A Diketopiperazine Dimer from a Marine-Derived Isolate of *Aspergillus niger*

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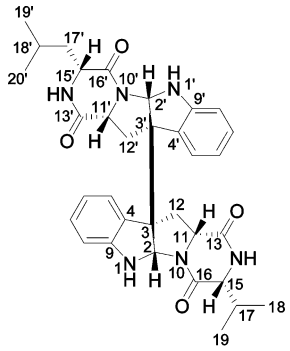
The new diketopiperazine dimer **1**, as well as the known compounds TMC-256A1 (**2**), TMC-256C1 (**3**), and demethylkotanin (**4**), were isolated from a culture of *Aspergillus niger*. The gross structure of **1** was determined by 2D NMR studies and comparison with literature data, and the absolute stereochemistry was elucidated by chiral HPLC analysis of the hydrolysis products.

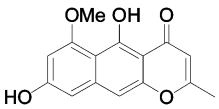
Initiation from most eukaryotic mRNAs occurs by ribosomal recognition of the 5′ cap of the RNA and subsequent scanning for the translational AUG start codon. However, translation is quite different for certain RNA viruses because their mRNAs are not capped. Flaviviruses and picornaviruses have retained a long 5′ nontranslated region (NTR), which has *cis*-acting elements that promote initiation of translation, independent of the uncapped 5′ end of the viral mRNA. The 5′ NTR contains a nucleotide segment that is the internal ribosomal entry site (IRES), which serves as a "landing site" for the host cell's protein synthesis machinery to bind to the highly structured RNA and begin synthesizing the viral polyprotein.[1] Because this mechanism is different from the processes of host RNA translation, it becomes an important molecular target to discover drugs that block virus translation without affecting host cell protein synthesis. In addition, since IRES-driven translation occurs in a number of medically important RNA viruses, this approach offers the potential of identifying an antiviral with broad-spectrum utility.[2]

While screening a library of natural product extracts for inhibitors of IRES, the crude MeOH extract of a marine-derived isolate of *Aspergillus niger* was found to be active. The isolate was cultured on a large scale (500 mL) and extracted with MeOH. This crude MeOH extract was filtered and dried, then subjected to bioassay-guided fractionation to yield the new diketopiperazine dimer (**1**). Also isolated were the known compounds TMC-256A1 (**2**),[3] TMC-256C1 (**3**),[3] and demethylkotanin (**4**),[4] whose structures were solved by comparison of spectroscopic data with the literature. Discussed below is the isolation, structure elucidation, and determination of the absolute stereochemistry of **1**, as well as the biological activities for compounds **1**−**4**.

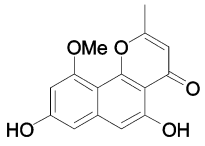
Compound **1** was isolated and showed a positive molecular ion at *m/z* 605.2859 [(M + Na)$^+$] in the HR/ESIMS spectrum indicative of a molecular formula of $C_{33}H_{38}N_6O_4$ [Δ −0.7 mmu] and consistent with 18 double bond equivalents. Analysis of the ^{1}H and ^{13}C NMR data for **1** (Table 1) showed that it was an almost symmetrical molecule consisting of two moieties that could be distinguished only by minor differences in the observed NMR data. Analysis of the 2D NMR data for **1** in CD_3OD (Table 1) allowed for the elucidation of two identical indoline moieties [^{13}C: 171.4, 171.2, 150.5 (×2), 131.7 (×2), 130.8 (×2), 126.0 (×2), 120.5 (×2), 111.0 (×2), 81.8, 81.4, 61.6 (×2), 57.6 (×2), 38.9, 37.9 ppm; ^{1}H: δ 7.44, 7.43, 7.12, 7.10, 6.78, 6.76, 6.65 (×2)]. Also evident were resonances consistent with leucine [^{13}C: 170.9, 56.8, 42.5, 25.5, 23.4, 22.0 ppm; ^{1}H: δ 3.75, 1.63, 1.50, 1.41, 0.88] and valine [^{13}C: 169.5, 64.4, 33.9, 19.7, 18.6 ppm; ^{1}H: δ 3.51, 2.05, 0.88, 0.77]. This accounted for 14 double bond equivalents and all but four exchangeable protons, implying the presence of an additional four rings. Further analysis of gHMBC data identified important



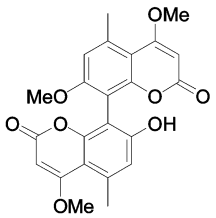
1



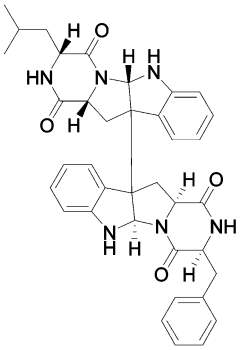
TMC-256A1 (**2**)



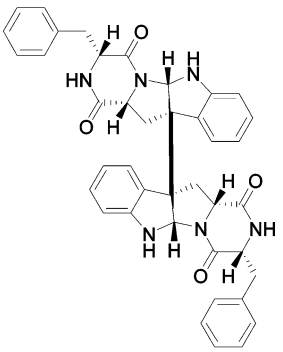
TMC-256C1 (**3**)



demethylkotanin (**4**)



WIN 64745 (**5**)



ent-WIN 64821 (**6**)

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Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHZ) NMR Data for **1** in CD_3OD and d_6-DMSO

	CD_3OD		d_6-DMSO	
no.	^{13}C (δ)	^{1}H (δ, mult, J in Hz)	^{13}C (δ)	^{1}H (δ, mult, J in Hz)
1				6.60[d]
2	81.4	5.10 (s)	78.7	4.87 (br s)
3	61.6		59.9	
4	131.7		131.0	
5	126.0	7.44 (dd, 7.9, 2.4)[a]	124.2	7.36 (d, 7.1)
6	120.5	6.78 (ddd, 7.9, 7.9, 2.4)[b]	117.5	6.63[d]
7	130.8	7.12 (ddd, 7.9, 7.9, 2.4)[c]	128.4	7.02 (ddd, 7.1, 7.1, 2.0)
8	111.0	6.65 (dd, 7.9, 2.4)	108.5	6.59[d]
9	150.5		149.0	
10				
11	57.6	4.24 (dd, 8.9, 8.9)	55.4	4.18 (t, 9.4)
12	38.9	2.58 (dd, 13.9, 8.9)	36.6	2.32 (dd, 14.3, 9.9)
		3.26 (dd, 13.9, 8.9)		3.07 (br s)
13	171.4		168.2	
14				8.24 (d, 4.0)
15	64.4	3.51 (d, 5.8)	62.0	3.36 (dd, 5.7, 4.0)
16	169.5		168.0	
17	33.9	2.05 (m)	31.6	1.95 (m)
18	18.6	0.77 (d, 6.7)	18.0	0.70 (d, 6.4)
19	19.7	0.88 (d, 6.7)	18.5	0.82 (d, 6.4)
1′				6.60[d]
2′	81.8	5.01 (s)	78.7	4.99 (br s)
3′	61.6		59.9	
4′	131.7		131.0	
5′	126.0	7.43 (dd, 7.9, 2.4)[a]	124.2	7.36 (d, 7.1)
6′	120.5	6.76 (ddd, 7.9, 7.9, 2.4)[b]	117.5	6.63[d]
7′	130.8	7.10 (ddd, 7.9, 7.9, 2.4)[c]	128.4	7.02 (ddd, 7.1, 7.1, 2.0)
8′	111.0	6.65 (dd, 7.9, 2.4)	108.5	6.59[d]
9′	150.5		149.0	
10′				
11′	57.6	4.26 (dd, 8.3, 8.3)	55.4	4.12 (t, 9.5)
12′	37.9	2.74 (dd, 14.4, 8.3)	36.0	2.50[e]
		3.26 (dd, 14.4, 8.3)		3.07 (br s)
13′	171.2		168.0	
14′				8.24 (d, 4.0)
15′	56.8	3.75 (dd, 9.5, 5.1)	54.9	3.56 (ddd, 9.4, 4.7, 4.0)
16′	170.9		168.2	
17′	42.5	1.50 (m)	40.7	1.44 (m)
		1.41 (m)		1.26 (m)
18′	25.5	1.63 (m)	23.5	1.58 (m)
19′	23.4	0.88 (d, 6.7)	22.6	0.82 (d, 6.4)
20′	22.0	0.88 (d, 6.7)	21.1	0.81 (d, 6.4)

[a–c] ^{1}H chemical shifts can be interchanged. [d] Overlapping signals. [e] Underneath DMSO signal.

correlations from H-15/15′ to the amide carbonyls at C-13/13′ and C-16/16′, allowing for two partial structures to be readily constructed: one consisting of an indoline moiety connected to leucine, and another of an indoline moiety connected to valine.

In an attempt to position the remaining four exchangeable protons, and hence solve the planar structure of **1**, the NMR data were re-collected in d_6-DMSO (Table 1). The observation of ^{1}H−^{1}H COSY correlations from two exchangeable protons at δ 8.24 to H-15/15′ positioned two of the remaining four exchangeable protons at N-14/14′. While adding further evidence for the connection of indoline moieties to valine and leucine, respectively, it did not allow for unambiguous determination of the planar structure of **1**. However, a survey of the literature pointed at similarities to several known diketopiperazine dimers, including WIN 64821 and WIN 64745 (**5**).[5] Although our NMR data were acquired in a different solvent (d_3-acetonitrile for **5** vs CD_3OD and d_6-DMSO for **1**), the reported chemical shifts were remarkably similar. Attempts to re-collect the data in d_3-acetonitrile failed, as **1** was insoluble in this solvent. The similarity of chemical shifts nevertheless allowed the planar structure of **1** to be proposed as shown.

Table 2. Biological Activities of Compounds **1**−**4** against IRES and Cap-Dependent Translation

compound	IC_{50} IRES (μM)	IC_{50} cap (μM)
1	>100	>100
2	44	80
3	>100	>100
4	>100	>100

The relative stereochemistry of C-2/2′, C-3/3′, C-11/11′, and C-15′ was established as shown by comparison of ^{13}C NMR chemical shifts for **1** with those for **5**[5] and for the synthetically derived *ent*-WIN 64821 (**6**).[6] However, the relative stereochemistry of **1** at C-15 could not be unambiguously assigned using NOESY or ^{13}C NMR data. Thus the absolute stereochemistry at C-15/15′ was determined by hydrolysis and comparison with authentic amino acid standards. A solution of **1** (0.4 mg) in 6 N HCl was stirred for 16 h at 100 °C. The resultant reaction mixture was concentrated and subjected to chiral HPLC analysis. Retention times of the hydrolyzed sample were compared with authentic standards of (D)- and (L)-leucine and (D)- and (L)-valine. This analysis confirmed the presence of (D)-valine and (D)-leucine and established a C-2/2′*S*, C-3/3′*S*, C-11/11′*R*, C-15/C-15′*R* absolute stereochemistry for **1**. It is interesting to note that **1** is of the same enantiomeric series as **6**, but has a substantially different specific rotation value ($[\alpha]_D$ **1**: +132; $[\alpha]_D$ **6**: −200).

There are many reports of diketopiperazine dimers related to **1**. Most of these compounds, such as the leptosins[7–9] and verticillins,[10] show *N*-methylation on the diketopiperazine ring as well as either di-, tri-, or tetrasulfide bridges across one or both of the diketopiperazine rings.[7–10] However, also evident in the literature are examples with only *N*-methylation, such as ditryptophenaline,[11] and examples lacking both the *N*-methylation and polysulfide bridges, such as **5**.[5] This compound class has also been the subject of several patents, one describing a series of diketopiperazines as substance P antagonists,[12] while another five describe a related series of diketopiperazines as cholecystokinin B/gastrin receptor antagonists.[13–17]

Compounds **1**−**4** were screened for their activity against viral IRES-mediated translation, as well as their selectivity for viral versus mammalian Cap-dependent translation (Cap), and the results are summarized in Table 2. As can be seen, only compound **2** displayed activity against IRES below 100 μM, while the remaining isolated compounds failed to register activity below this concentration.

Experimental Section

General Experimental Procedures. SPE was performed using Varian Megabond Elute C18 SPE cartridges (10 g, 50 μm). HPLC was performed on either a Waters Delta Prep 4000 chromatography system equipped with a Waters 2487 dual-wavelength UV detector, a Waters prep LC system controller and a Waters fraction collector, or a system equipped with a Waters 600 controller, a Waters 996 photodiode array detector, a Waters 717 plus autosampler, and a Waters fraction collector II. All data generated from these chromatographic systems were collected using the Waters Millenium[32] data collection package.

All NMR spectra were collected on a Varian Unity Inova 500 MHz spectrometer in the solvents indicated and referenced to residual ^{1}H in the deuterated NMR solvents.

Optical rotations were performed on a Jasco Dip-1000 digital polarimeter, while infrared spectra were acquired on a Bio-Rad FTS-165 Fourier transform infrared spectrometer.

Low-resolution mass spectral data were collected on a ThermoFinnigan LCQ ion trap mass spectrometer, with an ESI probe. High-resolution mass measurements were collected on a Bruker BioApex FT mass spectrometer.

Fungal Material. The fungal isolate was collected by the Australian Institute of Marine Sciences (AIMS) in Townsville and supplied to Cerylid Biosciences through a collection agreement. Its phenotypic characteristics were those of *Aspergillus niger*.

Fungal Growth Conditions. The isolate of *A. niger* was grown on a seed medium of malt extract, peptone, glycerol, and distilled water statically at 25 °C and 70% humidity for 2 days. It was then shaken for 3 h at 260 rpm to break up the mycelium and reincubated statically at 25 °C and 70% humidity for a further 1.5 days. A growth medium consisting of vermiculite soaked with a mixture of soybean meal and oil, cotton seed flour, glycerol, and distilled water was inoculated with the seed media and incubated statically at 25 °C and 70% humidity for 8 days.

Extract and isolation. A 500 mL culture of the isolate of *A. niger* was extracted with MeOH (500 mL), filtered, and concentrated in vacuo. The crude MeOH extract was then subjected to C18 SPE chromatography (15% gradient elution from 20% MeOH/H_2O to 80% MeOH/H_2O, and a flush with 100% MeOH), and the 50% and 65% MeOH/H_2O fractions were active. These were combined and further fractionated on HPLC [4 mL/min, gradient elution from 25% MeCN/H_2O to 70% MeCN/H_2O over 20 min through a Phenomenex Luna C18 150 mm × 10 mm, 5 μm semipreparative HPLC column] to yield the active known compounds TMC-256A1 (**2**, 0.6 mg), TMC-256C1 (**3**, 0.5 mg), and demethylkotanin (**4**, 0.4 mg). Another active fraction was subjected to a final purification through HPLC [4 mL/min, gradient elution from 20% MeCN/H_2O to 68% MeCN/H_2O over 12 min through an Alltech Ultima Phenyl 150 mm × 10 mm 5 μm HPLC column] to yield TMC-256A1 (**2**, 0.5 mg), as well as the new diketopiperazine dimer **1** (0.4 mg).

Compound 1: white powder. $[\alpha]_D$ +132 (*c* 0.004, MeOH); IR (film) ν_{max} 3584, 2960, 2922, 2851, 1666 cm^{-1}; UV (PDA, MeCN/H_2O) λ_{max} 240, 300 nm; ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; HRESIMS *m/z* 605.2859 $[M + Na]^+$ (calcd for $C_{33}H_{38}N_6O_4Na$, 605.2852).

Hydrolysis of 1. A solution of **1** (0.4 mg) in 6 N HCl (1 mL) was refluxed at 100 °C for 16 h. The reaction mixture was concentrated in vacuo and the resultant reaction mixture containing the free amino acids analyzed using chiral HPLC.

Amino Acid Analysis. Amino acid analysis was performed using a Phenomenex Chirex (D)-penicillamine 50 mm × 4.6 mm chiral HPLC column, with 2 mM $CuSO_4$ in 17:3 H_2O/MeOH as the eluent (flow rate 0.5 mL/min over 10 min, then 1.0 mL/min from 11 min). For authentic amino acids the retention times were 5.22 min [(L)-valine], 7.80 min [(D)-valine], 11.76 min [(L)-leucine], and 14.99 min [(D)-leucine]. Analysis of the hydrolysate of **1** using the above conditions showed peaks consistent with (D)-valine and (D)-leucine at retention times of 7.87 and 15.05 min, respectively.

IRES and Cap Assays. Viral IRES was linked to a luciferase reporter gene (IRES-Luc vector). In vitro transcribed RNA (Ambion Megascript kit) was generated from this plasmid and used in an in vitro translation reaction containing Hela cell extract, tRNA, complete amino acids, GTP, ATP, magnesium acetate, potassium acetate, creatine phosphate, creatine phosphokinase, and HEPES buffer (pH 7.6). Compounds were introduced by serial dilution into the reaction mix and incubated for 60 min at 30 °C. Plates were read for luminescence and data analyzed to determine the inhibition of luciferase production in the presence of compound compared to untreated control wells. To discriminate between compounds that nonselectively inhibit protein synthesis from those that selectively block viral translation, normal cellular translation was measured through a Cap-Luciferase-β-globin assay as a measure of host cell protein synthesis. Biochemical IC_{50} values were determined from a nonlinear regression analysis of six point concentration response curves.

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