Mycothiol disulfide reductase: solid phase synthesis and evaluation of alternative substrate analogues

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A solid phase synthesis of *des-myo*-inositol mycothiol disulfide and its alpha-configured methyl- and benzyl-glycoside derivatives has been developed. Kinetic characterisation of these compounds has demonstrated their viability as alternative substrates for use in mycothiol disulfide reductase enzyme assays.

Background

Glutathione (GSH) is the principle antioxidant thiol found in most eukaryotes and Gram negative bacteria. Most Actinomycetes (eg. *Mycobacterium tuberculosis, Streptomyces coelicolor*) lack GSH and instead utilise the cysteinyl *pseudo*-disaccharide mycothiol (MSH) (1-D-myo-inosityl-2(-*N*-acetyl-L-cysteinyl) amino-2-deoxy-α-D-glucopyranoside) as their principal low molecular weight thiol (Fig. 1).[1,2] Like GSH, MSH is believed to play a key role in the inactivation of potentially damaging radicals and reactive oxygen species and is oxidised to the symmetrical mycothiol disulfide (MSSM), in the process. NADPH-dependent mycothiol disulfide reductase (Mtr) (also referred to as mycothione reductase) helps to maintain an intracellular reducing environment by reducing MSSM back to MSH (Fig. 1).[3–5] MSH deficient bacteria show a significant increase in sensitivity to oxidative stress[6,7] making the mycothiol redox pathway a potential therapeutic target in *M. tuberculosis*.

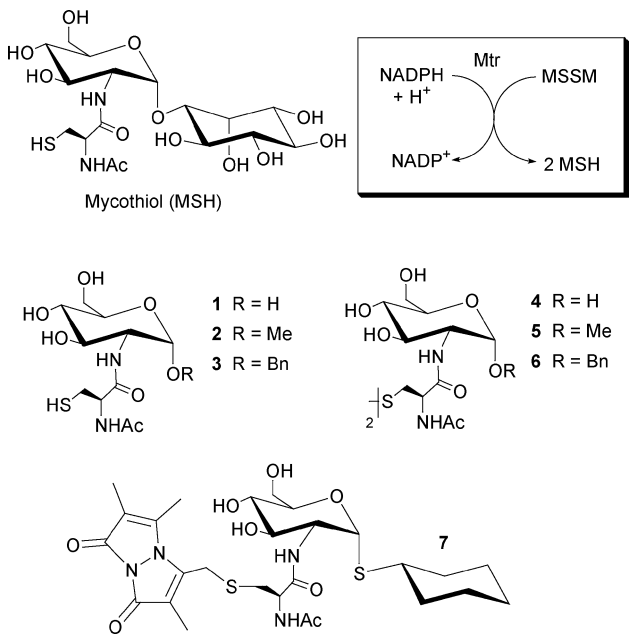

Fig. 1 Mycothiol, the mycothiol disulfide reductase pathway and mycothiol analogues.

Mtr inhibitor studies are severely restricted by the scarcity of MSH, which is difficult to prepare in sufficient quantity. Small quantities of MSH are routinely obtained by whole cell synthesis in typical yields of 1 mg per litre of *M. smegmatis* cell culture,[8,9] while chemical syntheses are convoluted.[10–13] Interestingly, the inositol portion of MSH is not always essential for substrate recognition by certain MSH-processing enzymes. The truncated substrate *des-myo*-inositol mycothiol disulfide **4** is a proven alternative substrate for Mtr[3] and the mycothiol-*S*-bimane analogue **7** (where inositol is replaced with *S*-cyclohexyl)[14,15] is recognised as an alternative substrate for mycothiol-*S*-conjugate amidase.[16] Such simplified analogues are more synthetically accessible than MSH and have potential as alternative substrates to facilitate inhibitor studies of Mtr, and (possibly) other MSH-dependent enzymes.

Routine access to sufficient quantities of **4** and/or other simplified mycothiol disulfide analogues as alternative substrates for Mtr inhibition assays is clearly desirable. Whilst solution phase syntheses of **1**[8] and its symmetrical disulfide **4**[3] have previously been reported, a solid phase approach would enable the preparation of parallel libraries of MSH analogues. Herein we report a solid phase method for the synthesis of **1** and the alpha-configured methyl- and benzyl-glycosides (**2** and **3**). Their subsequent oxidation to symmetrical disulfides **4–6** and evaluation as alternative Mtr substrates is also described.

Results and discussion

The methyl- and benzyl-glycosides of glucosamine (**10**[17] and **11**) were prepared by hydrazinolysis of their *N*-acetyl precursors **8**[18] and **9**[19] at 120 °C (Scheme 1a). The strategy for the solid phase reactions was to immobilise the cysteine motif onto a polystyrene resin *via* an *S*-trityl linkage as this would also serve to protect the thiol during the amide coupling reaction. Supported CysNAc **15** was prepared by loading CysNAc **12** onto polystyrene trityl chloride resin using standard procedures (Scheme 1b).[20] Immobilised *N*-Fmoc cysteine **16** and its pentafluorophenyl ester **17**

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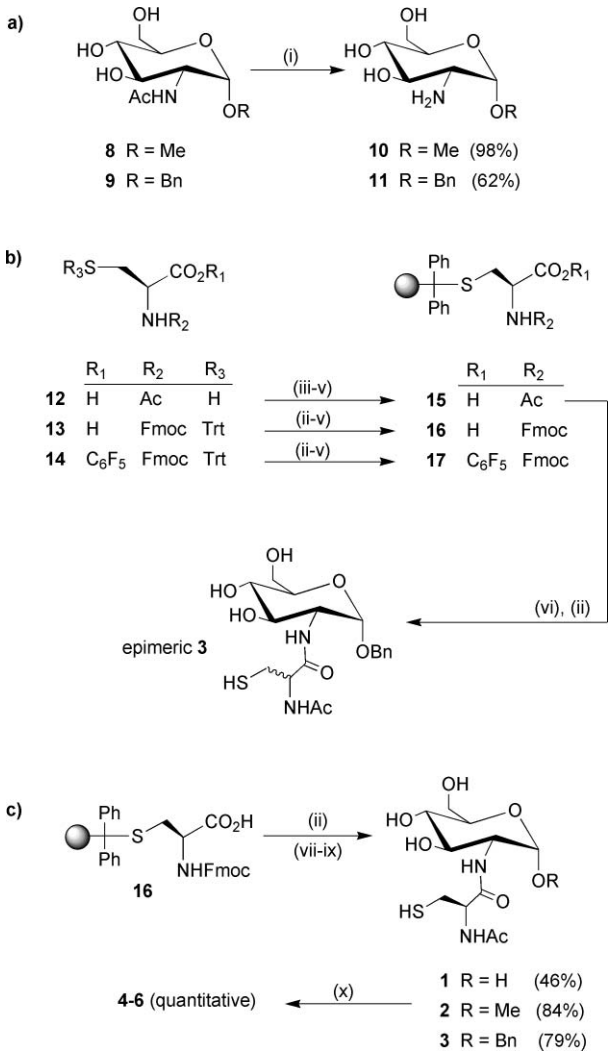

Scheme 1 *Reagents and conditions:* (i) $NH_2NH_2{\cdot}H_2O$, 120 °C; (ii) TFA–DCM–Et_3SiH (40 : 60 : 3); (iii) polystyrene tritylchloride, DIPEA, CH_2Cl_2–DMF; (iv) AcOH, trifluoroethanol, CH_2Cl_2; (v) MeOH, DIPEA, CH_2Cl_2; (vi) **11**, PyBOP, HOBt, base, DMF–CH_2Cl_2; (vii) **10**, **11** or glucosamine (2 eq.), PyBOP, HOBt, 2,6-di-*tert*-butylpyridine, DMF–CH_2Cl_2; (viii) DMF–piperidine (8 : 2); (ix) Ac_2O (1.1 eq.), pyridine (1.1 eq.), DMF; (x) NH_4HCO_3 (30 mM), shaking 24–60 h.

were prepared by acidic detritylation of their protected precursors **13–14** followed by removal of the trifluoroacetic acid (*in vacuo*) and direct loading onto the resin without further purification.

Solid phase coupling reactions were initially studied between immobilised CysNAc and aminosugar **11** (Scheme 1b). The use of HBTU or HATU and excess **11** failed to provide reaction product **3** (following TFA cleavage from resin). Presumably these aminium-based peptide coupling reagents react with the excess of aminosugar **11** to form guanidine byproducts instead. Reactions of pentafluorophenyl ester **17** with four equivalents of **11** were extremely sluggish and only trace quantities of **3** were isolated after the reaction had been agitated for four hours.

Racemisation of the cysteine side chain has previously been encountered when forming the cysteine-aminosugar amide bond in MSH, particularly when carbodiimides such as EDCI or DCC were used as the coupling reagent.[13] Racemisation is often caused by base-catalysed enolisation of the 5(4*H*)-oxazalone) intermediate, which itself forms *via* rearrangement of the pre-activated carboxylate intermediate. Usually the carboxylate is pre-mixed with the activating agent for five minutes prior to addition to the amine nucleophile. With cysteine, racemisation can be minimised by avoiding carboxylate pre-activation, using a weaker and/or more sterically hindered base and reducing the solvent polarity.[21]

A similar strategy was therefore explored for the solid phase synthesis of **3** using the phosphonium coupling reagent PyBOP. This involved pre-mixing the solvated aminosugar **11** with the cysteine-derivatised resin **15** prior to the addition of the activating reagents (*ie.* PyBOP, HOBt and base). When 2,6-collidine was used as a base, NMR analysis of the cleaved product revealed a 4 : 1 epimeric mixture of **3**. This was evident in the carbon NMR spectra where several of the carbons were represented by two closely spaced signals. Of particular note was the presence of two distinct anomeric carbon signals at 96.35 and 96.25 ppm for the major and minor epimers, respectively. In the proton NMR spectra two doublets were observed for the anomeric proton of the major and minor epimers at 4.84 and 4.79 ppm, respectively. Two closely overlapping singlets for the *N*-acetate methyl protons (1.91 ppm) were also observed. Since the anomeric proton signals shouldered the NMR solvent peak (in D_2O) and the acetate signals were too close together to allow the epimer ratio to be determined by their integration, epimer ratios were determined by integration of the anomeric carbon signals from an inverse gated coupling ^{13}C NMR spectra of epimeric **3**. When 2,6-collidine was replaced with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) the degree of epimerisation was reduced (7 : 2) and switching to 2,6-di-tert-butylpyridine reduced this even further to give a 9 : 1 ratio of epimeric **3**.

However, when 2,6-di-tert-butylpyridine was used as the base for coupling **11** with immobilised CysNFmoc **16** no detectable level of epimerisation was evident (presumably because the Fmoc carbamate moiety further disfavours oxazolone formation). These conditions were therefore employed for the preparation and purification of all three MSH analogues **1–3** (Scheme 1c). After coupling the amino sugar onto **16** the Fmoc protecting group was removed (20% piperidine) and the free amine was acetylated by treatment with acetic anhydride (1.1 equivalents). The product thiol was then cleaved from the resin by treatment with 40% trifluoroacetic acid (TFA). Sometimes, when the acetic anhydride was used in a small excess some additional *O*-acylation of the cleaved product was evident (by NMR). This was easily removed by treating the cleaved product with a catalytic amount of sodium methoxide under Zemplen conditions prior to purification. Under these conditions it was possible to obtain **2** and **3** in about 80% isolated yields (with respect to immobilised CysNFmoc **16**).

The DMF : CH_2Cl_2 solvent ratios used in the amide bond-forming steps for compounds **1–3** were dictated by the solubility of the respective aminosugars. The benzyl glycoside **11** was the most readily soluble (20% DMF) followed by the methyl glycoside **10** (25% DMF). Glucosamine was only sparingly soluble in DMF, which may explain why this reaction failed to go to completion, as evident from the significant amount of unreacted CysNAc **12** in the cleaved product mixture (by NMR). It is worth noting that the varying amounts of DMF used in all of the above procedures did not appear to compromise the epimeric purity of the products.

For evaluation as Mtr substrates compounds **1–3** needed to first be oxidised to their symmetrical disulfides **4–6**, ideally without

over-oxidation or the need for product purification. In our hands, oxidation using hemin adsorbed onto Celite[22] was not satisfactory as the oxidised product was heavily contaminated with hemin, which leached from the Celite solid phase when washing out the product. Treatment of the thiols with one equivalent of iodine in 50% aqueous acetonitrile[23] efficiently oxidised them to their disulfides, but required treatment with multiple batches of activated charcoal to remove any unconsumed iodine.[24] Thiols can be selectively oxidised to their symmetrical disulfides (without the risk of over-oxidation) under mildly basic conditions. In this manner, disulfides **4–6** were cleanly prepared by shaking a solution of thiols **1–3** in 30 mM ammonium hydrogen carbonate in an open sample vial until the oxidation was complete (Scheme 1c). Removal of the water and volatile salts by freeze-drying then provided the clean disulfides **4–6** in quantitative yield. Using this procedure, thiols **1** and **2** were completely oxidised within 24 h. Benzyl glycoside **3** was less readily soluble and kept precipitating out of solution and was periodically redissolved by warming the solution with a heat gun. This reaction took much longer to reach completion (60 h). It is worth noting that the epimer-free disulfide **6** gives a single anomeric carbon resonance whereas oxidation of an epimeric mixture of **3** gives a mixture of three diastereomeric disulfides, and therefore exhibits three closely spaced anomeric carbon resonances.

The substrate properties of **4–6** and authentic MSSM with recombinant hexahistidine-tagged *M. tuberculosis* Mtr[4] were determined (Table 1). In our hands, the substrate properties for **4**[5] and MSSM[5,25] are comparable to those previously reported. Compared to MSSM, removal of the inositol motif (*ie.* compound **4**) gives a four-fold increase in K_m but with no apparent effect on the steady state turnover rate (k_{cat}). The methyl glycoside **5** is just a subtle elaboration of the structure **4**, which results in only a small increase in K_m and a 3-fold reduction in k_{cat}. Replacement of the polyhydroxylated inositol motif of MSSM with a planar, non-polar, benzyl group **6** results in only a 4-fold increase in K_m and a 2-fold reduction in k_{cat}. The inositol ring of MSSM (as drawn in Fig. 1) has an apolar patch on the top face. Perhaps the planar face of the aromatic ring in **6** is able to replace some of the binding interactions that might occur between Mtr and the inositol hydrophobic patch in MSSM. If the benzyl group does occupy the inositol binding pocket it seems plausible that *myo*-inositol would be a much stronger inhibitor of Mtr using **6** as a substrate than with the *des-myo*-inositol derivative **4**. The inhibitory effects of *myo*-inositol (25 mM) on the Mtr catalysed turnover of **4**, **6**, and MSSM (at their respective K_m concentrations) were therefore studied (Fig. 2). Under these conditions *myo*-inositol displayed a small, but comparable level of inhibition (15%) when MSSM or its benzyl derivative **6** were the substrates. When compound **4** was the substrate, the level of inhibition was significantly less (3%).

Combined with their synthetic accessibility, any of **4–6** could be employed as alternative substrates for routine Mtr inhibition assays. We suggest the derivative **6** to be the most favourable alternative substrate for such applications as the benzyl moiety is likely to fill more the binding pocket within Mtr that is normally occupied by the inositol portion(s) of MSSM. Using this substrate it should be possible to also detect inhibitors which bind to Mtr but only overlap the inositol binding region of the active site. Such compounds could potentially be missed if assays were run using the more truncated substrate analogues **4** and **5**.

Table 1 Alternative substrate properties with Mtr

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
MSSM	113 (±10)	68.76 (±2.27)	0.61 (±0.08)
4	463 (±43)	70.05 (±5.88)	0.15 (±0.04)
5	610 (±82)	25.54 (±0.83)	0.04 (±0.01)
6	438 (±43)	36.99 (±1.57)	0.09 (±0.01)

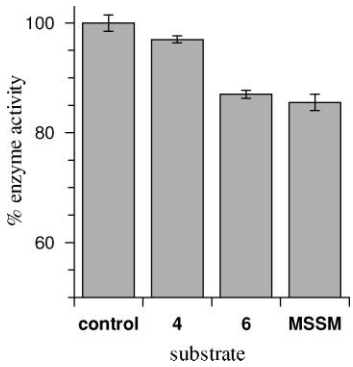

Fig. 2 The effects of myo-inositol on Mtr catalysed substrate turnover: assays were carried out with substrates **4** (400 μM), **6** (400 μM) and MSSM (105 μM) in the presence of myo-inositol (25 mM). Percentage enzyme activities are relative to control experiments (performed separately with each substrate) in the absence of myo-inositol. All assays were carried out in duplicate.

Conclusions

A convenient procedure for the solid phase synthesis of simplified mycothiol mimetics has been devloped which enables efficient coupling of the cysteine and aminosugar motifs without racemisation. The facile preparation of these alternative substrates will facilitate the screening of Mtr inhibitors. These solid phase synthesis protocols will also enable the synthesis of parallel libraries of mycothiol analogues as inhibitors and/or substrates of Mtr and other mycothiol-dependent enzymes.

Experimental

General

^{1}H-NMR spectra were recorded on a Bruker AV 300 or DRX 500 NMR FT-spectrometer at 300 or 500 MHz, ^{13}C-NMR were recorded on the same spectrometers at 75 or 125 MHz, respectively. Chemical shifts (δ) are expressed in ppm and coupling constants (J) are given in Hz. All NMR spectra were taken at 300 K except where specified. Electrospray mass spectra were recorded on a VG Quattro Triple Quadrupole Mass Spectrometer. Optical rotations ($[\alpha]_D$) were obtained with a Perkin-Elmer Model 341 Polarimeter, using the specified solvent and concentration, and are quoted in units of 10^{-1}deg cm^2 g^{-1}. Analytical TLC was carried out on glass backed Macherly-Nagel SIL G25 UV_{254} plates and visualised under UV light or by staining with a 9 : 1 mixture of ethanol : sulfuric acid followed by charring with a heat gun. Agitation of thiol oxidation reactions were carried out on a Stuart-SF1 flask shaker. Thiols were visualised on TLC plates by staining with 0.1% (w/v) Ellman's reagent dissolved

in a 10 : 1 solvent mixture of 0.45 M Tris-HCl (pH 8.5) and ethanol. Thiol titrations were carried out by addition of reaction samples to a 50 mM solution of Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid)) in Mtr assay buffer in a disposable cuvette and measuring the absorbance increase at 412 nm. Polystyrene trityl chloride resin (100–200 mesh) was purchased from Fluka (cat No. 93005). Resin loadings were determined by combustion analysis of sulfur. Compounds **8**,[18] **9**,[19] **10**,[17] **12**[26] and **13**[27] were prepared as previously described. Recombinant *M. tuberculosis* Mtr (hexahistidine-tagged) was over expressed and purified from an *M. smegmatis* mc²155 transformant as previously described.[4] Spectrophotometric assays of Mtr were carried out in using a temperature controlled PerkinElmer UV Lambda 25 spectrophotometer. Kinetic data were analysed (by non-linear regression) using Grafit Version 5 (Erithacus Software Ltd).

2(-*N*-Acetyl-L-cysteinyl) amino-2-deoxy-α-D-glucopyranoside (1). Resin **16** (0.122 mmol) was pre-swollen by agitation in DMF (5 cm^3) for 10 min and then filtered. A freshly prepared milky suspension of D-glucosamine (44 mg, 0.245 mmol) in DMF (5 cm^3) was then added and whilst agitating with nitrogen gas a solution of HOBt (33 mg, 0.244 mmol), PyBOP (127 mg, 0.245 mmol) and 2,6-di-*tert*-butylpyridine (46 mg, 0.245 mmol) was added and the resulting mixture was agitated for 3 h by which time the slurry had turned into a yellow/brown solution. The resin was washed successively with 6 cm^3 portions of DMF (4 × 3 min) followed by CH_2Cl_2 (3 × 3 min). The product was then cleaved from the resin by 3 × 3 min treatments with 8 cm^3 portions of TFA–CH_2Cl_2–Et_3SiH (40 : 60 : 3) and the resin finally washed with CH_2Cl_2 (6 cm^3). The combined filtrates were concentrated on a rotary evaporator to give a yellow/brown oil. The product was precipitated by the addition of ice cold Et_2O (4 cm^3). The ethereal solution was decanted and the precipitate washed with ice cold Et_2O (2 × 2 cm^3). The precipitate was then dissolved in water (8 cm^3) and extracted with ice cold Et_2O (3 × 2 cm^3). The acidic aqueous layer was then neutralised with 30 mM NH_4HCO_3 and passed through a Phenomenex Strata strong anion exchange solid phase extraction tube (1000 mg) which was then washed with 3 column volumes of water. The combined eluents were freeze dried to give **1** as a white solid (18 mg, 53%). δ_H (400 MHz, CD_3OD) 2.75–2.91 (2H, m, CH_2SH), 3.32–3.50 (1H, m) 3.56–3.88 (5H, m) 4.49–4.53 (1H, m, C*H*CH_2S), 4.64–4.66 (1H, d, *J* 8.0, H-1-α) 5.08 (1H, d, *J* 3.2, H-1-β); δ_C (100 MHz, CD_3OD) 22.78, (CH_3), 27.46 (CH_2SH), 36.27, 56.21, 57.46, 57.59, 63.00, 73.04, 73.46, 76.08, 78.31, 92.86, (C-1-α) 96.98 (C-1-β), 172.94 (C=O), 173.78 (C=O) *m/z* (ESI⁺) 347.0879 (M+Na⁺. $C_{11}H_{20}N_2O_7SNa$ requires 347.0883).

Methyl 2(-*N*-acetyl-L-cysteinyl) amino-2-deoxy-α-D-glucopyranoside (2). Resin **16** (0.148 mmol) was pre-swollen by agitation in DMF (10 cm^3) for 10 min and then filtered. A solution of **10** (58 mg, 0.297 mmol) in CH_2Cl_2–DMF (3 : 1) (5 cm^3) was then added followed by a solution of HOBt (41 mg, 0.297 mmol), PyBOP (154 mg, 0.297 mmol) and 2,6-di-tert-butylpyridine (57 mg, 0.297 mmol) in CH_2Cl–DMF (3 : 1) (2 cm^3) and the resulting mixture was agitated for 2 h. The resin was washed successively with 10 cm^3 portions of DMF (4 × 4 min), then agitated in 20% (v/v) of piperidine in DMF (40 cm^3). After washing with DMF (3 × 10 cm^3), a solution of acetic anhydride (30 mg, 0.297 mmol) and pyridine (24 mg 0.297 mmols) in DMF 10 cm^3 was then added and agitated for 60 min. The resin was washed with DMF (2 × 10 cm^3) then DCM (3 × 10 cm^3). The product was cleaved from the resin by 3 × 3 min treatments with 6 cm^3 portions of TFA–CH_2Cl_2–Et_3SiH (40 : 60 : 3) and the combined filtrates were concentrated on a rotary evaporator to give an oil. The product was triturated with ice cold ether (4 cm^3) to give a white precipitate which was dissolved in water (8 cm^3) and washed with cold ether (2 × 2 cm^3). The aqueous layer was then freeze-dried to give **2** as a white lyophilate (42 mg, 84%). $[\alpha]_D^{20}$ +57.1° (*c* 0.71 in H_2O); δ_H (300 MHz, D_2O); 1.91 (3H, s, CH_3), 2.73 (1H, dd, *J* 14.2, 6.3, C$H_A$$H_B$SH), 2.79 (1H, dd, *J* 14.2, 6.3, C$H_A$$H_B$SH), 3.24 (3H, s, OCH_3), 3.31 (1H, t, *J* 9.2, H-4), 3.50–3.66 (3H, m, H-3, H-5, H-6a), 3.72 (1H, dd, *J* 12.2, 2.3, H-6b), 3.79 (1H, dd, *J* 10.7, 3.6, H-2), 4.34 (1H, t, *J* 6.3, C*H*CH_2SH), 4.61 (1H, d, *J* 3.6, H-1); δ_C (75 MHz, D_2O); 22.63 (CH_3), 26.36 (CH_2SH), 56.23 (OCH_3), 54.71, 56.73 (C-2, *C*HCH_2SH), 61.53 (C-6), 71.05, 71.88, 72.70 (C-3, C-4, C-5), 99.03 (C-1), 173.18 (C=O), 175.23 (C=O); *m/z* (ESI⁺) 339.1245 (M+H⁺. $C_{12}H_{23}N_2O_7S$ requires 339.1226) 699 (88%, 2M + Na⁺), 361 (100, M+Na⁺), 339 (10, M+H⁺).

Benzyl 2(-*N*-acetyl-L-cysteinyl) amino-2-deoxy-α-D-glucopyranoside (3). Resin **16** (0.28 mmol) was pre-swollen by agitation in a 4 : 1 co-solvent mixture of CH_2Cl_2–DMF (20 cm^3) for 10 min and then filtered. A solution of **11** (148 mg, 0.56 mmol) in 4 : 1 CH_2Cl_2–DMF (0.5 cm^3) was then added followed by a solution of HOBt (74 mg, 0.56 mmol), PyBOP (288 mg, 0.56 mmol) and 2,6-di-tert-butylpyridine (124 μL, 0.56 mmol) in 4 : 1 CH_2Cl_2/DMF (1 cm^3) and the resulting mixture was agitated for 2 h. The resin was washed successively with 10 cm^3 portions of DMF (4 × 4 min), then agitated in 20% (v/v) of piperidine in DMF (40 cm^3). After washing with DMF (3 × 10 cm^3) a solution of acetic anhydride (30 μL, 0.297 mmol) and pyridine (24 μL, 0.297 mmols) in DMF 10 cm^3 was then added and agitated for 60 min. The resin was washed with DMF (2 × 10 cm^3) then DCM (3 × 10 cm^3). The product was cleaved from the resin by 3 × 3 min treatments with 6 cm^3 portions of TFA–CH_2Cl_2–Et_3SiH (40 : 60 : 3) and the combined filtrates were concentrated *in vacuo* to give an oil. The product was treated the NaOMe (45 mg, 0.84 mmol) in MeOH (5 cm^3) for 3 h then stirred with DOWEX H^+ (2 g) for 1 h. The resin was filtered and treated with hot water to obtain any product that had precipitated out during filtration. The aqueous layer was then freeze dried to give **3** as a white solid (91 mg, 79%). mp 217 °C (decomp); $[\alpha]_D^{20}$ +89.6; (c 1.0 in H_2O); δ_H (300 MHz, D_2O); 1.91 (3H, s, CH_3), 2.73 (1H, dd, *J* 14.1, 6.7 C$H_A$$H_B$SH), 2.80 (1H, dd, *J* 14.1, 5.7 C$H_A$$H_B$SH), 3.35 (1H, t, *J* 9.0, H-4), 3.59–3.65 (3H, m, H-3, H-5, H-6$_A$), 3.69 (1H, dd, *J* 12.0, 2.3 H-6$_B$) 3.79 (1H, dd, *J* 10.7, 3.7 H-2), 4.33 (1H, dd, *J* 6.7, 5.7 C*H*CH_2SH), 4.40 (1H, d, *J* 11.7, OC$H_A$$H_B$Ph), 4.60 (1H, d, *J* 11.7, OC$H_A$$H_B$Ph, 4.84 (1H, d, *J* 3.7, H-1), 7.29 (5H, m, 5 × ArH); δ_C (75 MHz, D_2O); 22.06 (CH_3), 25.91 (CH_2SH), 54.16, 55.94 (C-2, *C*HCH_2S), 60.78 (C-6), 70.07, 70.40, 71.06, 72.45 (CH_2Ph, C-3, C-4, C-5), 96.35 (C-1), 128.73 (CH), 128.91 (CH), 129.09 (CH), 137.20 (C), 172.35 (C=O), 174.49 (C=O); *m/z* (ESI⁺) 437.1355 (M+Na⁺. $C_{18}H_{26}N_2O_7SNa$ requires 437.1358).

2(-*N*-Acetyl-L-cysteinyl) amino-2-deoxy-α-D-glucopyranoside disulfide (4). A solution of **1** (34 mg, 0.105 mmol) dissolved in 30 mM aqueous NH_4HCO_3 (4 cm^3) was shaken for 24 h until all the thiol had been consumed (by titration with Ellman's reagent). The water was removed on a rotary evaporator and

the solids coevaporated three times with water before finally dissolving in water and freeze drying to give **4** as a white solid in quantitative yield. Spectral data was in agreement with that previously reported. [3]

Methyl 2(-*N*-acetyl-L-cysteinyl) amino-2-deoxy-α-D-glucopyranoside disulfide (5). A solution of **2** (26 mg, 0.077 mmol) dissolved in 30 mM aqueous NH_4HCO_3 (3 cm^3) was shaken for 24 h until all the thiol had been consumed (by titration with Ellman's reagent). The water and some of the volatile salts were removed on a rotary evaporator before dissolving in water and freeze drying to give **5** as a white solid (26 mg, quantitative). $[\alpha]_D^{20}$ +23.5° (*c* 0.74, DMSO); δ_H (300 MHz, DMSO-d_6); 1.85 (3H, s, CH_3), 2.7 (1H, dd, *J* 13.4, 9.6, CH_AH$_B$SH), 3.07–3.14 (2H, m), 3.22 (3H, s, OCH_3), 3.29–3.35 (1H, m), 3.40–3.48 (2H, m), 3.57–3.63 (2H, m), 4.48 (1H, d, *J* 3.3, H-1), 4.57 (1H, dd, *J* 9.6, 4.4, C*H*CH$_2$S); δ_C (75 MHz, DMSO-d_6); 22.74 (CH_3), 41.08 (CH_2SS), 52.20, 54.32, 54.95 (OCH_3, C-2, *C*HCH$_2$SS), 61.05 (C-6), 70.73, 70.88, 72.97 (C-3, C-4, C-5), 98.17 (C-1), 170.49 (C=O), 170.80 (C=O); *m*/*z* (ESI$^+$) 697.2038 (M+Na$^+$. $C_{24}H_{42}N_4O_{14}S_2Na$ requires 697.2037) 697 (55%), 483 (65), 143 (100).

Benzyl 2(-*N*-acetyl-L-cysteinyl) amino-2-deoxy-α-D-glucopyranoside disulfide (6). A solution of **3** (30 mg, 0.072 mmol) dissolved in a 1 : 1 mixture of hot 30 mM aqueous NH_4HCO_3 and MeOH (3 cm^3) and was shaken for 60 h until all the thiol had been consumed (by titration with Ellman's reagent). As the reaction progressed the reactants/products began to crash out of solution and were periodically re-dissolved by warming with a heat gun. The methanol was removed on a rotary evaporator and the remaining aqueous solution was freeze-dried to give **6** as a white solid in quantitative yield. At 300K, the NMR spectra of **3** was complicated by the detection of multiple conformations. The NMR analyses were therefore carried out at 363K. $[\alpha]_D^{20}$ 13.7° (*c* 0.51, DMSO; δ_H (500 MHz, DMSO-d_6); 1.84 (3H, s, CH_3), 2.83 (1H, dd, *J* 13.5, 10.0, CH_AH$_B$S), 3.11 (1H, dd, *J* 13.5, 4.2, CH$_A$$H_B$S), 3.13–3.18 (1H, m), 3.40–3.56 (3H, m), 3.60–3.69 (2H, m), 4.29 (1H, d, *J* 12.3, CH_AH$_B$Ph), 4.59 (1H, d, *J* 12.3, CH$_A$$H_B$Ph), 4.70 (1H, *J* 3.5, H-1), 4.70–4.75 (m, C*H*CH$_2$S); δ_C (125 MHz, DMSO-d_6); 22.32 (CH_3), 41.02 (CH_2S), 52.70, 54.35 (C-2, C*H*CH$_2$S), 61.23 (C-6), 68.78, 71.01, 71.13, 72.95 (C-3, C-4, C-5) 96.20 (C-1), 127.51 (CH), 127.67 (CH), 128.24 (CH), 137.66 (C), 170.55 (C=O); *m*/*z* (ESI$^+$) 849.2767 (M+Na$^+$. $C_{36}H_{50}N_4O_{14}S_2Na$ requires 849.2663.

Benzyl 2-amino-2-deoxy-α-D-glucopyranoside (11). Compound **9**[19] (1.5 g, 4.81 mmol) was dissolved in hydrazine hydrate (9 cm^3) and stirred at 120 °C for 2 days, then concentrated *in vacuo*, co-evaporated twice with toluene, and purified by chromatography (EtOAc–MeOH 7 : 3 as eluent) to yield **11** as a white solid (1.02 g, 78%), mp 110–114 °C; $[\alpha]_D^{20}$ +148.1 (c 1.0 in MeOH); δ_H (500 MHz, CD_3OD) 2.67 (1H, dd, H-2, *J* 10.1, 3.6), 3.31 (1H, m, H-4), 3.51 (1H, dd, *J* 10.1, 9.0, H-3), 3.63 (1H, ddd *J* 9.8, 5.7, 2.3 H-5), 3.69 (1H, dd, *J* 11.9, 5.7, H-6b), 3.80 (1H, dd, *J* 11.9, 2.3, H-6a), 4.50 (1H, d, *J* 11.5, PhCH$_A$CH_B), 4.76 (1H, d, *J* 11.5, PhCH_ACH$_B$), 4.93 (1H,d, *J* 3.6, H-1), 7.26–7.40 (5H, m, Ph), δ_C (125 MHz, CD_3OD) 57.03 (C2), 70.42 (CH_2Ph), 71.96 (C-4), 74.38 (C-5), 75.78 (C-3), 99.25 (C-1), 128.86 (CH), 129.31 (CH), 129.44 (CH), 138.95 (C); *m*/*z* (ESI$^+$) 270.1340 (M+H$^+$. $C_{13}H_{19}NO_5$ requires 270.1341.

Trityl polystyrene-supported cysteines (15–17): general procedure. For preparation of **16**, Trityl-*S*-CysNHFmoc (4.0 g, 6.8 mmol) was first detritylated by treatment with TFA–DCM–Et_3SiH (40 : 60 : 3) (20 cm^3) for 3 h. The solvents were removed *in vacuo*. The crude mixture and DIPEA (1.18 cm^3, 6.8 mmol) were then dissolved in anhydrous CH_2Cl_2–DMF (1 : 1, 20 cm^3) and added to polystyrene trityl chloride resin (1.7 mmol, pre-swollen in anhydrous DMF for 20 min). The mixture was agitated with a bubbling flow of argon for 2 h. The resin was filtered, washed with CH_2Cl_2 (20 cm^3, 4 × 3 min), treated with 10 cm^3 of CH_2Cl_2–trifluoroethanol–AcOH (7 : 2 : 1) for 15 min, then washed with four portions of DCM. The resin was finally treated with a 20 cm^3 of CH_2Cl_2–MeOH–DIPEA (16 : 3 : 1) for 30 min then washed (CH_2Cl_2, 4 × 20 cm^3) filtered and dried under vacuum (40 °C) overnight. Resin **17**, was prepared following detritylation of **14** in a similar manner and **15** was prepared from CysNAc **12** using the same loading procedures.

Enzyme assays

Prior to conducting detailed assays, the concentrations of the stock solutions of all the disulfide substrates (MSSM and **4–6**) were determined by titration against NADPH under the assay conditions outlined below, but in the presence of a large excess (25–50 nM) of Mtr. The amount of NADPH oxidised during complete substrate exhaustion using an extinction coefficient of 6220 M^{-1} (at 340 nm) was used to calculate the initial substrate concentration. Stock enzyme concentrations were determined by absorbance of the active site FAD chromophore at 462 nm with an extinction coefficient of 11 300 M^{-1}. Standard substrate assays were carried out in a final volume of 1000 μL in disposable cuvettes at 30 °C containing Mtr (5 nM), HEPES (50 mM), 0.1 mM EDTA, NADPH (0.14 mM) at pH 7.5 and varying concentrations of substrates. Substrate stock solutions were made up using co-solvent mixtures of assay buffer and DMSO; final assay mixtures contained 2% DMSO. Enzyme activity was monitored by the decrease in absorbance at 340 nm due to NADPH oxidation. Initial rates were measured from the linear region of product formation and K_m and k_{cat} values were determined by weighted non-linear regression analysis of the hyperbola plot of initial rate against substrate concentration using the Michaelis Menten equation.

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