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Research Paper

Intramolecular ditryptophan crosslinks enforce two types of antiparallel β structures

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Abstract

Background: Two types of biaryl crosslinks can be formed with natural protein sidechains: ditryptophan and dityrosine. Biaryl crosslinks have the same topology as disulfide crosslinks, yet little is known about their effect on local peptide structure.

Results: Three ditryptophan-linked peptide dimers based on the sequence Ac-Leu-Trp-Ala-COX were prepared. The tripeptide dimer with $-CONH_2$ termini was too insoluble to study, but the tripeptide dimer with –COOMe termini crystallized from methanol/chloroform as an antiparallel β-sheet. The tripeptide dimer with a $-CONMe_2$ termini adopted a slipped antiparallel β structure in methanol/chloroform.

Conclusions: These results suggest that intermolecular ditryptophan crosslinks that join the middle of peptide chains can confer a preference for antiparallel β-sheet structure. The effect is most dramatic when both the inside and outside edges of the dimer can form hydrogen bonds as in the crystal structure of dimer **3b**.

Keywords: Ditryptophan; Dityrosine; β-Sheet

1. Introduction

Tryptophan and tyrosine sidechains are subject to oxidative coupling to give fluorescent biaryl crosslinks known as ditryptophan [1] and dityrosine [2] crosslinks, respectively. The conditions required for ditryptophan and dityrosine formation are harsher and less efficient than the conditions required for disulfide formation, but unlike cystine disulfides, there are no simple mechanisms for the cleavage of biaryl crosslinks [3]. Ditryptophan crosslinks can have profound effects on local peptide structure. Intramolecular ditryptophan crosslinks have been shown to stabilize γ-turns in tripeptide sequences [4] and a β-turn in X-indolicidin [5]. Crosslinks generally have detrimental effects on protein structure and function, and these effects are probably more important when the crosslinks are persistent.

There are many examples of proteins in which cystine disulfides join neighboring strands in the middle of a β-sheet, and short antiparallel peptides with cystine disulfides at the terminus [6–10], but when placed in the middle of short peptides, cystine disulfides are too short to efficiently induce β structure [11,12]. One strategy for inducing peptides to adopt antiparallel β conformations is to put biphenyl [13,14] and bipyridyl [15,16] subunits at the ends of peptide chains. Peptides have been joined in the middle through flexible linkers using olefin metathesis [17–19], but it is not clear whether such linkers contribute to the stability of antiparallel β conformations [20]. Ditryptophans are topologically suited to join antiparallel β-strands, and long enough to do so in a strain-free manner. The stabilization of antiparallel β-strands by ditryptophans in short-peptide models would provide insight into their potential effects on protein structure and also provide the means to create short β-peptide dimers that could interfere with protein–protein interactions [21,22].

2. Results

2.1. Synthesis of ditryptophan peptide dimers

The simple tripeptide sequence Leu-Trp-Ala was chosen

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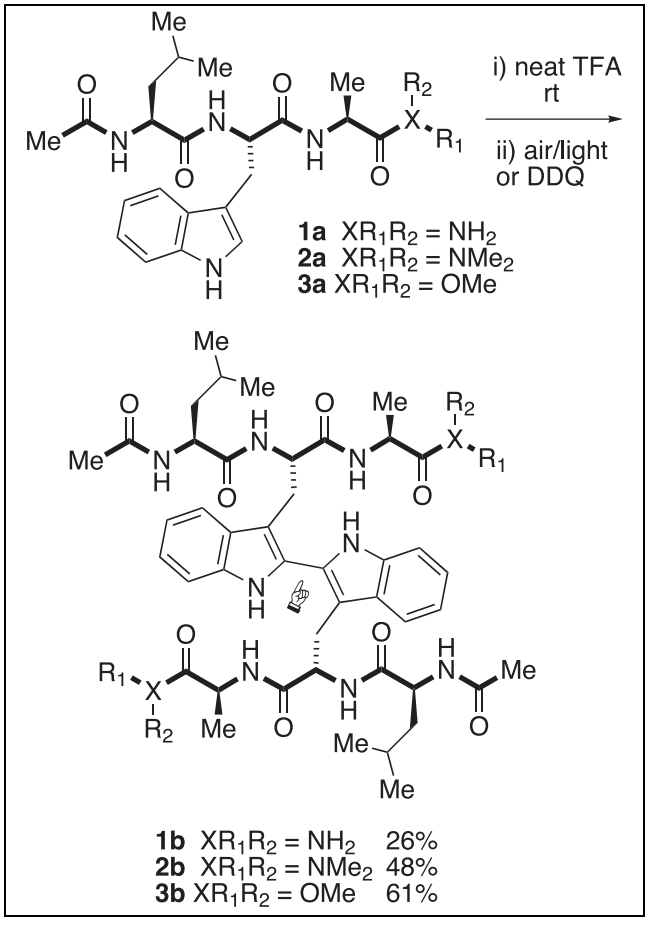
as a substrate for ditryptophan crosslinking. Leu-Trp-Ala is found on the edge of β-sheets in *Taq* adenine N^6-methyltransferase [23] and the capsid protein of bacteriophage ϕX174 [24], although ditryptophan crosslinks probably play no role in the function of these proteins. The tripeptide Ac-Leu-Trp-Ala-$CONH_2$, prepared by solid phase peptide synthesis from Rink amide resin, was subjected to Mannich dimerization in neat trifluoroacetic acid, followed by oxidative aromatization with DDQ in *N*-methylpyrrolidinone to give the fluorescent ditryptophan **1b**. Ditryptophan peptide dimer **1b** was reluctant to dissolve in solvents other than dimethyl formamide (DMF) or dimethyl sulfoxide (DMSO) (Scheme 1).

A common strategy for preventing aggregation is to place methyl groups on the edges of peptide chains. This was easily accomplished by converting the carboxy terminus of Ac-Leu-Trp-Ala-CO_2H to the corresponding *N,N*-dimethyl amide, followed by Mannich dimerization and oxidative aromatization to give dimer **2b**. The oxidative aromatization was accomplished with air and light in 9:1 dichloromethane/THF. As an additional strategy for reducing the number of hydrogen-bond donors, the corresponding methyl ester was prepared by solution phase synthesis of Ac-Leu-Trp-Ala-CO_2Me from *N*α-Boc-tryptophan, followed by the two-step process of ditryptophan



Scheme 1.

Table 1
^{1}H exchange rates in 1 M $CD_3OD/CDCl_3$

Proton	$t_{1/2}$	
	dimer **2b** (h)	dimer **3** (min)
LeuNHα	1	40
TrpNHα	0.5	~35
AlaNHα	9	~35
TrpNH (indole)	8	135

formation. In this case oxidative aromatization was accomplished using DDQ. The *N,N*-dimethylamide peptide dimer **2b** and the methyl ester dimer **3b** were soluble in mixtures of methanol and chloroform, but not neat chloroform.

2.2. ^{1}H NMR and IR data suggest intramolecular hydrogen-bonding in dimer *3b*

The ^{1}H NMR spectra of peptide dimers **2b** and **3b** in 1 M $CD_3OD/CDCl_3$ were assigned using COSY. None of the ^{1}H NMR data suggests a preferred conformation for the ester dimer **3b** in 1 M methanol/chloroform. In contrast, all of the spectroscopic evidence points to a decisive conformation for the *N,N*-dimethylamide dimer **2b**. The half-lives for exchange of the NH protons in the ester dimer **3b** were not substantially different, but the NH protons of dimer **2b** exhibited dramatic differences in exchange rates (Table 1). These effects are consistent with intramolecular hydrogen bonds to the alanine NHs and ditryptophan indole NHs in dimer **2b**. Stronger hydrogen bonding should be reflected in the downfield chemical shifts, and this is observed for the Ala amide NH and the Trp indole NHs. The Trp indole NH, Ala amide NH and the Trp amide NH are shifted 2.2, 0.8 and 0.3 ppm downfield in the dimer **3b** relative to the monomer **3a**. In theory, these differences might be attributable to conformationally induced differences in magnetic anisotropy, but concentration effects point to hydrogen bonding: the amide NH chemical shifts of dimer **2b** were invariant up to the solubility limit of 5 mM, whereas the amide NH chemical shifts of the monomer **2a** varied with concentration (Fig. 1). Monomers **2a** and **3a** exhibit an IR stretch characteristic of a free indole NH (3478 cm^{-1}), but dimers **2b** and **3b** do not (Fig. 2). Either the indole NHs of the peptide monomers are more resistant to hydrogen bonding or the biindole NHs of the dimers are more susceptible to hydrogen bonding.

2.3. Monte Carlo search and ROESY data converge on a single structure for dimer *2b*

Dimer **2b** has a molecular weight around 900 and ROESY is necessary to obtain spatial information. Since dimer **2b** is symmetrical, one can not distinguish intrachain ROEs from interchain ROEs. Thus, the ROESY data can not be used as a distance costraint in a Monte

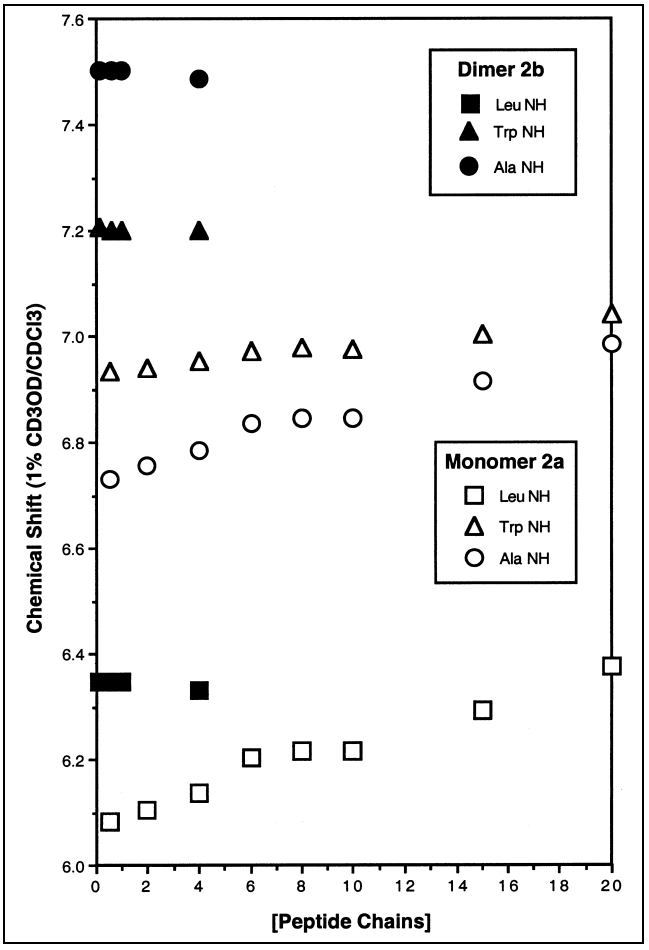


Fig. 1. Concentration dependence of amide NH shifts in $CDCl_3$.

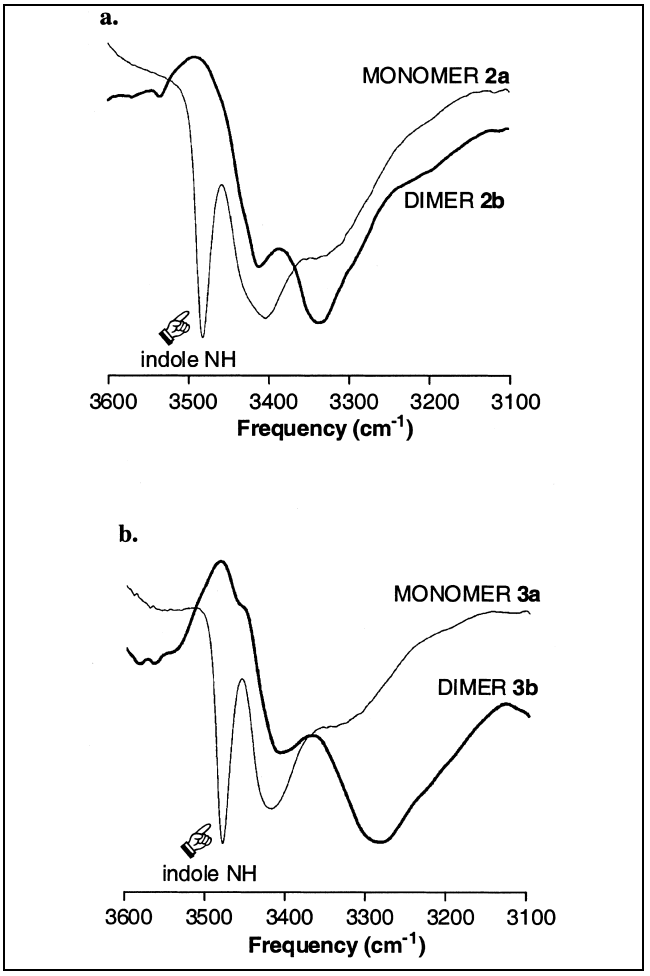
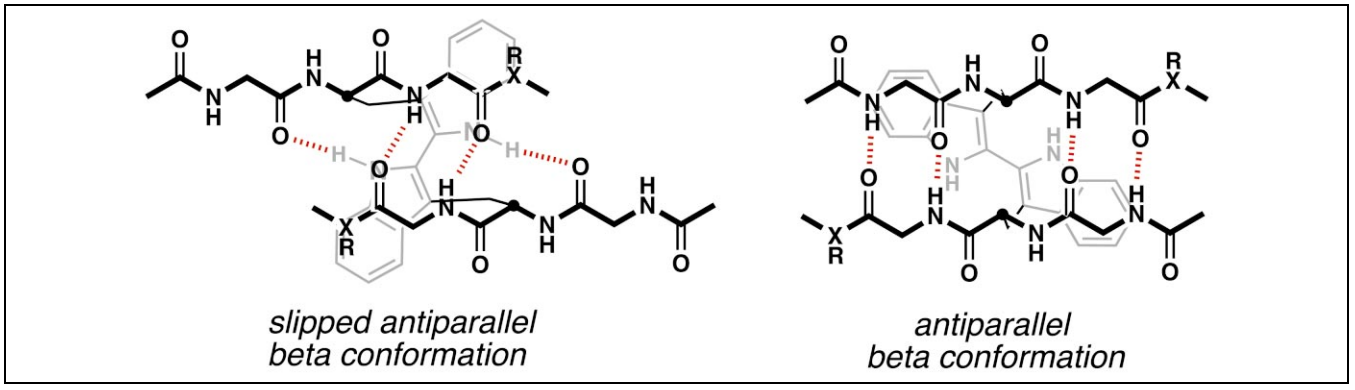


Fig. 2. Comparison of IR spectrum of (a) monomer **2a** (10 mM) vs. dimer **2b** (5 mM) in 1% (v/v) MeOH/$CHCl_3$, and (b) monomer **3a** (9 mM) vs. dimer **3b** (4.5 mM) in 1% (v/v) MeOH/$CHCl_3$.

Carlo conformational search. However, the ROESY data is powerful for distinguishing between plausible and implausible conformations generated by an unconstrained Monte Carlo search. These peptide dimers are particularly suitable for conformational searching because they have only six amino acids and the central crosslink substantially reduces the degrees of freedom in the system. Conformations for dimer **2b** were generated with Macromodel v.6.0 using the AMBER* force field ($CHCl_3$ solvent) starting from a random, non-β-sheet conformation. AMBER* is good for peptides, but it underestimates the slight barrier to ditryptophan planarity predicted by MM2 and AM1 in Fig. 2b. Thus, conformations with planar ditryptophans appear more often with AMBER* than with MM2. 1000 Monte Carlo cycles produced 123 unique conformations with good convergence. Fortunately, all 10 of the lowest energy conformations, within 4.24 kcal/mol of the global



Scheme 2.

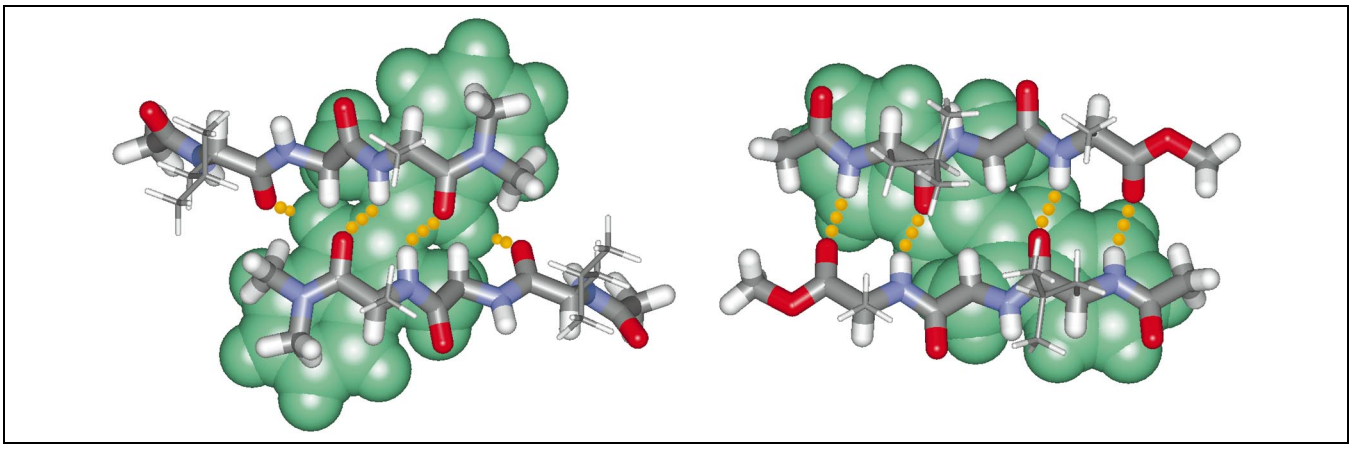


Fig. 3. Two antiparallel conformations. Unconstrained Monte Carlo searches (AMBER*) predicts two plausible low energy conformations for dimer **3b**, but only one low energy conformation (slipped) for dimer **2b**.

minimum, were nearly identical (Fig. 3). The picture that emerges for dimer **2b**, consistent with all of the spectroscopic data, is that the ditryptophan crosslink is holding the two peptide chains in a slipped antiparallel conformation, so that two of the four possible β-sheet hydrogen bonds are realized (Fig. 3). The alanine residues in opposite strands adopt an antiparallel hydrogen-bonded arrangement, consistent with IR and ^{1}H NMR data. The indole NHs are hydrogen bonded to the leucine carbonyl, which causes the antiparallel strands to twist downward from a flat sheet-like orientation. This slipped structure leaves the leucine and tryptophan amide NHs free to form intermolecular hydrogen bonds (Scheme 2). This explains the concentration dependence of their chemical shifts and why these NHs exchange faster than the corresponding tryptophan indole NH and the alanine amide NH in 1 M $CD_3OD/CDCl_3$.

The ROESY spectrum of dimer **2b** was obtained at 6 mM in a solution of 1 M $CD_3OD/CDCl_3$ at 30°C (400 ms mixing time). Under these conditions, the molar ratio of deuteromethanol to peptide dimer is about 166:1. The conformational search and ROESY data converge on the slipped antiparallel structure (Fig. 4). As expected, strong crosspeaks were observed for protons that were separated by fewer than four bonds. However, strong crosspeaks were also apparent between some non-neighboring protons. Fortunately, the two *N*-methyl groups were distinguishable on the ROE time scale. The *N*-methyl group with a strong crosspeak to the alanine $C_\alpha H$ also exhibits strong crosspeaks with protons at the 5 and 6 position of the indole ring. The other *N*-methyl group has a strong crosspeak to the proton at the 7 position of the indole ring. Thus, the carboxamide moieties of dimer **2b** seem to be positioned over the outer rims of the tryptophan benzene rings. One of the *N*-methyl groups also showed a strong NOE to leucine sidechain and the tryptophan $C_\alpha H$ as predicted by the slipped structure.

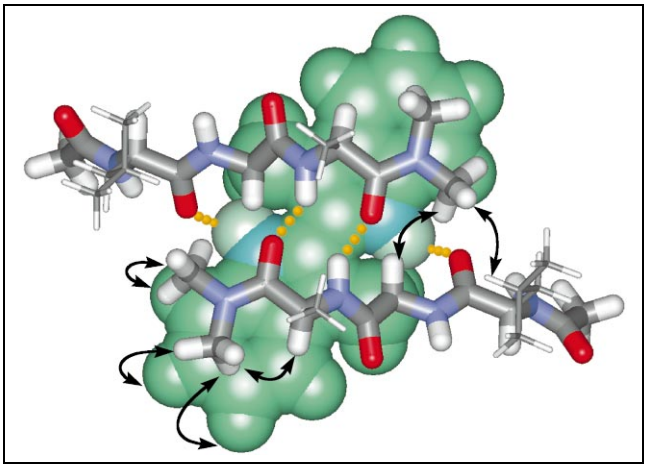


Fig. 4. Solution structure of peptide dimer **2b**. Diagnostic ROEs are consistent with the slipped antiparallel β conformation from the Monte Carlo search. The ditryptophan crosslink is shaded in green.

A Monte Carlo search for conformations of the ester dimer **3b** identified two low energy conformations. One of them was identical to the slipped β dimer of **2b**. The other conformation was a perfect antiparallel β-sheet with four intramolecular hydrogen bonds (Fig. 3). A ROESY spectrum of the ester dimer **3b** in 1 M $CD_3OD/CDCl_3$ generated some, but not all, of the ROEs seen for dimer **2b**, but the ROEs were not of sufficient quality or quantity to provide unambiguous information. It is possible that dimer **3b** may be rapidly (relative to the ^{1}H NMR time scale) interconverting between a slipped conformation and an antiparallel conformation in solution.

*2.4. Ditryptophan peptide dimer **3b** crystallizes as an antiparallel β-sheet*

Attempts were made to generate crystals of all three ditryptophan peptide dimers, but only the ester dimer **3b** gave X-ray quality crystals. Peptide dimer **3b** was crystallized from methanol/dichloromethane. X-ray diffraction revealed an antiparallel β-sheet structure partly predicted by the AMBER*/Monte Carlo search. In the crystal struc-

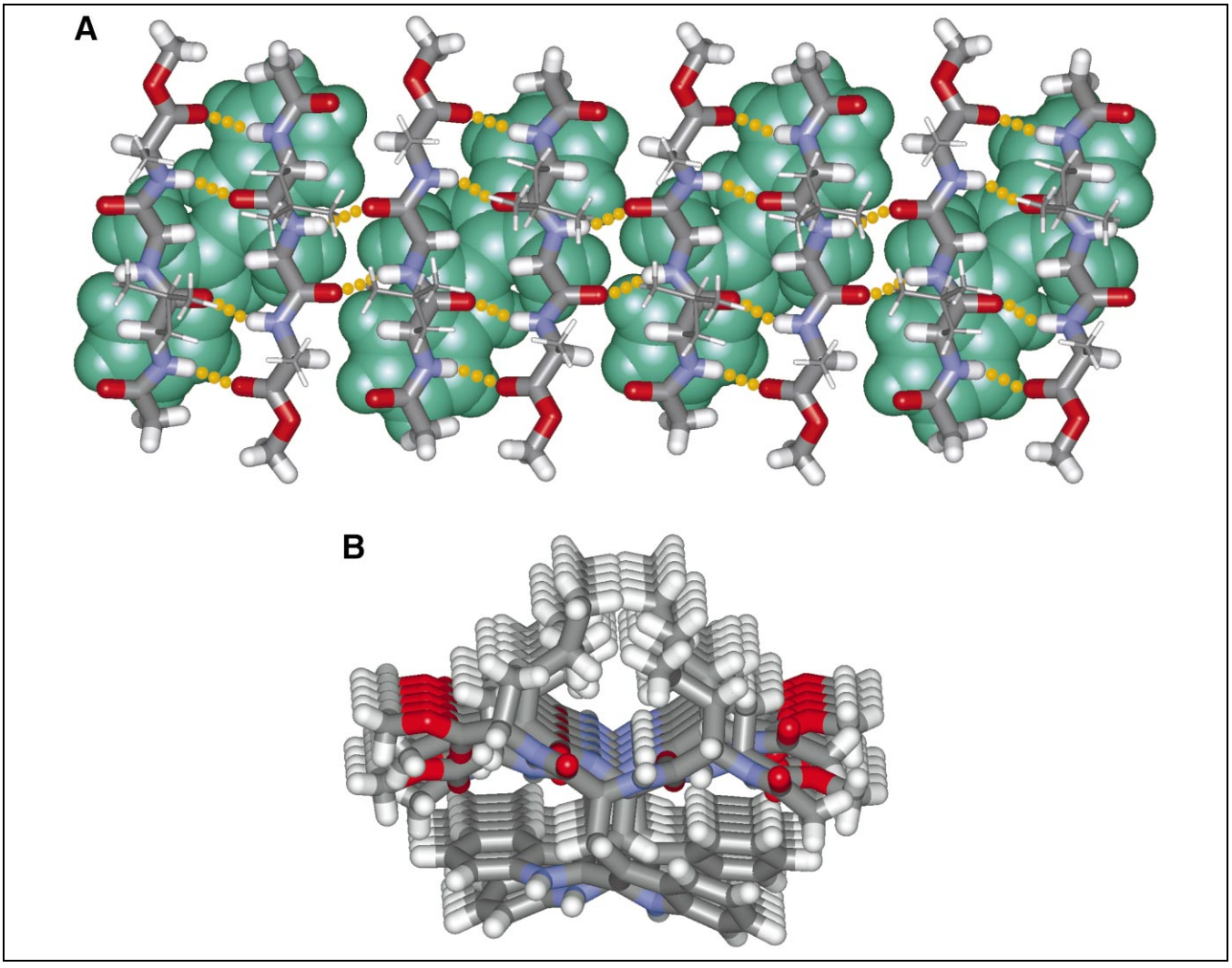


Fig. 5. Crystal structure of peptide dimer **3b**. Top (A) and end (B) views of X-ray crystal structure of the ditryptophan [Ac-Leu-Trp*-Ala-CO_2Me]$_2$ **3b** showing four unit cells. Leucine and alanine sidechains are rendered in line mode.

ture (Fig. 5), the ditryptophan adopts an *S*-biaryl configuration. Each peptide dimer possesses four intramolecular hydrogen bonds that polarize the outside edges. Each of the outside edges engages in the maximum number of hydrogen bonds, two on each side. The cooperative network of hydrogen bonds that holds this structure together has the same features as the cooperative networks of hydrogen bonds that are essential for the stability of globular proteins. The crosslinking effect of these hydrogen-bond networks provides a rationale for the intractability of peptide dimer **1b**.

Peptides do not generally crystallize as antiparallel β-sheets, but a variety of examples are presently known [25]. For example, Boc-(D)Val-Ala-Leu-Ala-CO_2Me crystallizes from aqueous butanol as an extended antiparallel β-sheet [26]. In addition, $+H_3N$-Ala-Ala-Ala-CO_2^- crystallizes from aqueous DMF as an antiparallel β-sheet in which each carboxylate is held in proximity to an ammonium group on an opposing strand [27].

3. Discussion

In conclusion, three ditryptophan-linked peptide dimers were studied. With a full complement of hydrogen bonds, the simple ditryptophan peptide dimer **1b** was highly insoluble. By adding two *N*-methyl groups to the carboxamide terminus, the resulting peptide dimer **2b** was made tractable. Modeling predicts a single preferred conformation for dimer **2b** and IR, ^{1}H exchange rates and ^{1}H chemical shifts, and ROEs in methanol/chloroform corroborate the model. In this slipped antiparallel β conformation, the two strands form only two hydrogen bonds. The peptide backbone of **2b** adopts a left-handed twist, allowing the Leu carbonyl groups to hydrogen bond with the tryptophan NHs. In contrast, most β-sheets are curved because extended peptide strands adopt a right-handed twist due to allylic 1,3 strain [28]. In dimer **3b**, the stability of the slipped structure is probably compromised because the two interstrand hydrogen bonds of **2b**,

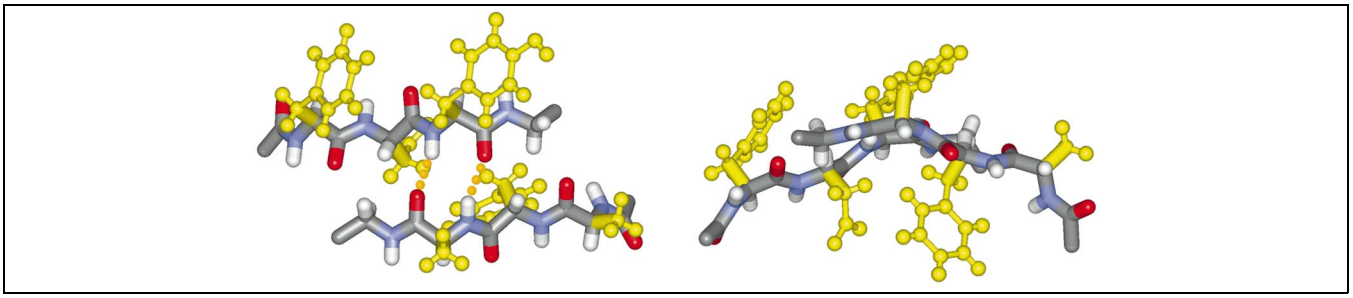


Fig. 6. A related structure from matrix porin outer membrane protein (2OMF.pdb; residues 83–87, 95–99).

involving amide carbonyls, would be replaced by interstrand hydrogen bonds involving ester carbonyls. The solubility of peptide dimer **3b** is better than the carboxamide analog **1b** because it reduces the number of potential hydrogen bonds on the outside edge of the antiparallel β-pleated structure. Recall that the IR data suggests that the indole NHs of amide dimer **2b** and ester dimer **3b** were hydrogen bonded. However, the exchange rates (Table 1) imply that the indole NHs in dimer **3b** hydrogen bond with less fidelity, raising the possibility the indole NHs of dimer **3b** may spend some time hydrogen bonding to the methanol co-solvent.

4. Significance

The antiparallel β-pleated sheet in the X-ray structure of dimer **3b** is a universal element of protein structure, but ditryptophans can have even more specific effects. The twisted conformation induced by the ditryptophan crosslink in dimer **2b** resembles conformations present in a variety of proteins. Using the program SPASM, similar peptide conformations were found in the X-ray structures of proteins MTCP-1 (1A1X), human rhinovirus 16 coat protein (1AYM), copper amine oxidase (1KSI), neocarzinostatin (1NOA), endo-1,4-β-xylanase (1XYP), sialidase (3SIL) and the matrix porin outer membrane protein (2OMF, Fig. 6) [29,30].

Biaryl crosslinks are distinct from disulfides because they are highly fluorescent and robust under reducing conditions. Ditryptophan crosslinks were first reported in 1995, but since that time they have not been identified in Nature. However, they appear to provide a promising way to construct protein-like architectures or peptide biomaterials. Since ditryptophans can have such profound effects on local peptide structure, it seems likely that dityrosine crosslinks, which occur in all humans, may have analogous consequences. Surprisingly, nothing is known about the effect of dityrosines on local peptide conformation. Dityrosines are longer than ditryptophans. If they span antiparallel β-sheets, then they are more likely to do so diagonally rather than directly across the peptide chains.

5. Materials and methods

5.1. Fmoc deprotection

In a solid phase reaction vessel, the Fmoc-resin was suspended in 1:5 piperdine/DMF (v/v, 50 ml/mmol). The reaction mixture was agitated by nitrogen bubbling for 1 h. The deprotection solution was forced out under a stream of air and the resin was dried under a stream of air.

5.2. Coupling with HOBt/HBTU

A solid phase reaction vessel was charged with solid phase resin with free amino termini. HOBt (3 equiv.) was added, followed by Fmoc-amino acid (3 equiv.), HBTU (3 equiv.), dry DMF (75 ml/mmol) and DIEA (3 equiv.). The solution was agitated by nitrogen bubbling until the Kaiser test indicated the disappearance of the terminal $-NH_2$ (about 45 min). The coupling solution was forced out under a stream of air and the resin was washed with DMF (3×50 ml) and CH_2Cl_2 (3×50 ml). The resin was then dried under a stream of air.

5.3. Acetylation of the N-terminus

To the resin-bound peptide in a solid-phase reaction vessel, pyridine (75 ml), acetic anhydride (5 equiv.), and DMAP (1 equiv.) were added. The suspended resin was agitated by nitrogen bubbling until the Kaiser test indicated the disappearance of the terminal $-NH_2$ (45 min). The pyridine solution was flushed out of the vessel under the air pressure. The resin-bound peptide was washed with DMF (3×50 ml) and CH_2Cl_2 (3×50 ml), and then dried under a stream of air.

5.4. Resin cleavage

To a solid-phase reaction vessel containing resin from Fmoc peptide synthesis was added a solution of 81:7:7:3:2 trifluoroacetic acid/phenol/H_2O/ethanedithiol/triisopropylsilane (v/v, 75 ml/mmol). The mixture was agitated by nitrogen bubbling for 1 h. The filtrate containing the cleaved, deprotected peptide was drained from the reaction vessel and the remaining resin was rinsed with TFA (2×20 ml). The combined filtrates were concentrated in vacuo.

5.4.1. AcNH-Leu-Trp*-Ala-NH_2

A 500 ml solid-phase reaction vessel was charged with Fmoc-Rink amide MBHA polystyrene resin (2.00 g, 0.54 mmol/g).

Fmoc deprotection and coupling were carried out iteratively using the following amino acids (in order): Fmoc-Ala, Fmoc-Trp and Fmoc-Leu. The peptide was acetylated and cleaved. After the cleavage step, the peptide was concentrated in vacuo and triturated with ether (3×50 ml). The solid was taken up in ether (50 ml) and filtered. The precipitate was dried in vacuo and purified by silica gel chromatography (10% MeOH/$CHCl_3$) to afford AcNH-Leu-Trp-Ala-NH_2 (0.34 g, 73%) as a white powder.

AcNH-Leu-Trp*-Ala-NH_2: mp 234–236°C ($CHCl_3$); $R_f = 0.26$ (10% MeOH/$CHCl_3$); IR (KBr) 3410, 3284, 3063, 2952, 1635, 1539 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 10.83 (s, 1H), 7.99 (d, $J = 8.0$ Hz, 1H), 7.92 (d, $J = 8.0$ Hz, 1H), 7.81 (d, $J = 8.0$ Hz, 1H), 7.54 (d, $J = 8.0$ Hz, 1H), 7.30 (d, $J = 8.0$ Hz, 1H), 7.18 (s, 1H), 7.12 (d, $J = 4.0$ Hz, 1H), 7.05 (t, $J = 8.0$ Hz, 1H), 7.03 (s, 1H), 6.96 (t, $J = 8.0$ Hz, 1H), 4.47 (dt, $J = 8.0$, 4.4 Hz, 1H), 4.18 (m, 2H), 3.15 (dd, $J = 14.8$, 4.4 Hz, 1H), 2.98 (dd, $J = 14.8$, 8.4 Hz, 1H), 1.80 (s, 3H), 1.52 (m, 1H), 1.35 (m, 2H), 1.17 (d, $J = 7.2$ Hz, 3H), 0.84 (d, $J = 6.4$ Hz, 3H), 0.79 (d, $J = 6.4$ Hz, 3H); ^{13}C NMR (125 Hz, DMSO-d_6) δ 174.1, 172.3, 170.8, 169.6, 136.0, 127.4, 123.6, 120.9, 118.4, 118.3, 111.3, 109.9, 53.3, 51.3, 48.1, 40.4, 27.1, 24.2, 23.0, 22.4, 21.6, 18.3; LRMS (FAB), *m/z* (intensity %): 452 (6) $[M+Na]^+$, 430 (100) $[M+H]^+$, 413 (23); HRMS (FAB): calcd for $C_{22}H_{31}N_5O_4$, 430.2454 $[M+H]^+$; found 430.2455.

5.4.2. *AcNH-Leu-Trp-Ala-OH*

A 500 ml solid-phase reaction vessel was charged with Fmoc-Ala-Wang polystyrene resin (2.3 g, 0.60 mmol/g). Fmoc deprotection and HBTU coupling of Fmoc-Trp were carried out using general procedures. The deprotection and coupling steps were then repeated for Fmoc-Leu. The N-terminus was acetylated and the peptide was deprotected and cleaved from the resin. Purification of the peptide residue by silica gel chromatography (1:5:94 AcOH/MeOH/$CHCl_3$) afforded AcNH-Leu-Trp-Ala-OH (0.35 g, 58%) as a white powder.

AcNH-Leu-Trp-Ala-OH: mp 212°C ($CHCl_3$, dec.); $R_f = 0.28$ (1% AcOH/10% MeOH/$CHCl_3$); IR (KBr) 3417, 3085, 2952, 1628, 1539 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 10.84 (s, 1H), 8.06 (d, $J = 7.2$ Hz, 1H), 8.04 (d, $J = 6.2$ Hz, 1H), 7.54 (m, 2H), 7.31 (d, $J = 8.0$ Hz, 1H), 7.11 (s, 1H), 7.02 (t, $J = 7.2$ Hz, 1H), 6.95 (t, $J = 7.6$ Hz, 1H), 4.49 (ddd, $J = 7.2$, 6.4, 4.8 Hz, 1H), 4.24 (ddd, $J = 8.6$, 8.4, 5.6 Hz, 1H), 3.98 (m, 1H), 3.20 (dd, $J = 14.8$, 4.4 Hz, 1H), 2.96 (dd, $J = 14.8$, 8.8 Hz, 1H), 1.82 (s, 3H), 1.50 (m, 1H), 1.37 (m, 2H), 1.21 (d, $J = 6.8$ Hz, 3H), 0.84 (d, $J = 6.4$ Hz, 3H), 0.79 (d, $J = 6.4$ Hz, 3H), (CO_2-H does not appear around 12 ppm); ^{13}C NMR (125 MHz, DMSO-d_6) δ 176.3, 172.1, 170.0, 169.6, 136.0, 127.4, 123.5, 120.8, 118.4, 118.2, 111.2, 110.2, 53.2, 51.2, 49.5, 40.5, 27.0, 24.2, 23.0, 22.5, 21.5, 18.7; LRMS (FAB), *m/z* (intensity %): 469 (30) $[M+K]^+$, 453 (90) $[M+Na]^+$, 449 (40), 345 (100), 430 (15) $[M]^+$, 413 (32); HRMS (FAB): calcd for $C_{22}H_{30}N_4O_5$, 453.2114 $[M+Na]^+$; found 453.2110.

5.4.3. *AcNH-Leu-Trp-Ala-NMe_2*

AcNH-Leu-Trp-Ala-CO_2H (260 mg, 0.60 mmol) and dimethylammonium chloride (59.1 mg, 0.725 mmol) were suspended in DMF (2 ml). DIPEA (0.32 ml, 1.82 mmol), HOBt (18.5 mg, 0.121 mmol) and HBTU (0.504 g, 1.33 mmol) were added and the mixture was stirred at room temperature for 21 h. The solvent was removed in vacuo and the residue was purified by silica gel chromatography (5% MeOH/$CHCl_3$) affording AcNH-Leu-Trp-Ala-NMe_2 (0.177 g, 64%) as a yellow solid.

AcNH-Leu-Trp-Ala-NMe_2: mp = 186–187°C ($CHCl_3$); $R_f = 0.34$ (10% MeOH/$CHCl_3$); IR ($CDCl_3$) 3478, 3403, 2961, 1644, 1501 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 10.83 (s, 1H), 8.02 (d, $J = 8.0$ Hz, 1H), 7.96 (d, $J = 8.0$ Hz, 1H), 7.90 (d, $J = 7.6$ Hz, 1H), 7.53 (d, $J = 8.0$, 1H), 7.30 (d, $J = 8.4$ Hz, 1H), 7.11 (d, $J = 2.0$ Hz, 1H), 7.04 (t, $J = 7.2$ Hz, 1H), 6.95 (t, $J = 8.0$ Hz, 1H), 4.67 (dddd, $J = 7.2$, 7.2, 6.8, 6.8 Hz, 1H), 4.50 (ddd, $J = 8.0$, 7.6, 5.2 Hz, 1H), 4.25 (ddd, $J = 10.8$, 8.8, 4.8 Hz, 1H), 3.11 (dd, $J = 14.8$, 5.2, Hz, 1H), 2.94 (dd, $J = 14.8$, 8.4 Hz, 1H), 2.91 (s, 3H), 2.81 (s, 3H), 1.80 (s, 3H), 1.51 (m, 1H), 1.37 (m,2H), 1.13 (d, $J = 6.8$ Hz, 3H), 0.85 (d, $J = 6.4$ Hz, 3H), 0.80 (d, $J = 6.4$ Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.0, 171.3, 170.4, 169.2, 136.0, 129.3, 123.6, 120.8, 118.4, 118.1, 111.2, 109.8, 53.1, 51.1, 44.5, 40.6, 36.3, 35.2, 27.3, 24.2, 23.0, 22.5, 21.6, 17.5; LRMS (FAB), *m/z* (intensity %): 458 (12) $[M+H]^+$, 307 (26), 289 (7), 170 (5), 154 (100), 136 (74), 124 (15); HRMS (FAB): calcd for $C_{24}H_{35}N_5O_4$, 458.2767 $[M+H]^+$, found 458.2776.

5.4.4. *AcNH-Leu-Trp-Ala-OMe*

AcNH-Leu-Trp-Ala-OMe was prepared using standard solution phase peptide synthesis methods using Boc-protected amino acids.

AcNH-Leu-Trp-Ala-OMe: mp 191–192°C; $R_f = 0.39$ (10% MeOH/$CHCl_3$); IR (KBr) 3412, 3261, 3072, 1743, 1629, 1547, 1459, 1371, 1280, 1215, 746 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 10.83 (s, 1H), 8.29 (d, $J = 7.0$ Hz, 1H), 7.94 (d, $J = 7.9$ Hz, 1H), 7.86 (d, $J = 8.0$ Hz, 1H), 7.56 (d, $J = 7.56$ Hz, 1H), 7.30 (d, $J = 8.0$ Hz, 1H), 7.11 (s, 1H), 7.04 (t, $J = 7.2$ Hz, 1H), 6.96 (t, $J = 8.1$ Hz, 1H), 4.51 (dd, $J = 7.9$, 4.8 Hz, 1H), 4.27 (dd, $J = 7.2$, 7.1 Hz, 1H), 4.21 (m, 1H), 3.62 (s, 3H), 3.12 (dd, $J = 14.7$, 10.2 Hz, 1H), 2.94 (dd, $J = 14.8$, 8.9 Hz, 1H), 1.79 (s, 3H), 1.50 (m, 1H), 1.32 (m, 2H), 1.25 (d, $J = 7.2$ Hz, 3H), 0.83 (d, $J = 6.5$ Hz, 3H), 0.78 (d, $J = 6.45$ Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.8, 171.9, 171.2, 169.3, 135.9, 127.4, 123.5, 120.7, 118.3, 118.1, 111.1, 109.8, 52.8, 51.8, 51.1, 47.6, 40.5, 27.4, 24.1, 22.9, 22.4, 21.5, 16.9; LRMS (FAB), *m/z* (intensity %): 445 (100), 342 (10), 307 (20), 290 (25), 272 (95); HRMS (FAB): calcd for $C_{23}H_{32}N_4O_5$, 444.2373; found 445.2457 $[M+H]^+$; anal. calcd for $C_{23}H_{32}N_4O_5$: C, 62.13; H, 7.26; N, 12.61; found: C, 62.08; H, 7.26; N, 12.51.

5.4.5. *Ditryptophan peptide dimer [AcNH-Leu-Trp*-Ala-NH_2]$_2$, **1b***

AcNH-Leu-Trp-Ala-NH_2 **1** (0.192 g, 0.447 mg) was dissolved in trifluoroacetic acid (3 ml) and stirred at room temperature for 31 h to promote Mannich crosslinking of the tryptophan side-chains. The reaction mixture was concentrated in vacuo and sat. aq. $NaHCO_3$ was added until the solution reached pH 7. The precipitate was filtered and washed with water (60 ml) and EtOAc (30 ml), and dried in vacuo. The mixture of tryptophan dimers was taken on to oxidative aromatization without removing the left-over peptide monomer.

The residue was dissolved in NMP (2.50 ml), DDQ (19.7 mg, 0.0870 mmol) was added, and the mixture was stirred at room temperature for 3 h. Aq. $NaHCO_3$ (5 ml, half saturated) was added and the mixture was stirred for 10 min. The precipitate was filtered and washed with aq. $NaHCO_3$ (200 ml, half saturated). The residue was purified by silica gel chromatography

(10% MeOH/$CHCl_3$) to afford the ditryptophan peptide dimer **1b** (50.3 mg, 26%) as a solid.

1b: mp 277°C ($CHCl_3$, dec); $R_f = 0.20$ (10% MeOH/$CHCl_3$); IR: (KBr) 3394, 2952, 1650, 1524 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 11.22 (s, 2H), 8.08 (d, $J = 7.5$ Hz, 2H), 7.99 (d, $J = 8.0$ Hz, 2H), 7.78 (d, $J = 7.5$ Hz, 2H), 7.72 (d, $J = 8.0$ Hz, 2H), 7.37 (d, $J = 8.5$ Hz, 2H), 7.13 (t, $J = 7.5$ Hz, 2H), 7.05 (m, 4H), 6.95 (s, 2H), 4.68 (ddd, $J = 7.8$, 7.5, 7.0 Hz, 2H), 4.18 (ddd, $J = 7.8$, 7.25, 7.0 Hz, 2H), 4.14 (p, $J = 7.5$ Hz, 2H), 2.95 (dd, $J = 14.5$, 7.5 Hz, 2H), 1.78 (s, 6H), 1.51 (m, 2H), 1.28 (t, $J = 7.5$ Hz, 4H), 1.11 (d, $J = 7.0$ Hz, 6H), 0.81 (d, $J = 6.5$ Hz, 6H), 0.76 (d, $J = 6.5$ Hz, 6H) (2H are overlapping with water between 3.27 and 3.37 ppm); ^{13}C NMR (125 MHz, DMSO-d_6) δ 174.2, 172.4, 171.2, 169.8, 136.2, 127.9, 187.8, 121.7, 119.2, 118.8, 111.3, 109.5, 53.5, 51.4, 48.2, 40.4, 27.3, 24.1, 22.9, 22.4, 21.5, 18.2; LRMS (FAB) *m/z* (intensity %): 901 (8) $[M-H+2Na]^+$, 889 (72) $[M+Na]^+$, 873 (24), 856 (100); HRMS (FAB): calcd for $C_{44}H_{60}N_{10}O_8$, 856.4595; found 856.4589.

5.4.6. Ditryptophan peptide dimer [AcNH-Leu-Trp*-Ala-NMe_2]$_2$, 2b

AcNH-Leu-Trp-Ala-NMe_2 **2a** (0.143 g, 0.314 mmol) was dissolved in TFA (1 ml) and stirred at room temperature for 25 h to effect Mannich crosslinking of the tryptophan sidechains. The trifluoroacetic acid was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (1 ml) and cooled to 0°C. Triethylamine (1 ml) was slowly introduced and the solution was stirred for 2 h. The solvent was evaporated in vacuo, and the remaining starting material was separated from the crude tryptophan dimer (77.2 mg) by silica gel chromatography (4.5% MeOH/$CHCl_3$).

In a pyrex round-bottom flask open to air, the crude tryptophan dimer was dissolved in a solution of 10% THF/CH_2Cl_2 (32 ml). The reaction mixture was illuminated with a 150 W light bulb while stirring vigorously for 24 h. The reaction mixture was concentrated in vacuo, and the residue was purified via silica gel chromatography (5% MeOH/$CHCl_3$) to afford ditryptophan peptide dimer **2b** (69.2 mg, 48%) as a solid.

2b: mp 287°C ($CHCl_3$, dec); $R_f = 0.27$ (10% MeOH/$CHCl_3$); IR ($CDCl_3$) 3416, 3343, 2923, 2856, 1635, 1499 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 11.23 (s, 2H), 8.15 (d, $J = 8.0$ Hz, 2H), 7.97 (d, $J = 8.0$ Hz, 2H), 7.94 (d, $J = 8.0$ Hz, 2H), 7.86 (d, $J = 7.6$ Hz, 2H), 7.38 (d, $J = 8.0$ Hz, 2H), 7.13 (t, $J = 6.8$ Hz, 2H), 7.03 (t, $J = 7.2$ Hz, 2H), 4.70 (q, $J = 7.2$ Hz, 2H), 4.54 (p, $J = 6.8$ Hz, 2H), 4.25 (ddd $J = 9.6$, 8.4, 7.6 Hz, 2H), 2.90 (m, 4H), 2.69 (s, 6H), 2.66 (s, 6H), 1.79 (s, 6H), 1.51 (m, 2H), 1.29 (t, $J = 7.6$ Hz, 4H), 1.04 (d, $J = 6.8$ Hz, 6H), 0.82 (d, $J = 6.8$ Hz, 6H), 0.78 (d, $J = 6.8$ Hz, 6H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.2, 170.6, 169.3, 136.1, 127.9, 127.8, 121.5, 119.0, 118.7, 111.2, 109.4, 53.5, 51.0, 44.6, 40.6, 36.0, 35.2, 37.5, 24.1, 23.0, 22.4, 21.8, 17.5 (one carbonyl peak missing between 167 and 175 ppm); LRMS (FAB) *m/z* (intensity %): 913 (78) $[M+H]^+$, 585 (10), 297 (24), 269 (100), 154 (10), 128 (24); HRMS (FAB): calcd for $C_{48}H_{68}N_{10}O_8$, 935.5119 $[M+Na]^+$; found 935.5119.

5.4.7. Ditryptophan peptide dimer [AcNH-Leu-Trp*-Ala-OMe]$_2$, 3b

AcNH-Leu-Trp-Ala-OMe **3a** (0.310 g, 0.700 mmol) was dissolved in trifluoroacetic acid (4.5 ml) and stirred at room temperature for 24 h. The reaction mixture was concentrated in vacuo. The residue was dissolved in ethyl acetate (50 ml) and washed with $NaHCO_3$ (3×25 ml) and brine (50 ml). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. Purification via chromatography on silica gel (2.5% MeOH/$CHCl_3$) afforded a diastereomeric mixture of tryptophan dimers as a light-yellow solid. This mixture of diastereomers was dissolved in dry dioxane (3 ml) and DDQ (0.077 g, 0.34 mmol) was added and the mixture was stirred for 5 min at room temperature. Solvent was removed in vacuo, and the resulting brown solid was dissolved in ethyl acetate and the dihydroquinone was removed by repeated extraction with saturated $NaHCO_3$ until the aqueous layer remained colorless. The organic layer was dried over $MgSO_4$ and concentrated in vacuo to afford the crude ditryptophan peptide dimer. Chromatography on silica gel (2.5% MeOH/$CHCl_3$) afforded **3b** (0.130 g, 42%). Dimer **3b** was recrystallized from 20% MeOH/CH_2Cl_2 at room temperature for X-ray analysis.

3b: 297–298°C; $R_f = 0.36$ (10% MeOH/$CHCl_3$); IR (KBr) 3386, 3268, 2948, 1743, 1635, 1524, 1449, 1381, 1339, 1212, 1156, 746 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 11.16 (s, 1H), 8.27 (d, $J = 6.7$ Hz, 1H), 7.97 (dd, $J = 15.6$, 7.9 Hz, 1H), 7.76 (d, $J = 7.9$ Hz, 1H), 7.36 (d, $J = 7.9$ Hz, 1H), 7.13 (t, $J = 7.0$ Hz, 1H), 7.03 (t, $J = 7.6$ Hz, 1H), 4.72 (dd, $J = 14.0$, 7.6 Hz, 1H), 4.19 (m, 2H), 3.38 (m, 1H), 3.36 (s, 3H), 2.94 (dd, $J = 14.0$, 7.0 Hz, 1H), 1.78 (s, 3H), 1.49 (m, 1H), 1.14 (d, $J = 6.8$ Hz, 3H), 0.79 (d, $J = 6.0$ Hz, 3H), 0.74 (d, $J = 6.4$ Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.4, 172.1, 171.6, 169.6, 136.2, 128.0, 127.9, 121.6, 119.2, 118.7, 111.2, 109.2, 53.2, 51.7, 51.3, 47.7, 40.4, 27.6, 24.1, 22.9, 22.4, 21.5, 16.8; LRMS (FAB), *m/z* (intensity %): 887 (100), 732 (20), 572 (60), 400 (10), 261 (100); HRMS (FAB): calcd for $C_{46}H_{62}N_8O_9$, 886.4589; found 886.4576.

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