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Stabilization of a C_7 Equatorial Gamma Turn in DMSO-d_6 by a Ditryptophan Crosslink

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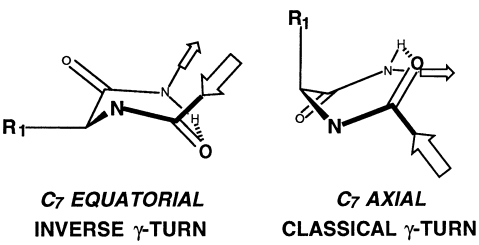
Abstract—Covalent crosslinks can control local peptide conformation. In tripeptide sequences of the general formula Cys-Xxx-Cys, cysteine disulfides have been previously shown to enforce a C_7 equatorial γ-turn conformation (also referred to as an inverse γ-turn). Much less is known about the effects of dityrosine and ditryptophan crosslinks on local peptide structure. In a series of tripeptides, ditryptophan crosslinks were formed using the two-step process of acid-promoted Mannich dimerization followed by oxidative aromatization. In these peptides, with the general formula Trp-Xxx-Trp (Xxx≠Gly), ditryptophan crosslinks were found to stabilize a C_7 equatorial γ-turn conformation in DMSO-d_6. Rigorous support for a C_7 equatorial conformation in the crosslinked sequence Trp-Pro-Trp came from a variety of ^{1}H NMR experiments and molecular modelling. Interproton distances were derived from NOE buildups that were determined through a series of double pulsed field gradient spin echo (DPFGSE) experiments. In addition, the small temperature dependence of the $i+2$ NH chemical shifts ($\Delta\delta/\Delta T < 2$ ppm/°C) provided further support for the intramolecular hydrogen bond which defines a γ-turn.

Introduction

In order for a protein to adopt a globular tertiary structure the polypeptide chain must change directions. These directional changes are often abrupt and serve to link different elements of secondary structure. Two conformational motifs for these directional changes are the three-residue γ-turn and the four-residue β-turn.[1,2] The importance of these turn structures is that they are often the focus of protein–protein recognition events because they are frequently found on the surface of proteins and unlike α-helices or β-sheets they do not satisfy the intrinsic hydrogen bonding capacity of the peptide chain.

There are two types of γ-turns: classical γ-turns and inverse γ-turns. Both types of γ-turns have a hydrogen bond between the CO of the i residue and the NH of the $i+2$ residue, leading to a 7-membered ring. The distinction between classical and inverse γ-turns is conformational. The classical γ-turn (C_7 axial) is characterized by a ϕ_{i+1} angle of 70° to 85° and a ψ_{i+1} angle of −60° to −70°, causing the α_{i+1} substituent to adopt an axial orientation with respect to the seven-membered ring. The inverse γ-turn (C_7 equatorial) is characterized by a ϕ_{i+1} angle of −70° to −85° and a ψ_{i+1} angle of 60° to 70° leading to an equatorial orientation for the α_{i+1} substituent. Ironically, classical γ-turns are extreme rarities in proteins whereas inverse γ-turns are common[3] so the C_7 terminology is preferred.



C_7 EQUATORIAL INVERSE γ-TURN **C_7 AXIAL CLASSICAL γ-TURN**

γ-Turns compete with β-turns for control of local peptide conformation. In nonpolar solvents, the seven-membered ring γ-turn is favored entropically over the 10-membered ring β-turn, but enthalpic effects and entropic effects oppose each other.[4] At room temperature the

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result of these combined effects is that γ-turns are disfavored relative to β-turns in nonpolar solvents whereas in polar solvents such as DMSO and water both of these conformations yield to solvent–peptide interactions.[5–7] A variety of synthetic constructs have been designed to stabilize or mimic γ-turn conformations,[8–17] but disulfide crosslinks in the sequence Cys-Xxx-Cys can also stabilize a C_7 equatorial γ-turn.[18,19] Unfortunately, this 11-membered ring disulfide suffers from sufficient angle strain to make it kinetically[20] and thermodynamically[21] unstable with respect to intermolecular disulfide exchange. Of the disulfide-containing peptides of the general sequence Cys-$(Gly)_n$-Cys ($n=0$–4) the tripeptide Cys-Gly-Cys is the least stable.[21] Surprisingly, only two of the 581 occurrences of the sequence Cys-Xxx-Cys in the Protein Databank are bridged with a disulfide and these two triads are not reverse turns![22,23]

There are two other homodimeric crosslinks which are available from the 20 common amino acids: dityrosines and ditryptophans. There have been no studies of the effects of intermolecular dityrosine crosslinks on peptide conformation, but molecular mechanics calculations (AMBER*) show that a γ-turn conformation is inaccessible in the sequence Tyr-Xxx-Tyr with a dityrosine crosslink. In contrast to dityrosines, we report here that ditryptophans can stabilize a C_7 equatorial γ-turn in the sequence Trp-Xxx-Trp, and the crosslink is kinetically and thermodynamically stable. Since the α positions of the i and i+2 positions are already substituted, ditryptophans are not likely to be very useful for sidechain display, rather, the importance of the ditryptophan in the sequence Trp-Xxx-Trp is that it reorients the direction of the peptide chain.

Synthesis of Cyclic Ditryptophans

Our studies were initiated by the previous observation that Ac-Trp-Pro-Trp-OMe displays two sets of ^{1}H NMR signals in DMSO-d_6 (due to cis and trans proline amides) but only one set of signals after tryptophan crosslinking.[24] In contrast, the tripeptide Ac-Trp-Gly-Trp-OMe displays one set of signals in DMSO-d_6, but three sets of signals after tryptophan crosslinking. It seemed likely that in both cases the effect of the ditryptophan was to restrict the conformations of the tripeptides. Our interest in the conformation of the ditryptophan derived from Trp-Pro-Trp was heightened by the biological relevance of this sequence. In the antibiotic peptide indolicidin, this tripeptide sequence occurs twice in succession: H_2N-Ile-Leu-Pro-Trp-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-$CONH_2$.[25]

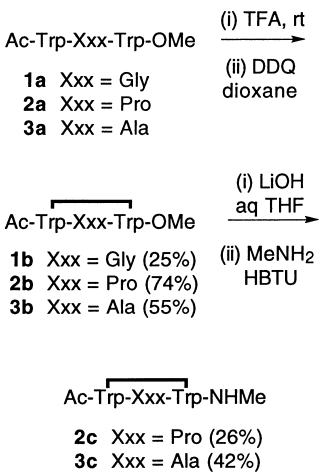
With respect to peptide conformation, proline and glycine are extremes—proline, for conformational restriction and glycine for conformational freedom. In order to establish the effects of the ditryptophan crosslink in more general terms, other ditryptophans were prepared which contained alanine and C-terminal amides rather than esters.

The peptide substrates were synthesized using solution phase BOC chemistry and DCC/HOBt couplings.[26] The *N*α-BOC deprotections were carried out with methanolic HCl to inhibit tryptophan dimerization. The carboxy termini were protected as methyl esters for ease of handling and the amino termini were acetylated to facilitate Mannich coupling of the tryptophan indole rings; tryptamine can not be dimerized unless the β-amino groups are acylated.[27] Mannich crosslinking of the indole sidechains was effected by stirring in neat trifluoroacetic acid (0.02 M) for 16 h (Scheme 1). After removal of the solvent and free basing with saturated sodium bicarbonate the mixture of tryptophan dimers was convergently oxidized to symmetrical ditryptophans with 1.2 equiv of DDQ in dioxane. The cyclic peptides were then purified by silica gel chromatography (methanol/chloroform). The *N*-methyl amides **2c** and **3c** were prepared from the corresponding esters by saponification and coupling with methylamine and HBTU (Scheme 1). The additional amide present in these substrates makes a type I β-turn topologically accessible where the ditryptophan would bridge the *i* and *i*+3 positions.

NMR Spectroscopy

Assignment of protons

Of the techniques used for determination of solution peptide conformations ^{1}H NMR is the most valuable because it allows quantitative estimates of interproton distances. The initial step in this process, the unambiguous



Scheme 1.

assignment of proton resonances, was accomplished by first assigning the peptide α and β protons using COSY. The spin system of Trp1 was distinguished from that of Trp3 by a strong NOE from the acetamido methyl group to the amide NH of Trp1 (Fig. 1(a)).

Assignment of the protons within the two independent aromatic spin systems was made difficult by the pseudo-symmetry of the ditryptophan crosslink. This pseudo-symmetry resulted in considerable overlap between aromatic protons preventing direct assignment using the COSY spectrum. The strong ^{1}H–^{1}H NOE from Hα of Trp1 to the isolated aromatic doublet at 7.75 ppm allowed this signal to be assigned as proton A (Fig. 1(a)). Assignment of the remaining protons in the Trp1 aromatic spin system was then made possible by following the excitation transfer in a series of 1D TOCSY experiments (Fig. 1(b)). These selective 1D experiments were considerably improved by using the excitation sculpting method[28,29] to select the target multiples, so that the TOCSY peaks were observed against essentially zero background.[30] Excitation of proton A spreads only to B, C, and D of Trp1, but not to protons a, b, c, and d of Trp3. The identity of protons a, b, c, and d were subsequently determined in a similar fashion. The downfield indole NH's (> 11 ppm) were distinguished by NOE's. The indole NH of Trp1 showed a strong NOE to proton D, allowing the indole NH of Trp3 to be assigned by default.

Temperature dependence of NH shifts

The temperature dependence of the amide NH resonances is often used to help distinguish between intra- and intermolecular hydrogen bonding.[1,2] In a strong hydrogen bond acceptor solvent such as DMSO-d_6, NH's which participate in intramolecular hydrogen bonds often display little variation in chemical shift with temperature, with $\Delta\delta/\Delta T$ typically less than 2 ppm/°C. In contrast, the chemical shift of NH's which participate in intermolecular hydrogen bonds with solvent show larger temperature variations, with $\Delta\delta/\Delta T$ typically greater than 4 ppm/°C. As shown in Table 1 $\Delta\delta/\Delta T$ for the amide NH's of cyclic ditryptophans **2b**, **3b**, **2c**, and **3c** fall neatly into these two general categories. The NH_{i+2} chemical shifts are relatively invariant with temperature, suggesting that they are involved in an intramolecular hydrogen bond typical of a γ-turn. The amide resonances of acyclic tripeptides **3a** and Ac-Trp-Ala-Trp-NHMe all display $\Delta\delta/\Delta T$ values over 4 ppm/°C suggesting little or no intramolecular hydrogen bonding. Since tripeptide **1b** shows several sets of signals at room temperature, the chemical shifts of the amide NH's were not assigned.

Determination of ^{1}H–^{1}H distances

For ditryptophan **2b**, intramolecular distances were obtained from NOE build-ups using a series of experiments based on the double pulsed field gradient spin echo (DPFGSE) NOE experiment.[29,31] There are two key features of the DPFGSE sequence which make it ideal for use in selective one-dimensional NOE experiments. Firstly, magnetization from all spins with offsets outside the excitation bandwidth is eliminated by the gradients. Thus, subtraction artifacts are less problematic allowing accurate measurement of the NOEs at short mixing time. Secondly, the phase of the resulting

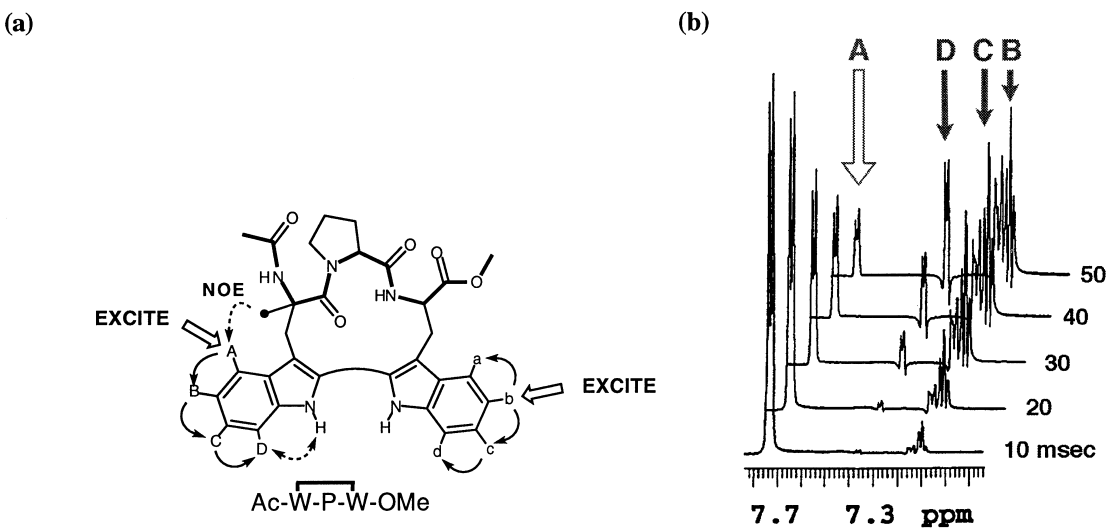


Figure 1. (a) General scheme for assignment of ^{1}H resonances of ditryptophan **2b** in DMSO-d_6; (b) time evolution of excitation transfer via a series of ^{1}D TOCSY experiments.

Table 1. Temperature dependence of amide NH shifts in DMSO-d_6-$\Delta\delta/\Delta T$ (ppm/°C)[a]

Sequence	i	$i+1$	$i+2$	$i+3$
Ac-Trp-Ala-Trp-OMe **3a**	−5.6	−6.7	−4.9	
Ac-Trp-Ala-Trp-NHMe	−6.4	−5.4	−4.4	−5.7
Ac-Trp-Pro-Trp-OMe **2b**	−5.9	—	−0.3	—
Ac-Trp-Pro-Trp-NHMe **2c**	−5.7	—	−1.8	−6.0
Ac-Trp-Ala-Trp-OMe **3b**	−5.8	−9.0	−0.2	—
Ac-Trp-Ala-Trp-NHMe **3c**	−6.0	−9.7	−0.4	−6.9
Ac-Trp-Gly-Trp-OMe[b] **1b**	*	*	*	*

[a]Measurements were made between 20 and 80 °C (500 MHz); substrate concentrations were between 0.01 and 0.1 mM.
[b]Three sets of signals are apparent in the ^{1}H NMR spectrum; these coalasce at high temperature.

magnetization does not vary with offset, an important feature when the target spin appears in the spectrum as a multiple. In such cases it is important to avoid exciting so-called 'anti-phase terms' as these can result in unwanted anti-phase multiplets (often called selective population transfer, SPT, effects) in the final NOE spectrum.[29] These features were used to make careful measurements of the various build-ups, from which the distances may be inferred under suitable assumptions.[29]

The buildup rates obtained from these experiments were compared with the buildup rates for the geminal β-protons of Trp1, for which a typical geminal proton distance of 1.73 Å was used. With this yardstick, distances were estimated for a number of vicinal and non-contiguous protons (Table 2). Prominent NOE's observed for **2b** were also seen in the NOESY spectra of **2c**, **3b**, and **3c**. From the combined results of temperature dependence studies and modeling, we believe that these compounds adopt similar conformations in DMSO-d_6.

Table 2. Distances obtained for **2b** (DMSO-d_6) from DPFGSE experiments

NOE	From:	To:	Distance (Å)
1	Trp1-NH(indole)	Trp3-CH$^\beta$	2.44 (±0.40)
2	Trp1-HA	Trp1-Hα	2.50 (±0.40)
3	Trp3-NαH	Trpl-O	2.00 (±0.20)
4	Trp3-NH(indole)	Trp1-CH$_\beta$	2.42 (±0.40)
5	Trp1-Ha	Pro2-H$_\delta$ (pro-S)	2.34 (±0.40)
6	Trp1-H$_A$	Trp1-CH$_\beta$	2.84 (±0.40)

Molecular Modeling

Plausible structures for parent ditryptophan **2b** were identified from an unconstrained Monte Carlo search using the AMBER* force field (H_2O solvent).[32,33] 10,000 Monte Carlo/TNCG cycles produced 40 unique conformations with good convergence. Of these naive conformations the global minimum (which possessed an *R*-biaryl configuration) was not consistent with the NOE-derived distances or the $J_{H\alpha\beta}$ coupling constants. Three of the 40 conformations were found to be consistent with the distance constraints shown in Table 2. These similar conformations were within 1.5 kcal/mol of the global minimum. As shown in Fig. 2, all three conformations possessed an *S*-biaryl configuration but differed in the pucker of the proline ring and the orientation of the carboxymethyl group. The ditryptophan crosslink clearly enforced a C7 equatorial γ-turn in the sequence Trp-Pro-Trp. The $J_{H\alpha\beta}$ coupling constants provided further support for the γ-turn conformation shown in Fig. 2. The $J_{H\alpha\beta}$ coupling constants of Trp1 were 3.4 Hz and 12.2 Hz for the pro-*R* and pro-*S* H_β protons, respectively. These values matched the modeled torsion angles of 50° and 170°, respectively. With respect to backbone conformation, the 7.7 Hz $J_{NH\text{-}H\alpha}$ coupling constant of Trp1 was consistent with the −160° ψ angle found by modeling. Likewise, the 9.2 Hz $J_{NH\text{-}H\alpha}$ coupling constant for Trp3 also corresponded to the −145° ψ angle in the γ-turn ensemble.

Unconstrained Monte Carlo searches for the other cyclic ditryptophans which showed evidence for an internal hydrogen bond with NH_1 (**2b**, **3b**, and **3c**) also predicted a C_7 equatorial γ-turn with an *S*-biaryl configuration to be the global minimum. Thus, in the competition for β- and γ-turn formation, the ditryptophan tips the balance in favor of the C_7 conformation. β-Branched amino acids have not been examined at the i+2 position but modeling shows sufficient room for β-branching without

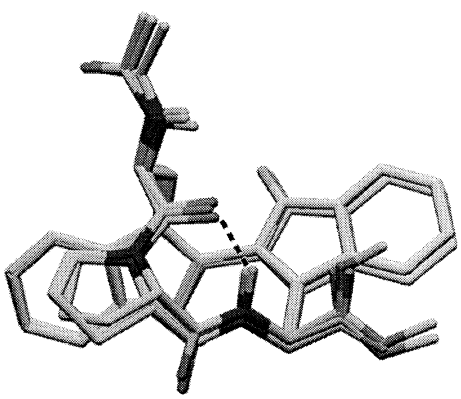


Figure 2. Ensemble of 3 lowest energy structures obtained from constrained Monte Carlo search and NOE distances.

interaction with the biindolyl chromophore. It seems likely that for peptides of the general sequence Trp-Xxx-Trp, where Xxx is not glycine, that the ditryptophan crosslink can serve as an unambiguous structural control element.

Conclusion

In the studies reported here we have examined the effects of ditryptophan crosslinks in tripeptide sequences using a variety of spectroscopic and modeling tools. These studies suggest that a ditryptophan crosslink enforces a C_7 equatorial γ-turn in the sequences Trp-Pro-Trp and Trp-Ala-Trp even in DMSO-d_6 which is a strong hydrogen bond acceptor. As with tyrosine, the crosslinking of tryptophan sidechains may eventually prove to be important in the detrimental aging of proteins or even in the biological activity of small peptides. Still, the effects of dityrosine or ditryptophan crosslinks on local peptide conformation remain largely unexplored and merit further attention.

Experimental

General methods

All reactions were run under an atmosphere of dry nitrogen unless otherwise indicated. Anhydrous solvents were transferred by oven-dried syringe or cannula. Flasks were flame dried under a stream of nitrogen. Dichloromethane, pyridine, and triethylamine were distilled from calcium hydride. Tetrahydrofuran (THF) was distilled from sodium benzophenenone ketyl. Dimethylformamide (DMF) and 1,4-dioxane were dried over activated 4 Å sieves. Analytical thin-layer chromatography (TLC) was performed using 0.25 mm commercial silica gel plates (EM science, silica gel 60 F_{254}). Solvents for chromatography are listed as volume/volume ratios. Melting points were determined in open capillaries and are uncorrected. *N*-L-Alanyl-L-tryptophan methy ester[34] was prepared from tBOC-Ala and L-tryptophan methyl ester by HOBt/DCC coupling.[26] Samples were judged to be >99% pure by HPLC analysis (C18 stationary phase, 20–100% H_2O/acetonitrile gradient over 30 min).

Ac-Trp-Ala-Trp-OMe (3a). To a solution of *N*-L-alanyl-L-tryptophan methy ester (2.51 g, 7.72 mmol) in dichloromethane (50 mL) was added triethylamine (0.86 g, 8.49 mmol). The solution was transferred via cannula to a flask containing ^{t}BOC-tryptophan (2.35 g, 7.72 mmol), DCC (1.75 g, 8.49 mmol), and HOBt (0.21 g, 1.54 mmol). The mixture was stirred for 12 h at room temperature. The mixture was diluted with ethyl acetate (250 mL), filtered to remove the dicyclohexylurea, and concentrated in vacuo. Ethyl acetate (250 mL) was added to the residue which was then washed with H_2O (100 mL), 1 N NaOH (100 mL), 1 N HCl (100 mL), and brine (100 mL) sequentially. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. Chromatography on silica gel (2.5% MeOH/$CHCl_3$) afforded ^{t}BOC-Trp-Ala-Trp-OMe as a white foam (4.43 g, 100%).

To a solution of *t*BOC-Trp-Ala-Trp-OMe (2.51 g, 7.71 mmol) in dry methanol (100 mL) and was added acetyl chloride (1.81 g, 23.1 mmol) dropwise. The reaction was stirred at room temperature for 12 h and concentrated in vacuo to afford the deprotected dipeptide as the hydrochloride salt (2.23 g, 100%).

The salt (2.08 g, 4.37 mmol) was then dissolved in pyridine (50 mL) and acetic anhydride (0.89 g, 8.74 mmol) was added. The reaction was stirred at room temperature for 4 h followed by concentration in vacuo. The residue was dissolved in ethyl acetate (200 mL) and washed with 1 N HCl (4×100 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. Chromatography on silica gel (2.5% MeOH/$CHCl_3$) afforded **3a** (2.07 g, 99%).

Ac-Trp-Ala-Trp-OMe (3a): mp 112–114 °C ($CHCl_3$). R_f=0.26 (10% MeOH/$CHCl_3$); IR (KBr) 3293, 1740, 1647, 1521, 1457, 1341, 1096, 743 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.87 (s, 1H), 10.77 (s, 1H), 8.23 (d, J=7.2 Hz, 1H), 8.11 (d, J=7.4 Hz, 1H), 8.01 (d, J=8.1 Hz, 1H), 7.61 (d, J=7.9 Hz, 1H), 7.48 (d, J=7.8 Hz, 1H), 7.34 (d, J=8.1 Hz, 1H), 7.31 (d, J=8.1 Hz, 1H), 7.19 (d, J=2.3 Hz, 1H), 7.13 (d, J=2.2 Hz, 1H), 7.09–6.93 (m 4H), 4.53 (m, 2H), 4.36 (pentet, J=7.1 Hz, M), 3.54 (s, 3H), 3.13 (m, 3H), 2.88 (dd, J=14.8, 9.5 Hz 1H), 1.75 (s, 3H), 1.21 (d, J=7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 172.3, 172.2, 171.5, 169.2,136.1, 136.0,127.1, 123.8, 123.6, 120.8, 118.4, 118.1, 118.0, 111.4, 111.2, 109.2, 53.3, 53.2, 51.8, 48.0, 27.7, 27.0, 22.6, 18.2 all of the aromatic signals could not be resolved within the limitations of the instrument; LRMS (+FAB): 518 (27), 290 (15), 257 (14), 219 (17), 201 (26), 159 (31), 130 (100); HRMS (+FAB): calcd for $C_{28}H_{31}N_5)_5$, 517.2325; found 518.2404 $[MH]^+$.

Ac-$\overline{\text{Trp-Ala-Trp}}$-OMe (3b). Compound **3a** (0.182 g, 0.352 mmol) was dissolved in trifluoroacetic acid (16 mL) and stirred at room temperature 16 h. The reaction mixture was concentrated in vacuo. The crude product was dissolved in dioxane (10 mL) followed by addition of DDQ (0.0.097 g, 0.410 mmol). The mixture was stirred for 5 h at room temperature after which the solvent was evaporated under reduced pressure. The brown solid was dissolved in ethyl acetate and washed repeatedly with 1 N NaOH until the aqueous layer

remained colorless. The organic layer was dried over $MgSO_4$ and concentrated in vacuo to afford the crude product. Chromatography on silica gel (2.5% MeOH/$CHCl_3$) followed by HPLC purification (C18 stationary phase, 20–100% acetonitrile/water gradient over 30 min) afforded **3b** (0.069 g, 38%).

Ac-Trp-Ala-Trp-OMe (3b): Mp 245 °C (dec, EtOAc). R_f=0.38 (10% Me0H/$CHCl_3$); IR (KBr) 3361, 3273, 1734, 1651, 1518, 1440, 749 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 11.26 (s, 1H), 11.16 (s, 1H), 8.34 (d, J=7.7 Hz, 1H), 8.17 (d, J=6.8 Hz, 1H), 7.91 (d, J=7.7 Hz, 1H), 7.39 (d, J=8.7 Hz, 1H), 7.37 (d, J=8.7 Hz, 1H), 7.26 (d, J=7.9 Hz, 1H), 7.18 (t, J=7.9 Hz, 1H), 7.14 (t, J=7.9 Hz, 1H), 7.11 (t, J=7.2 Hz, 1H), 7.04 (t, J=7.4 Hz, 1H), 6.72 (d, J=8.6 Hz, 1H), 4.76 (m, 1H), 4.09 (pentet, J=7.0 Hz, 1H), 3.09 (d, J=12.1 Hz, 1H), 1.96 (s, 3H), 0.98 (d, J=7.0 Hz, 3H); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 172.2, 171.8, 171.6, 169.1, 136.8, 136.5, 129.8, 128.3, 127.9, 129.3, 122.0, 121.7, 119.4, 119.0, 118.9, 118.4, 111.5, 111.3, 109.4, 106.1, 53.1, 52.2, 51.2, 47.4, 29.4, 26.0, 22.5, 14.6; LRMS (+FAB): 5 15 (100), 456 (5), 429 (7), 391 (8), 329 (14), 307 (19), 270 (37), 257 (35); HRMS (+FAB): calcd for $C_{28}H_{29}N_5O_5$, 515.2169; found 515.2159.

Ac-Trp-Ala-Trp-NHMe (3d). To a solution of **3a** (0.301 g, 0.582 mmol) in THF (5.8 mL) was added aq LiOH (0.1 N, 5.8 mL, 5.8 mmol). The solution was stirred at room temperature for 4 h. The reaction was then acidified with 0.1 N HC1 and extracted with ethyl acetate (3×30 mL). The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. The crude carboxylic acid (0.261, 0.518 mmol) was dissolved in THF (20 mL) and HBTU (0.393 g, 1.04 mmol) was added followed by DIPEA (0.27 g, 2.07 mmol). Methylamine (2.0 M in THF, 2.59 mmol) was added and the reaction was stirred at room temperature for 11 h. The solution was diluted with ethyl acetate (40 mL) and washed with water (30 mL). The aqueous layer was then extracted with ethyl acetate (3×30 mL). The combined organics were dried over $MgSO_4$ and concentration in vacuo. Chromatography on silica gel (15% MeOH/$CHCl_3$) afforded **3d** (0.245 g, 86%) as a white solid.

Ac-Trp-Ala-Trp-NHMe (3d): mp 256–258 °C (MeOH/$CHCl_3$). R_f=0.21 (10% MeOH/$CHCl_3$); IR (KBr) 3397, 3270, 3064, 1635, 1532, 1454, 741 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6 δ 10.79 (s, 1H), 10.78 (s, 1H), 8.18 (d, J=7.0 Hz, 1H), 8.06 (d, J=7.9 Hz, 1H), 7.81 (d, J=8.0 Hz, 1H), 7.78 (q, J=4.6 Hz, 1H), 7.62 (d, J=7.8 Hz, 1H), 7.54 (d, J=7.9 Hz, 1H), 7.32 (d, J=4.0 Hz, 1H), 7.30 (d, J=4.0 Hz, 1H), 7.15 (d, J=2.0 Hz, 1H), 7.11 (d, J=2.1 Hz, 1H), 7.04 (t, J=6.7 Hz, 2H), 6.96 (t, J=7.3 Hz, 2H), 4.53 (m, 1H), 4.42 (q, J=7.4 Hz, 1H), 4.23 (q, J=7.0 Hz, 1H), 3.11 (t, J=4.8 Hz, 1H), 3.08 (t, J=4.7 Hz, 1H), 2.97 (dd, J=14.5, 9.6 Hz, 1H), 2.89 (dd, J=14.8, 9.6 Hz, 1H), 2.53 (d, J=4.5 Hz, 3H), 1.77 (s, 3H), 1.17 (d, J=7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 171.83, 171.81, 171.5, 169.4, 136.03, 136.01, 127.3, 123.53, 123.49, 120.8, 118.6, 118.4, 118.2, 118.1, 111.2, 110.2, 109.9, 53.5, 53.4, 48.6, 27.8, 27.6, 25.6, 22.6, 17.8 all of the aromatic signals could not be resolved within the limitations of the instrument; LRMS (+FAB) 530 (16) $[M+Na]^+$, 517 (100), 387 (16), 307 (33), 289 (25), 257 (26), 229 (23), 218 (27), 201 (42): ; HRMS (+FAB): calcd for $C_{28}H_{32}N_6O_4$, 516.2485; found 517.2558 $[MH]^+$.

Ac-Trp-Pro-Trp-NHMe (2c). To a solution of **2b** (0.223 g, 0.413 mmol) in THF (5.8 mL) was added aq. LiOH (0.1 N, 5.8 mL, 5.8 mmol). The solution was stirred at room temperature for 4 h. The reaction was then acidified with 0.1 N HCl and extracted with ethyl acetate (3×30 mL). The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. The crude carboxylic acid (0.187, 0.355 mmol) was dissolved in THF (20 mL) and HBTU (0.270 g, 0.711 mmol) was added followed by DIPEA (0.184 g, 1.42 mmol). Methylamine (2.0 M in THF, 0.711 mmol) was added and the reaction was stirred at room temperature for 11 h. The solution was diluted with ethyl acetate (40 mL) and washed with water (30 mL). The aqueous layer was then extracted with ethyl acetate (3×30 mL). The combined organics were dried over $MgSO_4$ and concentration in vacuo. Chromatography on silica gel (3% MeOH/$CHCl_3$) afforded **2c** (0.057 g, 26%) as an off-white solid.

Ac-Trp-Pro-Trp-NHMe (2c): Mp >350 °C (EtOAc). R_f=0.34 (10% MeOH/$CHCl_3$); IR (KBr) 3319, 2954, 1663, 1630, 1508, 1442, 1337, 744 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 11.28 (s, 1H), 11.24 (s, 1H), 8.67 (d, J=7.7 Hz, 1H), 7.81 (d, J=7.6 Hz, 1H), 7.49 (d, J=7.9 Hz, 1H), 7.45 (d, J=8.0 Hz, 1H), 7.38 (d, J=8.0 Hz, 1H), 7.19 (m, 4H), 7.02 (t, J=7.5 Hz, 1H), 6.89 (d, J=8.6 Hz, 1H), 5.20 (m, 1H), 4.44 (m, 1H), 4.37 (d, J=5.3 Hz, 1H), 3.79 (t, J=12.7 Hz, 1H), 3.61 (t, J=12.7 Hz, 1H), 3.45 (m, 2H), 3.19 (d, J=10.0 Hz, 1H), 2.40 (d, J=4.4 Hz, 3H), 2.35 (m, 1H), 1.99 (m, 1H), 1.93 (s, 3H), 1.60 (m, 1H), 1.19 (m, 1H); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 172.5, 171.5, 169.9, 169.2, 136.5, 136.3, 128.8, 128.4, 128.0, 122.1, 121.6, 119.6, 119.4, 118.5, 118.44, 118.37, 111.7, 111.0, 108.3, 107.3, 59.3, 53.2, 49.9, 46.8, 28.5, 26.1, 26.0, 25.9, 23.9, 22.2; LRMS (+FAB): 541 (10), 461 (11), 401 (16), 355 (21), 341 (22), 327 (62), 307 (20); HRMS (+FAB): calcd for $C_{30}H_{32}N_6O_4$, 540.2485; found 540.2488.

Ac-Trp-Ala-Trp-NHMe (3c). To a solution of **3b** (0.190 g, 0.369 mmol) in THF (3.7 mL) was added aq.

LiOH (0.1 N, 3.7 mL, 3.7 mmol). The solution was stirred at room temperature for 4 h. The reaction was then acidified with 0.1 N HCl and extracted with ethyl acetate (3×30 mL). The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. The crude carboxylic acid (0.178, 0.355 mmol) was dissolved in THF (15 mL) and HBTU (0.270 g, 0.709 mmol) was added followed by DIPEA (0.183 g, 1.42 mmol). Methylamine (2.0 M in THF, 0.709 mmol) was added and the reaction was stirred at room temperature for 11 h. The solution was diluted with ethyl acetate (40 mL) and washed with water (30 mL). The aqueous layer was then extracted with ethyl acetate (3×30 mL). The combined organics were dried over $MgSO_4$ and concentration in vacuo. Chromatography on silica gel (15% MeOH/$CHCl_3$) afforded **3c** (0.079 g, 42%) as an off-white solid.

Ac-Trp-Ala-Trp-NHMe (3c): Mp >350 °C ($CHCl_3$). R_f=0.23 (10% MeOH/$CHCl_3$); IR (KBr) 3292, 3057, 2927, 1653, 1523, 1341, 741, 589, 471 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 11.22 (s, 1H), 11.06 (s, 1H), 8.30 (d, J=7.9 Hz, 1H), 8.15 (d, J=6.9 Hz, 1H), 7.89 (d, J=7.9 Hz, 1H), 7.48 (q(broad), J=4.5 Hz, 1H), 7.39 (d, J=8.0 Hz, 1H), 7.35 (d, J=8.0 Hz, 1H), 7.32 (d, J=8.1 Hz, 1H), 7.15 (t, J=7.5 Hz, 1H), 7.08 (m 2H), 6.96 (t, J=7.5 Hz, 1H), 6.43 (d, J=8.5 Hz, 1H), 4.67 (m, 1H), 4.32 (m, 1H), 4.27 (t, J=7.1 Hz, 1H), 3.69 (t, J=12.6 Hz, 1H), 3.63 (d, J=13.5 Hz, 1H), 3.35 (m, 1H), 3.04 (d, J=11.2, 1H), 2.48 (d, J=4.3, 3H), 1.90 (s, 3H), 0.92 (d, J=7.1, 3H); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 172.0, 171.7, 171.5, 169.1, 136.7, 136.5, 129.7, 128.3, 128.0, 127.3, 122.0, 121.6, 119.8, 119.3, 119.0, 118.4, 111.3, 111.1, 109.5, 107.0, 53.3, 52.1, 47.5, 28.9, 26.1, 26.0, 22.5, 143; LRMS (+FAB): 517 (100), 439 (11), 345 (64), 269 (81), 257 (67), 154 (75), 136 (89); HRMS (+FAB): calcd for $C_{28}H_{30}N_6O_4$, 514.2328; found 515.2402 $[MH]^+$.

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