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Synthesis of a new disulfide Fmoc monomer for creating biologically susceptible linkages in peptide nucleic acid oligomers

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ABSTRACT

Peptide nucleic acids (PNA) are one of many synthetic mimics of DNA and RNA that have found applications as biological probes, as nano-scaffold components, and in diagnostics. In an effort to use PNA as constructs for cellular delivery we investigated the possibility of installing a biologically susceptible disulfide bond in the backbone of a PNA oligomer. Here we report the synthesis of a new abasic Fmoc monomer containing a disulfide bond that can be incorporated into a PNA oligomer (DS-PNA) using standard solid phase peptide synthesis. The disulfide bond survives cleavage from the resin and DS-PNA forms duplexes with complementary PNA oligomers. Initial studies aimed at determining if the disulfide bond is cleavable to reducing agents while in a duplex are explored using UV thermal analysis and HPLC.

1. Introduction

Peptide nucleic acids [1], as well as a host of other non-natural DNA mimics, have been developed and adapted to fulfill roles where endogenous oligomers perform poorly or not at all. Central tenets to all of the mimics are (1) high affinity and selectivity for the target strand, (2) resistance to nucleases and degradation pathways, (3) cellular delivery, (4) low toxicity and binding of proteins, (5) synthetically accessible for large scale production, and (6) clinical relevance [2]. While current mimics satisfy many of the criteria above, all synthetic oligomers have pitfalls and shortcomings. Most notably for PNA is its inability to cross cell membranes, thus hindering its capacity to act as an antigene or antisense agent. In fact, the delivery of PNA is one of the most pressing issues of its utility as a therapeutic reagent [3,4].

Previously, researchers have investigated conjugating chemical modifications directly on the antigene or antisense PNA. These studies have included conjugation to cell penetrating peptides [5–8], adamantyl residues [9], nuclear localizing peptides [10], and lipophilic cations [11]. While these augmentations facilitate cellular uptake, the conjugates suffer from increased toxicity, entrapment in vesicles, limited bioavailability, and non-specific cellular sequestration [4]. Other delivery methods rely on non-covalent interactions between DNA carriers [12], lipophilic membranes [13,14], and mesoporous silica nanoparticles [15]. However, sequestration of end products, off target interactions, and clinical relevance remain a concern. To date, one of the most successful delivery systems is based on the co-encapsulation of PNA with a donor DNA in poly(lactic-*co*-glycolic acid) nanoparticles and has been shown to mediate site-specific gene recombination [16–18]. While these advancements are promising the need for alternative and/or potentially synergistic approaches is still warranted if PNA are to be used as viable therapeutic reagents.

We are currently pursuing a new strategy that will offer a means to deliver a pristine antisense PNA to the cytoplasm of a cell. The approach relies on a carrier PNA (blue strand), which is complementary to the antisense PNA (red strand), and can be decorated with cell localizing factors, cell penetrating peptide and/or other necessary moieties that will facilitate targeting, membrane penetration, and/or endosomal escape [19] (Fig. 1a and b). To enable the disassembly of the PNA:PNA duplex, a disulfide bond (orange circle), which is envisioned to be readily reduced in the cytoplasm, will be incorporated in the backbone of the temporary strand. Thus, once in the cytoplasm, the disulfide bond will be cleaved resulting in a destabilized multi-component duplex which is anticipated (*vide infra*) to dissociate to yield a free antisense strand (Fig. 1c and d). To begin this project we have synthesized a new Fmoc-disulfide monomer and then incorporated it into a PNA oligomer (DS-PNA) using standard Fmoc coupling, deprotection, and cleavage procedures. Duplexes of the DS-PNA with complementary PNA strands having G, C, A or T across from the abasic disulfide position were assessed using thermal denaturation studies and we performed initial analyses to confirm that the disulfide bond can be reduced while duplexed with a complementary antisense strand.

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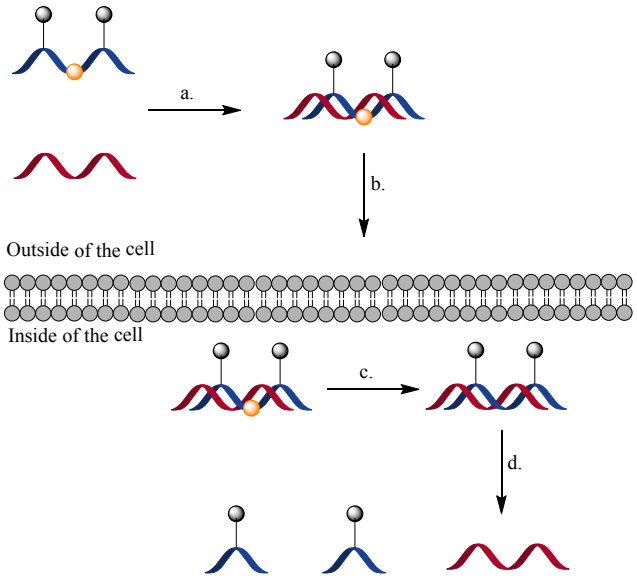


Fig. 1. Conceptual design of a temporary PNA containing a biologically susceptible disulfide bond strand that will assist with the delivery of an antigene or antisense strand to the cytoplasm of a cell. a. Duplex formation of antisense PNA (red) and a temporary PNA (blue) containing a disulfide bond (orange circle) in the backbone and cell penetrating peptides, endosomal escape and/or localization factors (black circles). b. Cellular entry meditated by the temporary strand. c. Reduction of the disulfide bond by endogenous reagents (glutathione). d. Release of a pristine antisense PNA.

2. Results and discussion

2.1. Synthesis of a disulfide containing monomer

A previously published route was followed to synthesize the Boc-based monomer and then the protecting group was exchanged for Fmoc [20] (Scheme 1). Efforts here focus on the *gem*-dimethyl derivative, which was theorized to hinder the reduction of the disulfide bond in later experiments, but, by the same token, would minimize cleavage or side reactions during solid phase peptide synthesis (SPPS). Commercially available 2-bromo-2-methylpropionic acid was converted to compound **1** through a substitution reaction with thiourea under basic conditions in 23% yield. Cystamine dihydrochloride was Boc protected and then treated with hydrogen peroxide to form an *in situ* sulfonate intermediate that was treated with **1** to yield the asymmetrical disulfide **3** in a 54% yield. Boc was exchanged for Fmoc in a two-step, one pot reaction where the Boc group was first cleaved using TFA (m-cresol as a scavenger), followed by removal of volatiles, and then treatment with Fmoc-Cl in the presence of DIPEA.

Table 1
PNA oligomers synthesized for thermal denaturation and HPLC cleavage studies. DS indicates where the disulfide monomer was incorporated into the oligomer and A/DS is the fully complementary strand to T-Comp oligomer.

PNA	Sequence	Expected mass	Found mass
DS-PNA	mPeg-gtatcg-DS-caaag-NHAc	3391.370 amu	3392.957 *m/z*
A/DS-PNA	mPeg-gtatcg-**a**-caaag-NHAc	3489.343 amu	3490.104 *m/z*
A-Comp	mPeg-ctttg-**a**-cgatac-NHAc	3431.293 amu	3432.035 *m/z*
T-Comp	mPeg-ctttg-**t**-cgatac-NHAc	3422.279 amu	3424.801 *m/z*
G-Comp	mPeg-ctttg-**g**-cgatac-NHAc	3447.292 amu	3448.726 *m/z*
C-Comp	mPeg-ctttg-**c**-cgatac-NHAc	3407.268 amu	3408.725 *m/z*

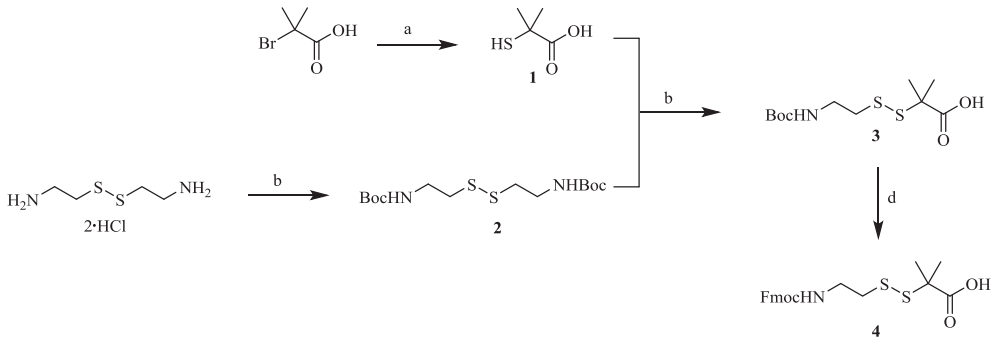
2.2. Incorporation of the monomer into PNA oligomers and thermal denaturation studies

Quite often, new or unique monomers require modified coupling conditions (double coupling, extended coupling times, etc). Fortunately, this was not the case for the new monomer, which was readily coupled using standard Fmoc-based SPPS conditions (HBTU activation, 1 h coupling). The disulfide bond proved to be stable throughout the synthesis and cleavage of the oligomer, which is evidenced by few truncated sequences observed during HPLC purification.

In our current investigation, the DS-PNA is a 12-mer oligomer and the disulfide monomer was incorporated at the 7th position. While short, the fully complementary duplex shows good stability, and cleavage near the middle of the oligonucleotide will yield 6- and 5-mer products. If a longer target sequence or smaller fragments are desired to promote efficient and complete release of the antisense PNA, multiple disulfide bonds can be incorporated into the temporary strand.

To determine if the incorporation of the disulfide monomer adversely affects duplex stability we performed a series of thermal melting experiments with the newly synthesized DS-PNA and four complementary strands (Table 1) where each nucleobase was paired across from the disulfide bond. Understanding any steric or electronic complications in terms of "pairing" is key for future designs if the disulfide happens to impart increased or decreased duplex stability. While not ideal, all of the DS-PNA duplexes showed a decreased (−10 °C) in stability compared to a fully complementary duplex (A/DS-PNA:T-Comp, Fig. 2b). While this drop in stability was anticipated, because the DS duplexes are deficient one base pair, the magnitude was more than expected, in particular the change in T_m for T-Comp was substantial (−19 °C) and more in the range observed with a mismatch in a PNA duplex [21].

One reason why all of the disulfide containing duplexes are less stable, in addition to lacking a base pair, could be because the new monomer contains an "extra" methylene in the backbone of the DS-PNA. This was not envisioned to be an issue, since the backbone of PNA is flexible and should be able to accommodate the slight increase in length. The significant drop in T_m for the thymine has no obvious steric



Scheme 1. Reaction scheme for synthesized compound 4. a. thiourea, NaOH, H_2O, 80 °C, 23% b. Di-*tert*-butyl-dicarbamate, dioxane, 2 M NaOH, 54% c. 30% H_2O_2, NaOH, H_2O, 49% d. (1) TFA, m-cresol; (2) DIPEA, Fmoc-Cl, DCM, 53%.

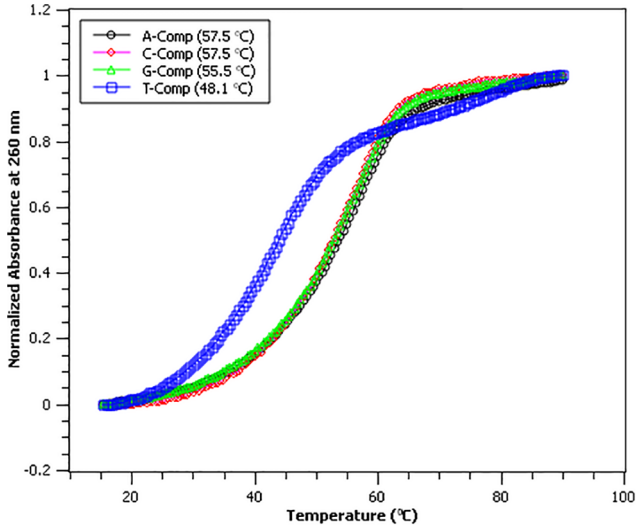


Fig. 2. Thermal denaturation studies of (a) DS-PNA with complementary strands having A, T, G or C opposite the disulfide monomer. Inset shows the T_m of all duplexes. Each strand was 1 μM.

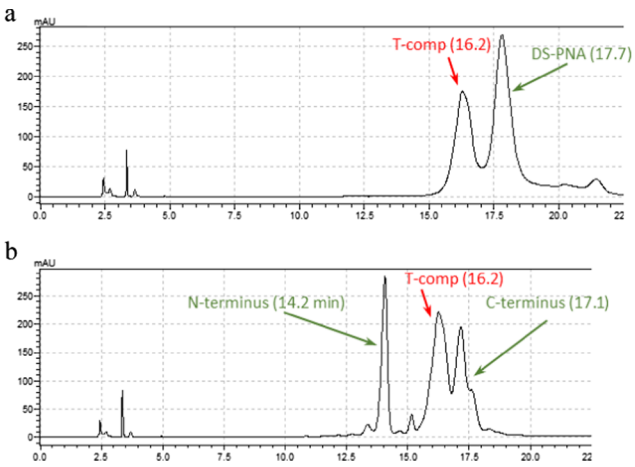


Fig. 4. Chromatograms of DS-PNA/T-Comp. Annealed samples a. before and b. after treatment with TCEP. Peaks are identified based on MS and retention times in minutes are in parenthesis.

or electronic foundation for why the stability shifted so dramatically. Further investigations (NMR or crystal structures) may provide information about the unforeseen interactions.

2.3. UV and HPLC cleavage studies of DS-PNA

Encouraged that a PNA oligomer containing a central disulfide bond can still form stable duplexes, we investigated the susceptibility of the disulfide to be reduced while duplexed to a complementary strand. While the T-Comp duplex was the most destabilized, we focused our investigation on this strand because it is the perfect match antisense probe for the mRNA start codon for β-galactosidase (*lacZ*) in Escherichia coli (*E. coli*) and will be used in subsequent *in vivo* proof of concept biological studies [15].

We repeated the thermal denaturation experiments in the presence of TCEP using a modified protocol. Complementary strands were first allowed to anneal and then TCEP was added before the melting phase was started. This ensured that the reduction was occurring while the two strands were hybridized and not when they were single stranded. While glutathione and other natural reducing agents would be preferred, they are not compatible with UV–vis studies. The thermal melting analysis of the DS-PNA with the complementary *T*-PNA strand with and without TCEP show marked differences (Fig. 3a). When the duplex is melted in the absence of TCEP the typical sigmoidal curve is observed, but in the presence of TCEP the resulting curve is ill defined and no discernable inflection point is observed, suggesting that no duplex is present under these conditions. In comparison, a PNA duplex having the complementary base (A) instead of the disulfide insertion (A/DS-PNA) shows no change under similar conditions (Fig. 3b).

To further support the idea that the DS-PNA is being cleaved we incubated annealed duplexes with and without TCEP at 37 °C for 1 h and then analyzed the products by HPLC (Fig. 4). In the absence of TCEP the two PNA oligomers show retention times of 16.2 min and 17.7 min. The strands were identified using MALDI as T-Comp and DS-PNA, respectively. In the presence of TCEP two new major peaks, at 14.2 and 17.1 min, are observed while concomitantly the peak identified as DS-PNA disappears. The mass of the peak at 14.2 min was found to be 1484.12 *m/z* which correlates to the *N*-terminus of the cleaved DS-PNA which has a calculated mass of 1474.47 amu, and the new peak at 17.1 min showed a mass of 1904.16 *m/z* which agrees well with the calculated mass of the C-terminus of 1904.93 amu. The shoulder at 17.6 was also analyzed since the retention time was near that of the DS-PNA, but only the mass of the cleaved C-terminus oligomer was observed. Importantly, mass analysis of the peak at 16.2 min showed that T-Comp was unchanged by the cleavage reaction. To confirm that the new peaks were a result of the cleavage of DS-PNA and not a phenomenon of the

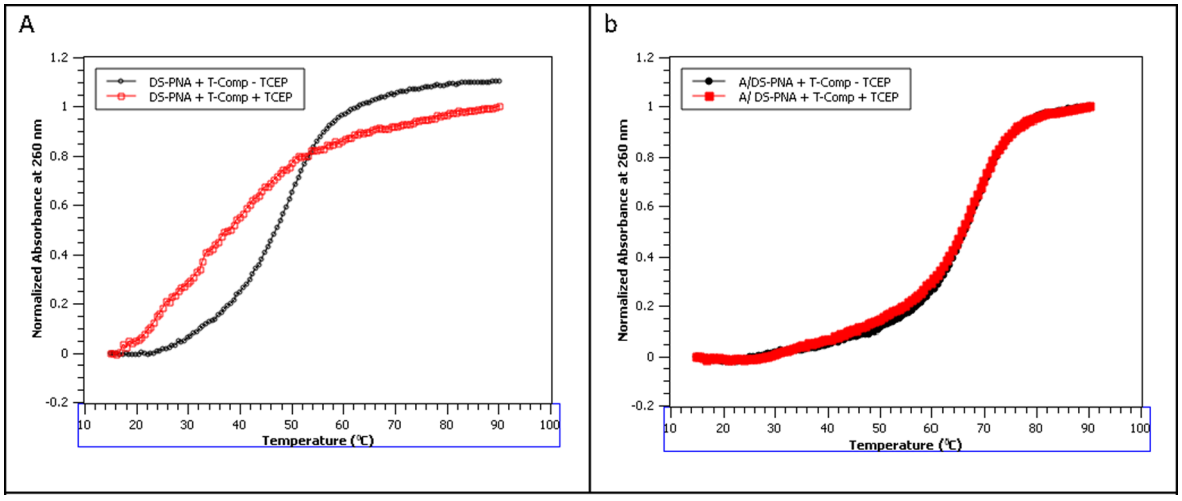


Fig. 3. Thermal melting curves of (a) DS-PNA:T-Comp with and without TCEP and (b) A/DS-PNA:T-Comp under the same conditions. Each strand was 1 μM.

chosen sequence the same experiments were performed on A/DS-PNA/T-Comp. As expected, the two fully intact PNA oligomers are observed in the presence or absence of TCEP.

Collectively, these observations indicate that the disulfide bond is likely cleaved by TCEP, which results in two smaller strands originating from DS-PNA that do not appear to form a stable duplex, even at low temperatures (the experiment was started at 15 °C). However, these results may not accurately reflect the availability of the antisense PNA to interact with a transient mRNA target that is typically present at a low concentration. Since PNA lack electrostatic repulsion, even short 5- or 6-mer oligomers are still capable of forming homoduplexes. Accordingly, additional measures may be necessary to ensure that the fragments of the DS-PNA are ushered clear of the antisense strand. One strategy would be to introduce additional disulfide linkages in the backbone of the DS-PNA, albeit maintaining duplex stability, to render small DS-PNA cleavage products once the disulfide bonds are cleaved. Alternatively, since the incorporation of the disulfide can have rather dramatic effects on the stability of the temporary but requisite duplex, the DS-PNA could be truncated on either termini to yield 4-mer or smaller cleavage produces. Future studies will be aimed at optimizing disulfide position, determining appropriate cleavage products lengths, and understanding the thermodynamic and kinetic parameters associated with these design elements as they pertain to binding an RNA target.

3. Conclusion

We have successfully synthesized a new Fmoc-disulfide monomer that can be incorporated into a PNA oligomer using standard Fmoc-based solid phase synthesis protocols. Incorporation of the monomer in the middle of a 12-mer PNA reduces the stability of the duplexes by roughly10 °C compared to a fully complementary strand, and interestingly, duplex stability is most effected when the disulfide is paired with T. Cleavage studies show that the disulfide bond is indeed susceptible to chemical reduction when hybridized with a complementary PNA strand. These findings provide the initial evidence that this approach may have the potential to release an antigene or antisense oligomer. Further investigations with different disulfide monomer derivatives and spacing of the cleavage sites will yield additional insights and provide direction towards designing second and third generation DS-PNA.

4. Materials and methods

Synthetic reagents were purchase from Fisher Scientific or Sigma Aldrich and used without further purification. PNA monomers were purchased from PolyOrg, Inc. Reactions were performed under a blanket of nitrogen with analytical grade solvents. Silica gel was obtained from Sorbent Technologies. ^{1}H and ^{13}C spectra were obtained on a JEOL 300 NMR spectrometer at 300 and 75 MHz, respectively, in $CDCl_3$. Mass spectrometry on compounds **1–4** was performed on an Agilent Infinity LC/MSD (G6125C) single quadrupole in the positive mode from 60 to 500 *m/z*.

4.1. PNA synthesis, purification, and characterization

PNA oligomers were synthesized using Fmoc conditions on PAL-mPeg resin. Fmoc protecting groups were removed with 20% piperidine in DMF followed and checked using a Kaiser test. Monomers (1 eqv) including compound **4**, were dissolved in 135 μLNMP and then activated using HBTU (0.92 eqv) in DMF in the presence of DIPEA (1 eqv) and lutidine (1 eqv) for 2.5 min before being added to the deprotected resin where they were allowed to react for 1 h. A mini-peg unit was added to the *N*-terminus of each PNA to increase water solubility [22]. Oligomers were cleaved from the resin using 5% m-cresol in TFA for 1 h (× 2).

PNA oligomers were purified using a Shimadzu LC-2-AD UFLC equipped with an X-Bridge column (BEH130PREP C18 5 μM, 10 × 250 mm) and eluted with water (solvent A) 0.1% TFA and an increasing amount of 9:1 ACN:water (solvent B). Typical gradient is 5% solvent B to 40% solvent B over 40 min. Samples collected from the HPLC were analyzed by Matrix Assisted Laser Dissociation Ionization-Time of Flight (MALDI-TOF) on a Bruker Daltonics Microflex LT. MALDI data analysis was performed with a Polytools 1.0 software package. UV–vis spectra (for PNA concentrations) and thermal denaturation studies were recorded on a Cary 100 UV–vis. PNA concentrations were determined at 260 nm at 90 °C using extinction coefficients obtained from Applied Biosystems extinction coefficients for PNA monomers were obtained from Applied Biosystems ($C = 6600\ M^{-1}\ cm^{-1}$; $T = 8600\ M^{-1}\ cm^{-1}$; $A = 13{,}700\ M^{-1}\ cm^{-1}$; $G = 11{,}700\ M^{-1}\ cm^{-1}$;).

4.2. Thermal denaturation studies

Samples were heated to 90 °C, held there for 5 min, and then cooled to 15 °C at 1 °C/min. Samples were held at this temperature for 5 min and then heated to 90 °C at 1 °C/min. Data points were collected at 260 nm every 0.5 °C. All strand concentrations were analyzed at 1 μM in 1 × PBS (pH 7.4).

4.3. HPLC cleavage studies

Complementary PNA strands were combined at a concentration of 10 μM, heated to 90 °C, and then allowed to cool to room temperature over 2 h. The samples were then transferred to a 37 °C oven and TCEP was added (final concentration 0.5 mM). After incubating for an hour the samples were analyzed by HPLC using a Shimadzu LC-2-AD UFLC equipped with a Phenomenex Kinetex colum (C 18 5 μ, 100 A, 250 × 4.6 mm) eluting with water with (solvent A) 0.1% TFA and an increasing amount of (solvent B) 9:1 ACN:water (5% solvent B to 40% solvent B over 40 min). Control samples were treated the same way except TCEP was not added before the 1 hr incubation period.

4.4. 2-Mercapto-2-methylpropanoic acid (*1*)

A flask equipped with a reflux condenser and a stir bar was charged with of 2-bromo-2-methylpropionic acid (8.0 g, 47.9 mmol, 1 eqv). The solid was melted gently with a heat gun (~44–47 °C) and then a solution of thiourea (4.56 g, 60 mmol, 1.25 eqv) in 8.00 mL of water was added while stirring vigorously. The reaction was heated to 80 °C, at which point the solution cleared (~25 min), and then a solution of NaOH (6 g, 150 mmol, 3.13 eqv) dissolved in 36 mL of water was added. The reaction was heated to 95 °C for two hours. The solvent was evaporated to leave a thick residue which was taken up in water (20 mL) and then the pH was adjusted to ~10 with HCl. The aqueous solution was extracted with DCM (50 mL). The aqueous layer was then acidified to pH 2–3 and extracted with DCM (3 × 50 mL). The organic washes were collected, dried with $MgSO_4$, and then the solvent was stripped to leave an oil. (Yield: 23%) ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.65 (s, 6H, 2xCH_3), 2.52 (s, 1H, SH), 9.88 (s, 1H, CO_2H) ppm. ESI-MS: *m/z* cacld. For $C_4H_8O_2S$, $[M]^+$: 120.17, found 121.00.

4.5. Di-tert-butyl (disulfanediylbis(ethane-2,1-diyl))dicarbamate (*2*)

Di-*tert*-butyl-dicarbonate (1.75 g, 8 mmol, 2 eqv) and cystamine dihydrochloride (1 g, 4 mmol, 1 eqv) were charged to a 50 mL flask with a stir bar and then the flask was sealed with a rubber septa and placed under a blanket of nitrogen. Dioxane (3.6 mL) was charged to the flask and the reactants were allowed to dissolve before adding NaOH (4.4 mL of 2 M) dropwise over 5 min. The reaction was allowed to stir for 90 min at room temperature. Dioxane and water were removed in vacuo and the residue was taken up in ethyl acetate (200 mL). The organic layer was washed with 1% HCl (3 × 50 mL) followed by brine, and then

dried with $MgSO_4$. The solvent was removed to yield white solid. (Yield: 54%) ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (s, 18H, Boc), 2.78 (t, 4H, J = 6.8 Hz, CH_2S), 3.43 (m, 2H, CH_2N), 5.03 (s, 2H, NH) ppm. ESI-MS: m/z cacld. for $C_{14}H_{28}N_2O_4S_2$, $[M]^+$: 352.51, found 353.20.

4.6. 2-((2-((tert-Butoxycarbonyl)amino)ethyl)disulfaneyl)-2-methylpropanoic acid (3)

Compound **2** (0.35 g, 1 mmol, 0.5 eqv) was dissolved in methanol (5.5 mL) in a 50 mL round bottom flask containing a stir bar and capped with a rubber septa and blanketed with nitrogen. Compound **1** (0.4 g, 2 mmol, 1 eqv) was dissolved in 0.1 M NaOH (3.67 mL) and slowly added to the reaction flask and allowed to stir until the solution became clear (~20 min). H_2O_2 (30%) was then added to the flask and the solution was heated at 70 °C for an hour. The reaction was cooled in an ice bath down to room temperature and the pH was adjusted with NaOH to ~9–10. Residual methanol was removed and then the aqueous solution was vacuum filtered. The basic solution was extracted with DCM (50 mL). The pH was then adjusted to ~2–3 with HCl and then extracted with DCM (2 × 50 mL). The collected organics were dried with $MgSO_4$, filtered, and then evaporated to leave a yellow oil. (Yield: 49%) ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.45 (s, 9H, Boc), 1.56 (m, 6H, 2 × CH_3), 2.82 (m, 2H, CH_2), 3.41 (m, 2H, CH_2), 4.99 (s, 1H, NH), 7.45 (s, 1H, CO_2H) ppm. ESI-MS: m/z cacld. for $C_{11}H_{21}NO_4S_2$ $[M + Na]^+$: 318.41, found 318.40.

4.7. 2-((2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)ethyl) disulfaneyl)-2-methylpropanoic acid (4)

Compound **3** (0.4 g, 1.35 mmol, 1 eqv) was dissolved in DCM (4 mL) followed by the dropwise addition of 95% TFA/5% m-cresol (1 mL). After stirring for 10 min, DCM and volatiles were removed to leave a yellowish oil. The oil was then dissolved in DCM (4 mL) and DIPEA (0.94 mL, 5.40 mmol, 4 eqv) was added dropwise while stirring vigorously. To this solution was added Fmoc chloride (0.384 g, 1.485 mmol, 1.1 eqv). The solution was stirred for an hour and then the volatiles were stripped to leave a yellow oil, which was taken up in water (30 mL) and the pH was adjusted to ~3 with HCl. The aqueous solution was extracted with ethyl acetate (3 × 50 mL). The combined organics were dried with MgSO4, filtered, and then evaporated to yield a yellow oil. The oil was purified via column chromatography eluting with DCM:MeOH (9.5:0.5). Removal of solvent gave a clear oil that solidified upon refrigeration. (Yield: 53%) ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.56 (s, 6H, 2 × CH_3), 2.83 (t, 2H, J = 6.8 Hz, CH_2S), 3.47 (m, 2H, CH_2N), 4.39 (d, J = 7.6 Hz, CH_2, Fmoc), 4.20 (t, 1H, J = 7.6 Hz, CH, Fmoc), 7.30 (d, 2H, J = 7.6 Hz, Fmoc), 7.40 (d, 2H, J = 7.2 Hz, Fmoc), 7.57 (d, 2H, J = 7.2 Hz, Fmoc), 7.74 (d, 2H, J = 7.5, Fmoc), 8.36 (br. s, 1H, CO_2H) ppm. Major rotamer reported. ^{13}C NMR (300 MHz, $CDCl_3$) δ: 24.5, 38.0, 39.9, 47.2, 51.1, 67.0, 120.1, 125.2, 127.2, 127.8, 141.4, 143.9, 156.6, 178.5 ppm. ESI-MS: m/z cacld. for $[M]^+$: 418.20, found 417.54.

Competing interests

The authors have no competing interests to declare.

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