Efficient, Traceless Semi-Synthesis of α-Synuclein Labeled with a Fluorophore/Thioamide FRET Pair

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Abstract: We have shown that thioamides can be incorporated into proteins through semi-synthesis and used as probes to monitor structural changes. To date, our methods have required the presence of a cysteine at the peptide ligation site, which may not be present in the native peptide sequence. Here, we present a strategy for the semi-synthesis of thioproteins using homocysteine as a ligation point with subsequent masking as methionine, making the ligation 'traceless'.

Key words: proteins, peptides, thiols, chromophores, bioorganic chemistry

Fluorescence spectroscopy is a powerful technique for studying protein dynamics and stability. Distance-dependent chromophore interactions, such as Förster resonance energy transfer (FRET) and photoinduced electron transfer (PET) are routinely employed for monitoring protein conformation in real time.[1] We have recently shown that a peptide backbone thioamide, a single-atom substitution of the carbonyl oxygen with sulfur, can quench a variety of natural and unnatural amino acids.[2] Unlike commonly used fluorescence quenchers, thioamides are sufficiently small that they can be placed at nearly any position of the protein sequence without significantly altering the secondary structure.[3] Our laboratory has developed semi-synthetic methods to incorporate these minimally perturbing fluorescence probe pairs into full-length proteins. Backbone thioamides cannot currently be installed into a protein by means of ribosomal expression. Therefore, we incorporate thioamides into full-length proteins using native chemical ligation (NCL), a fragment condensation reaction that typically takes place between peptides bearing a C-terminal thioester and an N-terminal cysteine, to form a native amide bond.[4] Expressed protein ligation (EPL), a variant of NCL, allows us to express a large portion of the protein in *E. coli* cells, to which we can append a small synthetic thioamide-containing fragment with high efficiency.[5]

Misfolding of the abundant neuronal protein α-synuclein (αS) has been implicated in the pathogenesis of several debilitating neurodegenerative disorders.[6] Under physiological conditions, αS is soluble, monomeric, and intrinsically disordered. In its pathological conformation, αS monomers associate with each other to form oligomers that eventually mature into β-sheet rich fibrils.[7] Despite recent developments of new methods to study αS dynamics, the molecular details underlying its aberrant oligomerization remain poorly understood.[8] Recently, we combined unnatural amino acid mutagenesis with native chemical ligation to install the FRET pair, *p*-cyanophenylalanine (Cnf) and a thioamide, into full-length αS for misfolding studies.[9] This FRET system provides reliable distance measurements over a range of 8–30 Å within a protein's three-dimensional structure. Using our double-labeled constructs, we were able to demonstrate that we can monitor conformational changes in monomeric αS using urea or trimethylamine oxide (TMAO) to denature or compact the protein, respectively.

Despite agreement of our data with other studies of αS in TMAO, we were somewhat concerned about the presence of the non-native Cys residues in our labeled αS constructs.[10] Cys mutants of αS display enhanced aggregation kinetics and altered fibril morphology as a result of inter-

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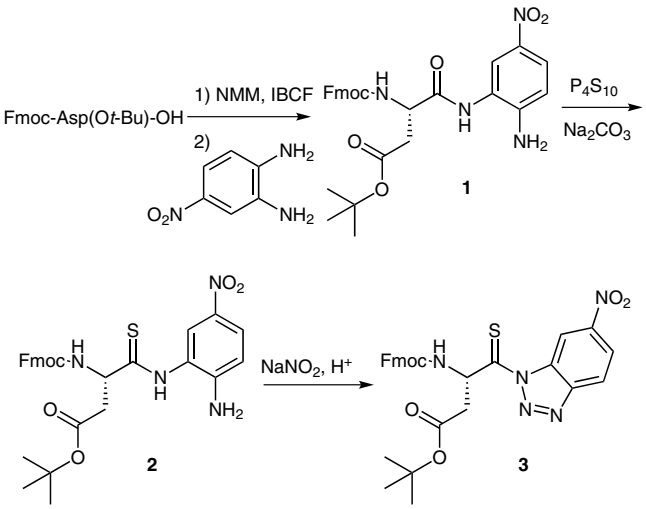
molecular disulfide bond formation.[11] Although we performed the previous FRET studies with our Cys-containing double-labeled constructs in the presence of a reducing agent, it is nonetheless possible that some amount of disulfide formation occurred during our experiments.

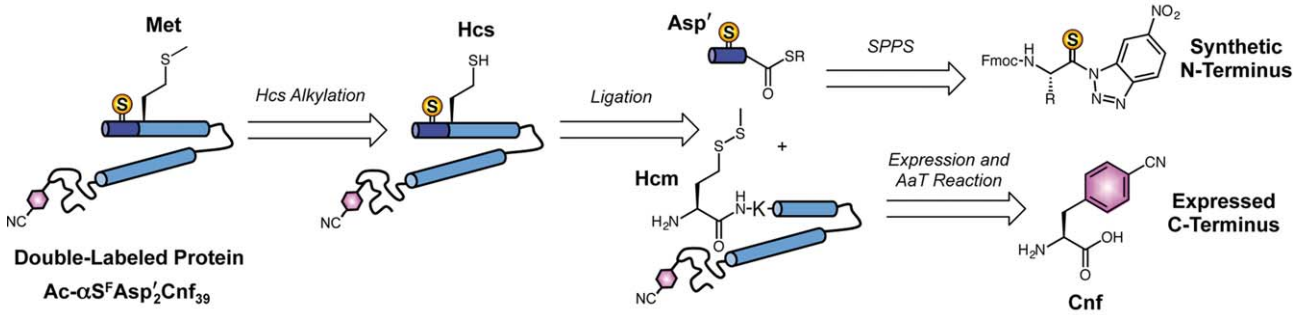
While many laboratories carry out protein synthesis with subsequent radical desulfurization of Cys to form Ala, this strategy is not viable for us as desulfurization of the thioamide would also occur.[12] Methods developed in our laboratory enable us to generate double-labeled αS for misfolding studies that does not contain cysteine at the ligation site. It has been shown that NCL can be performed with an N-terminal homocysteine (Hcs) in place of Cys. Hcs can then be selectively methylated to yield methionine at the ligation site.[13] Hcs-mediated ligation was previously restricted to short C-terminal peptides in which Hcs could be installed using solid-phase peptide synthesis (SPPS). We have recently established that we can use *E. coli* aminoacyl transferase (AaT) to deliver disulfide-protected Hcs [*S*-(thiomethyl)homocysteine, Hcm] to the N-terminus of a large expressed protein fragment.[14] In *E. coli* cells, AaT transfers Phe, Leu, or Met from an aminoacyl tRNA to the N-terminus of a protein.[15] By using a modified methionine aminoacyl tRNA synthetase enzyme (Met*RS), we can generate Hcm-tRNA in situ, which serves as a viable substrate for AaT.[16] Once Hcm is transferred to the N-terminus of a protein, it is easily deprotected by a reducing agent to form Hcs for ligation. By combining unnatural amino acid mutagenesis with EPL at homocysteine (which is converted into methionine), we can incorporate our minimal FRET pair into αS in a traceless manner that minimizes unnecessary synthesis of peptide fragments composed of natural amino acids. A retrosynthetic analysis of our target protein, Ac-$\alpha S^F Asp'_2 Cnf_{39}$, is shown in Scheme 1. The prime symbol is used to denote a backbone thioamide bond in an amino acid represented by the standard three-letter code, and αS^F represents a construct with all of the native Tyr residues (39, 125, 133, and 136) mutated to Phe to prevent chromophore cross talk.

Previously, we have shown that fluorenylmethoxycarbonyl (Fmoc)-protected thiocarboxybenzotriazoles can be used with conventional Fmoc-protected amino acids to synthesize thioamide-containing peptides.[5a,9] The procedure for the preparation of Fmoc-Asp′(O*t*-Bu)-nitrobenzotriazole **3** follows a previous report by Shallaby and is illustrated in Scheme 2.[17] Coupling of 4-nitro-1,2-phenylenediamine to Fmoc-Asp(O*t*-Bu)-OH was achieved using isobutyl chloroformate (IBCF) to generate **1** in 89% yield.[20] Amide **1** was then added to a solution of P_4S_{10} and anhydrous Na_2CO_3 at 0 ºC. Selective thionation of the Asp backbone carbonyl was completed within one hour to afford compound **2** in 79% yield.[21] Intramolecular diazonium cyclization of thioamide **2** gave the activated benzotriazole **3** in 67% yield.[22] Compound **3** can be isolated from the final reaction by precipitation with ice-cold water in sufficient purity for peptide coupling reactions. Since the activated benzotriazoles are subject to degradation through hydrolysis and intramolecular nucleophilic attack by the carbonyl oxygens, it is best to minimize handling of compound **3** prior to peptide coupling.



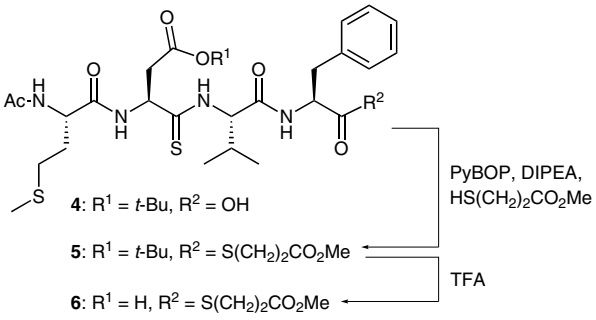
Scheme 2 Synthesis of aspartyl thioamide precursor **3**[20]

Although, we have previously used on-resin methods to prepare thioesters for NCL, we find that off-resin activation is simplest for short peptides. The N-terminal thioamide-containing fragment of αS was synthesized using standard Fmoc-based SPPS procedures with the exception of direct acylation of Val_3 by benzotriazole **3** (i.e., no activating agents were added). Thiopeptide **4** was synthesized on 2-chlorotrityl resin, cleaved under mild



Scheme 1 Retrosynthetic analysis of Ac-$\alpha S^F Asp'_2 Cnf_{39}$

conditions (10% AcOH) to retain the Asp side-chain protecting group, and then activated using benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) to form the mercaptopropionate thioester **5** (Scheme 3). Epimerization of the α-carbon of the C-terminal phenylalanine residue was eliminated by reducing the PyBOP activation time to 25 minutes.[18] Following acidic deprotection of Asp′(O*t*-Bu)$_2$, the thioester peptide **6** was isolated by HPLC (5.3 μmol, 5% yield) and its identity was confirmed by MALDI MS analysis.



Scheme 3 Synthesis of thioamide-containing thioester peptide **6** for native chemical ligation

The expressed C-terminal Cnf-containing αS fragment for NCL was generated as depicted in Figure 1. AaT selectively modifies the α-amine of proteins bearing a lysine or arginine as the N-terminal residue. Prior to this work, we demonstrated that Met$_5$Lys$_6$ could be used as a point of disconnection in the synthesis of αS.[14] To minimize perturbation of native αS dynamics resulting from Cnf incorporation, we chose to replace the similarly sized Tyr or Phe at position 39. A plasmid containing the C-terminal protein fragment (αS^F_{6-140}) with a TAG codon installed at position 39 was prepared. An N-terminal His$_{11}$ tag and a Factor Xa proteolysis site precede the αS gene. *E. coli* cells were transformed with two plasmids, one containing the Cnf-selective mutant synthetase (CnfRS) with tRNA and the other containing His$_{Tag}$-αS^F_{6-140}TAG$_{39}$.[19] Following an initial growth period, protein expression was induced by adding Cnf and isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture medium. The expressed protein, His$_{Tag}$-αS^F_{6-140}Cnf$_{39}$ (**7**), was purified by Ni-affinity chromatography and subsequently cleaved by Factor Xa to yield αS^F_{6-140}Cnf$_{39}$ (**8**). Following purification by ion-exchange chromatography, the truncated protein was incubated with AaT, Met*RS, *E. coli* total tRNA, Hcm, and ATP for two hours resulting in complete formation of αS^F_{5-140}Hcm$_5$Cnf$_{39}$ (**9**). αS^F_{5-140}Hcm$_5$Cnf$_{39}$ was subjected to an additional round of ion-exchange chromatography prior to use in the NCL reaction. Typically, about 1 μmol of pure **9** is obtained from a 1 L protein expression.

The ligation reaction was initiated by incubation of **9** with 1.5 equivalents of Ac-MetAsp′ValPhe-SR (**6**) in degassed ligation buffer [6 M guanidinium hydrochloride, 0.2 M sodium phosphate, 20 mM tris(2-carboxyethyl)phosphine, 2% thiophenol, at pH 7.5]. Disulfide deprotection of the Hcm residue was observed by MALDI MS within five minutes. The ligation reaction was allowed to proceed for 24 hours prior to buffer exchange into methylation reaction buffer (20 mM Tris, pH 8.6). Ac-αS^FAsp′$_2$Hcs$_5$Cnf$_{39}$ (**10**) was converted to Ac-αS^FAsp′$_2$Cnf$_{39}$ (**11**), with native Met$_5$, by a short treatment (10 min) with 3000 equivalents of MeI. Allowing the reaction to proceed for longer periods of time led to S-alkylation of the thioamide (data not shown). Ac-αS^FAsp′$_2$Cnf$_{39}$ was isolated from the NCL reaction by HPLC and characterized by MALDI MS, PAGE gel, and UV–Vis and spectroscopy (Figure 1 and Supporting Information). The conversion over the two-step ligation/methylation sequence was 41%. MALDI MS analysis of trypsin-digested **11** confirmed selective alkylation of Hcs to form Met in the purified product.

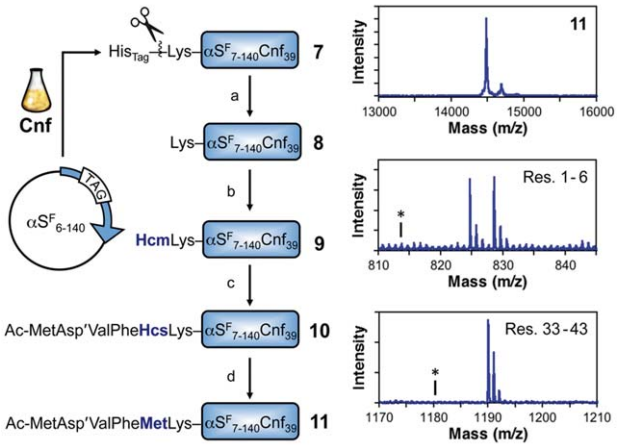


Figure 1 Ac-αS^FAsp′$_2$Cnf$_{39}$ synthesis. Left: Functionalization of αS^F_{6-140}Cnf$_{39}$ by (a) cleavage of His$_{11}$ tag at Factor Xa site; (b) attachment of Hcm by AaT-catalyzed modification; (c) ligation of an N-terminal thioester peptide (**6**), and; (d) conversion of Hcs to Met by methylation to form Ac-αS^FAsp′$_2$Cnf$_{39}$ (**11**). Top right: MALDI MS analysis of full length **11**. Calcd *m*/*z* [M + H]$^+$ 14480.1, found: 14479.6. Middle right: Trypsinized fragment corresponding to residues 1–6 of Ac-αS^FAsp′$_2$Cnf$_{39}$ confirms methylation. Calcd *m*/*z* [M + H]$^+$ 828.34; found: 828.46. The asterisk indicates the expected mass of the 1–6 fragment with unmethylated Hcs at position 5. Calcd *m*/*z* [M + H]$^+$ 814.39. Bottom right: Trypsinized fragment corresponding to residues 33–43 of Ac-αS^FAsp′$_2$Cnf$_{39}$ confirms Cnf incorporation. Calcd *m*/*z* [M + H]$^+$ 1189.66; found: 1189.85. The asterisk indicates the expected mass of the 33–43 fragment with Tyr at position 39. Calcd *m*/*z* [M + H]$^+$ 1180.65.

Using a similar double-labeled construct containing cysteine at the ligation site (Ac-αS^FPhe′$_4$Cys$_9$Cnf$_{39}$), we observed thioamide quenching of Cnf after compaction of the protein in high concentrations of TMAO.[9] Since the Ser-to-Cys mutation in this protein constituted a small deviation from the native αS protein sequence, we sought to investigate this phenomenon using our newly synthesized Ac-αS^FAsp′$_2$Cnf$_{39}$ construct (Asp′$_2$ used here because labeling at Phe′$_4$ would not be compatible with our ligation strategy using Hcs). To correct for any changes in Cnf fluorescence that are not due to the presence of the thioamide, an oxoamide control protein (αS^FCnf$_{39}$) was also

prepared for fluorescence experiments. In buffer without TMAO, thioamide quenching of Cnf in Ac-αS^{F}Asp′$_2$Cnf$_{39}$ (i.e., FRET) was minimal. In accordance with our previous observations, thioamide quenching of Cnf increased as a function of TMAO concentration (Figure 2, left axis). To assign distance constraints based on these measurements, the measured quenching efficiency (E_Q) values were converted to distances using Förster theory. In 2 M and 4 M TMAO, R_{FRET} was calculated as 15.4 Å and 12.6 Å, respectively (Figure 2, right axis). Taken together with our previous results, these data indicate that the N-terminal region of αS undergoes significant compaction in high TMAO concentrations. Furthermore, our new results conclusively demonstrate that the observed quenching is not due to Cys-mediated dimerization of αS.

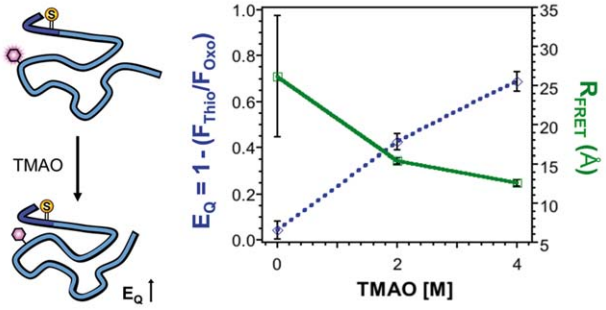


Figure 2 Refolding assay. Left: Monomeric double-labeled αS (Ac-αS^{F}Asp′$_2$Cnf$_{39}$) is mixed with TMAO to induce compaction and an increase in quenching efficiency (E_Q). Right: E_Q (blue dashed line) of Ac-αS^{F}Asp′$_2$Cnf$_{39}$ determined at varying concentrations of TMAO. Interchromophore distance (R_{FRET}, green solid line) computed using Förster theory as described in Supporting Information.

By combining unnatural amino acid mutagenesis with EPL at Met, we can install our minimal Cnf/thioamide FRET probe pair into αS without introducing a mutation at the ligation site. We have demonstrated that selective alkylation of Hcs can be performed in the presence of a thioamide, rendering our new ligation strategy compatible with the minimal probe pairs that we have developed. The αS refolding studies presented here demonstrate how this methodology can be used to study protein folding dynamics, though these techniques are by no means limited to αS. Over 20,000 proteins in the PDB contain MetArg or MetLys motifs that can be used as points of disconnection in their retrosynthetic analyses.[14] We are currently working to expand these methods even further by combining EPL at Met with multiple ligation strategies. This methodology will allow us to install thioamide-containing synthetic fragments in the middle of a large protein target in a traceless and efficient manner.

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Supporting Information for this article is available online at http://www.thieme-connect.com/ejournals/toc/synlett.

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(20) **Synthesis of α-*N*-Fmoc-L-aspartate-2-amino-5-nitroanilide (1)**: Fmoc-Asp(O*t*-Bu)-OH (2.67 g, 6.50 mmol) was dissolved in THF (35 mL) under argon flow and the solution was cooled to –10 °C in a NaCl/ice (1:3) bath. *N*-Methylmorpholine (NMM, 1.43 mL, 13 mmol) and isobutyl chloroformate (IBCF, 0.85 mL, 6.5 mmol) were added dropwise with stirring. After 15 min, 4-nitro-*o*-phenylenediamine (1.0 g, 6.5 mmol) was added and the reaction was allowed to proceed with stirring under argon flow at –10 °C for 2 h. The reaction was then allowed to proceed for an additional 6 h with stirring at r.t. The reaction mixture was dried by rotary evaporation, resuspended in DMF (20 mL), and then poured into sat. KCl solution (200 mL). The precipitated product was filtered and washed with cold H_2O. The precipitate was then dissolved in minimal EtOAc and purified over a silica gel column in hexanes–EtOAc (3:2) to afford **1** as a yellow solid in 88.9% yield; R_f 0.5 (hexanes–EtOAc, 1:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.1 (s, 1H), 8.0 (s, 1H), 7.93 (dd, *J* = 2.5, 9.0 Hz, 1 H), 7.74 (dd, *J* = 3.6, 7.5 Hz, 2 H), 7.57 (dd, *J* = 3.4, 7.4 Hz, 2 H), 7.36–7.41 (m, 2 H), 7.26–7.30 (m, 2 H), 6.62 (d, *J* = 9.0 Hz, 1 H), 5.98 (d, *J* = 8.2 Hz, 1 H), 4.73 (s, H) 4.63 (br s, 1 H), 4.45–4.53 (m, 2 H), 4.19 (t, *J* = 6.3 Hz, 1 H), 2.97 (dd, *J* = 4.6, 17.2 Hz, 1 H), 2.72–2.80 (m, 1 H), 1.46 (s, 9 H). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 172.5, 170.8, 157.3 , 149.0, 144.4, 142.3, 139.6, 128.8, 128.2, 125.9, 125.4, 124.5, 121.7, 121.1, 115.9, 83.6, 68.3, 52.7, 48.2, 38.3, 29.1. HRMS (ESI): *m*/*z* $[M + H]^+$ calcd for $C_{29}H_{31}N_4O_7$: 547.219; found: 547.218.

(21) **Synthesis of α-*N*-Fmoc-L-thioaspartate-2-amino-5-nitroanilide (2)**: P_2S_5 (2.47 g, 5.56 mmol) and anhyd Na_2CO_3 (0.589 g, 5.56 mmol) were stirred in THF (30 mL) at r.t. under argon flow until a clear yellow solution was obtained. After cooling the solution to 0 °C on ice, **1** (3.04 g, 5.56 mmol) was added, and the reaction was carefully monitored by TLC. After approximately 1 h, the reaction was filtered through Celite® (Sigma-Aldrich) and dried by rotary evaporation. The crude reaction material was dissolved in EtOAc and purified over a silica gel column in hexanes–EtOAc (1:1) to afford **2** as a yellow foam (2.46 g, 78.6% yield); R_f 0.7 (hexanes–EtOAc, 1:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.84 (br s, 1 H), 8.07 (br s, 1 H), 7.96 (d, *J* = 8.4 Hz, 1 H), 7.76 (d, *J* = 6.9 Hz, 2 H), 7.52 (dd, *J* = 7.3, 19.6 Hz, 2 H) 7.39–7.43 (m, 2 H), 7.28–7.32 (m, 2 H), 6.56 (d, *J* = 9.0 Hz, 1 H), 6.06 (d, *J* = 8.2 Hz, 1 H), 5.07 (br s, 1 H), 4.84 (br s, 2 H), 4.37 (br s, 2 H), 4.21–4.13 (m, 1 H), 3.18–3.05 (m, 2 H), 1.45 (s, 9 H). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 204.9, 172.1, 157.2, 149.6, 144.3, 142.2, 139.0, 128.8, 128.1, 126.5, 125.9, 125.8, 122.8, 121.0, 115.6, 83.5, 68.4, 58.4, 47.8, 41.6, 28.9. HRMS (ESI): *m*/*z* $[M + H]^+$ calcd for $C_{29}H_{31}N_4O_6S$: 563.196; found: 563.197.

(22) **Synthesis of α-*N*-Fmoc-L-thioaspartatenitrobenzotriazolide (3)**: Compound **2** (1.00 g, 1.78 mmol) was added to glacial acetic acid diluted with 5% H_2O (25 mL). $NaNO_2$ (0.16 g, 2.23 mmol) was added in small portions over 5 min with constant stirring at r.t. After 30 min, the reaction was quenched by the addition of ice water (500 mL). The resulting pale orange precipitate **3** was filtered, washed extensively with ice water, and allowed to dry under vacuum. The final product was characterized and used in peptide synthesis without any further purification; R_f 0.9 (hexanes–EtOAc, 1:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.64 (s, 1 H), 8.46 (d, *J* = 8.6 Hz, 1 H), 8.33 (d, *J* = 8.7 Hz, 1 H), 7.77–7.78 (m, 2 H), 7.59–7.64 (m, 2 H) 7.38–7.44 (m, 2 H), 7.29–7.35 (m, 2 H), 6.45–6.52 (m, 1 H), 6.23 (d, *J* = 8.7 Hz, 1 H), 4.48–4.54 (m, 1 H), 4.34–4.41 (m, 1 H), 4.21–4.26 (m, 1 H), 3.07–3.15 (m, 1 H), 2.86–2.97 (m, 1 H), 1.42 (s, 9 H). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 206.7, 169.3, 156.4, 150.6, 149.9, 144.6, 142.3, 132.8, 128.7, 128.0, 126.0, 123.2, 122.6, 121.0, 113.6, 83.4, 68.2, 59.0, 48.1, 41.9, 28.9. HRMS (ESI): *m*/*z* $[M + Na]^+$ calcd for $C_{29}H_{27}N_5NaO_6S$: 596.158; found: 596.158.

