2-Iminothiolane as a Useful Coupling Reagent for Polyamine Solid-Phase Synthesis

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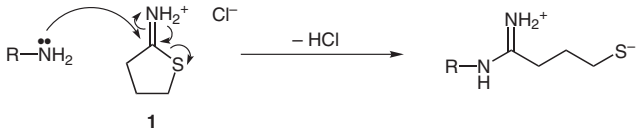
Received 9 May 2008

Abstract: Here, the versatility of 2-iminothiolane to act as coupling reagent for solid-phase synthesis of polyamine conjugates is shown. This method, which supersedes the use of elaborate protection group strategies, allows the mild coupling of biomacromolecules and other functional moieties. Spermine conjugates with diverse maleimido building blocks were synthesized by a quick and selective reaction.

Key words: solid-phase synthesis, chemoselectivity, conjugation, polyamines, 2-iminothiolane

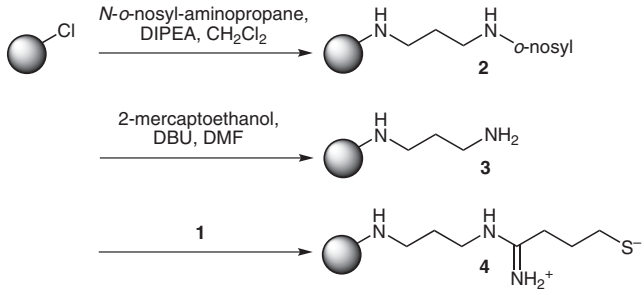
In order to increase their water solubility and bioavailability many therapeutically interesting drugs and nanoparticles have been covalently conjugated to polycationic moieties.[1] Besides the well-characterized polyarginines[2] also polyamines bearing primary and secondary amino groups have been shown to enhance the cellular uptake of different cargo.[1,3] They are protonated at physiological pH thereby tightly interacting with negatively charged cell-membrane components such as proteoglycans and the phosphate groups of phospholipids. Depending on the length of the polyamine backbone and the covalently coupled cargo, the cellular uptake is suggested to occur either via a polyamine transport system (PAT) or via endocytosis of proteoglycan-enriched membranes.[1]

The conjugation of polyamines with biomacromolecules is often a laborious task due to the elaborate protection-group strategy, which is required to differentiate between the amines and the mild chemistry, which should not harm the biomacromolecule. Furthermore, polyamine moieties are highly hydrophilic, making the conjugates often water soluble and difficult to handle in organic solvents. These problems can be greatly reduced by solid-phase chemistry.[4] Recently, we have established a novel protection-group strategy for polyamines on solid supports that permitted a mild cleavage procedure.[5] Now, we were looking for methods facilitating the on-bead coupling of the biomacromolecule or the nanomaterials. So far, one option is the coupling of a thiol to a maleimido group in a Michael addition yielding a thioether linkage. A very gentle method to introduce a thiol functionality in a biomacromolecule is the modification of a primary amino group with 2-iminothiolane hydrochloride (**1**, Traut's reagent), which is a popular crosslinking agent for peptides.[6] It has frequently been used for the conversion of the side-chain amino group of lysine into a thiol (Scheme 1). Eventually, the thiol-modified polyamines can be covalently coupled to other functionalities either to maleimido groups in a Michael addition or to other thiols in a disulfide bond as well as to a-bromocarbonyls in an S_N2 reaction.



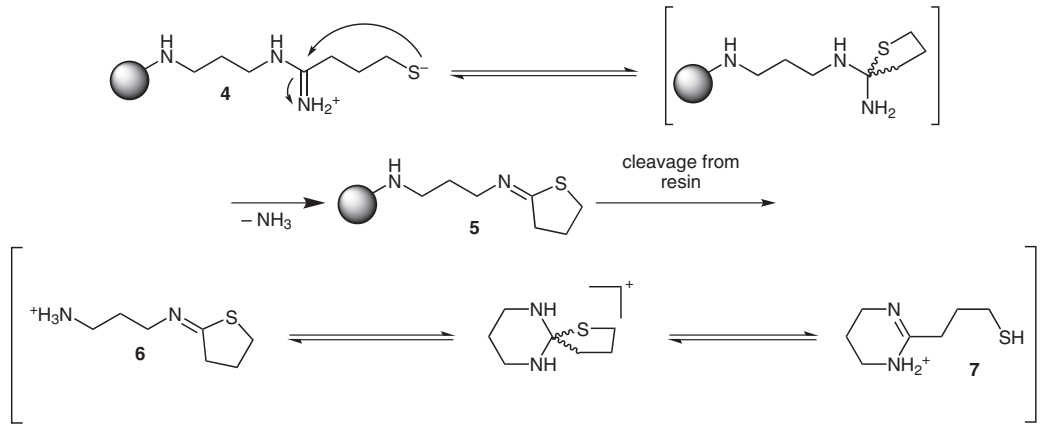
Scheme 1 Reaction of 2-iminothiolane with primary amines

Some features make 2-iminothiolane suitable for the use in solid-phase synthesis of polyamine conjugates. Namely, its selectivity for primary amines and the preservation of charge by the quick conversion of the amine into an amidine/ amidinium salt are attractive features. The reaction takes place under mild conditions and allows efficient synthesis on acid- and base-labile resins permitting mild cleavage conditions. Recently, this reaction was established for the solution-phase conjugation of spermine and cholesterol.[7] So far, it was not reported for solid-phase synthesis. To efficiently convert the reaction to solid phase, we established a model system with diaminopropane as a short diamine (Scheme 2). 2-Chlorotrityl resin (1.3 mmol/g) was loaded with N-monoprotected diaminopropane in order to avoid crosslinking of the symmetric diamine. The *o*-nosyl group was removed with 2-mercaptoethanol and DBU in DMF and the free primary amine was reacted with excess of 2-iminothiolane hydrochloride.



Scheme 2 Loading of the resin and subsequent deprotection

SYNLETT 2008, No. 18, pp 2785–2790
Advanced online publication: 15.10.2008
DOI: 10.1055/s-0028-1083566; Art ID: G14908ST

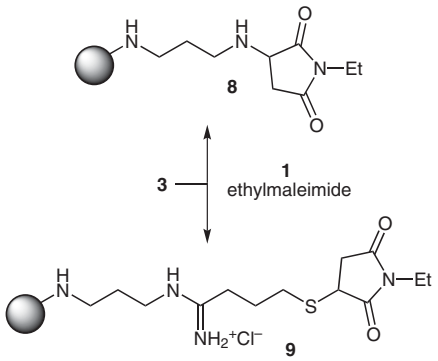


Scheme 3 Side reaction of **4** leading to restitution of a N-substituted iminothiolane

The thiol modification with 2-iminothiolane is usually carried out in DMSO or in aqueous buffers at pH values above 7.0.[7,8] However, both solvents are less suitable for reactions on polystyrene resins. Therefore, we investigated other solvent systems as described below. 2-Iminothiolane was added to the pre-swollen resin in the respective solvent. After 15 hours the resin was washed and the cleaved crude product was analyzed by ^{1}H NMR spectroscopy and mass spectrometry. As expected, the resins showed only minimal swelling in phosphate buffer (pH 8.0) and DMSO and the reaction between the 2-iminothiolane and the primary amine was prevented. Likewise, the reaction in THF proceeded very poorly, probably due to the low solubility of 2-iminothiolane. Although, the solubility in DMF was improved, not all primary amines reacted even after 15 hours. The addition of 2 equivalents DIPEA in order to accelerate the reaction did not markedly improve the conversion.

However, complete reaction was observed, if 2-iminothiolane was dissolved in water and was added to a sus-

Table 1 Optimization of Reaction Conditions



Entry	Reaction time (h)	2-Iminothiolane[a] (equiv/min)	*N*-Ethylmaleimide[a] (equiv/min)	Ratio[b,c]		
				5	**8**	**9**
1	15	2/0	5/780	1.69	0	1
2	15	2/0	5/2	0.13	0.47	1
3	15	1/0 1/5	1/0 1/5	0.10	0.10	1
4	15	1/0 1.5/1	1/1 0.3/2.5	0.08	0.10	1
5	15	0.5/0 1.5/2	1.3/2	0.10	0.31	1
6	15	3/0	2/1	0.06	0.04	1

[a] Amount (equiv), addition time (min), 1/5: addition of 1 equiv after 5 min
[b] The ratio between **5** and **9** was determined by peak integration of the characteristic triplets in the ^{1}H NMR spectra at δ = 3.7 and 3.4.
[c] The ratio of **8** and **5** was determined by integration of the characteristic ^{1}H NMR dd signals at δ = 4.5 and 3.9 in MeOD.

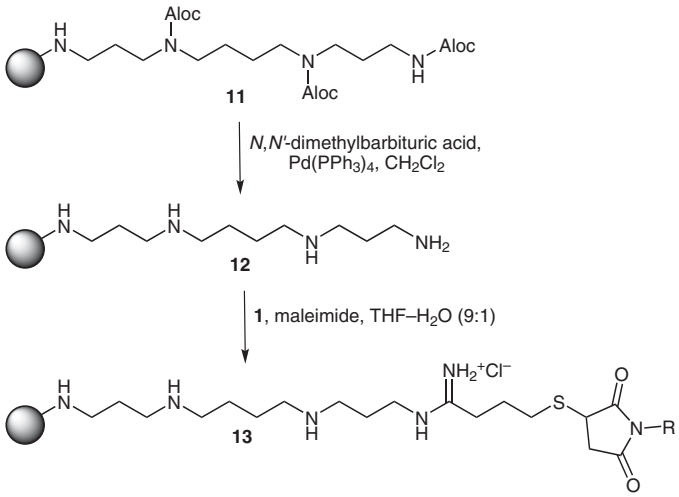
pension of the resin in THF, giving a solvent mixture of water–THF (1:9). The clear liquid phase indicated that the 2-iminothiolane had entirely dissolved, while the resin was sufficiently swollen.

Analysis of the crude mixture indeed showed the absence of diaminopropane but also the occurrence of a byproduct. The mass spectra showed an extra $[M - 16]^+$ peak at 159.1 u due to the loss of ammonia.[8] The formation of this byproduct arose from the nucleophilic attack of the liberated thiolate onto the amidite resulting in the N-substituted iminothiolane **5** (Scheme 3). If the reaction was carried out overnight in the presence of a weak base, only this byproduct can be detected by ^{1}H NMR spectroscopy (Scheme 2).[9] Addition of excess (5 equiv) of ethylmaleimide 2 hours prior cleavage from the resin did not result in any coupling indicating the absence of free thiols and also the irreversibility of the ring closure. After some time in a protic solvent and purification by preparative HPLC, **5** further showed severe decomposition arising from the attack of primary amines to the N-substituted iminothiolane. This attack seems to occur intra- as well as intermolecular, as indicated by ^{1}H NMR spectroscopy and ESI-MS.

To avoid the formation of this byproduct and to optimize the protocol for the maleimido–thiol coupling, the respective maleimide and 2-iminothiolane were simultaneously added to resin **3**. Although this mainly prevented the formation of the cyclization product, the simultaneaous coupling reaction of the amine to the maleimide resulted a second byproduct **8**.[10] Reaction time, excess of both components, and order of addition were varied in order to prevent both side reactions (Table 1). Finally, the desired product **9** could be obtained in 95% purity after a short reaction with 3 equivalents of **1** and 2 equivalents of ethylmaleimide.[11]

The formation of both byproducts could be suppressed by adding the maleimide two minutes after the 2-iminothiolane, since the recyclization occured slower than the coupling reaction. These conditions were used to perform the reaction with longer immobilized polyamine backbones such as spermine.

Table 2 Regioselective Synthesis of Spermine Conjugates



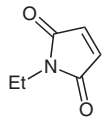
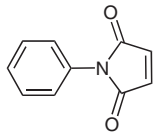
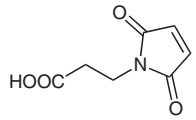
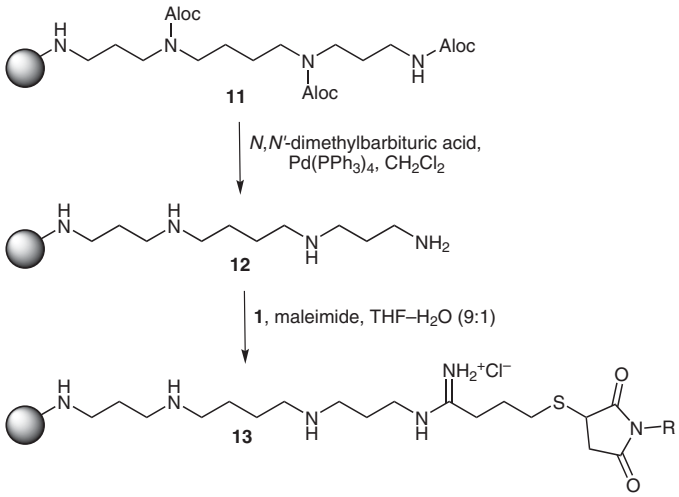
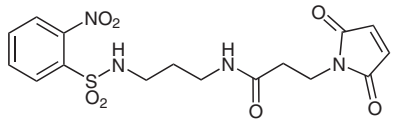
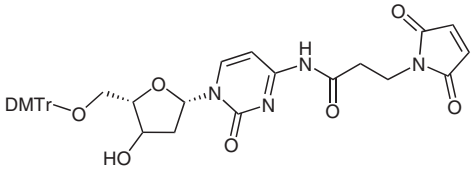
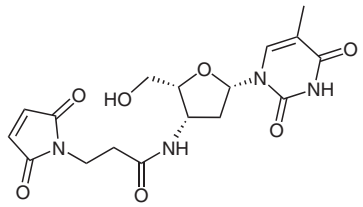
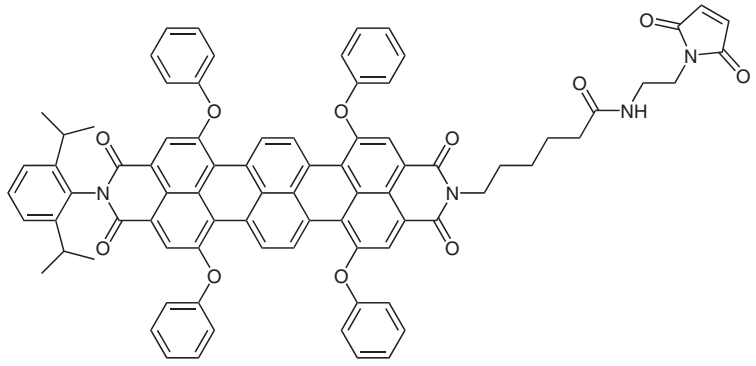
Entry	Maleimide (Mal-R)	Isolated yield (%)	Purity (%)[a]
1		75	93
2		82	94
3		72	98

Table 2 Regioselective Synthesis of Spermine Conjugates (continued)



Entry	Maleimide (Mal-R)	Isolated yield (%)	Purity (%)[a]
4		76	99
5		n.d.	62
6		n.d.	94
7[b]		63	55[c]

[a] The purities were calculated by integration of the relative peak area of the HPLC with UV detection at 280 and 215 nm.
[b] Only 1.7 equiv of the maleimide were applied to avoid aggregation on the polystyrene matrix.
[c] The high amphipilicity of the conjugate prevents a purification on RP-C_{18} as well as on silica gel columns. Therefore, the conversion was calculated from ^{1}H NMR spectra of the crude product; n.d. = not determined.

An alkoxytrityl resin[4b] was loaded with *tris*-Aloc-spermine (**11**). Protection of the primary amine prevented any coupling of **11** with 4 equivalents of **1** even after 4 hours. Deprotection resulted in nonsymmetrically immobilized spermine,[5] and a variety of maleimides were crosslinked using the established protocol (Table 2).[12,13] The alkoxytrityl resin was chosen due to its extremely mild cleavage conditions (0.1–0.5% TFA), which allow the solid-phase synthesis of acid- and base-labile polyamine conjugates. After one hour the resin was washed and the products

were cleaved from the resin and purified by preparative HPLC.[13] As shown in Table 2, the crosslink occurred efficiently with a variety of structurally diverse maleimides. The small maleimides from entries 1–3 reacted smoothly and the products were obtained in high purities. Entries 4–7 showed that also bulkier maleimido building blocks with a broad variation in polarity were tolerated.

The lower conversion rate in entry 7 (Table 2) is due to the lower excess of the strongly fluorescent maleimido building block (1.7 equiv) to avoid the aggregation of the compound on the resin.[14] Nonreacted substrate can be recovered.

In conclusion, we developed a protocol that allows the application of 2-iminothiolane for the chemoselective coupling of polyamines to maleimides for solid-phase chemistry. This method displays a quick and mild method that avoids both, laborious protection-group transformations and alkylation procedures, and allows the synthesis of base- and acid-labile conjugates if carried out on highly acid-labile alkoxytrityl resins.[15]

Acknowledgment

We would like to thank the DFG-Center for Functional Nanostructures (CFN), Karlsruhe, Prof. Bräse, Karlsruhe, and Prof. Sandhoff, Bonn for financial support.

References and Notes

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(9) **Spectroscopic Data for Side Product 6**
^{1}H NMR (MeOD, 400 MHz): δ = 3.69 (t, *J* = 7.2 Hz, 2 H), 3.65 (t, *J* = 6.5 Hz, 2 H), 3.26 (t, *J* = 7.2 Hz, 2 H), 3.09 (t, *J* = 7.5 Hz, 2 H), 2.44 (tt, J_1 = 6.9 Hz, J_2 = 6.8 Hz, 2 H), 2.18 (tt, J_1 = 7.4 Hz, J_2 = 7.4 Hz, 2 H). ESI-MS: *m/z* = 159.1 $[M + H]^+$.
(10) **Spectroscopic Data for Side Product 8**
^{1}H NMR (MeOD, 400 MHz): δ = 4.45 (dd, J_1 = 9.1 Hz, J_2 = 5.7 Hz, 1 H), 3.57 (q, *J* = 7.3 Hz), 3.16 (dd, J_1 = 17.9 Hz, J_2 = 9.2 Hz, 1 H), 3.08 (t, *J* = 7.6 Hz, 2 H), 3.05 (t, *J* = 7.7 Hz, 2H), 2.91 (dd, J_1 = 17.9 Hz, J_2 = 5.6 Hz, 1 H), 2.13 (m, 2 H), 1.17 (t, *J* = 7.2 Hz).
(11) **Spectroscopic Data for Compound 9**
^{1}H NMR (MeOD, 400 MHz): δ = 3.89 (dd, J_1 = 9.1 Hz, J_2 = 2.9 Hz, 1 H), 3.52 (q, *J* = 7.2 Hz, 2 H), 3.38 (t, *J* = 7.1 Hz, 2 H), 3.19 (dd, J_1 = 18.5 Hz, J_2 = 9.1 Hz, 1 H), 3.03 (t, *J* = 7.7 Hz, 2 H), 2.97 (dd, J_1 = 13.4 Hz, J_2 = 6.8 Hz, 1 H), 2.79 (dd, J_1 = 13.5 Hz, J_2 = 7.1 Hz, 1 H), 2.61 (t, *J* = 7.7 Hz, 2 H), 2.46 (dd, J_1 = 18.6 Hz, J_2 = 3.7 Hz, 1 H), 2.08 (tt, J_1 = 7.1 Hz, J_2 = 7.1 Hz, 1 H), 2.01 (tt, J_1 = 7.2 Hz, J_2 = 7.2 Hz, 1 H), 1.13 (t, *J* = 7.2 Hz, 2 H). ESI-HRMS: *m/z* calcd for $C_{13}H_{25}N_4O_2S^+$: 301.1693; found: 301.1686.
(12) Ethylmaleimide, phenylmaleimide, and 3-maleimidopropionic acid were purchased from Sigma-Aldrich. The maleimides from entries 4–6 were prepared by the following procedure: 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (3 equiv) and DIPEA (1 equiv) were dissolved in DMF, and amine (1 equiv) was added in the same volume of THF. The suspension was stirred for 8 h and THF was removed in vacuo. The remaining suspension was partitioned between CH_3Cl and H_2O, and the organic layer was washed with H_2O (3 ×). The organic layer was dried over Na_2SO_4, and the product was purified by flash chromatography. The products were isolated in 47%, 49%, and 35% yield and analyzed by HRMS and ^{1}H NMR spectroscopy. In entry 5, CH_2Cl_2 was used as solvent instead of THF–DMF and DMAP (1 equiv) was added for additional activation.
(13) **Experimental Procedure**
Alkoxytrityl resin (100 mg) loaded with *tris*-Aloc-spermine (0.027 mmol, 1 equiv) were swollen in CH_2Cl_2 and a solution of $Pd(PPh_3)_4$ (6 mg, 0.005 mmol, 0.2 equiv) and of *N*,*N*′-dimethylbarbituric acid (33 mg, 0.214 mmol, 8 equiv) in CH_2Cl_2 (2 mL) were added. The suspension was agitated for 16 h at 40 °C. The resin was alternately washed with a solution of sodium *N*,*N*-dimethylaminodithiocarbamate in CH_2Cl_2–MeOH (4:1) and MeOH (3 ×), THF and MeOH (3 ×), and CH_2Cl_2 (3 ×). The resin was swollen in THF (1 mL) for 10 min and 2-iminothiolane (11 mg, 0.081 mmol, 3 equiv.) in H_2O (200 mL) was added. The suspension was agitated for 2 min, then the respective maleimide (2 equiv) in THF (800 mL) was added, and the suspension was agitated for 1 h. The resin was washed with H_2O, THF, MeOH (3 ×), and CH_2Cl_2 (3 ×). The crude product was cleaved from the resin with 1% TFA in CH_2Cl_2. The filtrate was combined with the filtrate of the CH_2Cl_2 and MeOH wash. In entry 5 the 5′-DMTr group was removed by quick treatment of the resin with 1% TFA in CH_2Cl_2 and subsequent washing of the polymer with a minimal amount of CH_2Cl_2–Et_2O (2:1) until the filtrate is clear. The product was washed from the resin with CH_2Cl_2 and MeOH. The solvents were removed and the remaining compound was purified by preparative HPLC on C_{18} column with gradients of eluent A [95% TEAA (0.01 M, pH 7.0)–5% MeCN] and eluant B [5% TEAA (0.01 M, pH 7.0)–95% MeCN), or with eluant A (95% H_2O–5% MeCN–0.1% AcOH) and eluant B (5% H_2O–95% MeCN–0.1% AcOH).
(14) **Characterization of the Products of Entries 1–7 (Table 2)**
Entry 1: ^{1}H NMR (400 MHz, MeOD): δ = 1.14 (t, *J* = 7.2 Hz, 3 H), 1.79 (m, 4 H), 2.06 (tt, J_1 = 7.7 Hz, J_2 = 7.3 Hz, 4 H), 2.13 (m, 2 H), 2.47 (dd, J_1 = 18.5 Hz, J_2 = 3.9 Hz, 1 H), 2.62 (t, *J* = 7.7 Hz, 2 H), 2.80 (dt, J_1 = 13.5 Hz, J_2 = 7.0 Hz, 1 H), 2.98 (dt, J_1 = 13.6 Hz, J_2 = 6.8 Hz, 1 H), 3.00–3.15 (m,

10 H), 3.19 (dd, J_1 = 18.5 Hz, J_2 = 9.1 Hz, 1 H), 3.40 (t, J = 7.1 Hz, 2 H), 3.53 (q, J = 7.3 Hz, 2 H), 3.90 (dd, J_1 = 9.1 Hz, J_2 = 3.8 Hz, 1 H). ESI-HRMS: *m/z* calcd $[M + H]^+$: 429.3006; found: 429.3010.

Entry 2: ^{1}H NMR (400 MHz, MeOD): δ = 1.79 (br m, 4 H), 1.96–2.14 (m, 6 H), 2.60–2.70 (m, 3 H), 2.86 (dt, J_1 = 13.6 Hz, J_2 = 7.1 Hz, 1 H), 2.93–3.10 (m, 11 H), 3.39 (t, J = 6.5 Hz, 2 H), 3.32–3.40 (m, 1 H), 4.10 (dd, J_1 = 9.1 Hz, J_2 = 3.8 Hz, 1 H), 7.26–7.54 (m, 5 H). ESI-HRMS: *m/z* calcd $[M + H]^+$: 477.3006; found: 477.3002.

Entry 3: ESI-HRMS: *m/z* calcd $[M + H]^+$: 473.2905; found: 473.2907.

Entry 4: ^{1}H NMR (400 MHz, MeOD): δ = 1.67 (tt, J_1 = 6.7 Hz, J_2 = 6.7 Hz, 2 H), 1.73–1.88 (br m, 4 H), 2.00–2.14 (br m, 6 H), 2.50 (dd, J_1 = 17.9 Hz, J_2 = 3.6 Hz, 1 H), 2.44–2.70 (m, 2 H), 2.60–2.70 (m, 2 H), 2.82 (dt, J_1 = 13.9 Hz, J_2 = 6.5 Hz, 1 H), 2.93 (dt, J_1 = 13.8 Hz, J_2 = 6.9 Hz, 1 H), 3.04–3.24 (m, 15 H), 3.42 (t, J = 7.1 Hz, 2 H), 3.68–3.83 (m, 1 H), 3.90 (dd, J_1 = 9.2 Hz, J_2 = 3.9 Hz, 1 H), 7.80–8.11 (m, 4 H). ESI-HRMS: *m/z* calcd $[M + 2H]^{2+}$: 357.6749; found: 357.6736.

Entry 5: ^{1}H NMR (400 MHz, MeOD): δ = 1.80–1.90 (m, 4 H), 2.00–2.18 (br m, 6 H), 2.21 (m, 1 H), 2.50 (m, 1 H), 2.58–2.68 (m, 3 H), 2.81 (m, 2 H), 2.95 (dt, J_1 = 13.1 Hz, J_2 = 6.5 Hz, 1 H), 3.00–3.30 (m, 12 H), 3.42 (t, J = 6.9 Hz, 2 H), 3.70–3.90 (m, 4 H), 3.93 (dd, J_1 = 9.0 Hz, J_2 = 3.9 Hz, 1 H), 4.03 (dt, J_1 = 3.4 Hz, J_2 = 3.5 Hz, 1 H), 4.39 (dt, J_1 = 6.8 Hz, J_2 = 6.5 Hz, 1 H), 6.09 (d, J = 7.8 Hz, 1 H), 6.22 (m, 1 H), 8.39 (br, J = 7.8 Hz, 1 H). ESI-MS: *m/z* = 682.36 $[M + H]^+$, MS–FAB$^+$: 682.4 $[M + H]^+$.

Entry 6: ^{1}H NMR (400 MHz, MeOD): δ = 1.70–1.85 (m, 4 H), 1.89 (s, 3 H), 2.00–2.18 (m, 6 H), 2.25–2.40 (m, 2 H), 2.45–2.55 (m, 3 H), 2.63 (t, J = 7.6 Hz, 2 H), 2.81 (dt, J_1 = 13.3 Hz, J_2 = 7.2 Hz, 1 H), 2.91–3.28 (m, 12 H), 3.37–3.45 (m, 2 H), 3.70–3.97 (m, 7 H), 4.42 (dt, J_1 = 7.1 Hz, J_2 = 6.4, 1 H), 6.21 (m, 1 H), 7.87 (s, 1 H). ESI-HRMS: *m/z* calcd $[M + H]^+$: 696.3861; found: 696.3859.

Entry 7: MS (MALDI$^+$): *m/z* = 1583.16 $[M + H]^+$. ESI-HRMS: *m/z* calcd $[M + 2H]^{2+}$: 791.8509; found: 791.8456.

(15) A proposed sequence would be: (1) Selective introduction of a protection group at the primary amine; (2) protection of the secondary amines; (3) removal of the protection group on the primary amine; (4) elongation with an *S*-protected building block; (5) deprotection of the thiol; (6) coupling of the thiol to a maleimide; (7) deprotection of the secondary amine.