Engineering an unnatural N^{α}-anchored disulfide into BPTI by total chemical synthesis: structural and functional consequences

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Received 1 September 1999

Abstract A disulfide-engineered analogue of bovine pancreatic trypsin inhibitor (BPTI), ((N^{α}-$(CH_2)_2$S-)Gly^{38})BPTI, has been prepared using a thioester-mediated auxiliary functional group chemical ligation of a N^{α}-ethanethiol-containing peptide segment with a peptide-$^{\alpha}$COSR segment. In this study, N^{α}-(ethanethiol)Gly^{38} replaces the native Cys^{38}, providing the sulfhydryl group required for ligation and folding. Comparisons between ((N^{α}-$(CH_2)_2$SH)Gly^{38})BPTI, synthetic native BPTI and reference BPTI purchased from Sigma were made using mass spectroscopy, enzyme inhibitor association constant determination (K_a) and ^{1}H-nuclear magnetic resonance total correlated spectroscopy (^{1}H-NMR TOCSY) measurements. The K_a value for ((N^{α}-$(CH_2)_2$SH)Gly^{38})BPTI was approximately 20-fold lower than synthetic and reference BPTI, which was attributed to perturbations in the binding loop of the protein (near Cys^{14}). This hypothesis was confirmed by two-dimensional (2D) ^{1}H-NMR TOCSY experiments. The data reported here demonstrate that total chemical synthesis by auxiliary functional group chemical ligation is a practical method for the synthesis of a novel class of biologically active protein analogues containing additional functional groups linked to the protein backbone.

Key words: (N^{α}-$(CH_2)_2$SH)Gly^{38})BPTI; Trypsin; Binding loop

1. Introduction

Since its introduction in 1994 [1], thioester-mediated amide-forming chemical ligation of unprotected peptide segments, termed native chemical ligation, has greatly facilitated the total synthesis of proteins. Prior to this ligation strategy, the synthesis of native protein domains was an arduous task, most often undertaken by stepwise solid phase peptide synthesis methods. With few exceptions, the products of these syntheses were complex mixtures and were intractable to purification to homogeneous molecular preparations. With the advent of native chemical ligation, the routine synthesis of small, disulfide-containing proteins has become a reality [2].

Native chemical ligation requires a cysteine residue at the ligation site (Fig. 1a) [1]. Despite this limitation, the power of this technology has been demonstrated by the successful synthesis of biologically active proteins including a variety of chemokines such as interleukin-8 [1] and SDF1α [3], bovine pancreatic trypsin inhibitor (BPTI) [4], turkey ovomucoid third domain [5] and barnase [6]. Nonetheless, it would be of considerable utility to surmount the necessity for a cysteine residue at the ligation site.

A possible route for generalization to native chemical ligation at amino acids other than cysteine would be to separate the thiol moiety required for ligation from the functionality of the side-chain of the amino acid in the reacting peptide segment. Recently, Canne et al. [7] reported a ligation at Gly-Gly residues accomplished by employing a removable N^{α}-linked auxiliary thiol moiety to provide the functionality required in the ligation reaction. In the course of that study, an extremely facile chemical ligation reaction was observed utilizing a non-removable N^{α}-ethanethiol auxiliary functional group (Fig. 1b). The product of this ligation reaction contains a peptidomimetic backbone structure at the ligation site, a N^{α}-(ethanethiol) amide.

It occurred to us that use of a N^{α}-(ethanethiol)glycine residue to replace a cysteine involved in disulfide bond formation would result in a novel type of engineered protein (Fig. 1c). To our knowledge, the properties of such a N^{α} to side-chain disulfide bond have not been explored in the context of a protein molecule.

We chose BPTI (Fig. 2) as a model system in which to establish the use of the N^{α}-(ethanethiol) auxiliary functional group in the total chemical synthesis of a protein and to examine the properties of the resulting disulfide-engineered protein. BPTI is probably the most intensively studied protein extant and contains a Gly^{37}-Cys^{38} site appropriate for native chemical ligation. It is a small (6.5 kDa) protein first identified in 1936 because of its remarkable thermal stability and ability to strongly inhibit trypsin [8]. BPTI possesses 58 amino acids, six of them cysteine residues that form three disulfide bonds in the folded structure. The cysteine pairings are residues 5–51, 14–38 and 30–55 [9]. The three-dimensional (3D) structure of this protein has been solved both crystallographically [10] and by nuclear magnetic resonance (NMR) spectroscopy [11,12]. In addition, BPTI has a well-documented folding behavior [13,14]. Furthermore, this protein has previously been prepared in our laboratory by total synthesis using native chemical ligation [4].

Here, we report the use of the non-removable auxiliary functional group, N^{α}-(ethanethiol), in the synthesis of a biologically active, disulfide-engineered BPTI, (($N^{\alpha}(CH_2)_2$SH)-Gly^{38})BPTI. In this BPTI analogue, Cys^{38} has been replaced by a N^{α}-(ethylthiol)Gly^{38} residue. The chemically synthesized polypeptide folded to give a functional protein containing three disulfide bonds. The properties of this analogue were compared to native BPTI synthesized by chemical ligation and to a reference sample of native BPTI purchased from Sigma. These comparisons included electrospray mass spectrometry (ESMS), association constant binding studies (K_a)

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PII: S0014-5793(99)01310-1

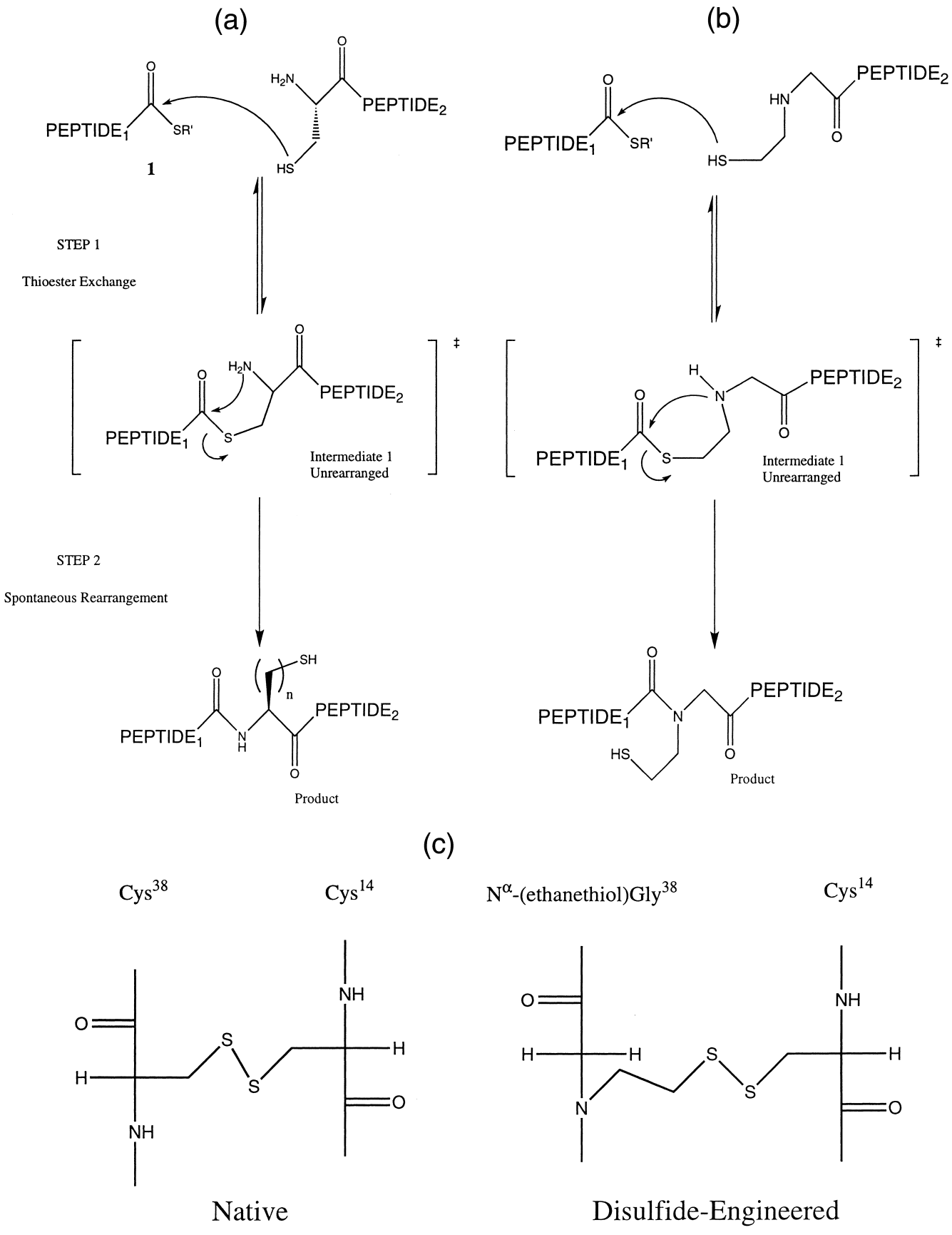


Fig. 1. Amide-forming ligation reactions: (a) native chemical ligation [1], (b) ligation using a N^{α}-(ethanethiol)glycine auxiliary functional group [7], (c) disulfide engineering using the N^{α}-ethanethiol auxiliary functional group. The disulfide-engineered system has an extra methylene in the disulfide linkage and the connection to the backbone has been shifted from the C^{α} to the amide nitrogen; the amino acid side-chain has been deleted.

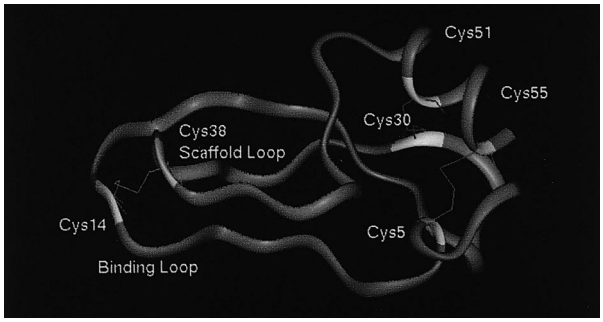


Fig. 2. Ribbon diagram of native BPTI. Positions of the disulfides (between cysteines 14–38, 30–51 and 5–55) are shown in lighter shade. The site of modification using the N^{α}-ethanethiol auxiliary functional group is at Cys^{38} on the scaffold loop.

with chymotrypsin and a detailed comparison of the fingerprint regions of the homonuclear two-dimensional (2D) ^{1}H-total correlated NMR spectroscopy (2D ^{1}H-NMR TOCSY) experiments. While all three of these BPTI proteins are potent inhibitors of both chymotrypsin and trypsin, the K_a of $((N^{\alpha}(CH_2)_2SH)Gly^{38})$BPTI with chymotrypsin was found to be 20-fold less than the affinity of the native protein, which suggested a perturbation in the binding loop of the BPTI analogue. This interpretation was confirmed by the fingerprint regions of the 2D-TOCSY spectra. These data unequivocally demonstrate the feasibility of auxiliary functional group chemical ligation in the synthesis of biologically active proteins and open protein design and engineering to modifications previously demonstrated only for smaller peptidomimetics [15,16].

2. Materials and methods

For flash-column chromatography of organic compounds, silica gel (230–400 mesh) was used in standard glass columns with gravity or air pressure. Analytical reversed phase high performance liquid chromatography (HPLC) was performed on a Rainin HPLC system with UV-detection at 214 nm using a Vydac C-18 analytical (5 μm, 0.46×15 cm) column. Semi-preparative reversed phase HPLC was performed on a Rainin HPLC system with UV-detection at 230 nm using a Waters RCM 8×10 module equipped with a Delta Pack C-18 Radial Pack cartridge (15 μm, 8×100 mm) column from Millipore. Preparative scale reversed phase HPLC was performed on a Waters Delta Prep 4000 system with UV-detection at 230 nm using a Vydac C-18 preparative (15–20 μm, 5.0×25 cm) column. Linear gradients of buffer B in buffer A (A = 0.1% trifluoroacetic acid (TFA) in water, B = 0.09% TFA in 90% acetonitrile/10% water) were used for chromatographic separations. Flow rates were 1 ml/min for analytical, 4 ml/min for semi-preparative and 30 ml/min for preparative scale HPLC. Affinity chromatography was performed on an anhydro-trypsin solid support resin in a Flex column. Adsorption solvent was 20 mM MES at pH 6.0. Non-specifically bound protein was eluted in 200 mM NaCl and the specifically bound protein was eluted in 200 mM NaCl with 100 mM HCl. Desalting was performed using 1% acetic acid in water with a Sephadex G-25 gel filtration column. All mass spectra of peptides and proteins were obtained on a Sciex API-III electrospray ionization triple-quadrupole mass spectrometer. The observed masses reported were derived from the experimental *m/z* states for all observed charge states of a molecular species, using the program MacSpec (Sciex, version 2.4.1). Calculated masses reported were derived from the program MacProMass (Terry Lee and Sunil Vemuri, Beckman Research Institute, Duarte, CA, USA) and reflect the average isotope composition. Phase sensitive 2D ^{1}H-NMR TOCSY spectra were recorded on a Brucker AMX 500 spectrometer using Watergate solvent suppression [21,27]. Ultraviolet-visible (UV-vis) spectroscopy was performed on a Hewlett-Packard 8452A photodiode array spectrophotometer. Boc-L-amino acids were purchased from Novabiochem (La Jolla, CA, USA) and Bachem Biosience (King of Prussia, PA, USA). 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was purchased from Richelieu Biotechnologies (Montreal, Canada). Boc-Ala-OCH_2-Pam-resin and diisopropylethylamine (DIEA) were purchased from Applied Biosystems (Foster City, CA, USA) and methylbenzhydrylamine (MBHA) resin was purchased from Peninsula Laboratories (San Carlos, CA, USA). JT-Baker Photrex grade dimethylformamide (DMF) and Baker Analyzed acetonitrile were obtained from Baker. HPLC grade methylene chloride (CH_2Cl_2) was purchased from Fisher. TFA was purchased from Halocarbon (New Jersey). Anhydrous hydrogen fluoride (HF) was purchased from Matheson Gas. Flex columns were purchased from Fisher Scientific (Tustin, CA, USA). Silica gel (230–400) mesh was purchased from Aldrich (Milwaukee, WI, USA). Sephadex G-25 was purchased from Pharmacia Biotech (Piscataway, NJ, USA). All other reagents were of analytical reagent grade or better and purchased from Aldrich (Milwaukee, WI, USA) or Lancaster (Windham, NH, USA).

2.1. Peptide segment synthesis

Synthesis of peptides was carried out manually by established methods using in situ neutralization and HBTU activation procedures for stepwise Boc chemistry solid phase peptide synthesis [18]. Synthesis was performed on either Boc-Ala-OCH_2-Pam-resin or Boc-Gly-thioester-resin. Boc-Gly-thioester-resin was prepared by coupling (4-((N^{α}-t-Boc-Gly-S)benzyl)phenoxy)-acetic acid, dicyclohexylamine salt (2.0 equivalents), to aminomethyl resin (one equivalent, washed with 10% DIEA in DMF) with HBTU (1.6 equivalents) added as an activating agent and DIEA (one equivalent) in DMF [28]. Protection of amino acid side-chain functional groups was as follows: Boc-Arg(*p*-toluenesulfonyl)-OH, Boc-Asn(xanthyl)-OH, Boc-Asp(O-cyclohexyl)-OH, Boc-His(dinitrophenyl)-OH, Boc-Thr(benzyl)-OH, Boc-Tyr(2-bromobenzyloxy-carbonyl)-OH, Boc-Cys(4-methylbenzyl)-OH, Boc-Glu(O-cyclohexyl)-OH, Boc-Lys(2-Cl-Z)-OH and Boc-Ser(benzyl)-OH. Other amino acids required no side-chain protection. Coupling was monitored by a quantitative ninhydrin assay after 10 min coupling cycles (typical coupling yields were >99%). After chain assembly, the N^{α}Boc group was removed with neat TFA (two 1 min treatments), the salt of the peptide-resin was neutralized with 10% DIEA in DMF (two 1 min treatments), then, N^{α}-deprotected peptide-resin was washed with DCM and dried in vacuo for >30 min. The peptides were simultaneously deprotected and cleaved from the resin support with anhydrous HF containing either 10% *p*-cresol or 10% anisole at 0°C for 1 h to yield the deprotected peptide-$^{\alpha}$COSH or -$^{\alpha}CO_2H$. Removal of HF was accomplished over 5–10 min under reduced pressure and the crude peptide was triturated with diethyl ether, dissolved in HPLC buffer (60% B), diluted to <20% acetonitrile by volume and lyophilized.

2.2. BPTI(1–37)-$^{\alpha}$thioester (**1**)

BPTI(1–37)-$^{\alpha}$acyl thioester was generated by reaction of the crude cleaved BPTI(1–37)-$^{\alpha}$COSH thioacid with >10 equivalents of 2-bromoacetic acid in 2 ml 6 M GnHCl, 100 mM sodium acetate, pH = 4.0 for 120 min at room temperature. The $^{\alpha}$thioester product was isolated by preparative HPLC (20–55% B in 60 min) in a 10% yield in respect to the crude HF-cleaved peptide. The identity of BPTI(1–37)-$^{\alpha}$acyl thioester (**1**) was confirmed by electrospray mass spectroscopy (observed mass 4291.5 ± 0.5 Da, calculated mass 4291.0 Da average isotope distribution).

2.3. BPTI $((N^{\alpha}(CH_2)_2SH)Gly^{38})(38–58)$-$^{\alpha}CO_2H$ peptide (**2a**)

BPTI(39–58)-$^{\alpha}CO_2H$ was synthesized on Boc-Ala-OCH_2-Pam-resin. After completion of chain assembly, the N^{α}Boc protecting group was removed by TFA treatment (two 1 min treatments with neat TFA). The peptide was then bromoacetylated by the Robey method [29]. Bromoacetic acid (236 mg, 2.0 mmol) was dissolved in CH_2Cl_2 (2 ml) and diisopropylcarbodiimide (156 μl, 1.0 mmol) was added. The solution turned cloudy with a floting precipitate. After activation for 15 min to form the symmetric anhydride, DMF (2 ml) was added and the resulting turbid solution was added to the deprotected peptide-resin and coupled for 20 min. The resin was washed with two 30 s flow washes of DMF and then a batch wash of DMSO. The DMSO-saturated BrAc-peptide-resin was then reacted with a 2 M solution of S-(4-methylbenzyl)-2-aminoethanethiol (181 mg, 1.0 mmol) in 300 μl DMSO and 200 μl DIEA for 14 h [7]. The resin was washed with two

30 s DMF flow washes, rinsed well with DCM/MeOH (1:1 v/v) and dried in vacuo for >30 min. Standard HF cleavage was performed with *p*-cresol as the scavenger. ((N$^{\alpha}$(CH$_2$)$_2$SH)Gly^{38})BPTI(38–58)-$^{\alpha}$CO$_2$H, peptide (2a), was purified by preparative HPLC (10–40% B in 45 min) in 30% yield from the crude HF-cleaved peptide (observed mass 2331.0 ± 0.5 Da, calculated mass 2331.7 Da average isotope distribution).

2.4. BPTI(38–58)-$^{\alpha}$CO$_2$H peptide (**2b**)

BPTI(38–58)-$^{\alpha}$CO$_2$H peptide (**2b**) was synthesized in the same manner as BPTI(39–58)-$^{\alpha}$CO$_2$H, incorporating a cysteine at position 38 (observed mass, calculated mass 2318.7 Da average isotope distribution).

2.5. Chemical ligations

Ligation reactions were performed in 6 M GnHCl, 100 mM phosphate, pH 7.5, at concentrations of 8–10 mg/ml for each peptide. Thiophenol (2–4% by volume) was added to maintain a reducing environment and accelerate the ligation reaction by exchanging the peptide-$^{\alpha}$thioacyl ester to the peptide-$^{\alpha}$thiophenyl ester [19]. The ligation reaction proceeded rapidly with significant product formation after 1–2 h. The clear reaction solution became progressively more cloudy with a white precipitate forming during the course of the ligation. To guarantee the completion of the reaction, ligations were allowed to react for 16–24 h prior to quenching with 20–40 mg dithiothreitol (DTT), mixing for 20 s and then letting the solution stand at room temperature for 30–45 min. The milky precipitate gradually dissolved into solution during this time. The ligation product was then purified by semi-preparative reversed phase HPLC (20–55% B in 45 min).

2.6. ((N$^{\alpha}$(CH$_2$)$_2$SH)Gly^{38})BPTI ligation (Fig. 4) [7]

15 mg BPTI(1–37)$^{\alpha}$COSCH$_2$CO$_2$H (**1**) (observed mass 4291.5 ± 0.5 Da, calculated mass 4291.9) and 15 mg BPTI ((N$^{\alpha}$(CH$_2$)$_2$SH)-Gly^{38})(38–58)-$^{\alpha}$CO$_2$H (**2a**) (observed mass 2331.0, calculated mass 2331.7) were dissolved in 2 ml of 6 M GnHCl, 100 mM phosphate, pH 7.5. After 5 μl of this solution was used for a zero time point HPLC analysis, thiophenol was added (4% by volume). The reaction was mixed for 20 s and was then left standing at room temperature for 14 h. The reaction was monitored by analytical HPLC analysis at various time points (Fig. 4). BPTI(1–37)-$^{\alpha}$COSCH$_2$CO$_2$H (**1**) disappeared rapidly after thiophenol was added and the thioester exchange product [6], BPTI(1–37)$^{\alpha}$COSC$_6$H$_5$ (observed mass 4323.5 ± 0.5 Da, calculated mass 4323.9), co-eluted with thiophenol under the chromatography conditions used. After 14 h, the ligated product, ((N$^{\alpha}$(CH$_2$)$_2$SH)Gly^{38})BPTI (observed mass 6530.0 ± 0.5 Da, calculated mass 6530.6 average isotope distribution), was recovered from the DTT-quenched reaction by semi-preparative reversed phase HPLC (20–55% B in 45 min, elution time 19 min) and lyophilized to yield 9 mg of pure unfolded ((N$^{\alpha}$(CH$_2$)$_2$SH)Gly^{38})BPTI (40%).

2.7. BPTI native ligation [1]

15 mg of BPTI(1–37)-$^{\alpha}$COSCH$_2$CO$_2$H (**1**) (observed mass 4291.5 ± 0.5 Da, calculated mass 4291.9 average isotope distribution) and 15 mg of BPTI(38–58)-$^{\alpha}$CO$_2$H (**2b**) (observed mass 2318.3 ± 0.5 Da, calculated mass 2318.7 average isotope distribution) were dissolved in 2 ml of 6 M GnHCl, 100 mM phosphate, pH 7.5. After a 5 μl aliquot was removed for HPLC analysis, thiophenol (4% by volume) was added. The reaction was mixed briefly for 20 s and was then left standing at room temperature. During the course of the reaction, the solution became increasingly opaque with a white, cloudy precipitate. After 16 h, the reaction was quenched for 40 min with 20–40 mg DTT and the product, native BPTI, was purified by semi-preparative HPLC (20–55% B in 45 min, elution time 19 min) and lyophilized to yield 9 mg of pure unfolded native BPTI (40% yield) (observed mass 6516.0 ± 1.0 Da, calculated mass 6517.6 average isotope distribution).

2.8. Folding [1]

Pure unfolded synthetic BPTI (9 mg) or ((N$^{\alpha}$(CH$_2$)$_2$SH)Gly^{38})BPTI (9 mg) was dissolved in 1 ml of 6 M GnHCl, 100 mM phosphate, pH 7.5. When the solution was complete, 49 ml of filtered water was rapidly added and the resulting solution mixed briefly. The concentration of folding protein is critical and should not exceed 0.3 mg/ml to reduce the chances of non-specific aggregation and precipitation. The reaction was left standing at room temperature overnight, vented to the atmosphere to permit air oxidation. The total crude folded protein preparations were subjected to affinity chromatography to recover active folded protein.

2.9. Affinity chromatography [20]

Solutions of folded synthetic native BPTI and ((N$^{\alpha}$(CH$_2$)$_2$SH)-Gly^{38})BPTI were bound to an anhydro-trypsin affinity column equilibrated in 20 mM MES, pH 6.0 buffer. After loading the sample onto the column, the column was washed with the equilibration buffer. When a stable base line was obtained, the column was then washed with 0.2 N NaCl in water to elute non-specifically bound folding by-products. Again, after a stable base line was obtained, the desired native BPTI or folded ((N$^{\alpha}$(CH$_2$)$_2$SH)Gly^{38})BPTI protein was eluted with 0.2 N NaCl in 0.1 N HCl. In a number of cases, the elution of protein from the affinity column resulted in two peaks that were not completely separable (these peaks were isolated and later demonstrated the same behavior in the K_a assay and mass spectrometry). This occurred with both proteins and is most likely the result of incomplete solubility in the low pH-high salt elution buffer. The eluted protein was diluted with an equal volume of filtered water and lyophilized.

2.10. Desalting

Proteins eluted from the affinity column contained considerable quantities of NaCl. The native BPTI or folded ((N$^{\alpha}$(CH$_2$)$_2$SH)-Gly^{38})BPTI was desalted using a Sephadex G-25 gel filtration column with a 1% acetic acid elution solvent. The eluted proteins were lyophilized to yield folded native BPTI (4 mg, 44% yield, observed mass 6511.3 Da, calculated mass 6511.6 Da average isotope distribution) and folded ((N$^{\alpha}$(CH$_2$)$_2$SH)Gly^{38})BPTI (3.7 mg, 41% yield, observed mass 6525.0, calculated mass 6525.5 average isotope distribution).

2.11. S-(4-Methylbenzyl)-2-aminoethane thiol

2-Aminoethane thiol (500 mg, 6.5 mmol) was dissolved in dry THF (10 ml) and DIEA (1.0 ml) and 0.75 equivalent 4-methylbenzyl bromide (906 mg, 4.9 mmol) was added to this solution while stirring at room temperature. After 6 h, the solvent was removed in vacuo and the resulting oil was purified by flash-column chromatography with a gradient of 1–10% methanol in chloroform to yield A as an oily solid (621 mg, 70% yield). R_f = 0.15–0.3 streak in 5% MeOH in chloroform, UV and ninhydrin active. ^{1}H-NMR (CDCl$_3$) δ 1.45 (s, 2H), 2.32 (s, 3H), 2.5 (t, J = 6.2Hz, 2H), 2.8 (t, J = 6.2 Hz, 2H), 3.66 (s, 2H), 7.14 (dd, J = 7.97 Hz, 24.0 Hz, 4H). FAB-MS for (C$_{10}$H$_{15}$NS-H$^+$) observed 182.1003 Da, calculated 182.1005 Da.

2.12. Chromogenic substrates [19]

The chromogenic substrates, Suc-GGF-pNa and Bz-VGR-pNa, were dissolved in 20 mM CaCl$_2$ with 2 mM HCl to a final concentration of 25 mg/ml. These solutions were used directly and were stable at room temperature in the dark for several months.

2.13. Standardization of BPTI solutions [19]

All BPTI stock solutions were dissolved at a concentration of 1 mg/ml in water with 20 mM CaCl$_2$ and 2 mM HCl. These BPTI stock solutions were diluted, one part stock solution/three parts water, prior to use. Two μl of 1 mg/ml trypsin dissolved in 20 mM CaCl$_2$ and 2 mM HCl was added to 3 ml of assay buffer (0.1 M Tris-HCl, 20 mM CaCl$_2$, 0.005% Triton X-100, pH 8.3) in a disposable cuvette. To this solution was added 0, 2, 4 and 6 μl of a solution of either ((N$^{\alpha}$(CH$_2$)$_2$SH)Gly^{38})BPTI, synthetic native BPTI or purchased native BPTI and the solution was mixed. The trypsin/inhibitor solution was incubated for 12 min at 23°C prior to adding 5 μl of a 25 mg/ml solution of the chromogenic substrate benzyl-VGR-pNa. After mixing for 5 s, the rate of cleavage of the chromogenic substrate was followed by UV-vis spectroscopy with average absorbance monitoring between 380 and 410 nm for 60 s at one measurement per second. The data were graphed and fitted to a line using least squares analysis. The volume required for complete inhibition was extrapolated and the concentration was determined assuming a trypsin/inhibitor stoichiometry of 1:1. In some cases, significant curvature of the fitted data was noted and further dilutions (1:10) of the inhibitor were required to obtain a linear response.

Another method for analysis of the concentration of protein in solution was proposed by Waddell [30]. This method uses UV-vis

spectroscopy to determine the concentration of protein solution by the formula:

$$(A_{214}-A_{224})\times 144 = C$$

where C is the concentration of protein in μg/ml. The range of accuracy for this method is between 10 and 100 μg/ml. Because all proteins in the stock solutions should be active inhibitors of trypsin, the titration and Waddell method should yield similar values of the protein concentration.

2.14. Association constant determination [19]

5 μl of a 4 mg/ml solution of chymotrypsin ($E_o = 2.67\times10^{-7}$ M final concentration) was dissolved in 3 ml of assay buffer (0.1 M Tris-HCl, 20 mM $CaCl_2$, 0.005% Triton X-100, pH 8.3). To this solution was added a solution of either (($N^{\alpha}(CH_2)_2SH$)Gly^{38})BPTI, synthetic native BPTI or native BPTI purchased from Sigma in the appropriate volumes for final concentrations of inhibitor ranging from 0.534×10^{-8} to 5.34×10^{-7} M in 10 steps ($0.2E_o$–$2E_o$ in 10 steps). The chymotrypsin-inhibitor mixture was incubated at 23°C for 5 min prior to adding 10 μl of a 25 mg/ml solution of the chromogenic substrate Suc-GGF-pNA. After mixing for 5 s, the rate of cleavage of the pNa substrate was followed by UV-vis spectroscopy with average absorbance monitoring at 380–410 nm over 120 s with a cycle time of 1 s and an integration time of 0.5 s. The derivation of the association constant, K_a, is derived from the equation:

$$K_a = [EI]/[E]\times[I] = [E_o]-[E]/[E]\times([I_o]-[E_o]+[E]) \quad (1)$$

where $[E]$, $[I]$ and $[EI]$ are the equilibrium concentrations of enzyme, inhibitor and enzyme-inhibitor complex and $[E_o]$ and $[I_o]$ are the total concentrations of enzyme and inhibitor. Eq. 1 is quadratic and has a physically meaningful root:

$$[E] = [E_o]-\frac{[E_o]+[I_o]+1/K_a-(([E_o]+[I_o]+1/K_a)^2-4[E_o][I_o])^{1/2}}{2} \quad (2)$$

The solution to Eq. 1 can be calculated directly by two- or three-parameter non-linear least squares fitting procedures with the caveat that the three-parameter fitting is not valid for K_a values of less than 1×10^6. This caveat is a function of the assay itself. For a valid measurement of K_a by three-parameter fit, $K_a\times E_o$ must be between five and 30. If this value is >30, the determined K_a will depend greatly on the last few data points which are inherently noisy. If $K_a\times E_o$ is <5, the inhibition reaction may not proceed stoichiometrically and three-parameter fitting is not valid [20].

The calculation for K_a can also be done at each inhibitor concentration as the slope of the reaction has a linear relationship to the quantity of free enzyme in the reaction (and, therefore, is a direct measurement of $[E]$). The ratio of the slope for the reaction at the inhibitor concentration $[I_o]$, and the reaction without inhibitor, yields a percentage value of the remaining free enzyme in solution. Multiplying this percentage by $[E_o]$ is a direct measure of $[E]$ at equilibrium at that inhibitor concentration $[I_o]$. In combination, there are 11 independent measurements of K_a in each experiment.

2.15. 2D ^{1}H-NMR spectroscopy

Protein samples were dissolved in 500 μl of 90% Millipore filtered water, 10% D_2O (99.9% deuterium) with the pH adjusted to 4.6 with minute amounts of HCl. Actual quantities of protein in solution were 3.8 mg for (($N^{\alpha}(CH_2)_2SH$)Gly^{38})BPTI, 6.8 mg for synthetic native BPTI and 5.1 mg for Sigma BPTI as determined by trypsin titration. The spectra were recorded on a Brucker AMX 500 NMR spectrometer at 23°C using a modified Brucker 2D ^{1}H-TOCSY pulse sequence [21]. Solvent signal suppression during acquisition was accomplished using the Watergate pulse sequence [27]. Data were accumulated over 6–12 h and processed using FELIX NMR software from Molecular Simulations (San Diego, CA, USA). Sequence assignments for these spectra were obtained by direct comparison with literature COSY spectra and assignment tables [11,12,22]. The spectra for the three proteins were then compared in the following regions: (1) C^{α}-NH amide ($\omega2 = 10.5$–6.5 parts per million (ppm), $\omega1 = 5.6$–3.0 ppm), (2) Ala C_α-C_β/Thr C_γ-C_β proton ($\omega2 = 2.0$–0.5 ppm, $\omega1 = 5.8$–3.0 ppm) and (3) aromatic proton ($\omega2 = \omega1 = 8.0$–6.0 ppm).

3. Results

3.1. Experimental design

The sequence of BPTI (Fig. 3) reveals a convenient native ligation site at Gly^{37}-Cys^{38} that was utilized in a previous synthesis of this protein [4]. Use of the N^{α}-(ethanethiol)Gly^{38} auxiliary functional group for the synthesis of BPTI at this ligation site would generate a modified backbone structure (Fig. 1c). While Cys^{38} forms a disulfide bridge with Cys^{14} in the folded protein (Fig. 2), it has previously been found that variants of BPTI in which Cys^{38} was mutated to alternate amino acids, thus preventing disulfide formation, showed retention of the 3D structure and possessed a significant bio-

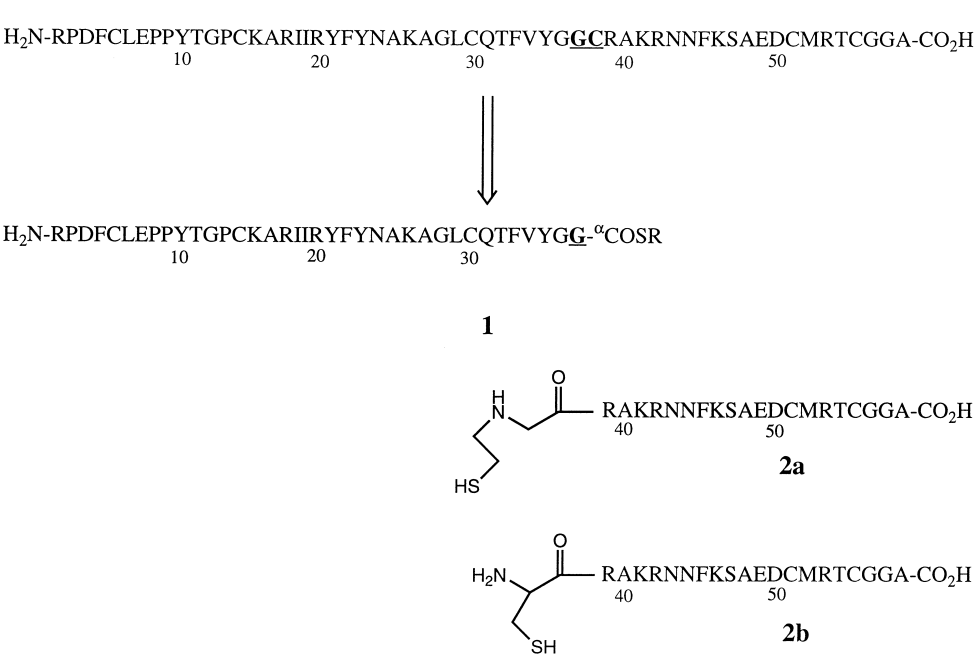


Fig. 3. Strategy used for the synthesis of the engineered protein corresponding to (($N^{\alpha}(CH_2)_2SH$)G^{38})BPTI (1+2a) and and for the synthesis of native BPTI (1+2b). Amino acids at the site of ligation are in bold and underlined.

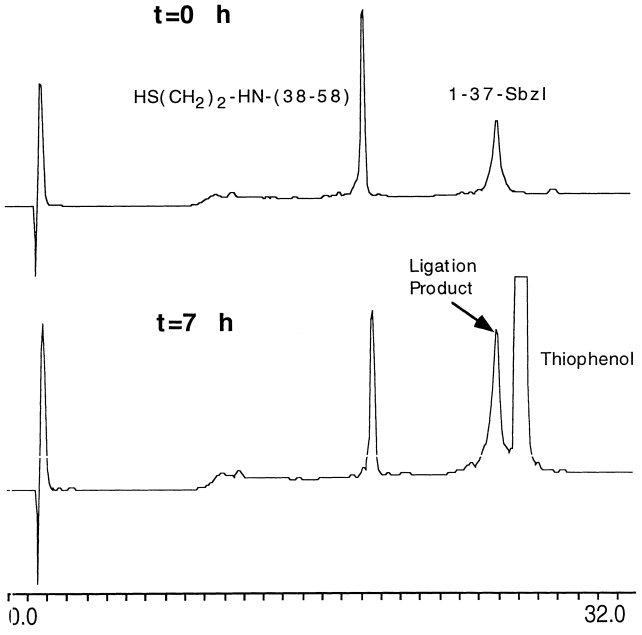


Fig. 4. Ligation of BPTI(1–37)-$COSCH_2C_6H_5$ with (($N^{\alpha}(CH_2)_2SH$)-Gly^{38})BPTI(38–58)-COOH. HPLC analyses shown are for the reaction mixture immediately prior to the addition of thiophenol and after 7 h. The phenyl thioester BPTI(1–37)-$COSC_6H_5$ co-elutes with free thiophenol under the conditions used. Ligations were performed in 100 mM phosphate buffer, 6 M GnHCl at pH 7.5 with 2–4% (v/v) added thiophenol as catalyst [6].

logical activity [17]. These data suggested to us that this protein was amenable to auxiliary functional group ligation and would tolerate the N^{α}-ethanethiol structural modification at the ligation site with a high probability of forming a functional, disulfide-crosslinked molecule.

The ligation strategy for the synthesis of both the native and disulfide-engineered BPTI proteins is outlined in Fig. 3. All peptide segments were synthesized by optimized in situ neutralization protocols for Boc chemistry stepwise solid phase peptide synthesis [18]. To generate the N^{α}-modified C-terminal peptide segment, ((N^{α}-$(CH_2)_2SH$))-Gly^{38})BPTI-(38–58)-$^{\alpha}CO_2H$ (2a), the resin-bound peptide BPTI(39–58) was further derivatized by bromoacetylation followed by displacement of the bromine using *S*-4-(methylbenzyl) protected aminoethane thiol as previously reported [7].

Electrospray ionization mass spectrometry was used to characterize the synthetic 58 residue polypeptide, before and after folding/disulfide formation. NMR spectroscopy and association constant determination (K_a) of the folded protein molecule were used to determine the effects of the N^{α}-ethanethiol backbone modification on the protein structure and function.

3.2. Ligation of BPTI fragments

The auxiliary functional group ligation of the BPTI(1–37)-$^{\alpha}COSR$ and (($N^{\alpha}(CH_2)_2SH$)-Gly^{38})BPTI(38–58)-$^{\alpha}CO_2H$ peptide segments proceeded in a rapid manner (Fig. 4) and was indistinguishable from standard native chemical ligation at cysteine both in rate and yield. The ligations were allowed to proceed at pH 7 in aqueous solution containing 6 M guanidine hydrochloride for 16–24 h to ensure complete reaction. Little or no starting N-terminal BPTI(1–37)-$^{\alpha}COSC_6H_5$ fragment was detectable after 8 h.

3.3. Folding the synthetic BPTI and (($N^{\alpha}(CH_2)_2SH$)Gly^{38})-BPTI proteins

Throughout ligation, folding and purification processes required to obtain these proteins, no discernible difference in behavior was noted. The folding efficiency was comparable and the correct number of disulfides formed was as indicated by ESMS (Fig. 5) and a negative DTNB test. While thermal stability studies have not yet been undertaken, (($N^{\alpha}(CH_2)_2SH$)Gly^{38})BPTI retained a high degree of chemical shift dispersion in a 2D ^{1}H-COSY experiment at 68°C (data not shown), which indicates retention of the folded structure at this temperature, in keeping with a high thermostability for this protein.

3.4. Standardization of BPTI solutions by trypsin titration [19]

BPTI stock solutions were made by dissolving desalted and lyophilized native protein (0.63 mg), Sigma BPTI (1.0 mg) and (($N^{\alpha}(CH_2)_2SH$)Gly^{38})BPTI (0.55 mg) in a solution of 20 mM $CaCl_2$ and 2 mM HCl to a concentration of 1 mg/ml. Native BPTI and Sigma BPTI stock solutions were diluted 10-fold with a 20 mM $CaCl_2$ and 2 mM HCl aqueous solution and titrated against trypsin to yield active protein concentrations of 0.68 (synthetic) and 0.51 mg/ml (Sigma). (($N^{\alpha}(CH_2)_2SH$)-Gly^{38})BPTI stock solution was diluted 4-fold with $CaCl_2$/HCl solution and titrated against trypsin to yield an active protein concentration of 0.38 mg/ml. These concentrations are corrected for dilution and are in excellent agreement with Waddel's method [20].

3.5. Association constant (K_a) determination [19]

The association constants of synthetic BPTI, native BPTI from Sigma and (($N^{\alpha}(CH_2)_2SH$)Gly^{38})BPTI for α-chymotrypsin were calculated by determining the quantity of free enzyme in solution in the presence of variable concentrations of inhibitor. The results are reported in Table 1. For each addition of inhibitor, the initial concentrations of inhibitor [I_o] and α-chymotrypsin [E_o] were known. K_a for each independent measurement was calculated as were the average and S.D. α-Chymotrypsin was used in these experiments to determine the relative activities of the three BPTI preparations because of the experimental difficulty of measuring the very strong BPTI-trypsin interaction.

Initial experiments with (($N^{\alpha}(CH_2)_2SH$)Gly^{38})BPTI did not meet the 50% criteria for valid K_a measurements (see Section 2), but all values obtained were consistent. Subsequent analyses with initial inhibitor concentrations of 1[E_o] to 5[E_o] resulted in only 63% inhibition at 5[E_o], but all K_a values obtained were in excellent agreement with one another and with the previous experiments, thus satisfying validity constraints. Three-parameter fitting could not be used for (($N^{\alpha}(CH_2)_2SH$)Gly^{38})BPTI because the measured K_a is less than 10^6. The analyses of the native BPTI prepared by chemical ligation and the native BPTI from Sigma demonstrated K_a values 20-fold greater than the N^{α}-(ethanethiol) disulfide-engineered BPTI variant.

3.6. 2D ^{1}H-NMR spectroscopy

2D ^{1}H-NMR TOCSY (^{1}H-TOCSY) experiments [21] were used as an indicator of the folded 3D structure and as a gauge of the similarities between the proteins. Four regions of the spectra were analyzed: backbone, Val/Leu/Ile methyls, Ala/

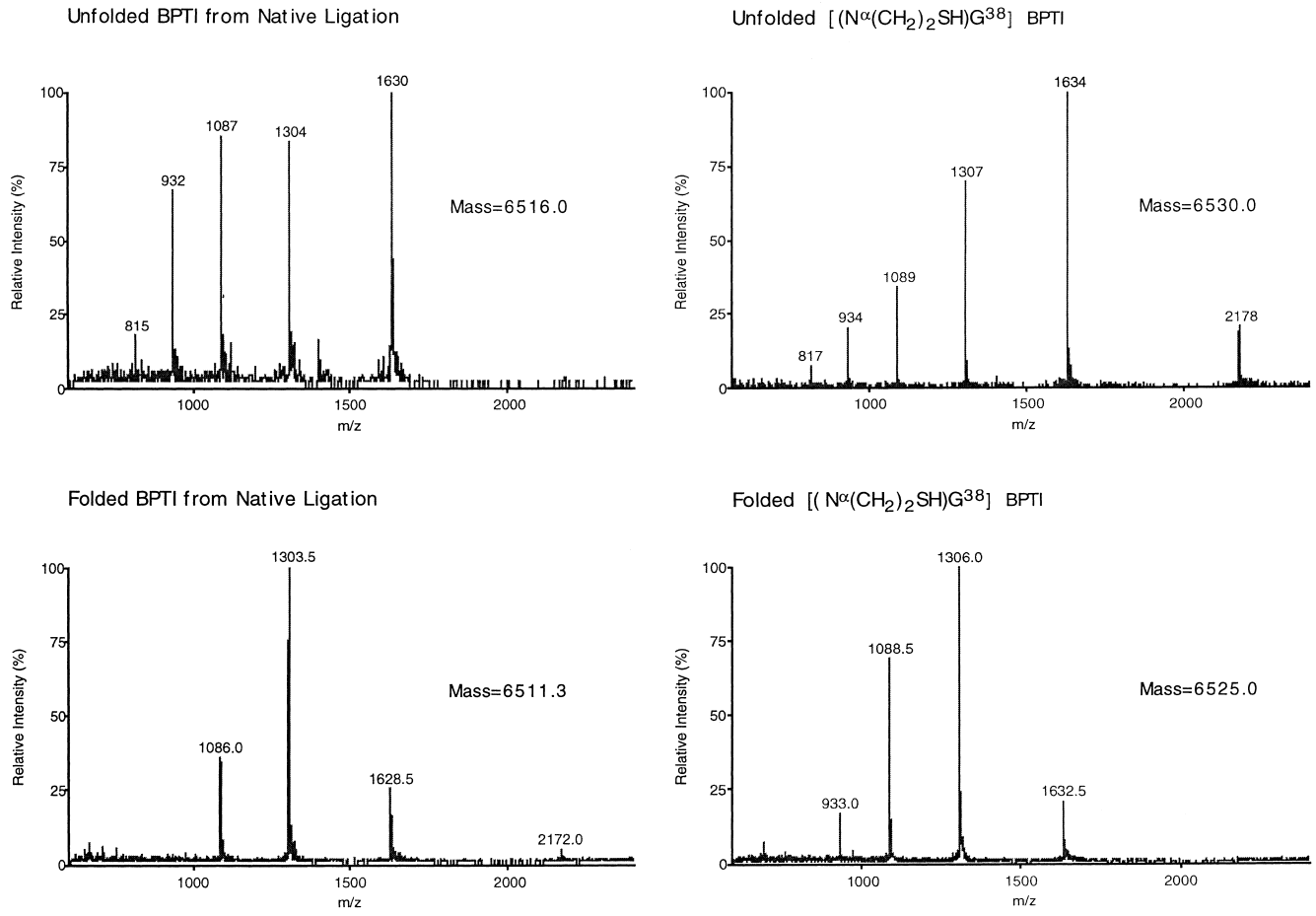


Fig. 5. Electrospray ionization mass spectrometry characterization of synthetic preparations before and after folding and disulfide formation. Note the change in charge distribution. Folded proteins have fewer sites that can acquire a proton.

Thr methyls and aromatic ring protons. Each protein analyzed exhibited a high degree of dispersion in all four TOCSY fingerprint regions, indicating a folded 3D protein structure. The backbone C^{α}-amide fingerprint region provides information about the backbone conformations. The other three fingerprint regions provide conformational information about the interior packing of the protein. After a number of sequence specific assignments were made for the spectra of native BPTI with the aid of published regions of COSY spectra [11,12] and assignment charts [22], the C_{α}-NH, Ala C_{α}-C_{β}/Thr C_{γ}-C_{β} proton and aromatic proton fingerprint regions were specifically analyzed between the spectra. These three fingerprint regions for synthetic and native BPTI were essentially identical to within the error of the experiment (± 0.05 ppm in $\omega 1$ and $\omega 2$, Fig. 6a,b).

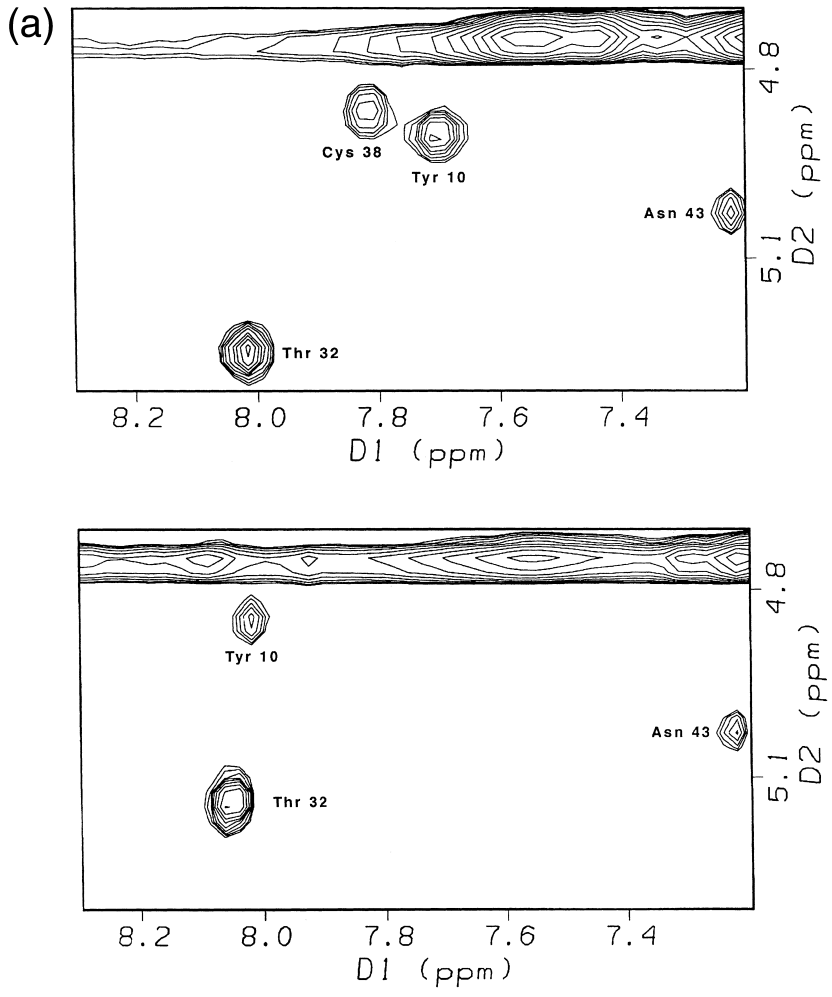
While direct comparison between the assigned native BPTI spectra and the spectra for ($(N^{\alpha}(CH_2)_2SH)Gly^{38}$)BPTI demonstrates a high degree of conservation of the core structure of the modified protein, localized deviations were evident and were concentrated in the region of the N^{α}-ethanethiol modification. The first region of difference that can be readily observed is in the vicinity of Cys^{38} in the C^{α}-amide fingerprint region (Fig. 6a). No amide signal was evident in the ($(N^{\alpha}(CH_2)_2SH)Gly^{38}$)BPTI spectrum, simply because there is no amide proton at this amino acid. The N^{α}-ethanethiol functional group has replaced the amide proton of this residue. The perturbation of another local environment can readily be documented by observing the shifted resonances for the binding loop protons (Fig. 6a,b). In the direct comparison of the aromatic, methyl, β- and γ-proton spectral regions between all three of the BPTI proteins, a high correspondence was found in the chemical shifts for the side-chain protons (data not shown). This is a strong indication of similar packing of the side-chains in the interior of the protein and corroborates the conservation of the core structure of the protein. Again, the main spectral differences were concentrated in the regions near the site of the N^{α}-ethanethiol modification.

Table 1

	Synthetic BPTI	($(N^{\alpha}(CH_2)_2SH)G^{38}$) BPTI
Values ($\times 10^7$)	1.29	0.080
	2.27	0.073
	1.41	0.089
	1.54	0.075
	1.89	0.058
Average ($\times 10^7$)	1.68 ± 0.16	0.078 ± 0.012

4. Discussion

In 1996, Canne et al. [7] reported the use of auxiliary functional groups for the ligation of model peptide fragments at sites other than Xxx-Cys. The present work extends these studies and demonstrates chemical ligation based on the use of auxiliary functional groups for the synthesis of a 'disulfide-



engineered' analogue of BPTI, ((N$^{\alpha}$(CH$_2$)$_2$SH)Gly^{38})BPTI. The auxiliary functional group used in this study, N$^{\alpha}$-ethanethiol, cannot be removed after ligation. This results in a product containing an ethanethiol-decorated amide bond at the ligation site (Fig. 1b). This peptidomimetic permutation was utilized to form a disulfide-engineered variant of BPTI with the objective of determining the synthetic feasibility and the structural and functional properties of the resulting unnatural protein.

The synthesis of native BPTI and ((N$^{\alpha}$(CH$_2$)$_2$SH)-Gly^{38})BPTI proved to be very similar. During the course of the ligation, folding and purification of both proteins, no significant difference in physical chemical behavior was observed. Folding proceeded under air oxidation with the appropriate number of disulfides formed as indicated by ESMS and a negative DTNB test. Each BPTI variant proved to be a potent inhibitor of trypsin, which further indicated a functional folded structure.

Only upon more detailed analysis was a functional difference between the native BPTI synthesized by chemical ligation at Cys^{38} and the ((N$^{\alpha}$(CH$_2$)$_2$SH)Gly^{38})BPTI detected. Determination of the association constant (K_a) with α-chymotrypsin for native and disulfide-engineered BPTI variants demonstrated a 20-fold weaker binding for the engineered protein. An alteration of binding affinity of this magnitude could not easily be detected for the interaction of this protein with trypsin because of the extraordinary strength of the trypsin-BPTI interaction [19,23]. These binding data suggested that a structural difference existed in the binding loop region of ((N$^{\alpha}$(CH$_2$)$_2$SH)Gly^{38})BPTI compared to native BPTI.

A direct corroboration of the structural perturbation in the binding loop region of ((N$^{\alpha}$(CH$_2$)$_2$SH)Gly^{38})BPTI compared to synthetic native BPTI was obtained by comparison of the fingerprint regions of the 2D ^{1}H-TOCSY NMR spectra. These spectra clearly demonstrated a nearly identical 3D structure for the core regions of these two proteins as evidenced by the nearly identical chemical shifts for core amide protons (Fig. 6a,b). The regions of dissimilar chemical shifts were confined to the residues near the N$^{\alpha}$-ethanethiol moiety in ((N$^{\alpha}$(CH$_2$)$_2$SH)Gly^{38})BPTI and, not surprisingly, in the binding loop (residues 8–13) which is attached to the N$^{\alpha}$-(ethanethiol)Gly^{38} by a disulfide bond to Cys^{14}. Chemical shifts for the amide protons of the loop residues 10–13 deviate significantly from those in the native protein (Fig. 6b). The significance of these differences can be readily seen when mapped onto the known 3D structure of native BPTI (Fig. 7).

Both K_a and 2D ^{1}H-TOCSY NMR data demonstrate that significant differences exist between the native and disulfide-engineered BPTI protein within the residues of the binding loop. The N$^{\alpha}$-ethanethiol modification is not itself contained in the binding loop, but in the adjacent scaffold loop connected to the binding loop by a disulfide bridge through Cys^{14} (Fig. 1c). It is intriguing that the local conformation

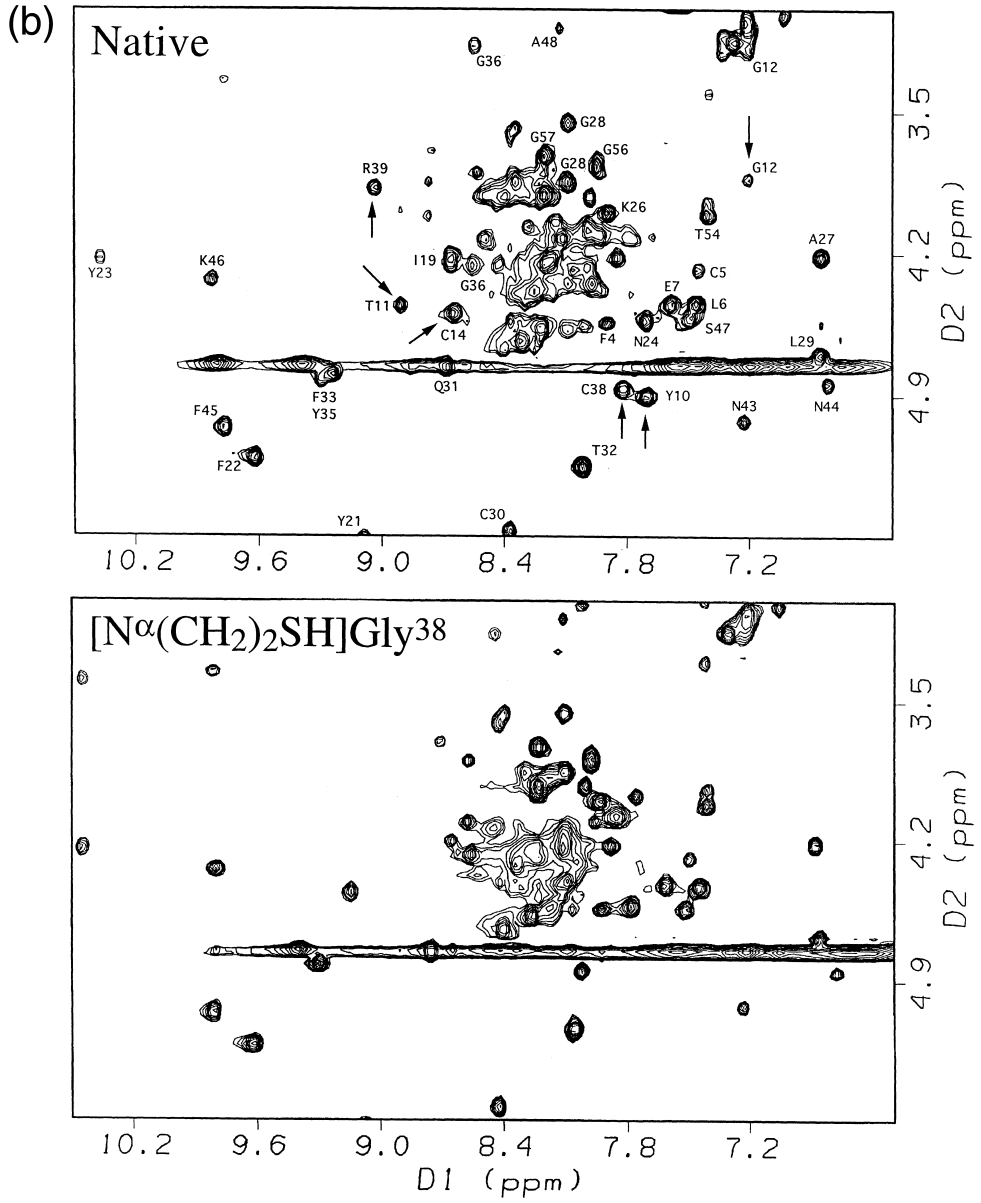


Fig. 6. (a) Enlarged area of ^{1}H-2D-TOCSY NMR spectra (taken in 90% H_2O, 10% D_2O, pH adjusted to 4.6 with trace HCl) for reference BPTI (top) and (($N^{\alpha}(CH_2)_2SH$)Gly^{38})BPTI (bottom). The amide-C$^{\alpha}$ crosspeak for Cys^{38} is not evident for the disulfide-engineered variant because the amide proton for this residue has been replaced with the N$^{\alpha}$-ethanethiol auxiliary functional group. The marked difference in chemical shift for the Tyr^{10} amide-C$^{\alpha}$ crosspeak in (($N^{\alpha}(CH_2)_2SH$)Gly^{38})BPTI compared to the native protein and the essentially unchanged positions for Thr^{32} ant Asn^{43} amide-C$^{\alpha}$ crosspeaks are consistent with only a localized difference in the structures of these two proteins. (b) Amide-C$^{\alpha}$ region of ^{1}H-NMR 2D-TOCSY spectra (taken in 90% H_2O, 10% D_2O, pH adjusted to 4.6 with trace HCl) for reference BPTI (top) and (($N^{\alpha}(CH_2)_2SH$)Gly^{38})BPTI (bottom). Assignments are given for the reference protein. The chemical shifts for both proteins show a high degree of identity, indicating a highly similar global structure. Differences in chemical shifts are localized to residues near the site of the N$^{\alpha}$-(ethanethiol)glycine^{38} in (($N^{\alpha}(CH_2)_2SH$)Gly^{38})BPTI (denoted by arrows).

of the binding residues has been perturbed as well as the conformation of the residues nearest in sequence to the N$^{\alpha}$-ethanethiol backbone modification. One explanation for these observations is that the binding loop is subject to less conformational constraints by adjacent regions of the protein molecule. This interpretation is consistent with the X-ray crystal structure of BPTI, which indicated higher B-factors in the binding loop (residues 8–13) region of the protein structure [10]. We can confidently say that the structural differences between the native and disulfide-engineered proteins are confined to the scaffold and binding loop regions. However, at this point, we cannot say anything further about the structure of the (($N^{\alpha}(CH_2)_2SH$)Gly^{38})BPTI molecule itself based on these data.

Clearly, the synthetic approach used to make the disulfide-engineered BPTI is a fruitful one. The ligation reaction reported here using the N$^{\alpha}$-ethanethiol auxiliary functional group has proven to be comparable to native chemical ligation at Cys^1, both in rate and yield as found in previous model studies [7]. The protein generated by this chemistry contained a N$^{\alpha}$-(ethanethiol)Gly^{38} replacing the native Cys^{38} at the ligation site. This N-substituted residue did not interfere with protein folding or disulfide formation and resulted in a functional 'disulfide-engineered' protein with a good biochem-

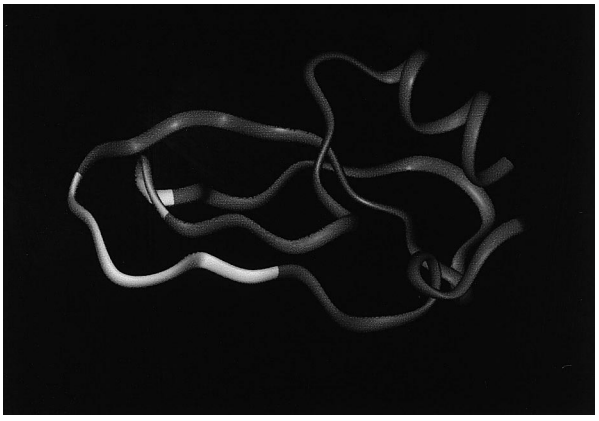


Fig. 7. Regions of the BPTI structure with identical (dark) or significantly different chemical shifts (light) between the residues in the native and $((N^{\alpha}(CH_2)_2SH)Gly^{38})$BPTI proteins. Gly^{37} was not assigned in the NMR data. The orientation of this figure is identical to that of Fig. 2.

ical activity and internal core structure similar to the native BPTI.

The feasibility of auxiliary functional group ligation for the synthesis of active backbone-engineered proteins has been demonstrated by these results. N^{α}-backbone modifications have previously proven their worth in the world of peptides for enhancing solubility of polypeptides both on resin and in solution [24,25] and as 'peptoids' (oligo(*N*-substituted glycines)) [15,16], for inhibiting proteolytic degradation [15], dimerization [16] and for enhancing peptide ligand affinities [26]. The work reported here extends 'peptoid' chemistry to the realm of backbone functionalized proteins. The introduction of novel chemically reactive moieties into defined sites in the primary sequence, independently of side-chain considerations, can be exploited for a wide range of other purposes including site specific labelling, development of new chemistry on a protein scaffold or immobilizing an active protein onto a solid support in a controlled fashion.

Acknowledgements: This work was supported by funds from the NIH (Grants PO1HL3195 and RO1GM43397, S.B.H.K.). We gratefully acknowledge Dr Wuyan Lu for assistance in the assays and purifications reported here. We also acknowledge the assistance of Dr John Chung, The Scripps Research Institute, for 2D ^{1}H-TOCSY NMR experiments, Dr Walter Chazin, The Scripps Research Institute, for providing NMR shift values and for aid in data interpretation and Dr Edelmira Cabezas and Dr Arnold Satterthwait, The Scripps Research Institute, for providing training and use of a Silicon Graphics workstation for NMR data processing.

References

[1] Dawson, P.E., Muir, T.W., Clark-Lewis, I. and Kent, S.B.H. (1994) Science 266, 776–779.
[2] Wilken, J. and Kent, S.B.H. (1998) Curr. Opin. Biotechnol. 9, 412–426.
[3] Ueda, H., Siani, M.A., Gong, W., Thompson, D.A., Brown, G.G. and Wang, J.M. (1997) J. Biol. Chem. 272, 24966–24970.
[4] Lu, W., Starovasnik, M. and Kent, S.B.H. (1998) FEBS Lett. 429, 31–35.
[5] Lu, W., Qasim, M.A., Laskowski Jr., M. and Kent, S.B.H. (1997) Biochemistry 36, 673–679.
[6] Dawson, P.E., Churchill, M., Ghadiri, M.R. and Kent, S.B.H. (1997) J. Am. Chem. Soc. 119, 4325.
[7] Canne, L.E., Bark, S.J. and Kent, S.B.H. (1996) J. Am. Chem. Soc. 118, 5891–5896.
[8] Kunitz, M. and Northrop, J.H. (1936) J. Gen. Physiol. 19, 991.
[9] Anderer, F.A. and Hornle, S. (1966) J. Biol. Chem. 241, 1568.
[10] Wlodawer, A., Walter, J., Huber, R. and Sjolin, L. (1984) J. Mol. Biol. 180, 301–329.
[11] Wagner, G. and Wuthrich, K. (1982) J. Mol. Biol. 155, 347–366.
[12] Stassinopoulou, C., Wagner, G. and Wuthrich, K. (1984) Eur. J. Biochem. 145, 423–430.
[13] Weissman, J.S. and Kim, P.S. (1995) Nat. Struct. Biol. 2, 1123.
[14] Creighton, T.E., Darby, N.J. and Kemmink, J. (1996) FASEB 10, 110–118.
[15] Zuckermann, R.N., Kerr, J.M., Kent, S.B.H. and Moos, W.H. (1992) J. Am. Chem. Soc. 114, 10646–10647.
[16] Rajarathnam, K., Sykes, B.D., Kay, C.M., Dewald, B., Geiser, T., Baggiolini, M. and Clark-Lewis, I. (1994) Science 264, 90–92.
[17] Staley, J.P. and Kim, P.S. (1992) Proc. Natl. Acad. Sci. USA 89, 1519–1523.
[18] Schnolzer, M., Alewood, P., Jones, A., Alewood, D. and Kent, S.B.H. (1992) Int. J. Pept. Protein Res. 40, 180–193.
[19] Wuyuan Lu, Thesis, Laskowski, M. Jr., Advisor, pp. 66–87, Purdue University.
[20] Chauvet, J. and Acher, R. (1972) FEBS Lett. 23, 317.
[21] Braunschweiler, L., Bodenhausen, G. and Ernst, R.R. (1983) J. Mol. Phys. 48, 535.
[22] Assignment table courtesy of Dr Walter Chazin, The Scripps Research Institute.
[23] Zollner (1993) Helmward Handbook of Enzyme Inhibitors, Part B, p. 572, VCH Publishers.
[24] Johnson, T., Quibell, M., Owen, D. and Sheppard, R.C. (1993) J. Chem. Soc. Chem. Commun., pp. 369–372.
[25] Quibell, M., Packman, L.C. and Johnson, T. (1995) J. Am. Chem. Soc. 117, 11656–11668.
[26] Nguyen, J.T., Turck, C.W., Cohen, F.E., Zuckermann, R.N. and Lim, W.A. (1998) Science 282, 2088–2092.
[27] Piotto, M., Saudek, V. and Skelnar, V. (1992) J. Biomol. NMR 2, 661–665.
[28] Canne, L.E., Walker, S.M. and Kent, S.B.H. (1995) Tetrahedron Lett. 36, 1217–1220.
[29] Robey, F.A. and Fields, R.L. (1989) Anal. Biochem. 177, 373–377.
[30] Waddel, W.J. (1956) J. Lab. Clin. Med. 48, 311–314.