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PAPER

Synthesis of 4′-aminopantetheine and derivatives to probe aminoglycoside *N*-6′-acetyltransferase†

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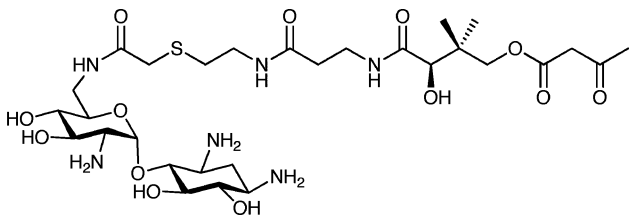
A convenient synthesis of 4′-aminopantetheine from commercial D-pantethine is reported. The amino group was introduced by reductive amination in order to avoid substitution at a sterically congested position. Derivatives of 4′-aminopantetheine were also prepared to evaluate the effect of *O*-to-*N* substitution on inhibitors of the resistance-causing enzyme aminoglycoside *N*-6′-acetyltransferase. The biological results combined with docking studies indicate that in spite of its reported unusual flexibility and ability to adopt different folds, this enzyme is highly specific for AcCoA.

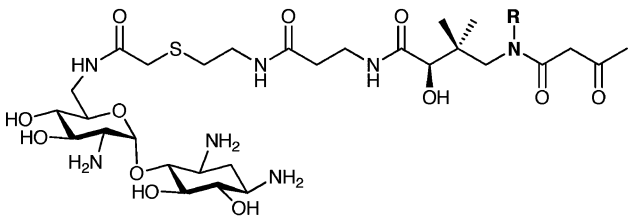
Introduction

Antibiotic resistance is a rising health concern. Aminoglycosides are a clinically important class of antibacterials, appreciated in particular for their activity against Gram-negative bacteria and mycobacteria.[1,2] Although different mechanisms of resistance to aminoglycosides have been observed in the clinics, the most common is drug modification by bacterial enzymes.[3–5] Expression of aminoglycoside *N*-6′-acetyltransferases (AAC(6′)s) is one of the most clinically relevant strategies used by bacteria to evade the currently used aminoglycosides.[6,7] Our group has reported the first molecule able to block aminoglycoside resistance in cells.[8] Compound **1** (Fig. 1) is an AAC(6′) inhibitor, and its synergistic activity with kanamycin A was demonstrated with a strain of *Enterococcus faecium* expressing AAC(6′)-Ii. The magnitude of the synergistic effect reported however was weaker than expected from the K_i, which we postulated may be explained by the low stability of the ester bond.[8] To verify this hypothesis, we report here the synthesis and biological evaluation of derivatives **2a–d** (Fig. 1). In compound **2a**, the biologically labile ester of **1** is replaced with a more stable amide bond. Based on the crystal structures reported for AAC(6′)-Ii,[9–11] compounds **2b–d** were expected to show increased affinity for the enzyme due to the amide substituents reaching into a nearby hydrophobic pocket (Fig. 2).

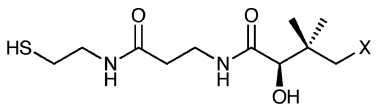
We also envisaged that a synthetic route to 4′-aminopantetheine (**3**) would find uses considering the critical biological role of its natural homologue pantetheine (**4**). For example, the disulfide form of **4**, D-pantethine (**5**, Scheme 1), is sold as a dietary supplement with claims of beneficial effects on blood lipid profiles amongst others. A number of pantetheine derivatives have been synthesized, however most are modified at the sulfur end.[12–14] The *N*-substituted pantothenamides are an interesting family of reported pantetheine derivatives, many of which show antibacterial activity.[15–23] With the importance of the pantetheinyl group in activating enzymes of the fatty acid, polyketide, and



1 (K_i = 11 ± 6 μM)



2a-d
R = H, CH_2Ph, CH_2CH_2Ph, $CH_2CH_2CH_2Ph$



3: X = NH_2
4: X = OH

Fig. 1 Chemical structure for compounds **1–4**. The K_i value provided is for AAC(6′)-Ii inhibition by **1**.

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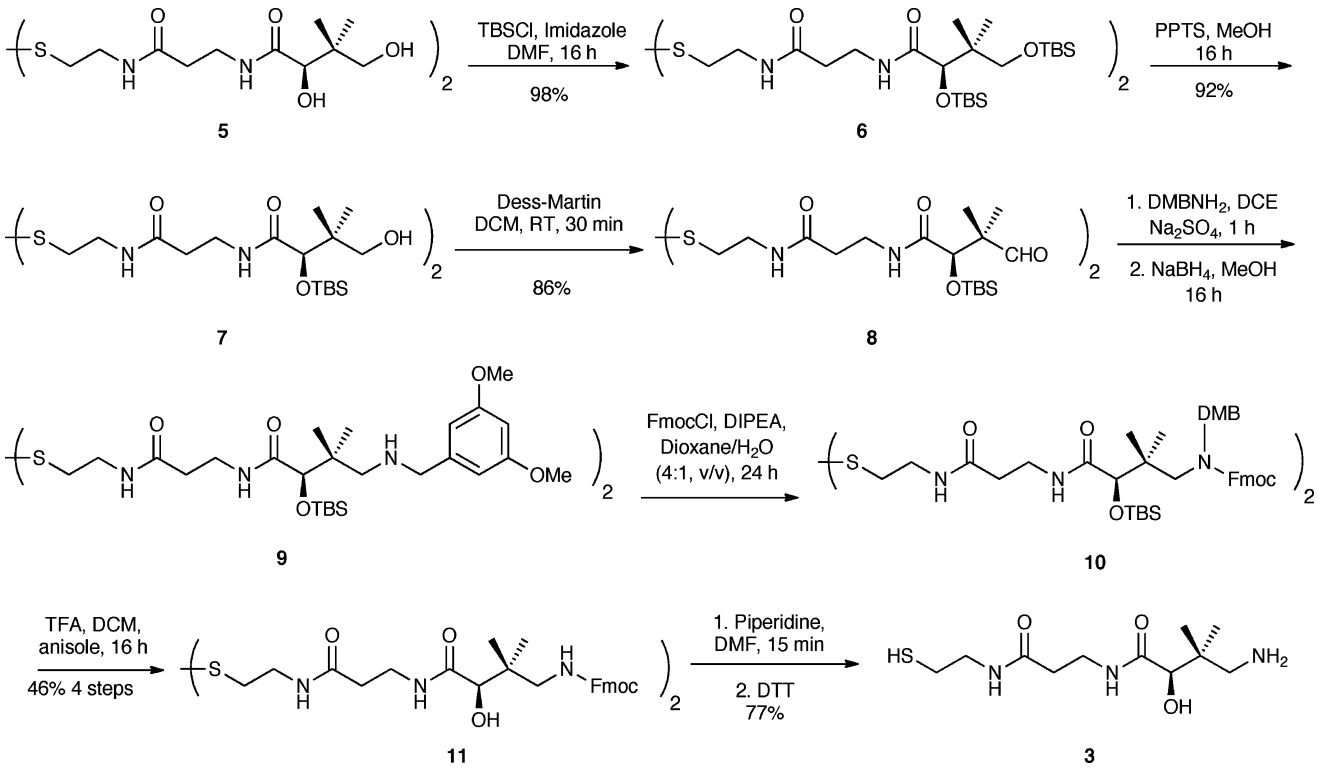


Scheme 1 Synthesis of compound **3**.

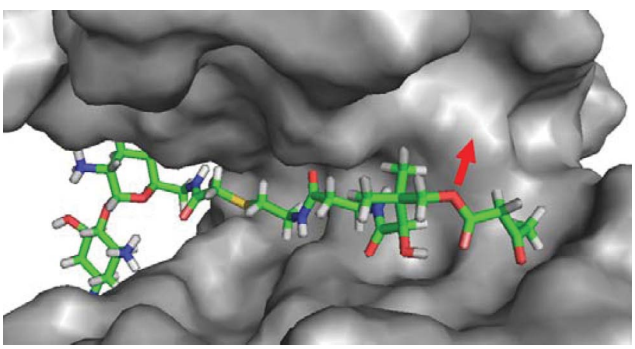


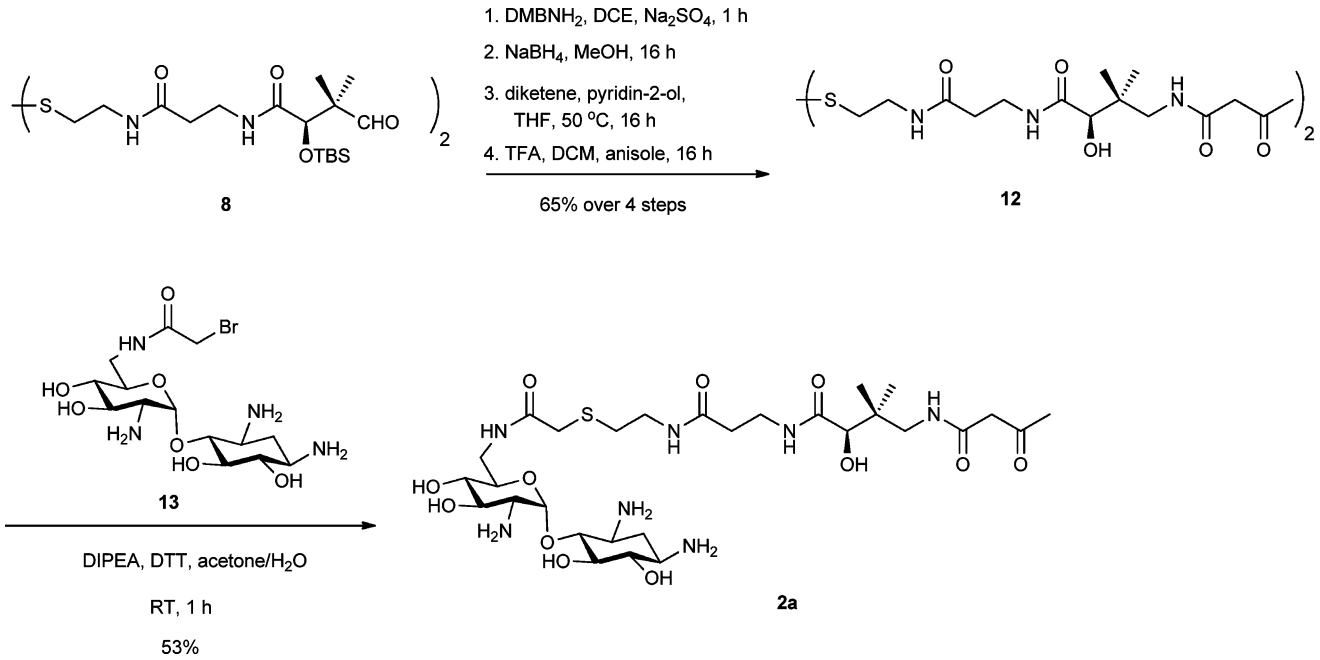
Fig. 2 Docking results for **1** into AAC(6′)-Ii, showing a binding pocket above the ester (highlighted with a red arrow) and potentially accessible by groups extending from the corresponding amide nitrogen.

non-ribosomal peptide biosyntheses,[24] pantetheine variants also find use in studies of these important biosynthetic pathways, and as such, have proven useful in protein labeling.[25–31] Although aminopantetheine[32] and 2′-aminopantothenic acid[33] have been reported, to our knowledge 4′-aminopantetheine (**3**) has not. We report here the first synthesis of **3** and derivatives **2a–d** which were used as probes for AAC(6′).

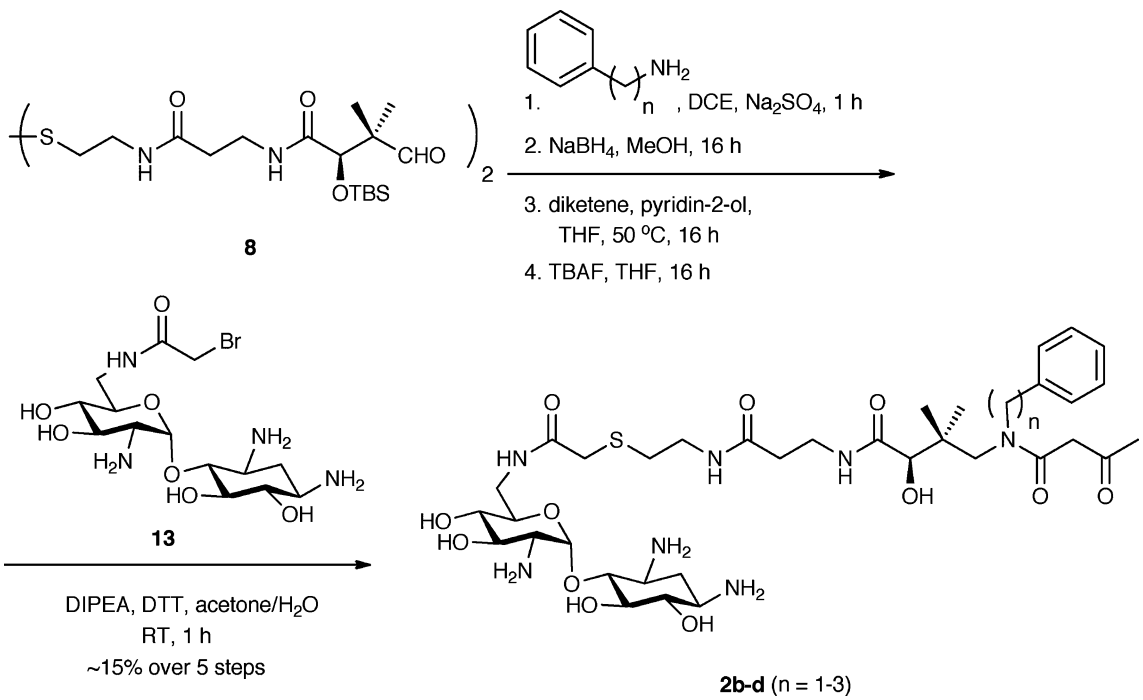
Results

Our first attempted route towards the synthesis of 4′-aminopantetheine (**3**) from commercial D-pantethine (**5**) involved substitution. Activation of the terminal hydroxyl group of **5** with 4-toluenesulfonyl chloride (TsCl) or 2,4,6-triisopropylbenzenesulfonyl chloride (TIPBSCl) were not successful. The more reactive methylsulfonyl chloride (MsCl) was successful at generating mesylated **5**, however, nucleophilic substitution with sodium azide failed, probably due to steric hindrance caused by the adjacent quaternary carbon.

Reductive amination was next explored to install an amine at the 4′ position of pantetheine (**1**). TEMPO/sodium hypochlorite, known to mediate selective oxidations of primary alcohols in 1,3-diols,[34] yielded mixtures when applied to **5** or its *S*-carboxybenzyl-protected analog. Thus, protection of the secondary alcohol of **5** was considered. This was achieved by protection of both the primary and secondary alcohols of **5** using *tert*-butyldimethylsilyl chloride (TBSCl), followed by selective cleavage of the primary alcohol Si–O bond with pyridinium *p*-toluenesulfonate (PPTS) in 92% yield and excellent regioselectivity (Scheme 1). Dess–Martin periodinane (DMP) was used next to generate aldehyde **8** as the sole product with the disulfide remaining intact. Reductive amination was carried out using a dimethoxybenzyl-protected amine ($DMBNH_2$) to afford **9**. The secondary amine of **9** was next transformed into the corresponding 9-fluorenylmethyl carbamate (Fmoc) in order to facilitate deprotection. Indeed, very few examples of DMB cleavage on secondary amines are reported, and all use harsh conditions.[35,36] On the other hand, DMB groups on amides are more easily removed.[37–40] Deprotection of the DMB and TBS groups were achieved using TFA under standard conditions to yield compound **11** in 46% yield over 4 steps. Deprotection of the Fmoc group using piperidine afforded compound **3** as the dimer in 77% yield after HPLC purification. Because of its instability, compound **3** was typically stored as the disulfide dimer and reduced *in situ* using dithiothreitol (DTT) when the free thiol was desired. This 7 step synthesis of 4′-aminopantetheine (**3**) proceeds with an overall yield of ~30%.



Scheme 2 Synthesis of target **2a**.



Scheme 3 Synthesis of targets **2b–d**.

Synthesis of derivative **2a** was achieved *via* a similar route (Scheme 2). After reductive amination, the crude amine was reacted with diketene to produce the desired acetoacetamidate. Complete deprotection to **12** and reaction with *N*-6′-bromoacetylneamine (**13**) under reducing conditions using our standard protocol[11] afforded target **2a** in 35% overall yield after HPLC purification.

This synthetic route was next adapted to prepare amide-functionalized compounds **2b–d** (Scheme 3). Thus the desired amine was used instead of $DMBNH_2$. Fmoc protection was not necessary, and TBS was removed with TBAF.

Compounds **2a–d** were tested for inhibition against *E. faecium* AAC(6′) using the standard enzyme assay.[11] Surprisingly, none of these derivatives showed any significant inhibition ($K_i > 200\ \mu M$).

Discussion

We have developed an efficient synthesis of 4′-aminopantetheine (**3**) from commercial D-pantetheine (**5**). In our hands, substitution

was not successful for replacing the 4′-OH with NH_2, yet oxidation to the aldehyde followed by reductive amination gave access to **3**. This route was next adapted to generate derivatives of the aminoglycoside resistance inhibitor **1**. Compounds **2a–d** can be prepared in one-pot from aldehyde **8**.

Amide **2a** was designed to overcome the suspected poor biological stability of its homolog ester **1**. Esters are rarely found in clinical drugs because they tend to be rapidly hydrolyzed *in vivo*. Amides on the other hand are fairly common functional groups in pharmaceutical compounds. Although the exchange of an ester for an amide has been successful at improving stability yet maintain activity with other systems,[41] to our surprise it was not the case for the inhibition of *E. faecium* AAC(6′). The K_i measured here for amide **2a** was below detection, compared to 11 μM for ester **1**. One possible explanation for this result may lie in the conformational differences between ester and amide bonds, which might orient the acetoacetyl groups differently. Based on structure–activity relationships (SARs) established by us,[8] the acetoacetyl functionality of compound **1** is postulated to mimic the biphosphate moiety of the natural substrate acetyl coenzyme A (AcCoA). Our earlier biological results strongly suggest that the proper orientation of the carbonyl groups is essential to maintain inhibition.[8] Docking studies and comparison of aligned structures (Fig. 3) validate our hypothesis that the 1,3-diketo functionality of compounds **1** and **2a** cannot physically point in the same direction because of the different geometry of the ester *versus* the amide (Fig. 3A). Moreover, when **2a** is docked into the enzyme active site it tends to adopt multiple conformations of similar energy (two examples are given in Fig. 3A and 3B), none of which ranks as favorably as the complex of **1** and AAC(6′)-Ii.

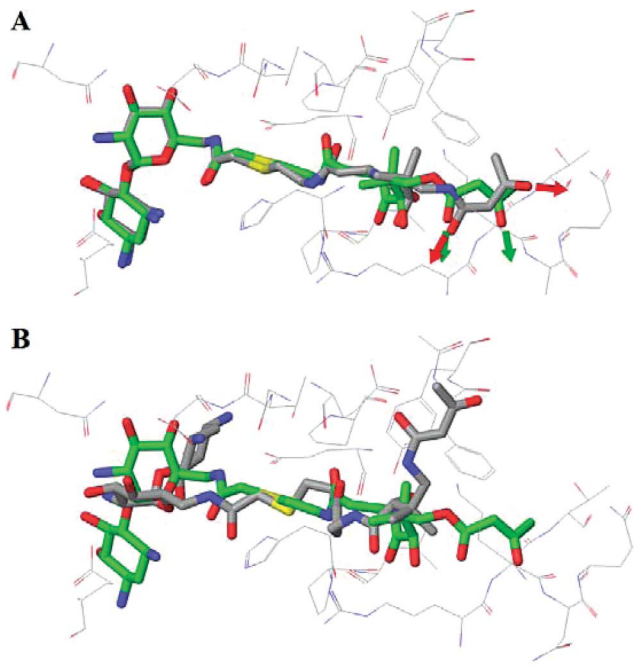


Fig. 3 Docking of the ester **1** (thick green backbone) and the amide **2a** (thick grey backbone) in AAC(6′)-Ii. Panel A shows that the 1,3-dicarbonyl moiety of **2a** does not point in the same direction as that of **1**. Directions are emphasized with red arrows for **2a** and green arrows for **1**. Panel B displays another of many binding configurations suggested by the docking studies for **2a** into AAC(6′)-Ii. Nearby enzyme residues are shown as thin grey wireframes. Atoms are colored by CPK.

As mentioned above, the different crystal structures of AAC(6′) and preliminary docking studies (Fig. 2) suggested that a binding pocket was accessible for groups extending from the amide nitrogen of **2a**. Based on electronic nature of this pocket (formed by Phe, Tyr and Leu residues), it was envisaged that aromatic groups would be ideal amide substituents. Compounds **2b–d** were therefore designed and expected to show improved affinity for AAC(6′)-Ii compared to **1** or **2a**. The lack of detectable inhibitory activity observed is rationalized based on the conformation adopted by the amide in the enzyme, which may prevent the aromatic ring from reaching into the available pocket, causing a clash with Glu141 and His142 (Fig. 4).

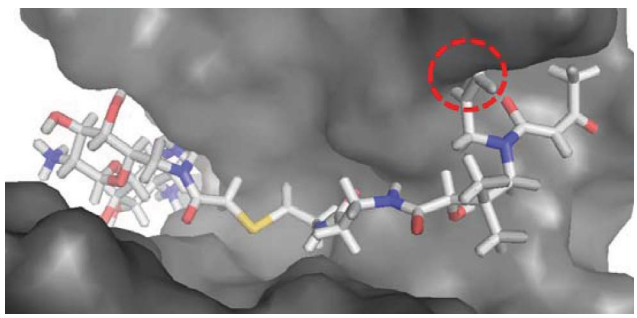


Fig. 4 The amide substituent of **2b–d** may clash with enzyme Glu141 and (highlighted with the red dashed circle). The enzyme is shown using grey space filling and **2b** is depicted in CPK colored wireframes on a grey backbone.

Conclusion

Considering the ubiquity of pantetheine, the above synthetic route to 4′-aminopantetheine (**3**) may prove useful to generate mechanistic and structural probes for numerous biological pathways. Although the *O*-to-*N* substitution reported here had a negative effect on AAC(6′) inhibition, it provided more information about the enzyme interactions with its ligands. AAC(6′)-Ii has been reported to bind a large variety of amine-containing acetyl-acceptor substrates, ranging from 4,5- and 4,6-linked aminoglycosides,[42] to positively charged peptides and proteins.[9] Such promiscuity is only possible if the enzyme structure can adapt to different substrates. In contrast, as suggested by our previous SAR data[8] and the current study, the enzyme seems to have evolved a much tighter specificity for its acetyl-donor substrate AcCoA. We have recently reported that binding of AcCoA to AAC(6′)-Ii requires partial folding of the protein and major structural changes, and proceeds with cooperativity at the dimer interface.[43] On the other hand, the conformational changes undergone by AAC(6′)-Ii are minimal (unpublished data) when binding the aminoglycoside after AcCoA (order necessary for catalysis[42,44]). Since substrate promiscuity suggests protein flexibility, the opposite might have been expected. We show here that in spite of this enzyme's exceptional flexibility, it folds with high accuracy upon encountering AcCoA. This study points to an interesting example of paradoxical enzyme behavior and warrants more studies on the mechanisms used to achieve specificity and promiscuity.

Experimental

Determination of AAC(6′) enzyme inhibition

Enterococcus faecium aminoglycoside *N*-6′-acetyltransferase Type Ii was expressed and purified as previously described.[8] The enzyme assay was performed using a procedure reported earlier.[8]

Docking methods

Docking studies were performed using the default settings of the Maestro v2007 suite of docking softwares developed by Schrodinger. The pdb file of the crystal structure for the bisubstrate inhibitor bound to AAC(6′)-Ii was provided by Prof. Albert M. Berghuis (McGill University). This pdb file was prepared using the Protein Preparation Wizard and the ligands to be docked were prepared by LigPrep; both modules within the Maestro suite. Some images generated from the docking studies were generated using a registered copy of the 'Educational-Use-Only' edition of PyMOL.

Materials and methods for synthesis

Unless otherwise mentioned, all reagents were purchased from Sigma–Aldrich Canada Ltd. (Oakville, Ontario). Reagents and solvents were used without further purification except where stated. Reactions with air or moisture sensitive reagents were carried out in dry glassware under an atmosphere of nitrogen. Flash chromatography and TLC analyses (F-254) were performed with 60 Å silica gel from Silicycle (Quebec, Canada). Purification by reversed-phase HPLC was achieved using an Agilent 1100 modular system equipped with an autosampler, a quaternary pump system, a photodiode array detector, a thermostatted column compartment and a ChemStation (for LC 3D A.09.03) data system. The columns used were a semi-preparative 4.6 × 250 mm, 5 µm Zorbax SB-CN (Agilent, Palo Alto, CA), a preparative 15.0 × 250 mm, 5 µm Luna 5u C8(2) 100A (Phenomenex, CA), or a preparative 21.2 × 250 mm, 5 µm Luna 5u CN 100A (Phenomenex, CA). Samples were eluted at a flow rate of 2 mL min^{-1}, using a combination of mobile phase A (0.1% aqueous TFA) and mobile phase B (acetonitrile containing 0.1% TFA). The detector was set to 214 nm. The different HPLC elution gradients used are detailed in Table 1. The purity of the targets was evaluated by HPLC using the same equipment described above with a Phenomenex semi-preparative Zorbax SB-CN column using gradient elution methods D, E, F, and G. The purity of all target molecules used in the biological assay ranged from 86 to 96%.

Table 1 Linear gradient profiles used for HPLC purification

Method A: with the preparative Luna 5u CN 100A column

Time (min)	%A	%B
0	99	1
25	60	40
37	1	99
55	99	1

Method B: with the semi-preparative Zorbax SB-CN column

Time (min)	%A	%B
0	99	1
15	60	40
25	99	1

Method C: with the preparative Luna 5u C8(2) 100A column

Time (min)	%A	%B
0	99	1
15	60	40
25	99	1

Method D: with the semi-preparative Zorbax SB-CN column

Time (min)	%A	%B
0	99	1
15	60	40
20	10	90
35	99	1

Method E: with the semi-preparative Zorbax SB-CN column

Time (min)	%A	%B
0	99	1
7	60	40
15	10	90
35	99	1

Method F: with the semi-preparative Zorbax SB-CN column

Time (min)	%A	%B
isocratic	65	35

Method G: with the semi-preparative Zorbax SB-CN column

Time (min)	%A	%B
Isocratic	75	25

Instruments used for compounds characterization

High-resolution mass (HRMS) spectra were acquired at the McGill University Mass Spectral facility on an EXACTIVE instrument in orbitrap mode. Low-resolution mass spectra (LRMS) were recorded using a Finnigan LCQDUO mass spectrometer with ESI without fragmentation. ^{1}H and ^{13}C NMR spectra were acquired using Varian mercury 400 or 300 or a Unity 500 spectrometers. The chemical shifts (δ) are reported in parts per million (ppm) and are referenced on residual solvent peaks ($CDCl_3$, $\delta = 7.26$ for ^{1}H NMR and 77.00 for ^{13}C NMR; D_2O, $\delta = 4.79$ for ^{1}H NMR; CD_3OD, $\delta = 3.31$ for ^{1}H NMR and 49.00 for ^{13}C NMR). The peak patterns are indicated as follows: s, singlet; d, doublet; t, triplet; dt, doublet of triplet; ddd, doublet of doublet of doublet; td, triplet of doublet; m, multiplet; q, quartet; p, pentet; and br, broad singlet. The coupling constants, *J*, are reported in hertz (Hz).

Synthetic procedures and compound characterization

Neamine 6′-*N*-(*R*)-(3-((2-((2-amino-2-oxoethyl)thio)ethyl)-amino)-3-oxopropyl)-2-hydroxy-3,3-dimethyl-4-(3-oxobutanamido)butanamide (2a). Compound **12** (22 mg, 0.030 mmol) was dissolved in deoxygenated acetone/H_2O (2 mL, 1/1 v/v).

Dithiothreitol (5.0 mg, 0.03 mmol) and DIPEA (1 mL, 5.7 mmol) were added and the resulting mixture was sonicated for 2 min. The mixture was transferred into a solution of **13**[11] (0.060 mmol) in acetone/H_2O (4 mL, 1/1 v/v). The resulting mixture was sonicated for 2 min and then stirred for 1 h at RT. The reaction mixture was then evaporated *in vacuo* to ~1 ml, diluted with H_2O (10 mL), and acidified to pH 4 using TFA. The solution was washed with ethyl acetate (5 mL × 3), concentrated and purified by reversed phase HPLC (method B, t_R = 10.86 min). The desired product was collected and lyophilized to yield a white fluffy powder (18 mg, 53%). ^{1}H NMR (D_2O, 400 MHz) δ 5.69 (d, J = 3.6, 1H), 3.89–3.83 (m, 4H), 3.64–3.45 (m, 7H), 3.41–3.27 (m, 8H), 3.06 (d, J = 14, 1H), 2.68 (t, J = 6.4, 2H), 2.51–2.45 (m, 3H), 2.24 (s, 3H), 2.18 (s, 2H), 1.84 (q, J = 12.8, 1H), 0.89 (s, 3H), 0.86 (s, 3H); ^{13}C NMR (D_2O, 126 MHz) δ 207.92, 174.47, 173.91, 173.18, 169.45, 96.73, 78.88, 76.06, 74.94, 72.41, 71.39, 70.31, 68.62, 53.76, 49.60, 48.46, 46.71, 39.48, 38.36, 38.19, 35.40, 35.27, 34.63, 31.23, 30.17, 29.75, 28.24, 21.30, 20.11; HRMS for $C_{29}H_{54}N_7O_{12}S$ [M + H]$^+$ calcd. 724.3546, found 724.3525. Purity, 95% (method F, t_R = 5.15 min; method G, t_R = 5.99 min).

Neamine 6′-*N*-(*R*)-(3-((2-((2-amino-2-oxoethyl)thio)ethyl)-amino)-3-oxopropyl)-4-(*N*-benzyl-3-oxobutanamido)-2-hydroxy-3,3-dimethylbutanamide (2b). To aldehyde **8** (80 mg, 0.10 mmol) in 1,2-dichloroethane (3 mL) was added anhydrous sodium sulfate (0.20 g) and benzylamine (0.045 mL, 0.40 mmol). The reaction mixture was stirred at RT for 1 h then filtered. The filtrate was diluted with toluene (2 × 20 mL) and evaporated to dryness under reduced pressure to get the imine intermediate as a clear oil. This crude product was dissolved in anhydrous MeOH (3 mL). Sodium borohydride (39 mg, 1.0 mmol) was added and the reaction mixture was stirred overnight at RT, before quenching with saturated aqueous sodium bicarbonate (5 mL), concentration under reduced pressure, and extraction with CH_2Cl_2 (4 × 20 mL). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to afford a colorless oil. ESI-MS for $C_{48}H_{85}N_6O_6S_2Si_2$ [M + H]$^+$ calcd. 961.5, found 961.6. This amine in THF (3 mL) was treated with 2-hydroxypyridine (20 mg, 0.20 mmol) and diketene (0.080 mL, 1.0 mmol). The reaction mixture was warmed up to 50 °C and stirred overnight at the same temperature. The reaction was monitored by ESI-MS for the disappearance of the amine and the emergence of the amide product; ESI-MS for the amide $C_{56}H_{92}N_6O_{10}S_2Si_2Na$ [M + Na]$^+$ calcd. 1151.6, found 1151.6. The reaction mixture was concentrated under reduced pressure, then redissolved in THF (3 mL). *tetra-n*-Butylammonium fluoride (TBAF, 1 M in THF, 1 mL, 1 mmol) was added and the resulting solution was allowed to stir at RT overnight. The reaction was quenched with saturated aqueous sodium bicarbonate (5 mL). Water (3 mL) was added, and the mixture extracted with ethyl acetate (3 × 10 mL). The combined organic layer was dried over anhydrous sodium sulfate, concentrated under reduced pressure, and redissolved in acetone/H_2O (2 mL, 1/1 v/v). Dithiothreitol (15 mg, 0.10 mmol) and DIPEA (1 mL, 5.7 mmol) were added and the resulting mixture was sonicated for 2 min. The mixture was transferred into a solution of **13**[11] (0.20 mmol) in acetone/H_2O (4 mL, 1/1 v/v). The resulting mixture was sonicated for 2 min and then stirred for 16 h at RT. The reaction mixture was then evaporated *in vacuo* to ~1 ml, diluted with H_2O (10 mL), and acidified to pH 4 using TFA. The solution was washed with ethyl acetate (5 mL × 3), concentrated and purified by reversed phase HPLC (method C, t_R = 27.72 min). The desired product was collected and lyophilized to yield a white fluffy powder (17.2 mg, 15% over 5 steps). ^{1}H NMR (D_2O, 400 MHz) δ 7.42–7.26 (m, 3H), 7.17 (d, J = 7.2, 2H), 5.69 (d, J = 3.6, 1H), 4.70 (s, 2H), 3.89–3.82 (m, 4H), 3.73–3.42 (m, 10H), 3.40–3.28 (m, 7H), 2.67 (t, J = 6.4, 2H), 2.51–2.39 (m, 3H), 2.25 (s, 1H), 2.08 (s, 3H), 1.84 (q, J = 12.4, 1H), 1.02 (s, 3H), 0.93 (s, 3H); ^{13}C NMR (D_2O, 126 MHz) δ 207.28, 174.14, 173.87, 173.15, 171.79, 136.47, 129.01, 127.74, 126.14, 96.70, 78.84, 76.13, 74.93, 72.40, 71.38, 70.30, 68.60, 54.28, 53.98, 53.75, 49.59, 48.45, 40.23, 39.70, 39.46, 38.16, 35.39, 35.26, 34.61, 31.24, 29.68, 28.22, 22.47, 20.64; HRMS for $C_{36}H_{60}N_7O_{12}S$ [M + H]$^+$ calcd. 814.4026, found 814.3997. Purity, 86% (method D, t_R = 18.29 min; method E, t_R = 13.14 min).

Neamine 6′-*N*-(*R*)-(3-((2-((2-amino-2-oxoethyl)thio)ethyl)amino)-3-oxopropyl)-2-hydroxy-3,3-dimethyl-4-(3-oxo-*N*-phenethylbutanamido)butanamide (2c)

To aldehyde **8** (80 mg, 0.10 mmol) in 1,2-dichloroethane (3 mL) was added anhydrous sodium sulfate (0.20 g) and 2-phenylethanamine (0.052 mL, 0.40 mmol). The reaction mixture was stirred at RT for 1 h then filtered. The filtrate was diluted with toluene (2 × 20 mL) and evaporated to dryness under reduced pressure to get the imine intermediate as a clear oil. This crude product was dissolved in anhydrous MeOH (3 mL). Sodium borohydride (39 mg, 1.0 mmol) was added. The reaction mixture was stirred overnight at RT, quenched with saturated aqueous sodium bicarbonate (5 mL), concentrated under reduced pressure, and extracted with CH_2Cl_2 (4 × 20 mL). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to afford a colorless oil. ESI-MS for $C_{50}H_{88}N_6O_6S_2Si_2$ [M + H]+ calcd. 989.6, found 989.6. This amine in THF (3.0 mL) was treated with 2-hydroxypyridine (20 mg, 0.20 mmol) and diketene (0.080 mL, 1.0 mmol). The reaction mixture was warmed up to 50 °C and stirred overnight at the same temperature. The reaction was monitored by ESI-MS for the disappearance of the amine and the emergence of the amide product; ESI-MS for the amide $C_{58}H_{96}N_6O_{10}S_2Si_2$ [M + Na]$^+$ calcd. 1179.6, found 1179.6. The reaction mixture was concentrated under reduced pressure, then redissolved in THF (3 mL). TBAF (1 M in THF, 1 mL, 1 mmol) was added and the resulting solution was allowed to stir at RT overnight. The reaction was quenched with saturated aqueous sodium bicarbonate (5 mL). Water (3 mL) was added, and the mixture was extracted in ethyl acetate (3 × 10 mL). The combined organic layer was dried over anhydrous sodium sulfate, concentrated under reduced pressure, and redissolved in acetone/H_2O (2 mL, 1/1 v/v). Dithiothreitol (15 mg, 0.10 mmol) and DIPEA (1 mL, 5.7 mmol) were added and the resulting mixture was sonicated for 2 min. The mixture was transferred into a solution of **13**[11] (0.2 mmol) in acetone/H_2O (4 mL, 1/1 v/v). The resulting mixture was sonicated for 2 min and then stirred for 16 h at RT. The reaction mixture was then evaporated *in vacuo* to ~1 ml, diluted with H_2O (10 mL), and acidified to pH 4 using TFA. The solution was washed with ethyl acetate (3 × 5 mL), concentrated and purified by reversed phase HPLC (method C,

t_R = 28.67 min). The desired product was collected and lyophilized to yield a white fluffy powder (17.4 mg, 15% over 5 steps). ^{1}H NMR (D_2O, 400 MHz) δ 7.23–7.12 (m, 3H), 7.05 (d, J = 6.8, 2H), 5.55 (d, J = 4.0, 1H), 3.75–3.68 (m, 4H), 3.51–3.12 (m, 19H), 2.73 (t, J = 6.4, 2H), 2.54 (t, J = 6.4, 2H), 2.37–2.27 (m, 3H), 2.07 (s, 1H), 1.96 (s, 3H), 1.70 (q, J = 12.4, 1H), 0.80 (s, 3H), 0.74 (s, 3H); ^{13}C NMR (D_2O, 126 MHz) δ 207.67, 174.12, 173.84, 173.12, 171.20, 138.06, 129.15, 128.84, 126.89, 96.64, 78.76, 75.98, 74.89, 72.36, 71.32, 70.24, 68.55, 53.69, 51.78, 51.62, 49.54, 48.39, 40.10, 39.39, 38.58, 38.11, 35.37, 35.21, 34.54, 33.61, 31.18, 29.82, 28.17, 22.41, 20.62; HRMS for $C_{37}H_{62}N_7O_{12}S$ $[M + H]^+$ calcd. 828.4172, found 828.4169. Purity, 96% (method F, t_R = 7.01 min; method G, t_R = 12.61 min).

Neamine 6′-*N*-(*R*)-(3-((2-((2-amino-2-oxoethyl)thio)ethyl)-amino)-3-oxopropyl)-2-hydroxy-3,3-dimethyl-4-(3-oxo-*N*-(3-phenylpropyl)butanamido)butanamide (2d). To aldehyde **8** (80 mg, 0.10 mmol) in 1,2-dichloroethane (3 mL) was added anhydrous sodium sulfate (0.20 g) and 3-phenylpropan-1-amine (0.059 mL, 0.40 mmol). The reaction mixture was stirred at RT for 1 h then filtered. The filtrate was diluted with toluene (2 × 20 mL) and evaporated to dryness under reduced pressure to get the imine intermediate as a clear oil. This crude product was dissolved in anhydrous MeOH (3 mL). Sodium borohydride (39 mg, 1.0 mmol) was added. The reaction mixture was stirred overnight at RT, quenched with saturated aqueous sodium bicarbonate (5 mL), concentrated under reduced pressure, and extracted with CH_2Cl_2 (4 × 20 mL). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to get the amine as a colorless oil. ESI-MS for $C_{52}H_{92}N_6O_6S_2Si_2Na$ $[M+Na]^+$ calcd. 1039.6, found 1039.7. The amine in THF (3 mL) was treated with 2-hydroxypyridine (20 mg, 0.20 mmol) and diketene (0.080 mL, 1.0 mmol). The reaction mixture was warmed up to 50 °C and stirred overnight. The reaction was monitored by ESI-MS for the disappearance of the amine and the emergence of the amide product; ESI-MS for the amide $C_{60}H_{100}N_6O_{10}S_2Si_2Na$ $[M + Na]^+$ calcd. 1207.6, found 1207.6. The reaction mixture was concentrated under reduced pressure, then redissolved in THF (3 mL). TBAF (1 M in THF, 1 mL, 1 mmol) was added and the resulting solution was allowed to stir at RT overnight. The reaction was quenched with saturated aqueous sodium bicarbonate (5 mL). Water (3 mL) was added, and the mixture was extracted with ethyl acetate (3 × 10 mL). The combined organic layer was dried over anhydrous sodium sulfate, concentrated under reduced pressure, and redissolved in acetone/H_2O (2 mL, 1/1 v/v). Dithiothreitol (15 mg, 0.10 mmol) and DIPEA (1 mL, 5.7 mmol) were added and the resulting mixture was sonicated for 2 min. The mixture was transferred into a solution of **13**[11] (0.20 mmol) in acetone/H_2O (4 mL, 1/1 v/v). The resulting mixture was sonicated for 2 min and then stirred for 16 h at RT. The reaction mixture was then evaporated *in vacuo* to ~1 mL, diluted with H_2O (10 mL), and acidified to pH 4 using TFA. The solution was washed with ethyl acetate (3 × 5 mL), concentrated and purified by reversed phase HPLC (method C, t_R = 29.13 min). The desired product was collected and lyophilized to yield a white fluffy powder (19 mg, 16% over 5 steps). ^{1}H NMR (D_2O, 400 MHz) δ 7.38–7.33 (m, 2H), 7.29–7.24 (m, 3H), 5.70 (d, J = 4.0, 1H), 3.90–3.76 (m, 4H), 3.66–3.24 (m, 19H), 2.69 (t, J = 6.4, 2H), 2.62 (t, J = 6.8, 2H), 2.51 (t, J = 4.0, 1H), 2.47 (t, J = 5.8, 2H), 2.25 (s, 1H), 2.13 (s, 3H), 1.97–1.81 (m, 3H), 0.90 (s, 3H), 0.86 (s, 3H); ^{13}C NMR (D_2O, 126 MHz) δ 207.77, 174.10, 173.89, 173.16, 170.70, 141.30, 128.72, 128.51, 126.27, 96.70, 78.85, 76.06, 74.94, 72.41, 71.38, 70.31, 68.61, 53.75, 52.11, 49.91, 49.60, 48.46, 39.99, 39.47, 38.18, 38.13, 35.39, 35.26, 34.62, 31.78, 31.24, 29.81, 29.25, 28.22, 22.38, 20.61; HRMS for $C_{38}H_{64}N_7O_{12}S$ $[M + H]^+$ calcd. 842.4328, found 842.4306. Purity, 90% (method D, t_R = 20.17 min; method E, t_R = 13.85 min).

4′-Aminopantetheine (3). To Fmoc protected amine **11** (33 mg, 0.03 mmol) dissolved in DMF (5 mL) was added piperidine (0.25 mL) followed by evaporation of the liquids under high reduced pressure. The process was repeated once more and the residue dissolved in H_2O (5 mL). The solution was washed with diethyl ether (5 mL × 3), concentrated and purified by reversed phase HPLC (method A, t_R = 35.87 min). The desired product (as the disulfide dimer) was collected and lyophilized to yield a white powder (14 mg, 77%). The compound was fully characterized as the *more stable* dimer, but the free thiol was easily accessible by treatment with dithiothreitol (DTT, ~1 mM). ^{1}H NMR (D_2O, 500 MHz) δ 4.08 (s, 1H), 3.61–3.58 (m, 4H), 3.10 (s, 2H), 2.92 (t, J = 6.5, 2H), 2.59 (t, J = 6.5, 2H), 1.16 (s, 3H), 1.09 (s, 3H); ^{13}C NMR (D_2O, 125 MHz) δ 173.75, 173.74, 77.38, 47.70, 37.83, 36.24, 35.67, 35.40, 34.99, 21.77, 19.76; HRMS for $C_{22}H_{45}N_6O_6S_2$ $[M + H]^+$ calcd. 553.2837, found 553.2848. Purity, 98% (method D, t_R = 10.15 min; method E, t_R = 7.41 min).

D-Pantetheine (4)[45]. D-Pantethine (**5**) (1.0 g, 1.8 mmol) was dissolved in degassed H_2O (10 mL) and MeOH (10 mL). The mixture was cooled to 4 °C and D/L-dithiothreitol (DTT, 0.44 g, 2.8 mmol) was added. The reaction mixture was stirred at 4 °C for 16 h under N_2. After evaporation of the solvent, the residue was purified by silica gel column chromatography with a mixture of $CHCl_3$–MeOH (10 : 1 → 9 : 1) as the eluent to give compound **4** as a white solid (0.99 g, 99%). R_f 0.31 (CH_3Cl–MeOH 7 : 1); ^{1}H NMR (D_2O, 500 MHz) δ 3.97 (s, 1H), 3.55–3.46 (m, 3H), 3.38–3.34 (m, 3H), 2.63 (t, J = 6.5, 2H), 2.50 (t, J = 6.5, 2H), 0.90 (s, 3H), 0.87 (s, 3H); ^{13}C NMR (D_2O, 126 MHz) δ 175.26, 174.18, 75.87, 68.47, 42.41, 38.72, 35.60, 35.41, 23.25, 20.61, 19.20; ESI-MS for $C_{11}H_{22}N_2O_4SNa$ $[M + Na]^+$ calcd. 301.1, found 301.2.

(*R*)-*S*-2-(3-(2,4-*tert*-butyldimethylsilyloxy-3,3-dimethylbutanamido)propanamido)ethyl disulfide (6). D-Pantethine **5** (1.05 g, 1.89 mmol) in anhydrous DMF (10 mL) was treated with imidazole (1.54 g, 22.6 mmol) and *tert*-butyldimethylsilyl chloride (3.42 g, 22.6 mmol) at RT. The reaction mixture was stirred overnight, and then quenched with water (60 mL). The mixture was extracted with EtOAc (3 × 100 mL). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The resulting colorless oil was purified by flash column chromatography on silica gel (EtOAc–hexane 1 : 1 → EtOAc) to get the title compound as a clear oil (1.86 g, 98%). R_f 0.40 (EtOAc); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.91 (t, J = 6.0, 1H), 6.84 (t, J = 6.0, 1H), 3.98 (s, 1H), 3.58–3.46 (m, 4H), 3.40 (d, J = 6.3), 3.29 (d, J = 6.3), 2.77 (t, J = 6.6, 2H), 2.44 (t, J = 6.0, 2H), 0.91 (s, 9H), 0.87 (s, 9H), 0.85 (s, 3H), 0.81 (s, 3H), 0.06 (s, 3H), 0.02 (s, 3H), 0.01 (s, 3H), –0.02 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 172.95, 171.34, 76.49, 68.76, 40.07, 38.33, 37.52, 35.88, 34.60, 25.96, 25.79, 20.97, 19.85, 18.33, 17.96, –5.08, –5.28, –5.36,

–5.47; HRMS for $C_{46}H_{99}N_4O_8S_2Si_4$ [M + H]$^+$ calcd. 1101.5981, found 1011.5977.

(*R*)-*S*-2-(3-(2-*tert*-butyldimethylsilyloxy-4-hydroxyl-3,3-dimethylbutanamido)propanamido)ethyl disulfide (7). Compound **6** (1.91 g, 1.89 mmol) was dissolved in MeOH (20 mL) at 0 °C. Pyridinium *p*-toluenesulfonate (1.02 g, 3.98 mmol) was added. The reaction mixture was allowed to warm up slowly to RT and stirred overnight. The mixture was concentrated under reduced pressure and was purified by flash column chromatography on silica gel (acetone–hexane 1 : 1) to provide the desired compound as a clear oil (1.37 g, 92%). R_f 0.20 (acetone–hexane 1 : 1); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.05 (t, J = 6.0, 1H), 6.91 (t, J = 5.7, 1H), 3.96 (s, 1H), 3.65–3.43 (m, 4H), 3.41 (d, J = 12.0), 3.35 (d, J = 12.0), 2.78 (t, J = 6.6, 2H), 2.54–2.40 (m, 2H), 0.98 (s, 3H), 0.93 (s, 9H), 0.78 (s, 3H), 0.08 (s, 3H), 0.00 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 174.04, 171.29, 78.24, 70.08, 40.50, 38.36, 37.56, 35.49, 34.87, 25.77, 23.54, 19.00, 17.96, –5.10, –5.27; HRMS for $C_{34}H_{70}N_4O_8NaS_2Si_2$ [M + Na]$^+$ calcd. 805.4071, found 805.4056.

(*R*)-*S*-2-(3-(2-*tert*-butyldimethylsilyloxy-4-oxo-3,3-dimethylbutanamido)propanamido)ethyl disulfide (8). To Dess–Martin periodinane (0.043 g, 0.10 mmol) in CH_2Cl_2 (3 mL) was added compound **7** (0.073 g, 0.090 mmol) in wet CH_2Cl_2 (3 mL). The reaction mixture was stirred at RT for 15 min. Another portion of Dess–Martin periodinane (0.043 g, 0.10 mmol) was added. The reaction mixture was stirred at RT for another 15 min. A mixture of saturated aqueous sodium thiosulfate and saturated aqueous sodium bicarbonate (1 : 1, 3 mL) was added. The mixture was extracted with CH_2Cl_2 (3 × 10 mL), and the combined organic layer was washed with saturated aqueous sodium bicarbonate (2 mL), dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to yield the title compound (0.062 g, 86%) as a clear oil. This crude product was directly used in the next reaction. R_f 0.38 (acetone–hexane 1 : 1); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.55 (s, 1H), 7.02 (t, J = 6.0, 1H), 6.91 (bs, 1H), 4.21 (s, 1H), 3.59–3.44 (m, 4H), 2.77 (t, J = 6.4, 2H), 2.43 (t, J = 6.4, 2H), 1.05 (s, 3H), 0.97 (s, 3H), 0.93 (s, 9H), 0.10 (s, 3H), 0.05 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 201.90, 171.61, 171.30, 76.11, 50.65, 38.35, 37.53, 35.34, 34.85, 25.70, 19.24, 17.93, 16.06, –4.97, –5.12; HRMS for $C_{34}H_{66}N_4O_8NaS_2Si_2$ [M + Na]$^+$ calcd. 801.3758, found 801.3745.

(2*R*,2′*R*)-*N*,*N*′3,3′-(2,2′-disulfanediylbis(ethane-2,1-diyl)bis(azanediyl))bis(3-oxopropane-3,1-diyl))bis(2,4-dihydroxy-3,3-dimetylbutanamide) (11). To the aldehyde **8** (370 mg, 0.475 mmol) in 1,2-dichloroethane (6 mL) was added anhydrous sodium sulfate (0.50 g) and 2,4-dimethoxybenzylamine (0.157 mL, 1.05 mmol). The reaction mixture was stirred at RT for 1 h then filtered. The filtrate was diluted with toluene (2 × 30 mL) and evaporated to dryness under reduced pressure to get the imine intermediate as a clear oil. This crude product was dissolved in anhydrous MeOH (15 mL). Sodium borohydride (180 mg, 4.75 mmol) was added. The reaction mixture was stirred overnight at RT, quenched with saturated aqueous sodium bicarbonate (15 mL), concentrated under reduced pressure, and extracted in CH_2Cl_2 (4 × 30 mL). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to get a colorless oil. R_f 0.23 ($CHCl_3$–MeOH–NH_4OH 10 : 1 : 0.25); ESI-MS for $C_{52}H_{92}N_6O_{10}S_2Si_2$ [M + Na]$^+$ calcd. 1103.6, found 1103.5. To the amine in dioxane–water (4 : 1, v/v) (5 mL) was added FmocCl (983 mg, 3.80 mmol) followed by DIPEA (1.66 mL, 9.50 mmol) and the reaction mixture stirred for 24 h. The reaction mixture was poured into water (50 mL), extracted with CH_2Cl_2 (4 × 30 mL), the combined organic layer was dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residue loaded on a short silica pad and the excess reagent was washed off with 50% EtOAc/hexanes (v/v). The product was washed off the column with 10% methanol–CH_2Cl_2 (v/v) and concentrated under reduced pressure to give an off white solid ESI-MS for $C_{82}H_{112}N_6O_{14}S_2Si_2$ [M + Na]$^+$ calcd. 1547.7, found 1547.6. The solid was dissolved in CH_2Cl_2 (3 mL), Trifluoroacetic acid (3 mL) and anisole (2–3 drops) were added and the mixture turned red. The solution was allowed to stir at RT overnight. CH_2Cl_2 (50 mL) was added, and the resulting solution was extracted with H_2O (3 × 20 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure to give a solid residue that was purified by FCC on silica gel using 5% Methanol in CH_2Cl_2 (v/v). (235 mg, 45% over 4 steps). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.75 (d, 4H), 7.58–7.52 (m, 6H), 7.41–7.36 (m, 4H), 7.33–7.27 (m, 4H), 6.87 (t, J = 5.5, 2H) 5.41 (t, J = 6.5, 2H) 4.42 (d, J = 6.5, 4, 4H), 4.19 (t, J = 6.5, 2H), 3.84–3.36(m, 14H), 2.83–2.76 (m, 6H), 2.54–2.42 (m, 4H), 1.00 (s, 3H), 0.87 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.00, 171.77, 157.94, 143.70, 141.29, 127.75, 127.07, 124.98, 124.91, 120.00, 74.83, 67.97, 66.89, 49.56, 47.20, 39.35, 38.50, 37.75, 35.93, 34.97, 25.60, 21.91, 20.64; HRMS for $C_{52}H_{64}N_6O_{10}NaS_2$ [M + Na]$^+$ calcd. 1019.4018, found 1019.4014.

(*R*)-*S*-(2-(3-(4-(3-oxobutanamido)-2-hydroxyl-3,3-dimethylbutanamido)propanamido)ethyl) disulfide (12). To the aldehyde **8** (40 mg, 0.050 mmol) in 1,2-dichloroethane (3 mL) was added anhydrous sodium sulfate (0.20 g) and 2,4-dimethoxybenzylamine (0.017 mL, 0.11 mmol). The reaction mixture was stirred at RT for 1 h then filtered. The filtrate was diluted with toluene (2 × 20 mL) and evaporated to dryness under reduced pressure to get the imine intermediate as a clear oil. This crude product was dissolved in anhydrous MeOH (3 mL). Sodium borohydride (19 mg, 0.50 mmol) was added. The reaction mixture was stirred overnight at RT, quenched with saturated aqueous sodium bicarbonate (5 mL), concentrated under reduced pressure, and extracted in CH_2Cl_2 (4 × 20 mL). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to get a colorless oil. R_f 0.23 ($CHCl_3$–MeOH–NH_4OH 10 : 1 : 0.25); ESI-MS for $C_{52}H_{92}N_6O_{10}S_2Si_2$ [M + Na]$^+$ calcd. 1103.6, found 1103.5. This amine in THF (3 mL) was treated with 2-hydroxypyridine (10 mg, 0.10 mmol) and diketene (0.040 mL, 0.52 mmol). The reaction mixture was warmed up to 50 °C and stirred overnight. The reaction was monitored by ESI-MS for the disappearance of the amine and the emergence of the amide product; ESI-MS for the amide $C_{60}H_{100}N_6O_{14}S_2Si_2$ [M + Na]$^+$ calcd. 1271.6, found 1271.5. The reaction mixture was concentrated under reduced pressure, then suspended in CH_2Cl_2 (1.5 mL). Trifluoroacetic acid (1.5 mL) and anisole (2–3 drops) were added and the mixture turned red. The solution was allowed to stir at RT overnight. CH_2Cl_2 (10 mL) was added, and the resulting solution was extracted with H_2O (3 × 10 mL). The aqueous layer was combined and evaporated under high vacuum

to obtain the title compound as a clear oil (24 mg, 65% over 4 steps). ^{1}H NMR (D_2O, 300 MHz) δ 3.84 (s, 1H), 3.55–3.40 (m, 4H), 3.30 (d, J = 13.8, 1H), 3.07 (d, J = 13.8, 1H), 2.80 (t, J = 6.3, 2H), 2.47 (t, J = 6.3, 2H), 2.26 (bs, 5H), 0.91 (s, 3H), 0.87 (s, 3H); ^{13}C NMR (D_2O, 75 MHz) δ 207.99, 174.65, 174.02, 169.58, 76.21, 46.92, 38.57, 38.18, 36.59, 35.60, 35.40, 29.94, 29.87, 21.52, 20.32; HRMS for $C_{30}H_{52}N_6O_{10}NaS_2$ $[M + Na]^+$ calcd. 743.3084, found 743.3068.

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