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First Total Synthesis of a Fluorescent Didemnin

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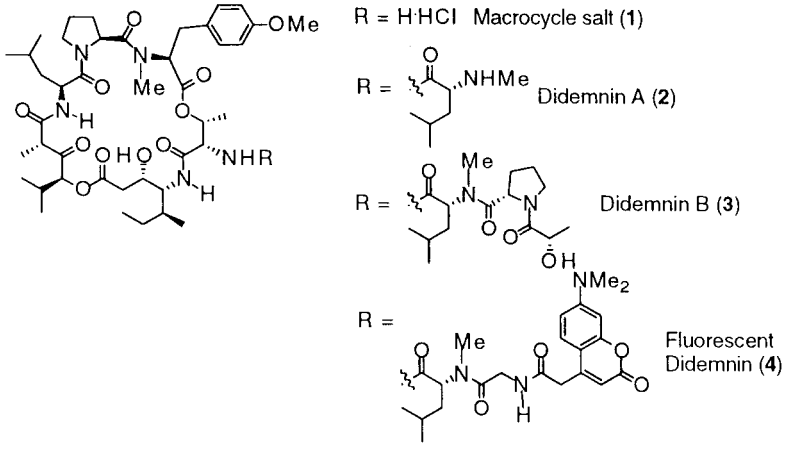
Abstract—A dimethylaminocoumarin fluorophore was linked to didemnin A through a glycine tether. The resultant fluorescent didemnin will be used to study cellular localization as detected by fluorescence microscopy.

The didemnins are a class of macrocyclic depsipeptides that were isolated in 1981 by Rinehart and co-workers from a Caribbean tunicate of the family Didemnidae (*Tridemnum* genus).[1] These marine natural products exhibit a wide variety of biological activities including potent antitumor,[2–4] antiviral,[5] and immunosuppressive[6,7] properties. Most didemnins share a common cyclic depsipeptide core, composed of a tetrapeptide portion and a nonpeptidic unit, and differ only in the side chains attached to the threonine unit. Didemnin A has *N*-methyl-D-leucine attached to the amino group of threonine. *N*-Methyl-D-leucine and the amino acids attached to it will be referred to as the side chains.

Most didemnins are derivatives of didemnin A, in which different amino acid residues are attached to the *N*-methylamino group of D-leucine. Didemnin B, one of the most potent natural members of the didemnin family, contains a L-lactyl-L-prolyl residue (Scheme 1).

Although the mechanism of action of the didemnins has not yet been established, some progress has been made toward disentangling the three activities.[8–10] However, SAR studies suggest that structural modifications within the didemnin framework do not correlate antitumor or antiproliferative activities with inhibition of protein synthesis. For instance, it has been shown with nordidemnins that the stereochemistry of the side chain leucine serves as a switch for antitumor activity, while leaving the protein biosynthesis inhibition essentially unaffected.[11] Comprehension of the mechanism(s) underlying these diverse biological activities would enlarge the understanding of cell biology and provide new tools for pharmaceutical discovery.

An attractive approach to identify the components responsible for the different bioactivities is the use of fluorescent probes. The cellular localization of the fluorescent analogs could be detected by fluorescence microscopy. As an example, fluorescent nordidemnin analogs differing in the



Scheme 1.

Keywords: fluorescence; peptide analog; macrocycle.

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stereochemistry of the D-leucine side chain would provide an opportunity to observe differences in localization factors and correlate these differences with the individual facets of the biological activity profile. In the future, further insight could be obtained by comparison studies among analogs with different bioactivities.

A fluorescent didemnin analog could be prepared by attaching a fluorophore to didemnin A via a glycine tether (**4**). This *N*-acylation is not expected to be detrimental to the biological activity based on the finding that several *N*-acyl derivatives of didemnin A were shown to be more potent than didemnin A and comparable to didemnin B.[12] To this end, 7-dimethylaminocoumarin-4-acetic acid (DACA) was chosen as the first fluorophore for the synthesis of a fluorescent didemnin. This coumarin has been widely employed in biological studies serving as an acceptor chromophore in the estimation of intramolecular distance distributions in bovine pancreatic trypsin inhibitor,[13,14] in the preparation of fluorescent taxol derivatives,[15] in the preparation of fluorescence-labelled oligo[(R)-3-hydroxybutanoic acid] derivatives,[16] and in the preparation of a fluorescent derivative of α-bungarotoxin.[17] It has also seen application in fluorescence in situ hybridization as a method for assessing the severity of prostatic cancer,[18] in the preparation of a conjugate with a polyhalogenated aryl moiety to assay glutathione/glutathione transferase,[19] in the preparation of fluorescent chloramphenicol derivatives,[20] and in fluorescent energy transfer immunoassays.[21]

En route to the fluorescent didemnin, DACA (**5b**) was prepared according to the method of Dey[22] from commercially available and affordable precursors. EDAC coupling with a glycine linker and hydrolysis of the *tert*-butyl ester to give **6b** was followed by BOP-Cl mediated coupling to *N*-methyl leucine methyl ester. Saponification of the methyl ester gave the fluorescent side chain (**7b**). The side chain was coupled with the macrocycle salt using BOP to give the fluorescent didemnin (**4**) (Scheme 2).

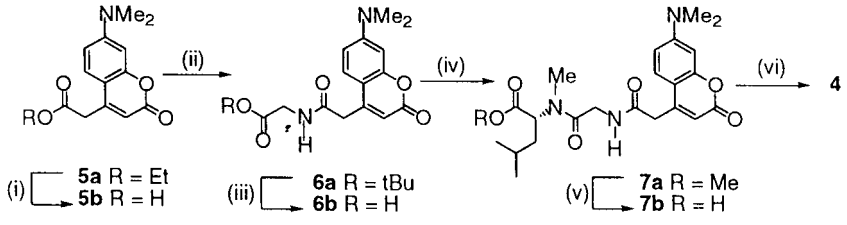
Experimental

General

All solvents were reagent grade and were distilled before use. Tetrahydrofuran (THF) was distilled from sodium and benzophenone. Methylene chloride was distilled from calcium hydride (CaH_2). Anhydrous zinc chloride was purchased from Fisher. 3-Dimethylaminophenol and diethyl 1,3-acetonedicarboxylate were purchased from Aldrich. The fluorescent probe 7-dimethylaminocoumarin-4-acetic acid (DACA) was prepared by a known procedure.[22] Melting points (°C) were determined using a Thomas–Hoover melting point apparatus and are uncorrected. Proton magnetic resonance spectra (^{1}H NMR) and carbon magnetic resonance spectra (^{13}C NMR) were recorded on a Bruker AMX-500 spectrometer operating at 500 MHz. Infrared spectra (IR) were obtained on a Perkin–Elmer Model 281-B spectrometer. Optical rotations were measured with a Perkin–Elmer Model 241 polarimeter. High resolution mass spectra (HRMS) were obtained on a Micromass AutoSpec. Elemental analyses were performed on a Perkin–Elmer 2400 Series II CHNS/O Analyzer at the University of Pennsylvania. Analytical thin-layer chromatography (TLC) was performed on Merk silica gel (60F-254) plates (0.25 mm) precoated with a fluorescent indicator. Visualization was effected with ultraviolet light and phosphomolybdic acid (7% w/v) in 95% ethanol. Flash column chromatography was carried out on E. Merck Silica gel 60 (240–400 mesh) using the solvent systems listed under individual experiments. The macrocycle salt (**1**) was prepared according to a method developed in our laboratory.[23]

Ethyl 7-dimethylaminocoumarin-4-acetate (5a). A mixture of diethyl 1,3-acetonedicarboxylate (10.00 g, 49.5 mmol), *m*-dimethylaminophenol (7.12 g, 51.9 mmol), and zinc chloride (8.56 g, 62.8 mmol) was maintained at reflux in absolute ethanol (25 mL) for 13 h. The reaction mixture was diluted with EtOAc (50 mL), washed with H_2O (25 mL), and the aqueous layer extracted with EtOAc (3×50 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated. The resulting crude solid was recrystallized from absolute ethanol to afford the coumarin (2.64 g, 20%) as orange crystals: mp 131–132°C, lit.[22] mp 133°C; R_f 0.20 acetone/hexane (30:70); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.22 (3H, t, J=7.1 Hz), 3.01 (6H, s), 3.64 (2H, s), 4.15 (2H, q, J=7.2 Hz), 6.02 (1H, s), 6.48 (1H, d, J=2.6 Hz), 6.58 (1H, dd, J^1=2.6 Hz, J^2=8.9 Hz), 7.37 (1H, d, J=8.9 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.02, 38.16, 40.01, 61.49, 98.28, 108.45, 108.93, 110.63, 125.22, 148.39, 152.91, 155.89, 161.65, 169.01; IR (KBr) 2907 (w), 1720 (s), 1600 (s), 1534 (m), 1484 (m), 1427 (m), 1405 (s), 1371 (m), 1330 (m), 1232 (m) cm^{-1}; HRMS (ESI) Calcd for $C_{15}H_{18}NO_4$ (M+H): 276.1236, Found 276.1244. Anal. Calcd for $C_{15}H_{17}NO_4$: C, 65.43; H, 6.23; N, 5.09. Found: C, 65.26; H, 6.36; N, 5.03.

7-Dimethylaminocoumarin-4-acetic acid (5b). To a suspension of **5a** (1.50 g, 5.5 mmol) in THF (10 mL) was added a solution of $LiOH{\cdot}H_2O$ (0.46 g, 11.0 mmol) in H_2O (25 mL). The reaction mixture was stirred for 2.5 h. The resulting solution was washed with ether (10 mL) and acidified to



Scheme 2. (i) LiOH, H_2O, THF (quant.); (ii) Gly-OtBu, EDAC·HCl, DMAP, CH_2Cl_2 (27%); (iii) TFA, CH_2Cl_2 (quant.); (iv) *N*-MeLeu-OMe, BOP-Cl, NMM, CH_2Cl_2 (50%); (v) LiOH, H_2O, THF (quant.); (vi) **1**, BOP, NMM, CH_2Cl_2 (30%)

pH 2. The precipitate formed was collected by filtration. Compound **5** was obtained as yellow crystals: mp 166–167°C, lit.[22] mp 168°C; ^{1}H NMR (500 MHz, $CDCl_3$/DMSO) δ 3.01 (6H, s), 3.62 (2H, s), 6.00 (1H, s), 6.44 (1H, d, J=2.3 Hz), 6.58 (1H, dd, J^1=2.4 Hz, J^2=8.9 Hz), 7.40 (1H, d, J=9.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$/DMSO) δ 38.42, 40.25, 98.34, 108.89, 109.35, 110.67, 125.84, 149.59, 153.22, 156.15, 162.00, 171.33; IR (KBr) 2923 (m), 1690 (s), 1619 (s), 1534 (m), 1404 (m), 1248 (m), 1145 (m), 1055 (m) cm^{-1}; HRMS (ESI) Calcd for $C_{13}H_{14}NO_4$ (M+H): 248.0922. Found: 248.0929.

Fluorescent Gly-DACA *tert*-butyl ester (6a). DACA (**5b**, 0.364 g, 1.47 mmol) was dissolved in freshly distilled CH_2Cl_2 (5 mL) under argon, cooled to 0°C, followed by the addition of EDAC·HCl (0.282 g, 1.47 mmol) and DMAP (0.035 g, 0.29 mmol) to the reaction mixture. After 10 min, glycine *tert*-butyl ester (0.246 g, 1.47 mmol) was added. The reaction was stirred at 0°C for 2 h and then warmed to rt overnight. The reaction was diluted with CH_2Cl_2, washed with 10% HCl (5 mL), water (5 mL) and brine (5 mL) solution. The organic layer was dried ($MgSO_4$), filtered and concentrated. The resulting residue was purified by flash column chromatography eluting with EtOAc:CH_2Cl_2 (50:50) to provide the coupled product as a yellow solid (0.250 g, 42%): mp 178.5–179.5°C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.42 (s, 9H), 3.03 (s, 6H), 3.64 (s, 2H), 3.88 (d, J=5.12 Hz, 2H), 6.05 (s, 1H), 6.48 (brs, 1H), 6.58 (d, J=2.5 Hz, 1H), 6.60 (dd, J^1=2.6 Hz, J^2=8.9 Hz, 1H), 7.45 (d, J=8.9 Hz, 1H); ^{13}C δ 168.5, 167.8, 161.6, 156.0, 153.1, 149.2, 125.6, 110.5, 109.2, 108.3, 98.3, 82.5, 42.3, 40.3, 40.1, 27.9; IR ($CHCl_3$) 3450, 1679, 1618, 1530, 1405, 1370, 1230, 1157 cm^{-1}; HRMS (CI) Calcd for $C_{19}H_{24}N_2O_5$ (M^+): 360.1685, Found 360.1686. Anal. Calcd for $C_{19}H_{24}N_2O_5$: C, 63.30; H, 6.72; N, 7.78. Found: C, 62.99; H, 6.60; N, 7.52.

Dipeptide (+)-(*R*)-*N*-Me-Leu(OMe)-Gly-DACA ester (7a). Gly-DACA-*tert*-butyl ester (0.250 g, 0.69 mmol) was dissolved in CH_2Cl_2 and cooled to 0°C. After 10 min, 10% trifluoroacetic acid (TFA) was added dropwise to the reaction mixture over a period of 10 min. After stirring at rt overnight, the reaction mixture was evaporated to dryness on a rotary evaporator and directly used in the next step. The TFA salt of **6b** (0.033 g, 0.108 mmol) was dissolved in CH_2Cl_2 and cooled to 0°C. BOP-Cl (0.027 g, 0.108 mmol) and NMM (0.012 g, 0.108 mmol) were added to the reaction mixture. After 30 min, *N*-Me-Leu-(OMe) was added, and the reaction was stirred at 0°C overnight. The reaction was diluted with CH_2Cl_2, washed with 10% HCl (5 mL), water (5 mL) and brine (5 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated. The resulting residue was purified by flash column chromatography eluting with EtOAc/CH_2Cl_2 (50:50) to provide the dipeptide as a yellow solid (0.030 g, 63%): mp 102–103°C; $[\alpha]_D^{20}$=+19.08 (c 0.865, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87 (d, J=6.52 Hz, 3H), 0.91 (d, J=6.79 Hz, 3H), 1.40 (m, 1H), 1.69 (m, 2H), 2.85 (s, 3H), 3.01 (s, 6H), 3.63 (s, 2H), 3.67 (s, 3H), 4.06 (m, 2H), 5.22 (dd, J^1=10.6 Hz, J^2=5.2 Hz, 1H), 6.04 (s, 1H), 6.47 (d, J=2.5 Hz, 1H), 6.57 (dd, J^1=2.5 Hz, J^2=8.9 Hz, 1H), 6.76 (brs, 1H), 7.42 (d, J=8.9 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.7, 168.6, 167.8, 161.6, 155.9, 152.9, 149.1, 125.4, 110.5, 109.1, 108.4, 98.3, 54.7, 52.3, 41.8, 40.1, 39.9, 37.1, 30.0, 24.8, 23.1, 21.3 cm^{-1}. HRMS (ESI) Calcd for $C_{23}H_{31}N_3O_6$ (M+Na$^+$): 468.2111, Found 468.2133.

Didemnin A-(*R*)-*N*-Me-Leu-Gly-DACA (4). Dipeptide methyl ester **7a** (0.135 g, 0.304 mmol) was dissolved in THF cooled to 0°C, and $LiOH·H_2O$ (0.025 g, 0.606 mmol) in water (2 mL) was added to the reaction over a period of 5 min. After stirring at 0°C for 30 min, the reaction mixture was warmed to rt. After 1.5 h, the reaction mixture was washed with ether (2×4 mL). The aqueous layer was evaporated to dryness. The crude mixture was dissolved in water (2 mL) and EtOAc (4 mL), cooled to 0°C and acidified to pH 2 (1 N, $KHSO_4$). The separated aqueous layer was washed with EtOAc (2×4 mL). The combined organic layers were dried ($MgSO_4$), filtered and concentrated to dryness and used directly in the next step.

The dipeptide free acid (**7b**, 0.010 g, 0.023 mmol) was dissolved in CH_2Cl_2 cooled to 0°C, followed by the addition of BOP (0.010 g, 0.023 mmol) and NMM (0.010 mL, 0.092 mmol). After stirring for 10 min, the macrocycle amine hydrochloride salt (0.018 g, 0.023 mmol) was added. The reaction was stirred at 0°C for 1 h and stirred at rt overnight. The solution was later treated with brine (1 mL) and extracted with EtOAc (2×2 mL). The organic layer was washed with 10% aqueous HCl (1 mL), water (2×1 mL), dried ($MgSO_4$), filtered, and concentrated. The crude residue was purified by flash chromatography eluting with acetone: hexane (30:70) to obtain the fluorescent didemnin B (**4**) as a yellow solid (0.008 g, 30%). $[\alpha]_D^{20}$=−160.1 (c 0.3, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.73–0.93 (m, 24H), 1.07–1.74 (m, 15H), 2.04 (m, 2H), 2.14 (s, 3H), 2.15 (m, 2H), 2.31 (m, 2H), 2.53 (s, 4H), 2.84 (s, 2H), 3.03 (s, 6H), 3.17 (brd, J=10.4 Hz, 2H), 3.35 (m, 1H), 3.57 (m, 2H), 3.68 (s, 2H), 3.78 (s, 3H), 3.99 (brd, 2H), 4.08–4.12 (m, 3H), 4.55–4.56 (m, 1H), 4.78–4.80 (m, 1H), 4.82–5.01 (m, 2H), 5.13–5.14 (brd, 1H), 6.08 (s, 1H), 6.51 (s, 1H), 6.61 (d, J=9.1 Hz, 1H), 6.83 (d, J=8.54 Hz, 2H), 7.07 (d, J=8.35 Hz, 2H), 7.24–7.41 (m, 3H), 7.51 (d, J=8.90 Hz, 1H), 7.81 (d, J=8.65 Hz, 1H); IR (KBr) 3462, 2953, 2358, 1732, 1658, 1632, 1555, 1538, 1456 cm^{-1}; HRMS (ESI) Calcd for $C_{64}H_{92}N_8O_{16}$ (M+Na$^+$): 1251.6529, Found 1251.6528.

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