To a solution of **22 (**135 mg, 0.61 mmol, 1.0 equiv) and Et_3N (0.2 mL) in CH_2Cl_2 (5 mL) was added TsCl (173 mg, 0.91 mmol, 1.5 equiv) at 0 °C. The mixture was then warmed to 23 °C and stirred for 16 h. The solvent was removed in vacuo and the residue was purified by flash chromatography (10:1 petroleum ether–EtOAc) to afford the title compound (190 mg, 83%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, *J* = 8.0 Hz, 2H), 7.48–7.34 (m, 5H), 7.27 (d, *J* = 8.0 Hz, 2H), 5.43 (s, 1H), 4.28 (dd, *J* = 10.8, 2.0 Hz, 1H), 4.11–4.03 (m, 1H), 3.91–3.85 (m, 1H), 3.65 (dd, *J* = 30.0, 11.2 Hz, 2H), 2.43 (s, 3H), 1.10 (s, 3H), 0.87 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.8, 138.0, 132.9, 129.8, 129.0, 128.2, 128.0, 126.2, 101.6, 82.5,

78.5, 69.5, 31.8, 21.7, 21.4, 18.8; MS (ESI+) calcd for $C_{20}H_{24}O_5SNa$ $[M+Na]^+$ 399.1, found 399.0.

4.1.17. (4*R*)-4-Azidomethyl-5,5-dimethyl-2-phenyl-1,3-dioxane (24)—To a solution of **23 (**185 mg, 0.49 mmol, 1.0 equiv) in DMF (3 mL) was added NaN_3 (96 mg, 1.47 mmol, 3.0 equiv) and the mixture was refluxed at 100 °C for 16 h. The mixture was extracted with diethyl ether and the combined organic layers were dried ($NaSO_4$), and concentrated. Purification by flash chromatography (10:1 petroleum ether–EtOAc) afforded the title compound (113 mg, 93%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55 (d, *J* = 6.8 Hz, 2H), 7.43–7.32 (m, 3H), 5.58 (s, 1H), 3.81 (d, *J* = 9.2 Hz, 1H), 3.74 (d, *J* = 11.2 Hz, 1H), 3.65 (d, *J* = 11.2 Hz, 1H), 3.46 (dd, *J* = 12.8, 9.2 Hz, 1H), 3.20 (d, *J* = 13.2 Hz, 1H), 1.16 (s, 3H), 0.84 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.1, 128.9, 128.3, 126.1, 101.7, 84.7, 78.8, 50.7, 32.3, 21.5, 18.9; MS (ESI+) calcd for $C_{13}H_{17}N_3O_2Na$ $[M+Na]^+$ 270.1, found 270.0.

4.1.18. (4*R*)-4-Aminomethyl-5,5-dimethyl-2-phenyl-1,3-dioxane (25)—To a solution of **24** (100 mg, 0.4 mmol, 1.0 equiv) in MeOH (3 mL) under nitrogen was added 10% w/w Pd/C (20 mg). The nitrogen atmosphere was replaced with an atmosphere of H_2 and the mixture was stirred at 23 °C for 2 h. The reaction mixture was then filtered through Celite washing with MeOH and the filtrate was concentrated to afford the title compound (67 mg, 75%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.56–7.28 (m, 5H), 5.47 (s, 1H), 3.89 (br s, 2H), 3.60–3.52 (m, 3H), 2.88–2.67 (m, 2H), 1.08 (s, 3H), 0.78 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.6, 128.8, 128.2, 126.1, 101.9, 87.5, 78.8, 41.4, 32.0, 21.4, 19.0; MS (ESI+) calcd for $C_{13}H_{20}NO_2$ $[M+H]^+$ 222.1, found 222.2.

4.1.19. 4-Nitrophenyl-[((4*R*)-5,5-dimethyl-2-phenyl-1,3-dioxan-4-yl)methyl]sulfamate (26)—To a solution of **25 (**60 mg, 0.27 mmol, 1.0 equiv) and 4-nitrophenol (375 mg, 2.7 mmol, 10.0 equiv) in CH_2Cl_2 (3 mL) was added 4-nitrophenyl chlorosulfate (192 mg, 0.81 mmol, 3.0 equiv) dropwise in CH_2Cl_2 (3 mL) at −78 °C. The reaction was stirred 2 h at −78 °C, then diluted with CH_2Cl_2 and warmed to 23 °C followed by consecutive washes with 1 M NaH_2PO_4 and H_2O. The organic layer was dried (Na_2SO_4) and concentrated. Purification by flash chromatography (50:1 CH_2Cl_2–MeOH) afforded the title compound (60 mg, 50%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (d, *J* = 9.2 Hz, 2H), 7.50–7.33 (m, 7H), 5.45 (s, 1H), 3.80–3.70 (m, 2H), 3.61 (d, *J* = 11.2 Hz, 1H), 3.57–3.49 (m, 1H), 3.32–3.21 (m, 1H), 1.20 (s, 3H), 0.89 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.3, 145.8, 137.7, 129.5, 128.4, 126.4, 125.4, 122.6, 102.1, 82.8, 78.3, 44.1, 31.9, 21.1, 18.7; MS (ESI+) calcd for $C_{19}H_{22}N_2O_7SNa$ $[M+Na]^+$ 445.1, found 444.8.

4.1.20. 5′-Amino-5′-deoxy-5′-*N*-[*N*-((*R*)-2,2-dimethylbutane-1,3-diol-4-yl)sulfamoyl] adenosine (5)—To a mixture of **26** (120 mg, 0.28 mmol, 1.0 equiv) and **27**[33] (167 mg, 0.56 mmol, 2.0 equiv) in CH_2Cl_2 (10 mL) at 23 °C was added 4Å molecular sieves (80 mg) and Et_3N (0.40 mL, 2.80 mmol, 10 equiv). The reaction was stirred for 24 h, then filtered and the filtrate concentrated. Purification by flash chromatography (40:1 CH_2Cl_2–MeOH) afforded 5′-amino-5′-deoxy-5′-*N*-(*N*-{[(2*R*,4*R*)-5,5-dimethyl-2-phenyl-1,3-dioxan-4-yl]methylamino} sulfamoyl)-2′,3′-*O*-isopropylideneadenosine (100 mg) as a white

solid. The compound was dissolved in 80% aqueous TFA (2 mL) at −35 °C. After stirring 4 h at −35 °C, the solution was concentrated in vacuo. Purification by flash chromatography (10:1 CH_2Cl_2–MeOH) afforded the title compound (5 mg, 4% yield from **26**) as a white solid: ^{1}H NMR (400 MHz, CD_3OD) δ 8.28 (s, 1H), 8.23 (s, 1H), 5.90 (d, *J* = 6.8 Hz, 1H), 4.91–4.89 (m, 1H), 4.36 (dd, *J* = 5.2, 2.0 Hz, 1H), 4.32–4.28 (m, 1H), 3.64–3.55 (m, 2H), 3.40–3.34 (m, 3H), 3.16 (dd, *J* = 12.4, 2.0 Hz, 1H), 2.85 (dd, *J* = 12.8, 10.0 Hz, 1H), 0.81 (s, 3H), 0.78 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 157.6, 153.9, 150.0, 142.5, 121.2, 91.6, 85.9, 76.3, 74.3, 73.0, 70.2, 46.1, 45.8, 39.6, 21.8, 20.1; HRMS (ESI–) calcd for $C_{16}H_{26}N_7O_7S$ $[M–H]^-$ 460.1620, found 460.1635.

4.2 Pantothenate Synthetase Assay

4.2.1. Materials—Pantoic acid was synthesized as described by Rychlik,[42] 7-methyl-6-thioguanosine (MesG) was purchased from Berry and Associates (Dexter, MI, USA), *E. coli* TOPO and BL21(DE3) cells are from Invitrogen (Carlsbad, CA, USA), restriction enzymes and Taq polymerase are from New England Biolabs (Ipswich, MA, USA), the vectorpET28b is from EMD Biosciences (San Diego, CA, USA), the primers for PCR are from Integrated DNA Technologies (Coralville, IA, USA) and the PFU polymerase is from Agilent Technologies (Wilmington, DE, USA). All other chemicals, biological buffers, and the coupling enzymes inorganic pyrophosphatase (I1643) and purine nucleoside phosphorylase (N8264) were purchased from Sigma-Aldrich (St. Louis, MO).

4.2.2. Cloning, Expression and Purification of Recombinant PanC from *Mycobacterium tuberculosis*—PanC (Rv3602c) was amplified from the H37Rv BAC Rv222 using PFU turbo and the primers GCGAGCAACCACATCGTCAC and CGACTTCAGCATCGTCCGTAAC. The PCR product was treated with Taq using standard procedures to add 3′ overhanging adenosine residues and then cloned into pCR2.1-TOPO. The resulting plasmid (pCDD22) was used as template to amplify the expression construct using the primers GGCATATGACGATTCCTGCGTTCCATCCC and GCTCAAGCTTCAGTTTCTCCAATGTGATTCGAGGATTGCCCGG containing the restriction sites *NdeI* and *HindIII* respectively (underlined). The resulting PCR product was cloned into pCR2.1 giving plasmid pCDD23. The construct was digested using *NdeI* and *HindIII* and ligated into similarly digested pET28b yielding pCDD24. The recombinant plasmid pCDD24 was transformed into *E. coli* BL21 (DE3) electrocompetent cells and streaked-out on a Luria Bertani (LB) agar plate with 100 μg mL^{-1} of kanamycin and incubated overnight at 37 °C. A single colony was selected to inoculate 50 mL of LB with 100 μgmL^{-1} of kanamycin to be used as a starting culture.

Terrific Broth (TB) cultures (4 L) were supplemented with 100 μg mL^{-1} kanamycin, and 10 mL of an overnight starting culture added to the media. The cultures were grown at 37 °C until an OD_{600} of 0.5 and then induced with 0.5 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 30 °C. The cells were centrifuged at 6000*g* for 30 min and the pellets were stored at −80 °C. The frozen pellets were thawed in lysis buffer (50 mM HEPES, 300 mM NaCl, 10 mM imidazole, pH 8.0) and the cells were disrupted by sonication on a Branson Sonifier (5 × 2min, 30% duty cycle, power 8) and centrifuged for 30 min at 50,000*g* to remove cell debris. To the supernatant was added 1 mL of 50% Ni-

NTA agarose resin in 30% ethanol (Qiagen) and the final solution was incubated at 4 °C for 1 h. The sample was then loaded onto a gravity column and the resin was washed with 15 mL of wash buffer (50 mM HEPES, 300 NaCl, 20 mM imidazole, pH 8.0) and eluted with 2.5 mL of elution buffer (50 mM HEPES, 300 NaCl, 250 mM imidazole, pH 8.0). In order to remove the imidazole from the sample, a desalting column (PD-10, GE HealthCare, Piscataway NJ, USA) was used with the storage buffer (10 mM HEPES [pH 8.0], 1 mM EDTA, 5% glycerol). Protein concentrations were determined using ε_{280} = 15,930 M^{-1} cm^{-1} for the native PanC with a hexa-histidine tag and the final yield of this protocol provided approximately 45 mg per liter of cell culture.

4.2.3. *In vitro* Inhibition Studies—Kinetic studies to evaluate PanC inhibition of each compound were performed under initial velocity conditions using the MesG coupled assay (EnzChek pyrophosphatase assay, Invitrogen) with 400 nM PanC, 100 mM HEPES (pH 8.0), 2.4 mM β-alanine, 10 mM $MgCl_2$, 0.04 unit of pyrophosphatase, 0.1 unit of purine nucleoside phosphorylase, 0.2 mM 7-methyl-6-thioguanosine (MesG) and varying concentrations of inhibitor, pantoic acid, and ATP in a total volume of 100 μL containing up to 5% DMSO. Reactions were initiated by addition of PanC. In this coupled assay, pyrophosphate generated from the PanC reaction is converted to phosphate by pyrophosphatase. Purine nucleoside phosphorylase then catalyzes the phosphorolysis of the substrate 7-methyl-6-thioguanosine (MesG) to the chromogenic product 7-methyl-6-thioguaninethat is measured by an increase in absorbance at 360 nm. Experiments were performed in 96-well plates (UV Half Area plate with Transparent Bottom-Corning) and formation of 7-methyl-6-thioguaninewas measured at 360 nm (ε_{360} = 11,000 M^{-1} cm^{-1}) at 25 °C on a microplate reader.

For experiments to determine the apparent inhibition constants with respect to pantoic acid, the initial rates were measured at varying concentrations (50, 100, 130, 180, 240 and 300μM) of pantoic acid with 2.6 mM ATP and 2.4 mM β-alanine at different fixed levels of the inhibitors [inhibitor **1a** (0, 0.4, 0.8 and 1 μM), inhibitor **2** (0, 0.08, 0.12 and 0.16 μM), inhibitor **3** (0, 0.2, 0.4 and 0.6 μM), inhibitor **4** (0, 0.2, 0.4 and 0.5 μM) and inhibitor **5** (0, 0.8, 1.6 and 2 μM)]. Inhibition data were fit to equation 1:

$$v = \frac{V_{max}[S]}{[S] + K_m\left(1 + {}^{[I]}/_{K_i}\right)} \quad (1)$$

where *I* is the inhibitor concentration, *S* is the substrate concentration, *K*m is the Michaelis-Menten constant, V_{max} is the maximal velocity, K_i is the competitive inhibition constant.[37]

4.3. Modeling Studies

The three-dimensional structures of the aforementioned compounds were constructed using Chem. 3D ultra 12.0 software [Chemical Structure Drawing Standard; Cambridge Soft corporation, USA (2010)], which were then energetically minimized by using Tripos force field with 5000 iterations and minimum RMS gradient of 0.05 prior to docking. The crystal structures of pantothenate synthetase (*M. tuberculosis*. and *E. coli*) (PDB code: 3IVG.pdb) complex were retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/

home.do). All bound waters were eliminated from the protein and hydrogens were added to the protein. Each ligand was docked into the active site of pantothenate synthetase using the Surflex-Dock suite of SYBYL 1.3.

4.4. *Mtb* whole-cell assays

Whole-cell screening of **1–5** was carried out against wildtype H37RvMA[43] and the *panC*-depleted strain as previously described.[38] Briefly, cells were grown to an OD_{600} of ~0.2 in 7H9 medium prior to 500-fold dilution. In the case of the *panC* knockdown, the medium was supplemented with Hygromycin B (Hyg; 50 μg/mL), Kanamycin (Km; 25 μg/mL) and Gentamicin (Gm; 5 μg/mL) in order to facilitate maintenance of the regulatory vectors. Using a starting concentration of 4 mM, 2-fold serial dilutions of each inhibitor were performed in 96-well microplates containing 50 μL 7H9 medium, supplemented with Hyg (25 μg/mL), Km (12.5 μg/mL) and Gm (2.5 μg/mL) where appropriate, in both the presence and absence of anhydrotetracycline (ATc; 20 ng/mL) alone or ATc (20 ng/mL) together with pantothenate (50 μg/mL). A volume of 50 μL of the diluted cell suspension was added to each well, yielding a final volume of 100 μL per well. Plates were incubated at 37 °C and growth was observed visually following 7, 10 and 14 days of incubation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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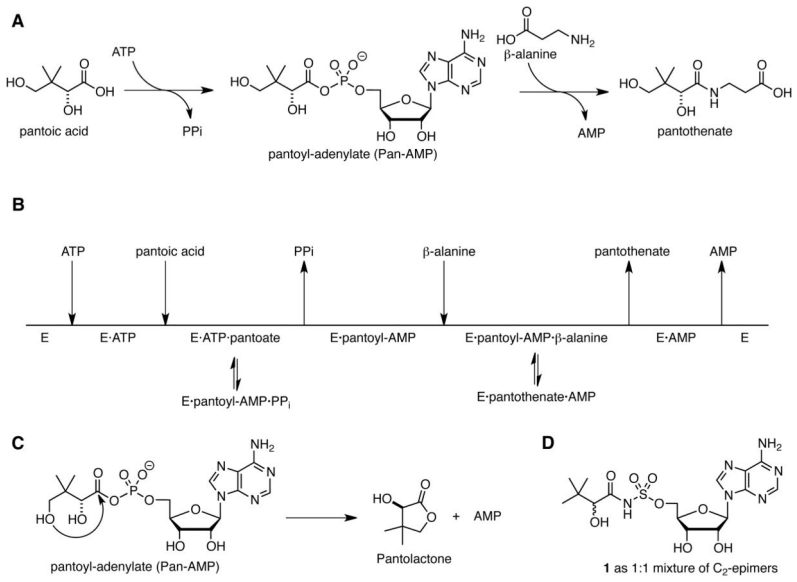
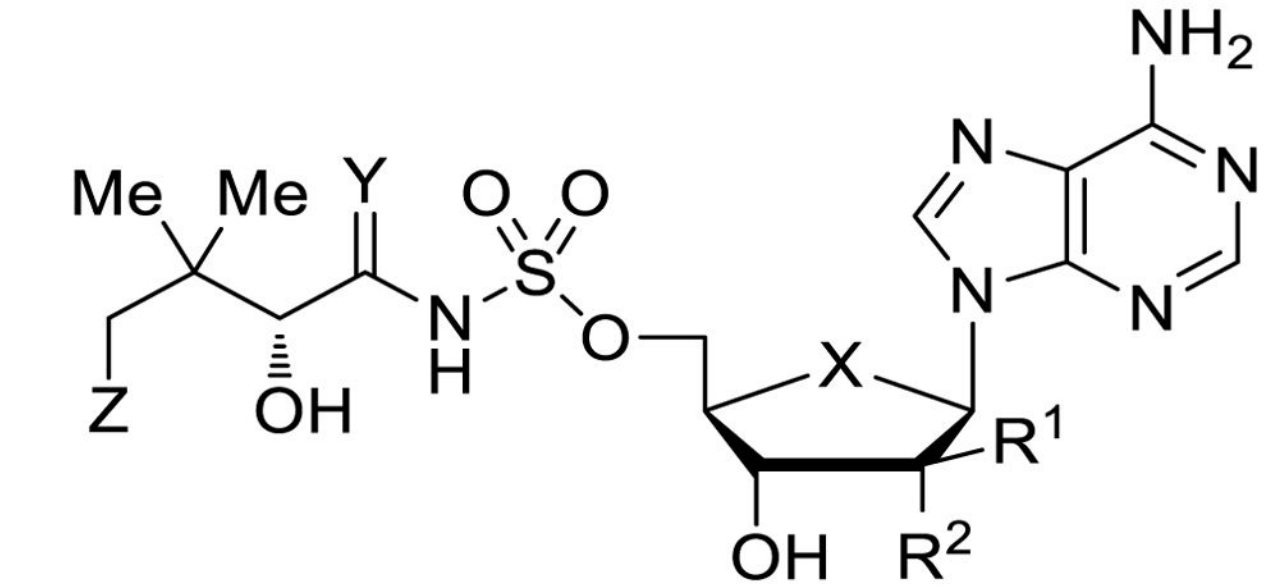


Figure 1.
Pantothenate synthetase catalyzed reactions.



1a, $R^1 = H$, $R^2 = OH$, X = Y = O, Z = H

2, $R^1 = OH$, $R^2 = H$, X = Y = O, Z = H

3, $R^1 = H$, $R^2 = F$, X = Y = O, Z = H

4, $R^1 = H$, $R^2 = OH$, $X = CH_2$, Y = O, Z = H

5, $R^1 = H$, $R^2 = OH$, X = O, $Y = H_2$, Z = OH

Figure 2.
Reaction intermediate analogues of pantoyl-adenylate

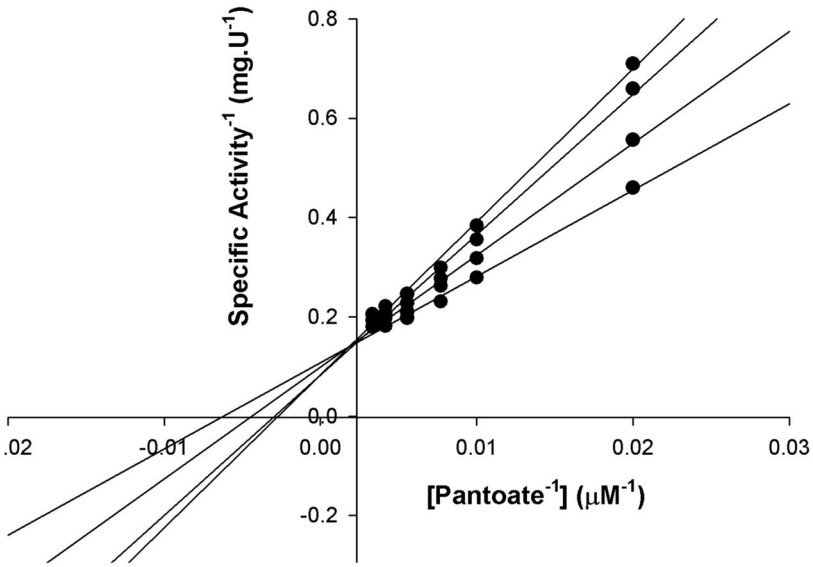


Figure 3.

Inhibition of PanC with inhibitor **1a**. Assays were performed at varying concentrations of inhibitor **1** (0–0.8 μM) and pantoic acid, fixed concentrations of ATP (2.6 mM) and β-alanine (2.4 mM).

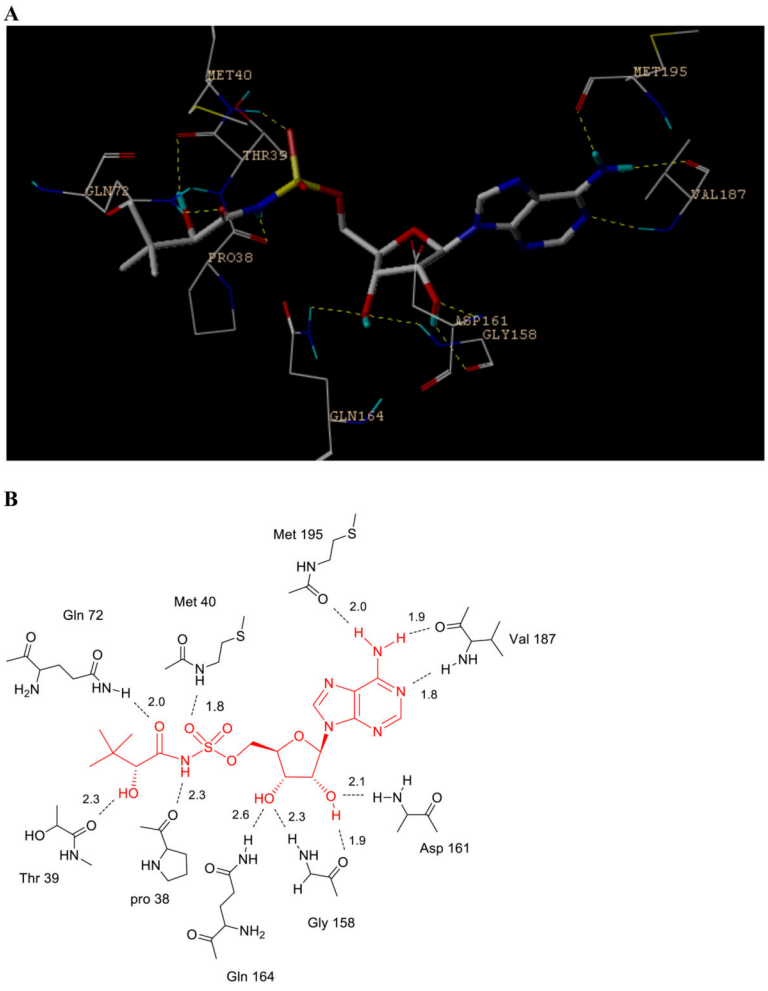
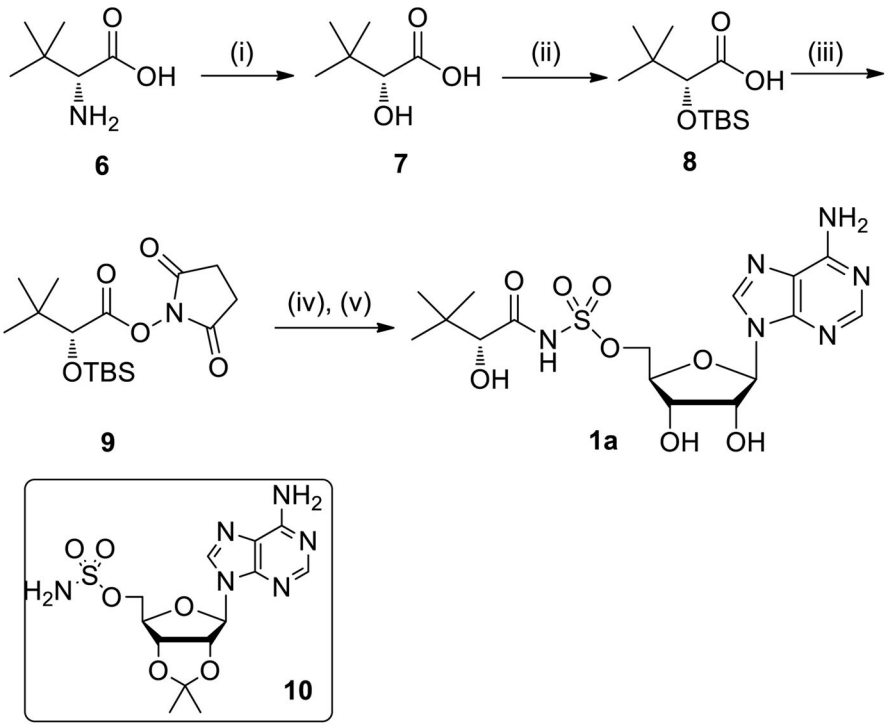


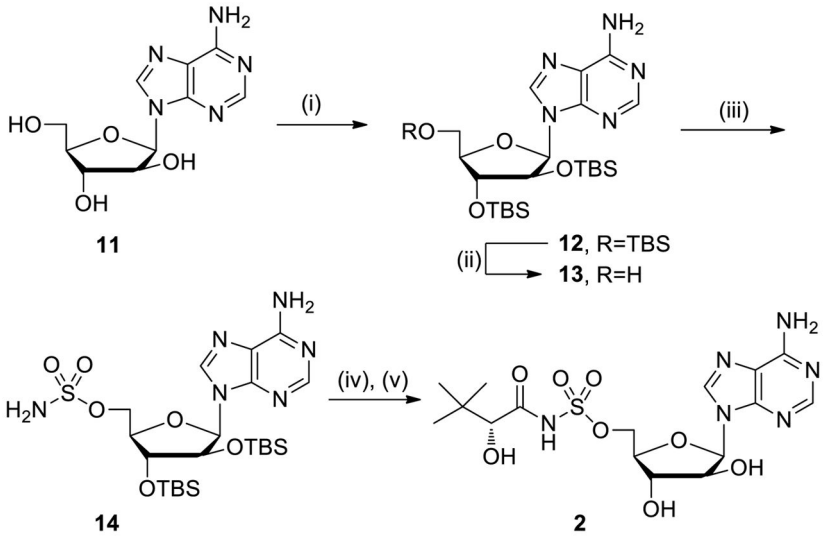
Figure 4.

Docking results of the inhibitors in the active site of the closed model of *E. coli* pantothenate synthetase. (A) The detailed binding interactions of inhibitor **1** in the active site of the protein. The inhibitor is shown as sticks with grey carbons. (B) The detailed hydrogen bonds are indicated with dashed lines and H-bond distances are given.



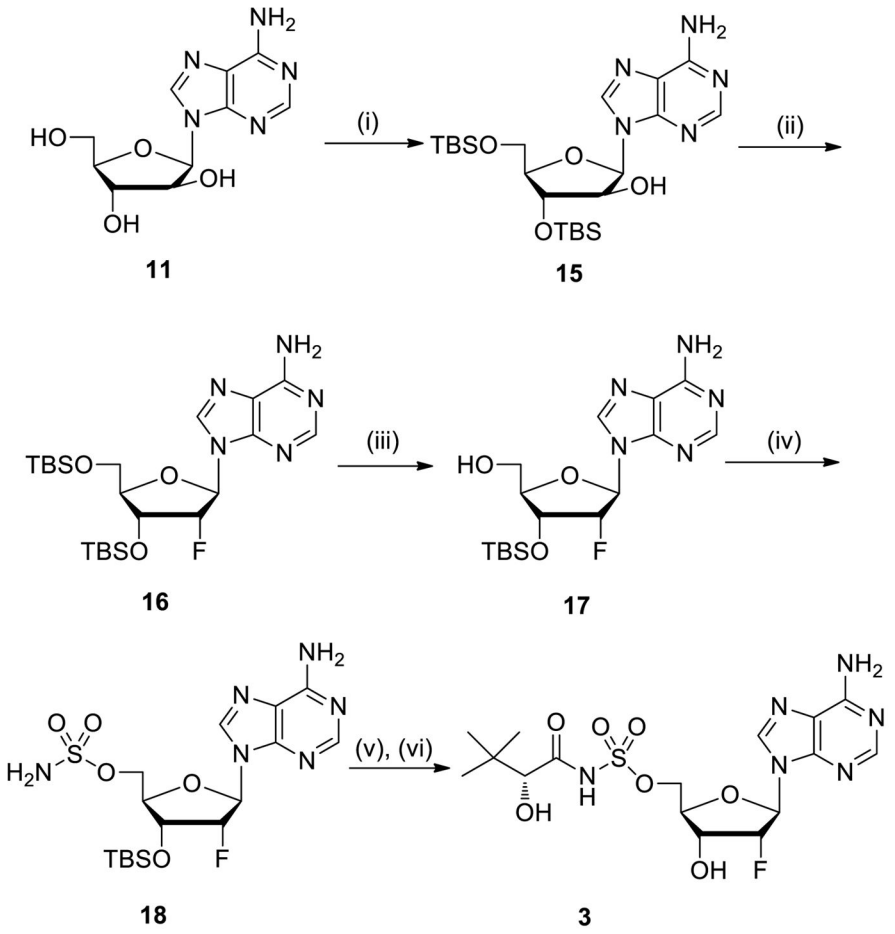
Scheme 1.

Reagents and conditions: (i) H_2SO_4, $NaNO_2$, H_2O, 0 °C→rt, 16 h, 81%; (ii) TBSCl, imidazole, DMF, 16 h, 81%; (iii) NHS, DCC, DME, 16 h, 60%; (iv) **10,** Cs_2CO_3, DMF, 24 h; (v) 4:1 TFA–H_2O, 8 °C, 48 h, 7% from **10**.



Scheme 2.

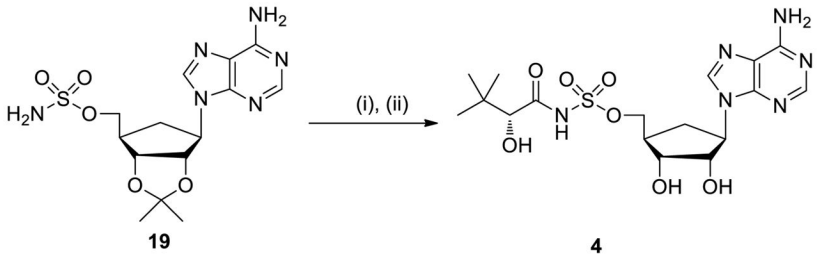
Reagents and conditions: (i) TBSCl (6.0 equiv), imidazole, DMF, 16 h, 70%; (ii) 2:1:1 H_2O–THF–TFA, 2 h, 81%; (iii) NaH, H_2NSO_2Cl, dioxane, 24 h, 60%; (iv) **9,** Cs_2CO_3, DMF, 24 h; (v) 4:1 TFA–H_2O, 20 °C, 60 h, 3% from **14**.



11 **15** **16** **17** **18** **3**

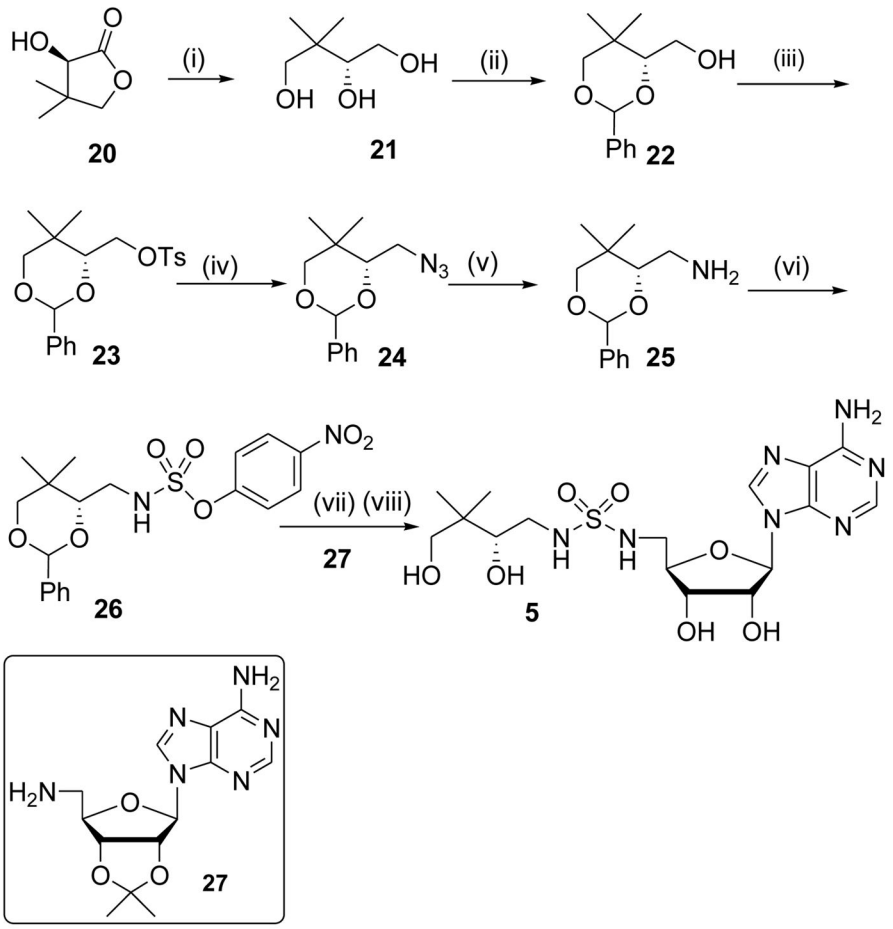
Scheme 3.

Reagents and conditions: (i) TBSCl, Et_3N, DMF, 16 h, 65%; (ii) DAST, pyridine, CH_2Cl_2, 6 h, 31%; (iii) 2:1:1 H_2O–THF–TFA, 20 min, 92%; (iv) NaH, H_2NSO_2Cl, dioxane, 16 h, 60%; (v) **9**, Cs_2CO_3, DMF, 20 h; (vi) 4:1 TFA–H_2O, 20 °C, 72 h, 4% from **18**.



Scheme 4.

Reagents and conditions: (i) **9**, Cs_2CO_3, DMF, 20 h; (ii) 4:1 TFA–H_2O, 8 °C, 48 h, 7% from **19**.



Scheme 5.

Reagents and conditions: (i) $LiAlH_4$, THF, 0 °C, 2 h, 58%; (ii) $PhCH(OEt)_2$, CSA, CH_2Cl_2, reflux, 24 h, 85%; (iii) TsCl, Et_3N, CH_2Cl_2, 16 h, 83%; (iv) NaN_3, DMF, reflux, 16 h, 93%; (v) H_2, Pd/C, MeOH, 2 h, 75%; (vi) 4-nitrophenyl chlorosulfate, CH_2Cl_2, −78 °C, 2 h, 50%; (vii) **27**, Et_3N, CH_2Cl_2, 4 Å molecular sieves; (viii) 4:1 TFA–H_2O, −35 °C, 4 h, 4% from **26**.

Table 1

Inhibition constants for **1a–5**.

Inhibitor	K_i (μM)
1a	0.27 ± 0.04
2	1.73 ± 0.24
3	0.99 ± 0.30
4	0.87 ± 0.12
5	3.47 ± 0.48