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Synthesis and Study of a Gramicidin B Mutant Possessing a Ditryptophan Crosslink

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Abstract: Recent studies of peptide dimers linked by Trp-Trp (ditryptophan) crosslinks suggest that the crosslinks can reinforce antiparallel β-structure. Depending on environment, gramicidins A, B and C form either helical ion channels with parallel β-structure or non-functional pores with antiparallel β-structure. In the channel conformation of the gramicidins Trp9 and Trp15 are close in space, but in the pore conformation Trp9 and Trp15 are far apart. We hypothesized that a ditryptophan crosslink between Trp9 and Trp15 could pre-organize gramicidin in an active conformation. To test the potential for pre-organization, an intramolecular ditryptophan crosslink was formed between Trp9 and Trp15 in a W13F mutant of gramicidin B. Photooxidative conditions were shown to generate ditryptophan crosslinks in low yields. While not preparatively useful, photooxidative tryptophan crosslinking may have implications for protein aging processes like cataract formation. The ditryptophan crosslink in the gramicidin B mutant substantially lowered the antibiotic activity of the gramicidin B mutant, unlike the ditryptophan crosslink in the antibiotic X-indolicidin. The biaryl chromophore generated diagnostic Cotton effects in the CD spectrum that revealed the absolute stereochemistry of the biaryl chromophore, but the biaryl chromophore obscured diagnostic features below 220 nm. However, changes in peptide conformation were reflected in changes in the biaryl region of the CD spectrum above 240 nm.

Keywords: tryptophan; ditryptophan; crosslink; gramicidin A; Cotton effect

BACKGROUND

In the human body, fluorescent dityrosine crosslinks form as a function of oxidative damage. Dityrosines are difficult to form efficiently in short peptides, but analogous ditryptophan crosslinks can be readily prepared using a Mannich reaction [1]. Unlike disulfides, the carbon–carbon bonds that join dityrosine and ditryptophan crosslinks can not be reductively cleaved. Ditryptophan crosslinks have profound effects on the shape of the peptide backbone: ditryptophans that bridge tripeptide sequences have been shown to induce γ-turn formation [2]. In peptide dimers, ditryptophan crosslinks have been shown to reinforce antiparallel β-sheets [3].

Recently, a derivative of the peptide indolicidin, X-indolicidin, was shown to possess a ditryptophan crosslink that mimics the active conformation of indolicidin [4]. Five of the 13 amino acids in indolicidin are tryptophans, making it highly susceptible to tryptophan crosslinking. X-Indolicidin has about the same level of antibiotic activity as indolicidin, but the ditryptophan crosslink confers protease resistance. Gramicidins A, B and C (GmA, GmB, GmC), from *Bacillus brevis*, constitute another class of tryptophan-rich peptides with

Abbreviations: CD, circular dichroism; DDQ, 2,3-dichloro-5,6-dicyano-quinone; GmA, gramicidin A; GmB, gramicidin B; GmC, gramicidin C; HPLC, high performance liquid chromatography; LPC, lysophosphatidylcholine; mequiv, milliequivalents; MIC, minimum inhibitory concentration.

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antibiotic activity. GmA, GmB and GmC are pentadecapeptides that differ by a single amino acid in the sequence HCO-Val-Gly-Ala-^{D}Leu-Ala-^{D}Val-Val-^{D}Val-Trp-^{D}Leu-**Xxx**-^{D}Leu-Trp-^{D}Leu-Trp-CONHCH$_2$ CH$_2$OH [5]. In GmA, GmB and GmC, the aromatic amino acid **Xxx** is Trp, Phe and Tyr, respectively. The mechanism of action of GmA, GmB and GmC is different from the mechanism of action of indolicidin and X-indolicidin. Gramicidins A, B and C form transient ion channels in membranes through end-to-end stacking of $\beta^{6.3}$ helical conformations [6]. The channel conformation is made possible by the alternating *S* and *R* stereochemistry starting from Val1 to Trp15 (Gly2 has no stereogenic center). There are other dimeric conformations of gramicidin A in which the two strands an antiparallel β-helices (referred to as a pore). In the active channel conformation, the sidechains of Trp9 and Trp15 are close in space and seem poised to crosslink, but in the pore conformation, Trp9 and Trp15 are far apart (Figure 1). Thus a ditryptophan crosslink between Trp9 and Trp15 would certainly disfavor the pore conformation and might favor the channel conformation. We formed a ditryptophan crosslink between Trp9 and Trp15 in a mutant of gramicidin B. The chiroptic properties of the ditryptophan chromophore were shown to correlate with local conformation. CD spectroscopy revealed that the configuration of the biaryl chromophore was different in LPC micelles versus methanol. Unfortunately, the ditryptophan crosslink reduced the antimicrobial activity of the gramicidin B mutant suggesting that the molecule is not restricted to the active helical conformation.

MATERIALS AND METHODS

Analytical and preparative HPLC were carried out using C18 Microsorb stationary phase. CD spectra were acquired on a Jasco J-700 spectropolarimeter at 25 °C in a quartz cuvette with a 0.1 cm pathlength. CD measurements were expressed as mean residue ellipticities, [Θ], in deg cm^2 dmol^{-1}. Conversion from observed ellipticities, (θ), in millidegrees, was performed by using the relationship $[\Theta] = (\theta)/10cnl$, where c is the peptide concentration in molar, n is the number of amino acid residues and l is the path length of the cell in centimeters [7].

Photooxidation of Poly-(D/L)-Tryptophan

To a solution of poly-(D/L)-tryptophan (20 mg, 4000–5700 MW) in pyridine (4.2 ml) in a 250 Pyrex

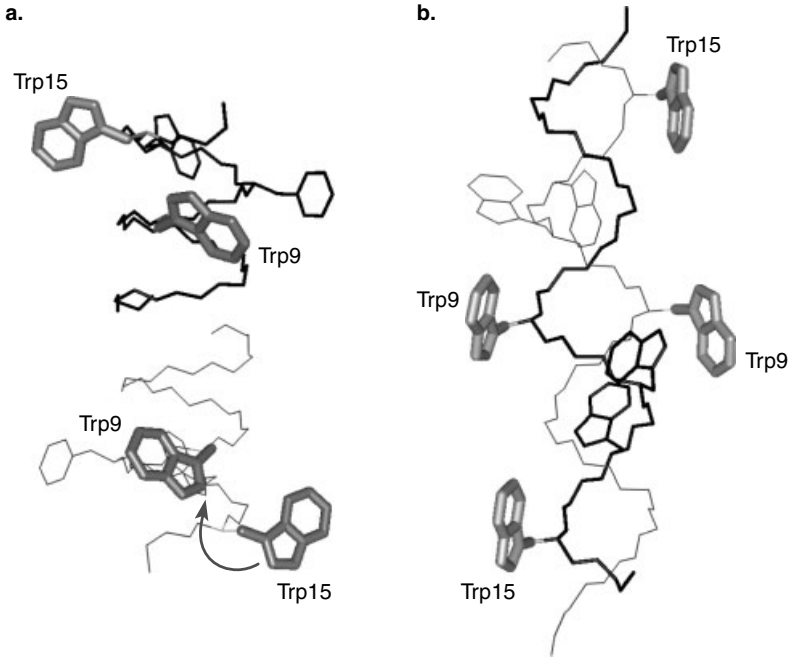

Figure 1 Two common conformations of gramicidin with non-aromatic sidechains removed for clarity. (a) In the channel conformation (1JO3.pdb) Trp9 and Trp15 are close. A change in the χ_1 the angle of Trp15 would facilitate crosslinking. (b) In the pore conformation (1AL4.pdb) Trp9 and Trp15 can not reach each other regardless of sidechain conformation.

photoreactor was added methanol (24 ml) and rose bengal (0.005 g). Oxygen was bubbled through the reaction for 12 h while irradiating with an external 500 W halogen lamp. The reaction was maintained at 0 °C by circulation of isopropanol (−10 °C) through the internal immersion well. Peroxides were quenched by addition of dimethylsulfide (1 ml) and stirring for 5 h at room temperature. The reaction mixture was concentrated *in vacuo* and passed through a plug of neutral alumina using 10% $MeOH/CH_2Cl_2$ to remove the rose bengal. After removal of solvent the residue was taken up in 1.25 ml $CHCl_3$/THF/MeOH (2 : 2 : 1) and a catalytic amount of acetic acid (3 drops) was added. The mixture was stirred for 12 h at room temperature and solvent was removed *in vacuo*.

The residue was taken up in 4 N NaOH (2 ml) in a glass round bottom flask and stirred at reflux under argon over 12 h. After cooling to room temperature, the pH was adjusted between 3 and 4 with 1 N HCl. The reaction was concentrated *in vacuo* and subjected to analytical reverse phase HPLC using a fluorescence detector.

Ac-Trp-^{D}Leu-Ala-^{D}Leu-Ala-^{D}Leu-Trp-CONHMe, 1

Peptide **1** was synthesized manually on 1.08 mequiv Merrifield (Advanced ChemTech) resin using Boc-protected amino acids. Boc deprotections were carried out using a mixture of TFA : phenol : water : ethanedithiol : triisopropylsilane (80.1 : 7.3 : 7.3 : 3.3 : 1.9) by bubbling with nitrogen for 1 h. The resin was then rinsed three times with 20 ml portions of dichloromethane followed by air drying. Peptide couplings were carried out by adding, in order, 10 ml DMF (dried over molecular sieves), 3 equiv of Boc-protected amino acid, 3 equiv of HBTU and 6 equiv of diisopropylethylamine. The sides of the vessel were then rinsed with an additional 10 ml dry DMF, making sure that all resin beads were in solution. This mixture was bubbled with nitrogen for 3 h or until a small sample of beads produced a negative Kaiser test [8]. The solvent was then removed and the resin rinsed three times with 20 ml portions each of DMF, dichloromethane and methanol. The resin was then air dried.

After the final Boc deprotection step, 20 ml of dry DMF was added to the dry resin followed by 10 equiv of DIPEA and 20 equiv acetic anhydride and nitrogen was bubbled through the mixture for 15 min or until a negative Kaiser test resulted. The solvent was then removed, and rinsed three times with 20 ml portions each of DMF, dichloromethane and methanol. The resin was then air dried.

The peptide was cleaved from the beads by aminolysis. The resin was placed in an airtight round bottom flask and stirred 24–72 h at room temperature with a 2.0 M solution of methylamine in THF. The peptide was collected by rinsing the resin with THF through a filter. The filtrate was concentrated *in vacuo* to yield 1.185 g crude peptide. Purification by HPLC (0–100% acetonitrile/0.1% aqueous TFA, 30 min ramp, 8 ml/min, 254 nm; elution time = 28.5 min) in 50 mg aliquots gave **1** as a white solid in 51% yield.

(1): m.p. 171°–173 °C (decomposed) (white solid from acetonitrile/0.1% aqueous TFA solution); R_f 0.40 (10% methanol/chloroform); IR (KBr plate) 3281(b), 2957, 1627, 1541, 1458, 1236, 1025, 744 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 10.83 (s, 1H), 10.78 (s, 1H), 8.26 (m, 3H), 7.98 (m, 3H), 7.91 (d, $J = 7.8$ Hz, 1H), 7.87 (d, $J = 4.6$ Hz, 1H), 7.56 (t, $J = 6.6$ Hz, 2H), 7.31 (t, $J = 6.7$ Hz, 2H), 7.13 (s, 1H), 7.09 (s, 1H), 7.04 (q, $J = 6.7$ Hz, 2H), 6.96 (q, $J = 7.2$ Hz, 2H), 4.50 (q, $J = 7.2$ Hz, 1H), 4.41 (m, 1H), 4.23 (m, 3H), 4.11 (m, 2H), 3.06 (m, 2H), 2.92 (m, 2H), 2.58 (d, $J = 4.3$ Hz, 3H), 1.81 (s, 3H), 1.51 (m, 1H), 1.43 (t, $J = 6.9$ Hz, 1H), 1.32 (t, $J = 7.2$ Hz, 1H), 1.19 (m, 12H), 0.80 (d, $J = 6.3$ Hz, 3H), 0.77 (d, $J = 6.6$ Hz, 3H), 0.73 (d, $J = 6.4$ Hz, 3H), 0.71 (d, $J = 7.1$ Hz, 3H), 0.70 (d, $J = 6.9$ Hz, 3H), 0.60 (d, $J = 6.4$ Hz, 3H); ^{13}C NMR (CD_3OD, 125 MHz) δ 175.61, 175.41, 175.17, 175.05, 174.76, 174.67, 174.51, 173.19, 138.09, 138.06, 128.61, 128.57, 124.64, 124.54, 122.45, 122.37, 119.94, 119.77, 119.34, 119.31, 112.34, 112.26, 111.23, 110.28, 57.01, 55.87, 53.76, 53.61, 53.39, 51.45, 50.96, 41.25, 41.14, 40.94, 28.72, 28.54, 26.51, 25.89, 25.57, 24.96, 23.47, 23.15, 22.50, 22.10, 21.65, 21.36, 17.75, 17.23, 9.19; HRMS calcd. for $C_{49}H_{70}N_{10}O_8$: 926.5378; found: 927.5451 $[M + H]^+$.

Ac-Trp-^{D}Leu-Ala-^{D}Leu-Ala-^{D}Leu-Trp-CONHMe, 2

To a 50 ml receiving flask containing heptapeptide **1** (0.108 g, 0.117 mmol) was added 25 ml neat TFA and stirred, capped, at room temperature for 36 h. The TFA was removed *in vacuo* and the residual material dissolved in 75 ml of ethyl acetate and extracted three times with 20 ml 1 N NaOH. The organic layer was dried with anhydrous $MgSO_4$, filtered and concentrated *in vacuo*.

The resulting white solid (80 mg, 0.086 mmol) was placed in a 25 ml receiving flask under a nitrogen atmosphere with stirring in 2.5 ml of dioxane (dried over molecular sieves). To this flask was added 21 mg (0.091 mmol) of DDQ in one portion and allowed to stir at room temperature for 45 min. The dioxane was then removed *in vacuo* and the resulting material taken up in 75 ml of ethyl acetate and extracted four times with 25 ml portions of saturated sodium bicarbonate solution. The combined organic layers were then dried with anhydrous Mg_2SO_4, filtered and concentrated *in vacuo*. The resulting white solid (78 mg) was purified by preparative HPLC (50%–60% acetonitrile/0.1% aqueous TFA, 8 ml/min, 30 min ramp, 280 nm; elution time = 16.6 min) to give 53 mg (49%) of **2.**

Ditryptophan, **(2)**: m.p. 174°–176°C (decomposed) (white powder from acetonitrile/0.1% TFA buffer); R_f 0.40 (10% methanol/chloroform); IR (KBr plate) 3285(b), 2958, 1648, 1542, 1450, 1339, 1203, 1025, 746 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz, 80°C) δ 10.93 (s, 1H), 10.90 (s, 1H), 8.11 (d, $J = 7.3$ Hz, 1H), 8.04 (d, $J = 5.9$ Hz, 1H), 7.82 (d, $J = 8.3$ Hz, 2H), 7.78 (d, $J = 7.9$ Hz, 1H), 7.72 (d, $J = 7.5$ Hz, 2H), 7.60 (d, $J = 5.5$ Hz, 1H), 7.53 (d, $J = 4.2$ Hz, 1H), 7.32 (d, $J = 8.0$ Hz, 1H), 7.26 (d, $J = 8.0$ Hz, 2H), 7.02 (m, 4H), 4.83 (bs, 1H), 4.65 (m, 1H), 4.27 (m, 2H), 4.11 (m, 3H), 3.31 (m, 2H), 3.04 (m, 2H), 2.42 (d, $J = 4.2$ Hz, 3H), 1.48 (s, 3H), 1.44 (m, 5H), 1.22 (m, 2H), 1.16 (d, $J = 7.1$ Hz, 3H), 1.14 (d, $J = 7.6$ Hz, 3H), 1.07 (m, 2H), 0.78 (m, 12H), 0.67 (d, $J = 6.5$ Hz, 3H), 0.63 (d, $J = 6.5$ Hz, 3H); ^{13}C (DMSO-d_6, 125 MHz, 80°C) δ 171.55, 171.35, 171.25, 171.18, 171.15, 170.85, 170.79, 169.04, 135.86, 128.17, 128.01, 127.58, 127.53, 120.77, 120.68, 118.74, 118.45, 118.16, 110.61, 110.24, 109.99, 55.08, 54.29, 51.98, 51.63, 51.21, 50.49, 48.99, 48.96, 47.68, 28.46, 28.36, 28.30, 24.83, 23.91, 23.61, 23.00, 22.78, 22.65, 22.25, 21.32, 21.15, 20.99, 17.78, 16.74: HRMS calcd. for $C_{49}H_{68}N_{10}O_8$: 924.5221; found: 925.5316 $[M + H]^+$.

HCO-Val-Gly-Ala-^{D}Leu-Ala-^{D}Val-Val-^{D}Val-Trp-^{D}Leu-Phe-^{D}Leu-Phe-^{D}Leu-Trp-CONHMe, 3

Peptide **3** was prepared manually on Fmoc-Trp-Wang resin (0.40–0.60 mequiv/g, Advanced ChemTech). Couplings and deprotections were carried out with freshly distilled DMF as solvent. Fmoc deprotections were carried out using enough 20% piperidine/DMF mixture to cover the resin with nitrogen agitation. The resin was then rinsed three times with 20 ml portions each of DMF and dichloromethane followed by N_2 and vacuum drying. Amino acid couplings were carried by adding, in order, 20 ml DMF, 3 equiv of Fmoc protected amino acid, 0.3 equiv of HOBt, 3 equiv HBTU and 3 equiv of DIPEA. The sides of the vessel were then rinsed with an additional 20 ml DMF, making sure that all resin beads were covered by DMF. This mixture was bubbled with nitrogen for 1 h. The solvent was removed and the resin rinsed three times with 20 ml portions each of DMF and dichloromethane. The resin was then dried with N_2 and vacuum.

After the final Fmoc deprotection step, *N*-formyl valine was coupled according to the above peptide coupling procedure, except that the coupling time was shortened to 30 min. To cleave the peptide from the resin, the dry resin beads were placed in a 250 ml flask and 50 ml 2.0 M methylamine/THF was added. The flask tightly capped and the reaction mixture was stirred at room temperature for 12 h. The beads were collected by filtration and rinsed with 100 ml THF followed by 100 ml DMF. The filtrate was evaporated until only DMF remained. The peptide was precipitated from solution with water and collected by filtration to give 1.098 g (31%) of a white solid. The resin was resubjected to cleavage conditions for 96 h to provide an additional 1.650 g (77% overall) of white solid. The peptide was purified by preparative HPLC in 50 mg batches (85% MeOH–50 mM AcOH/Et_3N, pH 6, isocratic 21 min then 5 min ramp to 100% MeOH, 15 ml/min, 280 nm; elution time = 26–36 min) to give 41 mg (82%) of pentadecapeptide **3**.

Pentadecapeptide, **(3)**: m.p. 266–268°C (white solid from methanol); R_f 0.35 (10% methanol/chloroform); IR (KBr plate) 3278 (b), 3065, 2960, 2922, 1636, 1541 cm^{-1}, ^1H NMR (DMSO-d_6, 400 MHz) δ 10.78 (s, 1H), 10.72 (s, 1H), 8.37 (m, 2H), 8.31 (m, 2H), 8.24 (d, $J = 8.4$ Hz, 1H), 8.14 (m, 3H), 8.05 (s, 1H), 8.00 (t, $J = 6.4$ Hz, 2H), 7.94 (m, 4H), 7.78 (d, $J = 8.8$, 1H), 7.74 (d, $J = 8.8$ Hz, 1H), 7.57 (d, $J = 8.4$ Hz, 1H), 7.55 (d, $J = 9.6$ Hz, 1H), 7.29 (t, $J = 7.8$ Hz, 2H), 7.15 (m, 8H), 7.14 (m, 2H), 7.09 (m, 2H), 7.03 (m, 2H), 6.94 (m, 2H), 4.56 (m, 3H), 4.44 (m, 1H), 4.30 (m, 3H), 4.24 (m, 7H), 3.71 (m, 2H), 3.18 (dd, $J = 12.3, 4.3$ Hz, 1H), 3.10 (dd, $J = 13.3, 4.7$ Hz, 1H), 3.01 (m, 2H), 2.90 (m, 2H), 2.73 (m, 2H), 2.60 (d, $J = 4.4$ Hz, 3H), 1.99 (m, 3H), 1.78 (m, 1H), 1.55 (m, 1H), 1.45 (m, 2H), 1.20 (m, 17H), 0.82 (m, 25H), 0.69 (d, $J = 6.4$ Hz, 3H), 0.61 (m, 18H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ

172.3, 172.2, 171.8, 171.73, 171.65, 171.2, 171.09, 171.06, 171.00, 170.98, 170.73, 170.70, 168.5, 161.2, 137.6, 136.1, 129.3, 129.2, 127.9, 127.13, 127.11, 126.1, 123.8, 123.7, 120.7, 118.4, 118.3, 118.14, 118.07, 111.2, 110.3, 109.6, 78.0, 69.5, 57.7, 57.6, 57.4, 56.3, 54.15, 54.09, 53.73, 53.66, 51.40, 51.37, 51.29, 51.2, 48.6, 48.5, 41.8, 40.9, 40.6, 38.1, 37.7, 30.8, 30.6, 30.4, 28.5, 27.9, 27.6, 25.7, 24.3, 23.7, 23.6, 23.0, 22.7, 22.65, 22.62, 21.93, 21.88, 21.8, 21.2, 20.6, 19.33, 19.28, 19.21, 19.0, 18.4, 18.2, 17.8, 17.7, 17.6, 17.5, 17.0; LRMS calcd. for $C_{94}H_{136}N_{18}O_{16}$: 1797 $[M + Na]^+$; found: 1797 $[M + Na]^+$.

HCO-Val-Gly-Ala-^{D}Leu-Ala-^{D}Val-Val-^{D}Val-Trp-^{D}Leu-Phe-^{D}Leu-Phe-^{D}Leu-Trp-CONHMe, 4

Pentadecapeptide **3** (0.200 g) was placed in a 250 ml round bottom flask and stirred with 112 ml neat trifluoroacetic acid (0.001 M) under an N_2 environment with a condensing column for 84 h. The TFA was evaporated *in vacuo* to give a purple solid. This solid was stirred in 10 ml refluxing THF for 1.5 h producing a gelatinous slurry. 0.025 mg DDQ was then added in one portion. After stirring for 30 min the reaction was removed from heat and the THF evaporated *in vacuo*. The resulting yellow solid was stirred with 100 ml ethyl acetate for 5 min to remove residual DDQ and dihydroquinone. The EtOAc solution was filtered to collect the insoluble bisindole. The collected brown solid (148 mg, 74%) contained 2% of peptide **3** and 98% of ditryptophan **4** (by NMR comparison of the indole N-H peak integrations). A methanol-chloroform solution fluoresces light blue when subjected to long wave UV light.

Ditryptophan, **(4)**: m.p. 296–300 °C (brown solid from methanol-chloroform); R_f 0.43 (10% methanol/chloroform); IR (KBr plate) 3405 (b), 3288 (b), 3059, 2960, 1638, 1540 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz, 340 K) δ 10.89 (s, 1H), 10.87 (s, 1H), 8.12 (d, $J = 8.5$ Hz, 1H), 8.08 (s, 1H), 7.98 (m, 3H), 7.92 (d, $J = 8.2$ Hz, 1H), 7.88 (d, $J = 8.7$ Hz, 1H), 7.80 (d, $J = 7.9$ Hz, 1H), 7.69 (m, 5H), 7.61 (d, $J = 8.5$ Hz, 1H), 7.58 (d, $J = 6.1$ Hz, 1H), 7.49 (d, $J = 8.7$ Hz, 1H), 7.41 (m, 3H), 7.29 (t, $J = 7.9$ Hz, 2H), 7.15 (m, 8H), 7.06 (m, 4H), 6.98 (t, $J = 7.4$ Hz, 2H), 4.84 (bs, 1H), 4.72 (m, 1H), 4.29 (m, 10H), 4.11 (dd, $J = 8.4, 5.5$ Hz, 1H), 3.95 (q, $J = 6.9$ Hz, 1H), 3.73 (m, 2H), 3.34 (dd, $J = 14.5, 5.5$ Hz, 1H), 3.28 (dd, $J = 14.3, 6.0$ Hz, 1H), 3.10 (m, 3H), 2.89 (dd, $J = 13.5, 6.6$ Hz, 1H), 2.81 (dd, $J = 13.5, 7.0$ Hz, 1H), 2.66 (dd, $J = 13.8, 10.3$ Hz, 1H), 2.46 (d, $J =$ 4.6 Hz, 3H), 2.03 (m, 3H), 1.73 (m, 1H), 1.60 (m, 1H), 1.51 (m, 2H), 1.28 (m, 3H), 1.23 (d, $J = 3.4$ Hz, 3H), 1.22 (d, $J = 3.4$ Hz, 3H), 1.19 (m, 2H), 1.07 (m, 2H), 0.99 (m, 1H), 0.85 (m, 25H), 0.74 (d, $J = 6.1$ Hz, 3H), 0.71 (d, $J = 5.6$ Hz, 3H), 0.66 (d, $J = 3.2$ Hz, 3H), 0.65 (d, $J = 3.3$ Hz, 3H), 0.62 (s, 3H), 0.60 (s, 3H), 0.54 (d, $J = 6.7$ Hz, 3H), 0.40 (d, 6.6 Hz, 3H); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 172.2, 171.8, 171.6, 171.1, 170.8, 170.5, 170.3, 168.5, 161.3, 161.2, 138.0, 136.1, 136.0, 129.2, 129.1, 128.4, 127.9, 127.8, 126.4, 126.1, 121.4, 119.3, 118.8, 118.6, 111.1, 110.9, 110.6, 59.8, 57.3, 57.2, 56.4, 54.8, 53.4, 51.3, 50.3, 48.7, 48.5, 41.9, 40.6, 37.6, 31.2, 31.0, 30.4, 30.0, 28.7, 25.7, 24.2, 24.0, 23.5, 23.3, 23.0, 22.6, 22.4, 22.2, 21.8, 21.5, 21.4, 21.2, 20.8, 19.4, 19.2, 18.5, 18.3, 18.2, 17.9, 17.8, 17.7, 17.6, 17.4, 16.4, 14.1; LRMS calcd. for $C_{94}H_{134}N_{18}O_{16}$: 1772 $[M]^+$; found: 1772 $[M]^+$ (100), 1794 $[M + Na]^+$ (10).

General Procedure for Gramicidin Analog Incorporation into LPC Micelle

To 1.0 ml of a 1.13 mM solution of LPC micelle (prepared by sonication of LPC in deionized water for 5 min), was added an excess of peptide (3–10 mg) and sonicated for 12–24 h. The sample was then centrifuged at 14 000 rpm for 5 min and the supernatant poured off. The approximate peptide incorporation was calculated by an ultraviolet A_{280} comparison to the peptide dissolved in methanol. The solution was then diluted with additional 1.13 mM LPC solution until the A_{190} was approximately 1.0. This solution was allowed to sit at room temperature for 7 days to ensure full equilibration.

RESULTS

Photochemical Formation of Ditryptophan Crosslinks

An authentic ditryptophan standard was prepared by alkaline hydrolysis [9] of $[AcTrp^*CO_2Me]_2$ **6** (* = ditryptophan). Both tryptophan and ditryptophan are stable to alkaline hydrolysis but the stereogenic centers undergo epimerization to give a mixture of meso and racemic isomers that appear as two peaks with retention times around 15 min in the HPLC chromatogram. Tryptophan does not form ditryptophans under alkaline hydrolysis conditions. The presence of these two peaks,

in addition to retention time and chromophoric properties, are diagnostic for the presence of ditryptophan.

To test for ditryptophan formation under photooxidative conditions, oxygen was bubbled through a solution of poly-(D/L)-tryptophan (4000–5700 MW) and 10 mol% rose bengal in methanol (at or below 5 °C) while irradiated with a 500 W tungsten-halogen lamp. After 12 h, the peroxides were quenched with dimethylsulfide and the reaction products were then hydrolysed using 4.2 N NaOH under argon. A large number of UV active and fluorescent compounds were formed in the reaction but free ditryptophan was not detectable by TLC. The reaction mixture was subjected to analytical HPLC and monitored by fluorescence. Free ditryptophan (meso and racemate) was easily detected as a pair of peaks with retention times around 15 min (Figure 2a). The identity of the peaks was confirmed by coinjection with an authentic sample of ditryptophan (Figure 2b). The much larger peaks in the chromatogram could not be assigned.

Formation of Ditryptophan Crosslinks Through a Two-Step Reaction Sequence

Before attempting to form a ditryptophan in a gramicidin analog, we first carried out crosslinking studies on a smaller heptapeptide **1**. When the tryptophan side chains of peptide **1** were crosslinked, the 25-membered macrocyclic ring **2** was generated in a satisfying 49% yield (Figure 3). With confidence in the efficiency of the process, we next crosslinked the tryptophan sidechains in gramicidin analog **3** and were delighted to obtain an even higher yield in the crosslinking process (74%). Unfortunately,

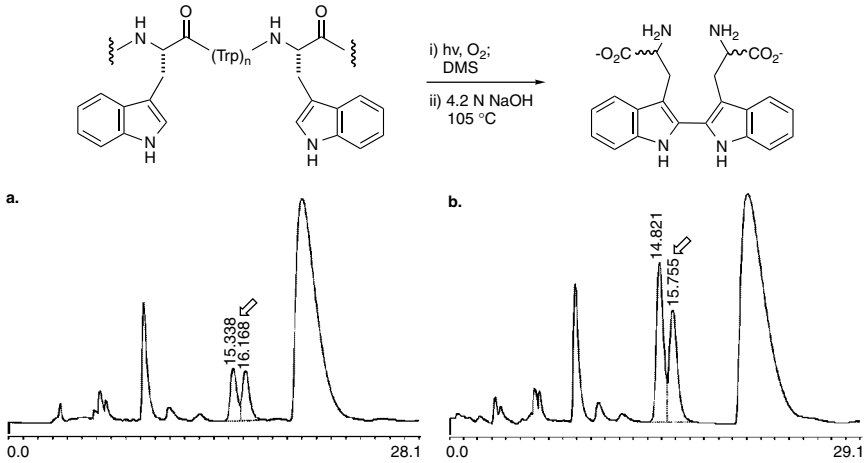

Figure 2 Photooxidation of $[(D/L)\text{-Trp}]_n$ and hydrolysis generates free ditryptophan. (a) Fluorescence HPLC chromatogram of the crude reaction mixture. (b) Fluorescence HPLC chromatogram of coinjection with an authentic ditryptophan standard. HPLC conditions: 25% methanol/H_2O, isocratic, 1 ml/min, excitation: 342 nm, emission: 389 nm.

1 Ac-**Trp**-^{D}Leu-Ala-^{D}Leu-Ala-^{D}Leu-**Trp**-CONHMe

(45%, 2 steps) i) CF_3CO_2H, rt, 36 h; ii) DDQ, dioxane, rt, 45 min

2 Ac-**Trp**-^{D}Leu-Ala-^{D}Leu-Ala-^{D}Leu-**Trp**-CONHMe

3 HCO-Val-Gly-Ala-^{D}Leu-Ala-^{D}Val-Val-^{D}Val-**Trp**-^{D}Leu-Phe-^{D}Leu-Phe-^{D}Leu-**Trp**-CONHMe

(79%, 2 steps) i) CF_3CO_2H, rt, 84 h; ii) DDQ, dioxane, 64 °C, 30 min

4 HCO-Val-Gly-Ala-^{D}Leu-Ala-^{D}Val-Val-^{D}Val-**Trp**-^{D}Leu-Phe-^{D}Leu-Phe-^{D}Leu-**Trp**-CONHMe

Figure 3 Ditryptophan crosslinking of a model peptide **1** and gramicidin mutant **3**.

crosslinked gramicidin **4** had low solubility in aqueous solution and organic solvents. Even in DMSO-d_6, aggregation was apparent above 5 mM, but as expected, there was no evidence for a single defined conformation.

CD Studies of a Model Ditryptophan with a Known Biaryl Configuration

Two ditryptophan molecules were studied using CD (Figure 4): an unconstrained ditryptophan with free rotation about the aryl-aryl bond (**6**) and a constrained ditryptophan with a known chiral biaryl configuration (**5**). The CD spectra were recorded at 55 μM in methanol at 25 °C. The unstructured ditryptophan exhibits weak signals, whereas the constrained ditryptophan with the biaryl locked in a *P* configuration, exhibits a large positive Cotton effect centered at 230 nm with a maximum around 245 nm and a minimum at 225 nm.

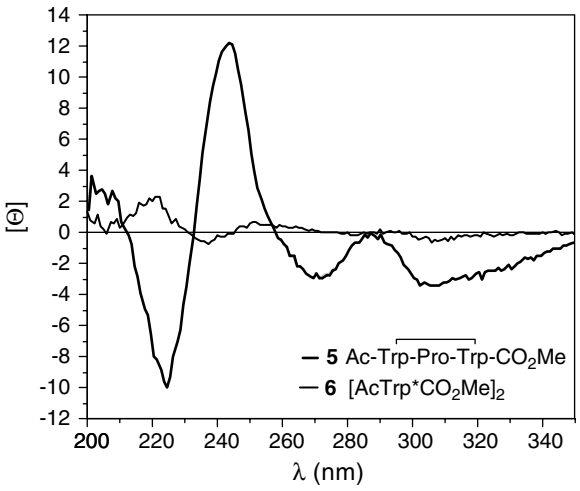

Figure 4 CD spectra of a locked *P* ditryptophan **5** and a freely rotatable ditryptophan **6** at 55 μM in methanol. The atom symbols and bond orders were omitted for clarity.

While the region below 240 nm is complicated by contributions from both the amides and the peptide backbone, the region above 240 nm is dominated by the biaryl chromophore. Like the *P* ditryptophan **5**, the crosslinked gramicidin analog **4** has negative peaks at 305 nm and 270 nm and a strong positive peak around 240 nm (Figure 5a). In micelles, gramicidin adopts an ion channel conformation, so a sample of gramicidin analog **4** was prepared in lysophosphatidylcholine (LPC) micelles according to the method of Massoti (Figure 5b) [10].

Biological Activity

Gramicidin analogs **3** and **4** were tested for growth inhibition against *S. aureus, S. epidermidis, E. faecalis, S. pyogenes, H. influenzae, E. coli, K. pneumoniae* and *P. aeruginosa* using the agar dilution method up to concentrations of 128 μg/ml. The stock solutions were prepared in 1 : 1 DMSO/H_2O at 2.56 mg/ml and serially diluted with equal volumes of water. Peptide **3** was not entirely soluble above 32 μg/ml. The only susceptible organism was *S. pyogenes* and in this cell line the GmB analog **3** (without a ditryptophan) inhibited growth with an

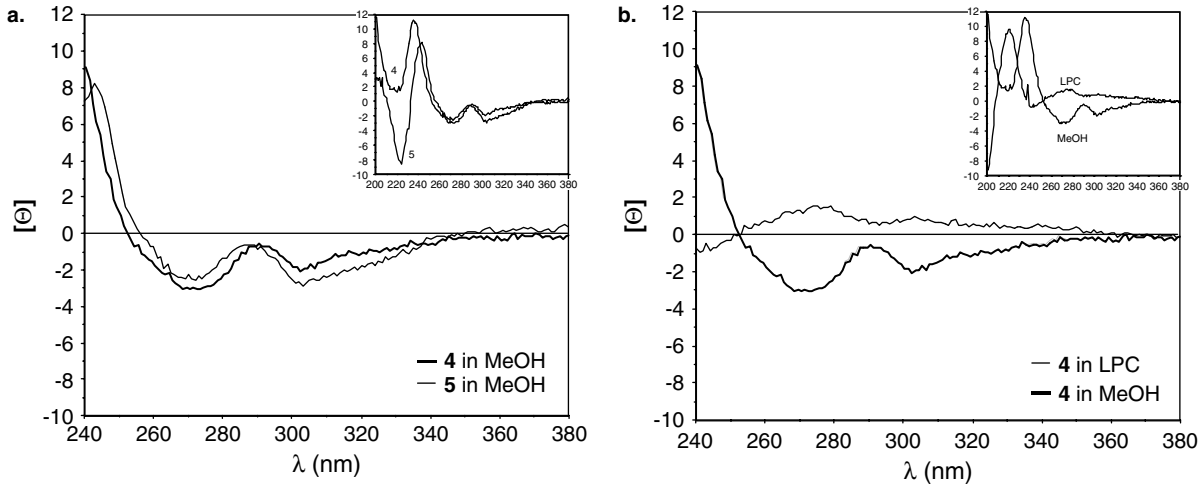

Figure 5 (a) CD spectra of ditryptophan cross-linked GmB **4** and locked *P* ditryptophan **5** in methanol. (b) CD spectra of GmB **4** in LPC and MeOH.

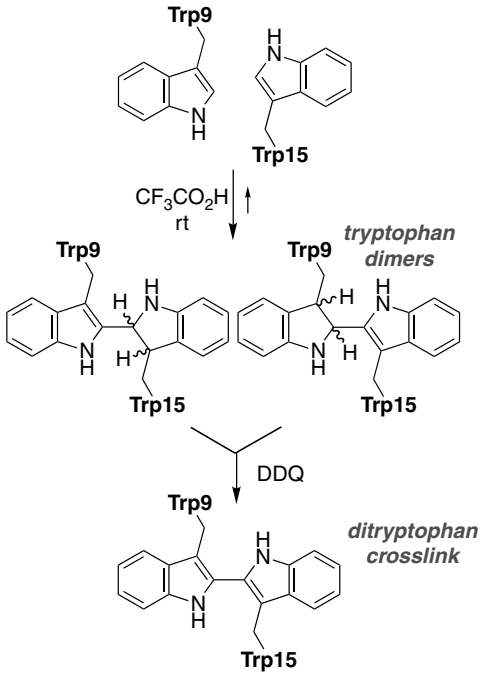

Figure 6 Two step process for ditryptophan crosslink formation.

MIC of 4 μg/ml whereas the crosslinked analog **4** inhibited growth with an MIC of 128 μg/ml.

DISCUSSION

In simple peptides, the two step sequence of Mannich crosslinking and oxidation is an effective chemical method for generating ditryptophan crosslinks (Figure 6). In our experience, the best conditions for Mannich crosslinking involve stirring the peptide in neat trifluoroacetic acid to generate an equilibrium mixture of isomeric tryptophan dimers. The diastereomeric crosslinks are made permanent and symmetrical by convergent oxidation with 2,3-dichloro-5,6-dicyanoquinone (DDQ). Unfortunately there is no way to selectively crosslink only two out of four tryptophans in a peptide. As expected, an attempt to form a ditryptophan crosslink in gramicidin A led to a large number of fluorescent products, probably consisting of oligomers and various regioisomers.

To overcome the lack of regiocontrol we designed a gramicidin model (**3**) in which Trp11 and Trp13 of GmA were replaced with Phe residues that are incapable of crosslinking. This design change has implications for biological activity. In previous studies of gramicidin analogs, when Trp11 and Trp13 of GmA were individually replaced with Phe, the single channel conductance was reduced to 60% and 78%, respectively, of the conductance of GmA [11]. When all four Trp residues were replaced by Phe, the single channel conductance was reduced to 5%–7% of the conductance of GmA [12]. It was presumed that replacing only Trp11 and Trp13 with Phe residues, leaving Trp9 and Trp15, would change the biological activity by less than an order of magnitude. To prevent *N* to *O* acyl migration in trifluoroacetic acid [13] we chose to add an *N*-methylcarboxamide terminus instead of the *N*-(2-hydroxyethyl)carboxamide; similar carboxamide derivatives of gramicidins A and C have activity equivalent to the wild type peptide [14]. Thus, the peptide chosen for crosslinking and study is best described as a W13F mutant of gramicidin B with an *N*-methylcarboxamide terminus.

Macrocyclic ring formation is often challenging in organic synthesis, especially when carbon–carbon bonds are formed. In irreversible reactions, high dilution can be used to favor cyclization over oligomerization. However, the advantage of dilution is less predictable in an equilibrium reaction, especially when the stability of the macrocyclic ring is difficult to predict. Tryptophan crosslinking was highly efficient for both the heptapeptide model **1** and the gramicidin analog **3**, leading to ditryptophan crosslinks in 49% and 74% yield, respectively. These ditryptophan yields are higher than those previously obtained with dipeptides, tripeptides and tetrapeptides [1]. In fact, the yields are comparable to macrocyclizations that generate amide bonds.

The tryptophan sidechains of gramicidin A in micellar solutions have previously been shown to be highly susceptible to UV light and air [15]. The 3-hydroperoxytryptophan intermediates expected from Trp photooxidation should be capable of forming ditryptophan crosslinks by a mechanism similar to that of the two-step chemical method (Figure 7) [16]. To test this hypothesis we subjected an oligomer of tryptophan to sensitized photooxidation and then hydrolysed the peptide bonds using alkaline hydrolysis conditions. Many products were formed, many of which were fluorescent. The presence of ditryptophan in the hydrolysis mixture was demonstrated using analytical HPLC and fluorescence detection. Ditryptophans hydrolysed under alkaline conditions give rise to two isomers: meso and racemate. Under carefully

selected HPLC conditions, the isomers are separable, giving rise to a two-peak 'signature' that confirms the presence of ditryptophan in an alkaline hydrolysis reaction. Based on TLC analysis, it is unlikely that ditrypophan accounts for more than 1% of the product mixture, but the actual quantity may be far lower. Thus, ditryptophan crosslink formation occurs under photochemical conditions, but yields are too low to be used for general preparative purposes. Photochemical crosslinking of lens proteins has been hypothesized as a cause of cataract formation [17]; ditryptophan crosslinks could be a contributing factor in cataractogenesis.

Biaryls can adopt atropisomeric conformations with axial chirality. The 'minus' and 'plus' conformations are designated a*R* and a*S*, or *M* and *P*, respectively, depending on the system of nomenclature [18]. CD spectra offer diagnostic information regarding the configuration of biaryl chromophores with polar ortho substituents. Biaryl chromophores with the *P* configuration tend to give positive Cotton effects, whereas biaryl chromophores with the *M* configuration tend to give negative Cotton effects. For example the CD spectrum of a*S*-2,2′-dimethoxy-1,1′binaphthyl exhibits a strong positive Cotton effect centered around 230 nm, whereas the CD spectrum of a*R*-1,1′-diamino-3,3′-dimethyl-2,2′-biphenyl exhibits a strong negative Cotton effect centered around 295 nm. However, unlike biphenyl and binaphthyl molecules with polar substituents, ditryptophans possess polar atoms within the aromatic rings; so the correlation between configuration and Cotton effect was unclear.

Generally, 2,2′-biindoles interconvert rapidly, even on the ^{1}H NMR timescale, however, intramolecular ditryptophan crosslinks can be constrained to a single biaryl configuration by the peptide backbone. For example, the ditryptophan peptides Trp*-Xxx-Trp* adopt a γ-turn with a hydrogen bond between in the i and $i+1$ positions (Figure 8); the γ-turn conformation is retained even in strongly disruptive solvents like DMSO [2]. The ditryptophan chromophore in tripeptide **5** prefers to adopt a chiral *P* configuration. The chiral non-planar conformation of the ditryptophan is consistent with x-ray structures [19] which suggest an optimal interplanar angle around ±30°. Ditryptophans do not always lock peptides in single conformations. Dipeptides and tetrapeptides containing ditryptophan crosslinks often exist in two or more conformations that interconvert slowly at room temperature relative to the ^{1}H NMR timescale.

It was expected that the unconstrained ditryptophan **6** would not give strong signals in the CD spectrum since there is no obvious bias toward the *P* or *M* biaryl rotamers. In contrast, we expected

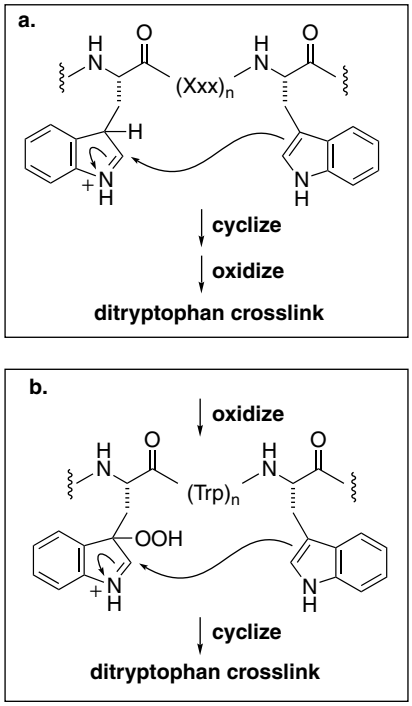

Figure 7 Ditryptophan formation via two types of processes: (a) Acid-catalysed cyclization and oxidation. (b) Photooxidation and cyclization.

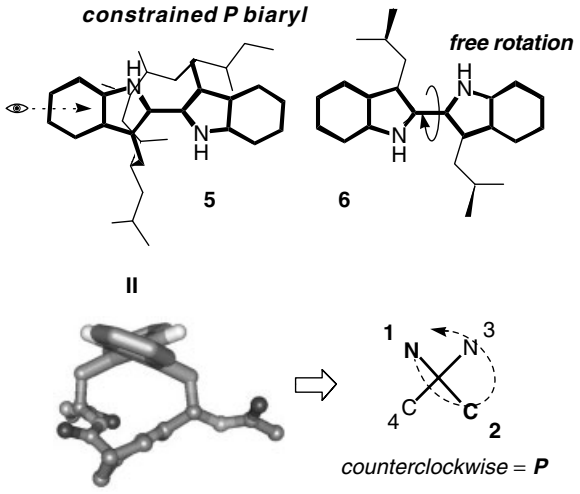

Figure 8 Two types of ditryptophan peptides: a ditryptophan locked in a *P* configuration **5** and a freely rotating ditryptophan **6**.

that the ditryptophan chromophore in peptide **5** would contribute strongly to the CD spectrum and it was satisfying to observe a large positive Cotton effect centered at 230 nm. Thus, like other biaryls, the *P* configuration of ditryptophan generates a large positive Cotton effect. It is true that constrained peptides without biaryl chromophores can exhibit strong CD signals in the near UV region, but not with maxima around 240 nm. These studies suggest that ditryptophan chromophores are responsive to, and report on, the biaryl conformation. Unfortunately, ditryptophan chromophores also dominate the near UV region of the CD spectrum, obscuring information about long-range secondary structure.

When the CD spectrum of crosslinked gramicidin **4** was measured in methanol, the CD signals above 240 nm closely match those of the constrained *P* ditryptophan model **5**. The close match suggests that the ditryptophan crosslink in gramicidin analog **4** prefers to adopt a *P* configuration in methanol. Unfortunately, derivative **4** was not sufficiently soluble in CD_3OD to obtain NMR spectra. In LPC micelles the minima and maxima are reversed above 240 nm, suggesting that the ditryptophan prefers the opposite configuration. Since the ditryptophan is covalently linked to the peptide backbone, it is likely that changes in biaryl configuration are correlated to changes in peptide conformation. The fact that gramicidins change conformation going from methanol to lipid environments is well known. However, the ability to use biaryl crosslinks to detect changes in conformation is new and may offer a way to sense conformational changes in peptides or proteins that have received less scrutiny.

While the ditryptophan crosslink in indolicidin did not compromise antibiotic activity, the ditryptophan crosslink in gramicidin analog **4** raised the MIC from 4 μg/ml to 128 μg/ml in *S. pyogenes*. Thus, even though Trp9 and Trp15 seem poised to form a crosslink in the active conformation of gramicidin A, the presence of the ditryptophan crosslink did not improve or even maintain the antibiotic activity.

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