Published in final edited form as:

ChemBiochem. 2009 November 23; 10(17): 2772–2779. doi:10.1002/cbic.200900537.

A Fragment-Based Approach to Probing Adenosine Recognition Sites by Using Dynamic Combinatorial Chemistry

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Abstract

A new strategy that combines the concepts of fragment-based drug design and dynamic combinatorial chemistry (DCC) for targeting adenosine recognition sites on enzymes is reported. We demonstrate the use of 5'-deoxy-5'-thioadenosine as a noncovalent anchor fragment in dynamic combinatorial libraries templated by *Mycobacterium tuberculosis* pantothenate synthetase. A benzyl disulfide derivative was identified upon library analysis by HPLC. Structural and binding studies of protein–ligand complexes by X-ray crystallography and isothermal titration calorimetry informed the subsequent optimisation of the DCC hit into a disulfide containing the novel *meta*-nitrobenzyl fragment that targets the pantoate binding site of pantothenate synthetase. Given the prevalence of adenosine-recognition motifs in enzymes, our results provide a proof-of-concept for using this strategy to probe adjacent pockets for a range of adenosine binding enzymes, including other related adenylate-forming ligases, kinases, and ATPases, as well as NAD(P)(H), CoA and FAD(H₂) binding proteins.

Keywords

drug design; dynamic chemistry; enzymes; inhibitors; protein X-ray crystallography

Introduction

The search for new small-molecule ligands for proteins is central to chemical biology and drug discovery. Traditional high-throughput screening (HTS) methods involve large libraries that are expensive to produce, to maintain and to screen. Furthermore, HTS can identify promiscuous compounds that appear to be inhibitors in the assays because they form nonspecific aggregates in solution rather than due to their specific binding to the target protein.^[1] The shortcomings of HTS mean there is a need for other ways to identify specific

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Accession codes: The atomic coordinates and structure factors for the enzyme–ligand complexes have been deposited in the Protein Data Bank with the following accession codes: 3iob (PS-6), 3ioc (PS-14a), 3iod (PS-14b) and 3ioe (PS-18).

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/cbic.200900537>.

binding ligands, including rational design,^[2] covalent tethering^[3,4] and fragment-based methods.^[5–7]

Dynamic combinatorial chemistry (DCC) is a powerful concept in supramolecular chemistry. It involves setting up a dynamic combinatorial library (DCL) by using reversible linker chemistries between library building blocks.^[8] Equilibria relating the library members allow the product mixture to redistribute upon the addition of a stabilising template, thus producing an amplification of the best-stabilised species through specific molecular interactions. Effectively, DCC combines the synthesis and assay steps into a single procedure. Combining inhibitor discovery and DCC together into a single stream is potentially a very powerful approach that has yet to fulfil its full promise.^[9,10] The use of proteins as templates in DCLs for ligand identification has been reported; however, few examples have shown the applicability of this method to assembling noncovalent binding fragments.^[11–14] Recently, Liénard et al. reported the use of DCC coupled with mass spectrometry to identify inhibitors of a metallo- β -lactamase, relying upon the formation of a stable complex between a thiol and two functional zinc ions.^[15] Cancilla et al. have modified previous “extended tethering” procedures to find inhibitors of Aurora A kinase, thus removing the necessity for a reactive “warhead” to modify the protein, but still requiring the presence of a wild-type or introduced cysteine residue.^[16]

Herein, we describe a general DCC approach coupled with structure-based design to probe adenosine binding sites. As a model system, we used *Mycobacterium tuberculosis* pantothenate synthetase, which catalyses the final step required for pantothenate (vitamin B₅) biosynthesis.^[17] The enzyme is thought to be required for virulence in *M. tuberculosis*,^[18] so is of potential therapeutic interest. Pantothenate synthetase catalyses the formation of pantothenate (**1**) from pantoate (**2**), β -alanine (**3**) and ATP (**4**) via formation of a pantoyladenylate intermediate **5**.^[19] A crystal structure of the pantoyladenylate intermediate bound in the active site of the *M. tuberculosis* enzyme has been solved.^[20] More recently, Ciulli et al. reported the cocrystal structures and binding characterisation of three potent sulfonamide inhibitors that mimic the pantoyladenylate reaction intermediate.^[21]

Results and Discussion

The strategy for our DCC experiments was to use 5'-deoxy-5'-thioadenosine (**6**) as an anchor building block and look for thiols that would form disulfides templated by pantothenate synthetase. The approach was based in part on the success of inhibitors containing the adenosine fragment rationally designed from the pantoyladenylate intermediate **5**,^[21] Scheme 1. The expectation was that, under suitable conditions, thiol–disulfide exchange would produce a library of disulfides of the heterodimer structure **7**. This exchange takes place under mild physiological conditions in weakly basic aqueous solution and can be stopped by acidifying the solution. We envisaged that heterodisulfides formed with thioadenosine **6** would be capable of accessing the pockets adjacent to the adenosine binding site, namely the pantoate and phosphate binding sites of the protein.

Synthesis, biophysical and structural studies of 5'-deoxy-5'-thioadenosine

5'-Deoxy-5'-thioadenosine **6** was synthesised according to a previously published procedure.^[22] From the commercially available acetonide-protected nucleotide **9**, a Mitsunobu reaction with thioacetic acid effected the transformation of the 5'-alcohol to the thioacetate **10** (Scheme 2). The acetonide **10** was then deprotected with a TFA/water mixture to give **11**, and the acetate group was removed with a methanolic ammonia solution to afford the thiol **6**. In order to investigate the feasibility of using thiol **6** as a DCL building block and hence the likelihood of asymmetric disulfides **7** based on **6** acting as ligands, the ability of thioadenosine **6** to bind noncovalently to *M. tuberculosis* pantothenate synthetase was assessed. A ¹H NMR WaterLOGSY experiment^[23,24] was used to show binding of ligand **6** to *M. tuberculosis* pantothenate synthetase (Figure 1A–D). The purine protons were distinct from other parts of the spectra and could be easily monitored (Figure 1A). In the absence of protein, the WaterLOGSY signals from the purine protons were negative, as expected (Figure 1B). In the presence of protein, the signals became strongly positive, that is, indicative of protein–ligand binding (Figure 1C). Upon addition of ATP, the purine signals of **6** were significantly diminished; this suggests competition with ATP for the ATP binding site (Figure 1D). The binding of thiol **6** to pantothenate synthetase was characterised quantitatively by using isothermal titration calorimetry (ITC), performed under low-*c*-value conditions.^[24] The thiol was titrated directly into a solution of protein, and a *K*_D of 380 μM was measured (Figure 1E). A titration of ATP into the resulting solution containing protein and **6** was then performed. A comparison of this titration with one in which ATP was titrated against enzyme in the absence of **6**, but under otherwise identical conditions, clearly indicated a competition between **6** and ATP for the same binding site (see Figure S1 in the Supporting Information). This was subsequently confirmed in the X-ray structure of thioadenosine bound in the active site of *M. tuberculosis* pantothenate synthetase (Figure 1F). Protein crystals of *M. tuberculosis* pantothenate synthetase were soaked with compound **6**, and the structure was solved by X-ray crystallography to a resolution of 1.8 Å (Table S1). Thioadenosine **6** was found to bind in the anticipated conformation, superimposing well with the ribonucleotide fragment of the pantoiladenylate intermediate.^[21] As expected, the interactions made between ligand **6** and the protein active site were very similar to those involved in binding the ribonucleotide moiety of the pantoiladenylate intermediate. In addition to **6**, an ion of sulfate and a molecule of glycerol from the crystallisation buffer were revealed to bind in the phosphate and pantoate pockets, respectively (Figure 1F).

DCL design and analysis by HPLC

Inspection of the crystal structure of thioadenosine **6** in complex with the protein suggested that disulfides formed with **6** would either be able to link to the phosphate binding pocket or bridge into the pantoate/glycerol binding site (Figure 1F). Aside from the polar interactions between the hydroxyl and carbonyl groups of pantoate and the side chains Gln72 and Gln164, the pantoate pocket is principally a hydrophobic pocket, lined with residues Pro38, Met40, Phe67, Val139, Val142, Val143 and Phe157. In contrast, the phosphate pocket is very polar, binding the magnesium phosphate ATP complex, with interactions dictated largely by electrostatics and hydrogen bonding between His44, His47, Lys160 and Ser197. On the basis of these observations, compounds **8a–d** were selected as hydrophobic thiols

likely to bind in the pantoate pocket, whilst thiols **8e–h** were selected for their potential ability to participate in charge–charge and hydrogen-bond interactions with residues within the phosphate binding site (Scheme 1).

In order to facilitate the rapid exchange of DCL members, a glutathione redox buffer was used.^[25–27] Under the exchange conditions (Tris buffer at pH 8.5 under an inert atmosphere), it was found that libraries equilibrated within 24 h (data not shown). In order to only observe the species containing anchor thiol **6**, the library mixtures were separated by HPLC and monitored by UV at 260 nm, the absorption maximum of **6**. The library of thiols **8a–h** does not absorb significantly at this wavelength. This selective monitoring greatly simplifies the library analysis and identification of the best binder. The identities of the DCL components were assigned by comparing retention times against control experiments. As the thiol–disulfide exchange reactions were performed in the absence of oxygen, the redox state of the system was defined at the start of the experiment by the ratio of reduced to oxidised glutathione. This ratio was set at 4:1, giving a bias towards thiols. Under these conditions, any amplified disulfides must be reasonable binders to the template, competing with weaker binding disulfides and thiols. This stringent selection helped to allow only the best disulfides to be amplified from a mixture of thiols and disulfides. Furthermore, it has been suggested that keeping the equilibrium of the untemplated library in favour of the monomers is a good strategy to ensure optimal amplification.^[28]

In one set of experiments, thiols **6** and **8a–h** were mixed in a glutathione redox buffer and allowed to exchange under anoxic conditions with and without protein as a template. After 24 h, the DCL mixtures were quenched with acid and filtered by ultrafiltration, and the libraries were analysed by HPLC (Figure 2). In the absence of protein, the major component of the library was the adduct thiol **6** formed with glutathione, present as 63% of the detected species. From a comparison of the DCLs equilibrated in the absence and presence of pantothenate synthetase, two peaks were found to be amplified in the presence of protein, thiol **6** and disulfide **14a**, formed between **6** and benzyl thiol **8a**. Docking studies suggested that the DCC hit **14a** would bind with the benzyl group buried in the hydrophobic pantoate pocket, and that some substituents might be tolerated on the aromatic ring. This hypothesis was subsequently confirmed by solving the X-ray crystal structure of the enzyme with bound disulfide **14a** (see below).

Synthesis, biophysical and structural studies of disulfide derivatives of thioadenosine

Based on these observations, analogues of the DCC hit **14a** were prepared. In addition, the presence of glycerol bound in the pantoate pocket of the protein crystal structure of **6** suggested that the disulfide **14c** and thioether **18**, formed by linking glycerol mimics to **6**, would be interesting compounds to investigate. To make these compounds, a general synthetic route to the novel asymmetric disulfides containing the adenosine building block **6** was devised (Scheme 2). The efficient synthesis of asymmetric disulfides can be a significant synthetic challenge, usually best effected by activation and isolation of one of the thiols followed by subsequent treatment with a second thiol.^[29] 4-(Pyridin-2-ylsulfanyl)morpholine (**12**) was synthesised from the chloroamine of morpholine, which was then treated with 2-mercaptopyridine. Reaction of the activated 2-thiopyridone **12** and thiol

6 gave the asymmetric 2-thiopyridyl disulfide **13**, which was sufficiently reactive to form the desired asymmetric disulfides **14a–c** in reasonable yields (65–89%). In addition, the primary alcohol **15** was converted to the alkyl bromide **16** by an Appel reaction, and **6** was alkylated to provide the diol-protected thioether **17**. Subsequent deprotection of the acetonide moiety under acid conditions gave the thioether **18**.

The binding of **14a–c** and **18** to the protein was assessed by ITC, and results were compared with those for **6**, as summarised in Table 1. DCC hit **14a** displayed a K_D of 210 μM , an approximately twofold improvement over thiol **6**. The introduction of a *meta*-nitro group in **14b** led to a further decrease in K_D to 80 μM . The designed glycerol-linked compounds **14c** and **18** (prepared as diastereomeric mixtures) bound to the protein less well than the original thiol **6**, possibly due to the introduction of the flexible glyceroyl side chain.

To better understand the compounds' binding modes, the heterodisulfides **14a** and **14b** and thioether **18** were soaked into protein crystals of *M. tuberculosis* pantothenate synthetase, and the structures of the complexes were solved to 2.50, 1.75 and 1.95 Å of resolution, respectively (Table S1). The omit electron density maps ($F_o - F_c$) showed clearly defined binding modes for all three ligands (Figure S2). In all cases, the position of the adenosine moiety was identical to that of **6**, and the different substituents were all placed in the pantoate pocket. Analysis of the electron-density map of the enzyme:**18** complex identified the bound ligand as the diastereoisomer with the R configuration at the chiral hydroxyl position (Figure S3). This configuration allows the glyceroyl side chain of **18** to superpose very well with the glycerol molecule bound in the enzyme:**6** complex, and to reproduce the key hydrogen bonds between the two hydroxyl groups and Gln72 and Gln164.^[21] No significant changes in the position of side chains and water molecules were observed in the active site. The benzyl substituent of compound **14a** interacts well with all of the hydrophobic residues in the pantoate pocket, thus contributing to the increase in affinity observed relative to **6**. However, the hydrogen bonds with Gln72 are now unsatisfied, pushing away the Gln72 side chain by approximately 3.5 Å. Finally, the X-ray structure of **14b** identified a favourable hydrogen bond between the benzyl *meta*-nitro group and a water molecule deep in the pantoate pocket (Figure 3). This molecule of water is conserved in all published crystal structures of pantothenate synthetase, and appears to be structurally important by forming a hydrogen bond network between the terminal hydroxyl of pantoate, the backbone carbonyls of Phe67 and Thr39, and the hydroxyl side chain of Ser65. To accommodate this important interaction, the benzyl ring of **14b** must be placed in a position that would clash with Gln72. As a result, electron density is lost for the flexible active site loop (residues 71–85), thus suggesting that this is displaced into a disordered conformation.

Conclusions

In conclusion, we have demonstrated the use of 5'-deoxy-5'-thioadenosine **6** as a building block in a dynamic combinatorial library templated with an adenosine-binding enzyme, *M. tuberculosis* pantothenate synthetase. The best binding component based upon thiol **6** was identified as the benzyl disulfide **14a**, and structural binding data informed its subsequent optimisation into derivative **14b**, which contained the novel *meta*-nitrobenzyl fragment binding at the pantoate site. The cocrystal structures here presented provide a structural basis

for targeting this binding pocket and will inform future inhibitor design. Furthermore, this is the first demonstration of a strategy combining DCC and fragment-based approaches for identifying novel adenosine-containing heterodisulfides that bind noncovalently to a target protein. Given the prevalence of adenosine-recognition motifs in enzymes, the approach here described could be more widely applicable as a tool to probe adjacent pockets for a range of other adenosine-binding enzymes, including other related adenylate-forming ligases, kinases and ATPases, as well as NAD(P)(H), CoA and FAD(H₂) binding proteins.

Experimental Section

Synthesis

All reactions were performed at ambient temperature unless stated otherwise. All reagents were purchased from commercial sources and used as supplied. The organic solvents were freshly distilled over the appropriate drying agent prior to use. NMR spectra (¹H and ¹³C) were recorded on either a Bruker AM-400 or an Avance-500 spectrometer in deuterated solvents as indicated. Analytical thin-layer chromatography (TLC) was performed on commercially prepared silica plates (Kieselgel 60 0.25 mm). Flash chromatography was performed by using 230–400 mesh Kieselgel 60 silica eluting with distilled solvents as described. Liquid chromatography mass spectrometry (LCMS) was carried out on an Alliance HT Waters 2795 Separations Module coupled to a Waters Micromass ZQ Quadrupole Mass Analyser. Samples were run on a gradient from ammonium acetate (10 mM) containing 0.1% formic acid to 95% acetonitrile over a period of 8 min. High-resolution mass spectrometry was carried out on a Micromass Quadrupole-Time of Flight (Q-Tof) spectrometer.

4-(Pyridin-2-ylsulfanyl)morpholine (**12**):^[29]

Morpholine (1.58 mL, 18.0 mmol) was added to aqueous sodium hypochlorite solution (50 mL). The reaction mixture was stirred at room temperature for 5 min, then extracted with chloroform (3×50 mL). The organic extracts were treated with morpholine (6.5 mL) and cooled to 4 °C. 2-Mercaptopyridine (1.0 g, 9.0 mmol) was added, and the mixture was stirred for 15 min. The reaction mixture was washed with saturated aqueous NaHCO₃ (80 mL), water (3×50 mL) and brine (50 mL), then dried over MgSO₄, and the solvent was removed in vacuo. The residue was purified by flash column chromatography (diethyl ether/hexane 1:1) to give the product as a clear oil (710 mg, 40%). *R*_f [EtOAc]=0.21; ¹H NMR (400 MHz, CDCl₃): δ=8.40 (dd, *J*=4.9, 1.1 Hz, 1H; ArH-6), 7.58 (td, *J*=7.8, 1.1 Hz, 1H; ArH-4), 7.42 (dd, *J*=7.8 Hz, 1.1 Hz, 1H; ArH-3), 6.96 (ddd, *J*=7.8, 4.9, 1.1 Hz, 1H; ArH-5), 3.75 (t, *J*=5.0 Hz, 4H; 2CH₂), 3.22 (t, *J*=5.0 Hz, 4H; 2CH₂); ¹³C NMR (100 MHz, CDCl₃): δ=164.6, 149.8, 136.9, 119.9, 118.5, 68.3, 56.6

5'-(Pyridin-2-ylthio)-5'-deoxyadenosine (**13**)

4-(Pyridin-2-ylsulfanyl)morpholine (**12**; 69 mg, 0.35 mmol) and 5'-deoxy-5'-thioadenosine (**6**; 100 mg, 0.35 mmol) were dissolved in chloroform (1.5 mL) and acetic acid (1 mL). After 40 min, the solvent was removed in vacuo, and the crude residue was purified by flash column chromatography (CHCl₃/MeOH 4:1) to give the product as a white solid (72 mg, 52%). *R*_f [CHCl₃/MeOH 4:1]=0.30; ¹H NMR (400 MHz, CO(CD₃)₂): δ=8.41 (dd, *J*=4.8, 1.7

Hz, 1H; ArH-6), 8.18 (s, 1H; H-8), 8.16 (s, 1H; H-2), 7.80 (dd, $J=8.1, 1.1$ Hz, 1H; ArH-3), 7.73 (ddd, $J=8.1, 7.3, 1.7$ Hz, 1H; ArH-4), 7.17 (ddd, $J=7.3, 4.8, 1.1$ Hz, 1H; ArH-5), 6.63 (brs, 2H; NH₂), 5.99 (d, $J=5.0$ Hz, 1H; H-1'), 5.01 (t, $J=5.0$ Hz, 1H; H-2'), 4.51 (dd, $J=5.0, 4.4$ Hz, 1H; H-3'), 4.28 (td, $J=6.5, 4.4$ Hz, 1H; H-4'), 3.34 (d, $J=6.5$ Hz, 2H; H-5'); ¹³C NMR (100 MHz, CO(CD₃)₂): $\delta=165.1, 163.9, 160.3, 156.8, 153.3, 150.0, 140.6, 137.8, 121.3, 119.7, 89.7, 83.2, 73.9, 73.8, 42.5$; LCMS ($[M+H]^+=393.0$), $t_R=3.03$ min; HRMS (+ESI) calcd for C₁₅H₁₇N₆O₃S₂: 393.0799 $[M+H]^+$, found: 393.0804.

5'-(Benzyldithio)-5'-deoxyadenosine (14a)

Benzyl mercaptan (3 μ L, 0.03 mmol) was added to a solution of 5'-(pyridin-2-ylthio)-5'-deoxyadenosine (**13**; 9.8 mg, 0.03 mmol) in methanol (1 mL). After 1 h of stirring at room temperature, the solvent was removed, and the residue was purified by flash column chromatography (CHCl₃/MeOH 9:1) to give the product as a white solid (9 mg, 89%). R_f [CHCl₃/MeOH 9:1]=0.23; ¹H NMR (400 MHz, CDCl₃): $\delta=8.28$ (s, 1H; H-8), 7.96 (s, 1H; H-2), 7.32–7.23 (m, 5H; Ph), 5.84 (d, $J=6.0$ Hz, 1H; H-1'), 5.75 (s, 2H; NH₂), 4.47–4.41 (m, 2H; CH), 4.31 (m, 1H; CH), 3.91 (s, 2H; PhCH₂), 2.56 (m, 2H; H-5'); ¹³C NMR (125 MHz, CD₃OD): $\delta=157.3, 153.9, 150.7, 141.6, 138.9, 130.4, 129.5, 128.4, 120.7, 90.1, 84.8, 74.7, 74.2, 44.3, 42.5$; LCMS ($[M+H]^+=406.0$), $t_R=3.43$ min; HRMS (+ESI) calcd for C₁₇H₂₀N₅O₃S₂: 406.1008 $[M+H]^+$, found: 406.1017.

5'-(3-Nitro benzyldithio)-5'-deoxyadenosine (14b)

3-Nitrobenzyl mercaptan (5.4 μ L, 0.04 mmol) was added to a solution of 5'-(pyridin-2-ylthio)-5'-deoxyadenosine (**13**; 16 mg, 0.04 mmol) in methanol (1 mL). After 40 min of stirring at room temperature, the solvent was removed, and the residue was purified by flash column chromatography (CHCl₃/MeOH 9:1) to give the product as a white solid (13 mg, 72%). R_f [CHCl₃/MeOH 9:1]=0.16; ¹H NMR (400 MHz, CD₃OD): $\delta=8.29$ (s, 1H; H-8), 8.23 (s, 1H; H-2), 8.20 (t, $J=1.9$ Hz, 1H; ArH), 8.13 (ddd, $J=8.2, 2.2, 1.0$ Hz, 1H; ArH), 7.70 (dt, $J=7.6, 1.6$ Hz, 1H; ArH), 7.54 (t, $J=7.9$ Hz, 1H; ArH), 6.01 (d, $J=5.3$ Hz, 1H; H-1'), 4.30 (m, 1H; CH), 4.25–4.21 (m, 1H; CH), 4.04 (s, 2H; ArCH₂), 3.02 (dd, $J=14.0, 5.2$ Hz, 1H; H-5'), 2.95 (dd, $J=14.0, 7.38$ Hz, 1H; H-5'); ¹³C NMR (125 MHz, CD₃OD) 156.6, 152.7, 150.6, 149.6, 142.1, 141.6, 136.7, 130.7, 125.1, 123.2, 90.3, 84.5, 74.7, 74.2, 42.6, 42.6; LCMS ($[M+H]^+=450.9$), $t_R=3.39$ min; HRMS (+ESI) calcd for C₁₇H₁₉N₆O₅S₂: 451.0858 $[M+H]^+$, found: 451.0867.

5'-(2,3-Dihydroxypropyldithio)-5'-deoxyadenosine (14c)

3-Mercaptopropane-1,2-diol (2.2 μ L, 0.02 mmol) was added to a solution of 5'-(pyridin-2-ylthio)-5'-deoxyadenosine (**13**; 10 mg, 0.02 mmol) and NaOMe (19 μ L, 1.0 M, 0.02 mmol) in methanol (1 mL). After 1 h of stirring at room temperature, the solvent was removed, and the residue was purified by flash column chromatography (CHCl₃/MeOH 4:1) to give the diastereomeric products as a white solid (9 mg, 65%). *Mixture of two diastereoisomers*: R_f [CHCl₃/MeOH 4:1]=0.19; ¹H NMR (400 MHz, CD₃OD): $\delta=8.29$ (s, 1H; H-8), 8.19 (s, 1H; H-2), 5.98 (m, 1H; H-1'), 4.86 (m, 1H; CH), 4.35–4.29 (m, 1H; CH), 3.89–3.82 (m, 2H; CH₂), 3.21 (m, 1H; CH), 3.12 (m, 1H; CH), 2.76 (m, 2H; CH₂S), 2.64 (dd, $J=13.6, 4.8$ Hz, 1H; CH₂S), 2.58 (dd, $J=13.6, 6.4$ Hz, 1H; CH₂S); ¹³C NMR (125 MHz, CD₃OD): $\delta=157.4$,

153.9, 153.9, 150.7, 141.7, 141.5, 120.7, 120.6, 90.2, 90.0, 86.8, 84.8, 74.9, 74.5, 74.2, 73.4, 65.9, 65.3, 43.6, 28.3, 23.7; LCMS ($[M+H]^+$ = 390.1), t_R =2.51 min; HRMS (+ESI) calcd for $C_{13}H_{19}N_5O_5S_2$: 412.0725 $[M+Na]^+$, found: 412.0734.

4-(2-Bromoethyl)-2,2-dimethyl-[1,3]dioxolane (16):^[30]

Triphenyl phosphine (1.20 g, 7.4 mmol) and carbon tetrabromide (1.52 g, 7.4 mmol) were added to 2-(2,2-dimethyl-[1,3]dioxolan-4-yl)ethanol (500 μ L, 3.53 mmol) in acetonitrile (11 mL) at 4 °C. The mixture was allowed to warm to room temperature and stirred for 2.5 h. The crude reaction mixture was concentrated under reduced pressure and purified by flash column chromatography (40–60 petroleum ether, then 40–60 petroleum ether/EtOAc 9:1) to give the product as a clear oil (580 mg, 81%). R_f [40–60 petroleum ether/EtOAc 9:1]=0.70; 1H NMR (400 MHz, CD_3Cl_3): δ =4.24 (m, 1H; H-4), 4.06 (dd, J =7.9, 6.1 Hz, 1H; H-5), 3.77 (td, J =5.8, 1.3 Hz, 2H; CH_2Br), 3.57 (t, J =7.8 Hz, 1H; H-5), 1.80 (m, 2H; CH_2CH_2Br), 1.40 (s, 3H; CH_3), 1.34 (s, 3H; CH_3).

5'-((2-(2,2-Dimethyl-[1,3]dioxolan-4-yl)ethyl)thio)-5'-deoxyadenosine (17)

A solution of 5'-deoxy-5'-thioadenosine **6** (87 mg, 0.31 mmol) and NaOMe (17 mg, 0.31 mmol) in methanol (10 mL) was degassed by being repeatedly frozen with liquid N_2 and allowed to thaw under vacuum (3 $\times$). 4-(2-Bromoethyl)-2,2-dimethyl-[1,3]dioxolane (**16**; 65 mg, 0.31 mmol) was added, and the reaction mixture was stirred under Ar for 21 h. The solvent was removed in vacuo, and the crude residue was purified by flash column chromatography (DCM/MeOH 85:15) to give the diastereomeric products as a white solid (60 mg, 48%). *Mixture of two diastereoisomers*: R_f [DCM/MeOH 9:1]=0.15; 1H NMR (500 MHz, CD_3OD): δ =8.30 (s, 1H; H-8), 8.20 (s, 1H; H-2), 6.00 (d, J =5.1 Hz, 1H; H-1'), 4.80 (t, J =5.1 Hz, 1H; H-2'), 4.34 (t, J =5.1 Hz, 1H; H-3'), 4.20 (m, 1H; H-4'), 4.10–4.03 (m, 1H; CH), 3.95 (dd, J =8.0, 6.1 Hz, 1H; CH), 3.44–3.39 (m, 1H; CH), 2.99–2.90 (m, 2H; H-5'), 2.68–2.53 (m, 2H; CH_2S), 1.78–1.68 (m, 2H; CH_2CH_2S), 1.29 (s, 3H; CH_3), 1.26 (s, 3H; CH_3); ^{13}C NMR (125 MHz, CD_3OD): δ =157.3, 153.9, 153.9, 150.7, 150.7, 141.5, 120.57, 110.0, 90.2, 86.0, 85.9, 76.1, 76.1, 74.8, 74.1, 74.0, 70.0, 70.0, 35.3, 35.2, 34.9, 34.9, 30.0, 30.0, 27.2, 25.9; LCMS ($[M+H]^+$ =412.2), t_R =2.92 min; HRMS (+ESI) calcd for $C_{17}H_{26}N_5O_5S$: 412.1656 $[M+H]^+$, found: 412.1655.

5'-(3,4-Dihydroxybutylthio)-5'-deoxyadenosine (18)

5'-((2-(2,2-Dimethyl-[1,3]dioxolan-4-yl)ethyl)thio)-5'-deoxyadenosine (**17**; 9 mg, 0.02 mmol) was added to a mixture of water (1 mL) and TFA (1 mL) at 4 °C. The reaction mixture was allowed to warm to room temperature and stirred for 1 h. The solvents were removed in vacuo, and trace amounts of TFA were removed by azeotroping with absolute ethanol (5 $\times$ 30 mL). After being dried in vacuo, the two diastereomeric products were isolated as a white solid (8 mg, 93%). *Mixture of two diastereoisomers*: 1H NMR (500 MHz, CD_3OD): δ =8.48 (s, 1H; ArH), 8.35 (s, 1H; ArH), 6.03 (m, 1H; H-1'), 4.72 (t, J =5.0 Hz, 1H; H-2'), 4.30 (t, J =5.0 Hz, 1H; H-3'), 4.21 (m, 1H; CH), 3.63 (m, 1H; CH), 3.40 (m, 1H; CH), 3.28 (qn, J =1.6 Hz, 1H; CH_2CH), 2.97 (m, 1H; H-5'), 2.89 (dd, J =14.1, 5.8 Hz, 1H; H-5''), 2.74–2.67 (m, 1H; CH_2CH_2S), 2.65–2.58 (m, 1H; CH_2CH_2S), 1.77–1.70 (m, 1H; CH_2CH_2S), 1.59 (m, J =1.4 Hz, 1H; CH_2CH_2S); ^{13}C NMR (125 MHz, CD_3OD): δ =153.0,

150.2, 147.1, 147.0, 143.6, 143.6, 120.6, 90.4, 86.0, 75.3, 75.3, 73.9, 73.9, 71.9, 71.8, 67.2, 35.3, 35.2, 34.6, 34.5, 30.1, 30.1; LCMS ($[M+H]^+$ = 372.1), t_R = 2.42 min; HRMS (+ESI) calcd for $C_{14}H_{21}N_5O_5S_2Na$: 394.1161 $[M+Na]^+$, found: 394.1175.

Protein expression and purification

The *M. tuberculosis* pantothenate synthetase His₆-tagged (His₆-PS, for DCL and biophysical solution studies) and untagged (for X-ray crystallography) proteins were expressed and purified as previously described.^[21]

Dynamic combinatorial chemistry

All buffered stock solutions were prepared in MgCl₂ (5 mM), NaCl (50 mM) and Tris buffer (50 mM, pH 8.5). Each thiol, including 5'-deoxy-5'-thioadenosine (**6**), was diluted to give a final concentration of 200 μM. Stock solutions of reduced and oxidised glutathione were freshly prepared in buffer and used at 1.5 mM and 375 μM, respectively. When present, *M. tuberculosis* pantothenate synthetase was used at a final concentration of 190 μM to make a final volume of 25 μL for each DCL. DCLs were prepared in PCR tubes and placed in a round-bottomed flask. Oxygen was removed by repeated cycles of placing the flask under vacuum and refilling with argon. Thiol–disulfide exchange was quenched by the addition of TFA (75 μL, 0.1%, v/v). Denatured protein was removed from the DCL by centrifugal filtration with a Vivaspin 10 kDa PES membrane, spinning at 11700g for 10 min. The protein-free filtrate was then analysed by HPLC at 260 nm by using a 5 μ C18(2) 150×4.60 mm column. The mobile phases were water, 0.1% TFA (eluent A) and acetonitrile, 0.1% TFA (eluent B). Eluent B was held at 2% for 5 min, increased to 12.5% over 15 min, held at this concentration for a further 5 min, increased to 100% over 15 min, then returned to 2% over 5 min and held for a further 10 min at 2%.

WaterLOGSY NMR spectroscopy

¹H NMR experiments were performed at 298 K on a 500 MHz Bruker NMR spectrometer equipped with a 5 mm triple TXI cryoprobe with z gradients. WaterLOGSY experiments employed a 20 ms selective Gaussian 180° pulse at the water frequency and an NOE mixing time of 1 s. The resulting spectra were analysed with Bruker TopSpin software. Samples of 5'-deoxy-5'-thioadenosine (500 μM) in HEPES (20 mM, pH 7.5), NaCl (20 mM), MgCl₂ (10 mM) in D₂O (10% v/v) were prepared with and without *M. tuberculosis* His₆-PS (12 μM) to a total volume of 700 μL. Competition experiments with ATP were performed with a final ATP concentration of 330 μM. [D₄](Trimethylsilyl)propionic acid (TSP; 20 μM) was present for calibration purposes.

Isothermal titration calorimetry

ITC experiments were performed at 25 °C with a MicroCal VP-ITC instrument, and all data were analysed with the software implemented in Origin (version 7). In all titrations, *M. tuberculosis* His₆-PS was at a concentration of 30 μM and buffered in HEPES (50 mM, pH 7.6), MgCl₂ (5 mM) and NaCl (50 mM). ATP and 5'-deoxy-5'-thioadenosine (**6**) were prepared and used in the same buffer, whereas **14a**, **14b**, **14c** and **18** were diluted from DMSO stocks into buffer to give a final concentration of DMSO of 5–15% (v/v). Care was

taken to match the concentration of DMSO in the ligand and protein samples as closely as possible. In a typical experiment, enzyme (30 μM) was loaded in the sample cell, and a total of 28 injections of 10 μL were made at 3–4 min intervals from a 300 μL syringe rotating at 300 rpm and loaded with ligand solution (1–4 mM). In all titrations, an initial injection of 2 μL ligand was made, and these data were discarded during data analysis. Control titrations of ligand to buffer were performed and subtracted from the ligand-to-protein titrations. The thermodynamic parameters were obtained by fitting the data to a single-site binding model with a stoichiometry of 1, as accurately determined from titrations with ATP under high c values.

Protein X-ray crystallography

Crystallisation of pantothenate synthetase was carried out as previously described.^[21] Briefly, the purified cleaved protein was exchanged into HEPES (5 mM, pH 7.6), NaCl (10 mM) and then concentrated to 20 mg mL^{-1} . Crystals were grown by the hanging-drop vapour diffusion method at 20 °C by mixing 1 μL of protein with an equal volume of a well solution containing PEG3000 (11–14%, w/v), Li_2SO_4 (100–150 mM), imidazole (100 mM, pH 8.0), ethanol (2–4%, v/v), glycerol (10%, v/v) and MgCl_2 (20 mM). Soaking of **6** and **18** was carried out overnight by adding well solution containing the ligand (1.5 μL) to give a final concentration of 5 (**6**) and 3.75 mM (**18**) in DMSO (5%, v/v). To perform the remaining soaking experiments, the crystals were transferred into a soak solution (2 μL) containing PEG3000 (14%, w/v), LiCl (0.15 M), imidazole (0.1 M, pH 8.0), and ethanol (4%, v/v). Crystals were then soaked overnight with soak solution containing the ligands (2 μL) to give a final concentration of 5 (**14a**) or 2.5 mM (**14b**) in DMSO (10%, v/v). The crystals were cryoprotected in soak solution containing glycerol (30%, v/v) and flash frozen in liquid nitrogen.

Diffractions were collected at the European Synchrotron Radiation Facility, beam station ID29 (Grenoble, France) for crystals soaked with **6** and **14a**; at the Synchrotron Radiation Source, beam station 14.1 (Daresbury, UK) for crystals soaked with **18**, and at the Diamond Light Source, station I03 (Oxfordshire, UK) for crystals soaked with **14b**. All derived data were indexed and scaled by using either Denzo and Scalepack or XDS. The structures of the complexes were solved by molecular replacement by using AMoRe, from the CCP4 suite,^[31] and using the crystal structure of apo pantothenate synthetase from *M. tuberculosis* (PDB ID: 3cov^[21]) as the molecular replacement search probe. The structures were refined by using successive rounds of manual rebuilding in Coot 0.0.33^[32] and maximum-likelihood refinement with Refmac 5 from the CCP4 suite.^[33,34] Data collection and final refinement statistics for all the refined coordinate sets are presented in Table S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This study was supported by the UK Biotechnology and Biological Sciences Research Council, the UK Engineering and Physical Sciences Research Council (D.E.S.), the Japan Patent Office (M.A.), and Homerton College (A.C.). We thank Dimitri Y. Chirgadze for his advice on X-ray crystallography.

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Figure 1.

Biophysical characterisation of 5'-deoxy-5'-thioadenosine (**6**) binding to *M. tuberculosis* pantothenate synthetase. A) ^1H NMR spectrum of **6**, observing the purine H-2 and H-8 signals. WaterLOGSY experiments B) with no protein, C) with 12 μM pantothenate synthetase, and D) displacement with 330 μM ATP. E) ITC titration of **6**. Data were fitted to a single-binding-site model. F) Crystal structure of **6** bound in the enzyme active site. Omit electron density $F_o - F_c$ around **6** is shown in green and contoured at 3.0σ . Electron density $2F_o - F_c$ is shown in blue contoured at 1.5σ and superimposed around sulfate and glycerol.

Carbon atoms are shown in green (ligands), pink (pantoate pocket) and cyan (phosphate pocket), nitrogen in blue, oxygen in red, and sulfur in yellow. The figure was generated and rendered by using Pymol v. 0.99.^[35]

Figure 2.

Analysis of dynamic combinatorial libraries. HPLC traces of DCLs with thiols **6** and **8a–h** in a glutathione redox buffer after 24 h; A) with no protein, the disulfide conjugate between **6** and glutathione forming the major constituent, and B) with 200 μ M *M. tuberculosis* pantothenate synthetase, showing amplification of thiol **6** and disulfide **14a**.

Figure 3.

Crystal structure of disulfide **14b** bound in the active site of *M. tuberculosis* pantothenate synthetase. Omit electron density $F_o - F_c$ is shown in green and contoured at 3.0σ around **14b**. The ligand is shown as sticks with green carbons, and hydrogen-bonded water is shown as a red sphere. Key protein residues are shown with pink carbon atoms, nitrogen in blue, oxygen in red, and sulfur in yellow. The figure was generated and rendered by using Pymol v. 0.99.^[35]

Scheme 1.

The pantoyladenylate intermediate **5** inspired the design of disulfides **7**, which are formed by the dynamic exchange of a library comprised of thiols **8a-h** and **6**.

Scheme 2.

Synthetic scheme for the synthesis of asymmetric disulfides and thioethers from thiol **6**. a) diethyl azodicarboxylate, AcSH, PPh₃, THF; b) TFA, H₂O, 4 °C; c) NH₃, MeOH; d) i: NaOCl, ii: pyridine-2-thiol; e) CHCl₃, CH₃CO₂H; f) NaOMe, RSH; g) PPh₃, CBr₄; h) NaOMe, MeOH; i) TFA, H₂O, 4 °C.

Table 1

Dissociation constants and enthalpies determined by ITC of compounds containing the 5'-deoxy-5'-thioadenosine scaffold binding to *M. tuberculosis* pantothenate synthetase.

Compound	R	K_D [μM]	H [kcalmol^{-1}]
6	H	380 $\pm$ 30	-22.4 $\pm$ 0.1
14a	PhCH ₂ S	210 $\pm$ 20	-14.3 $\pm$ 0.1
14b	3-NO ₂ PhCH ₂ S	80 $\pm$ 10	-4.5 $\pm$ 0.2
14c	HOCH ₂ CH(OH)CH ₂ S	570 $\pm$ 60	-11.8 $\pm$ 0.8
18	HOCH ₂ CH(OH)CH ₂ CH ₂	540 $\pm$ 20	-29.9 $\pm$ 0.5