

Integerrimides A and B, Cyclic Heptapeptides from the Latex of *Jatropha integerrima*

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Two new cyclic heptapeptides, integerrimides A and B, have been isolated from the latex of *Jatropha integerrima*. Their structures were elucidated by using extensive 1D and 2D NMR, MS, and chemical degradation. At 50 μ M both peptides **1** and **2** significantly inhibited neurite outgrowth in neuronal cell culture. They also partially inhibited proliferation of human IPC-298 melanoma cells as well as migration of human Capan II pancreatic carcinoma cells, but were inactive in HSV-1, antifungal, and antimalarial assays.

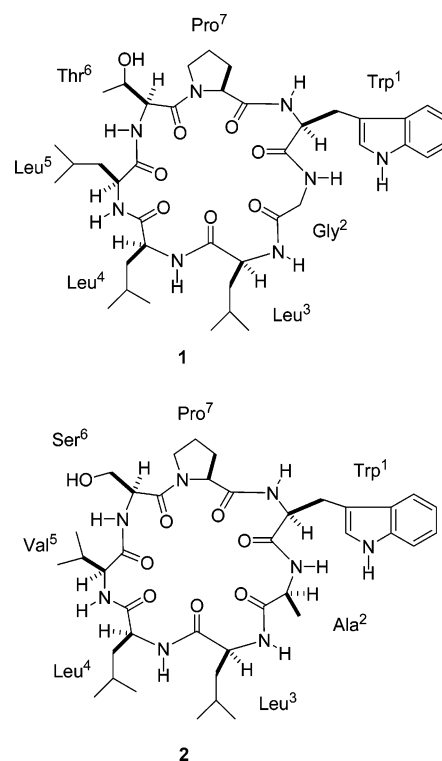
Jatropha species (Euphorbiaceae) are known to be a rich source of bioactive diterpenes and cyclic peptides.^{1–9} In a previous investigation of the roots of *Jatropha integerrima* Jacq. we isolated three rhamnofolane endoperoxide diterpenes together with integerrimide possessing a new 8,9-*seco*-rhamnofolane skeleton.¹ In the present paper we report the isolation and structure elucidation of two new cyclic heptapeptides, namely, integerrimides A (**1**) and B (**2**), from the latex of *J. integerrima*. We also report the biological effects of these compounds on nerve and cancer cells, as well as their activities in HSV-1, antifungal, and antimalarial assays.

The latex of *J. integerrima* of freshly cut leaf stalks was partitioned between a mixture of MeOH/H₂O and CH₂Cl₂. Successive column chromatography of the CH₂Cl₂ extract using normal-phase and C₁₈ reversed-phase silica gel followed by semipreparative HPLC on RP18 afforded integerrimides A (**1**) and B (**2**).

Integerrimide A (**1**) was obtained as a colorless solid (CH₂Cl₂/MeOH), mp 184–186 °C, [α]_D²⁵ –76.6 (c 0.5, MeOH). The ESIMS spectrum displayed the [M + H]⁺ and [M + Na]⁺ ions at *m/z* 781 and 803, respectively. The molecular formula was determined as C₄₀H₆₀N₈O₈ by HRFABMS (*m/z* 781.4605, [M + H]⁺, calcd for C₄₀H₆₁N₈O₈ 781.4612). ¹H NMR studies of **1** with different solvents revealed that **1** exists as an equilibrium mixture of at least two or more stable conformers at room temperature. When using pyridine-*d*₅, three conformers in the ratio 5:3:2 could be detected, whereas two conformers were observed in the case of acetone-*d*₆ (ratio 85:15), DMSO-*d*₆ (ratio 4:1), and a mixture of CDCl₃/DMSO-*d*₆ (ratio 92:8). Combining minimal signal overlapping and accurate peak sharpness, the latter two solvent systems were used for both the unambiguous structure elucidation of **1** and the identification of the different conformers. Analysis of the ¹H (Figure 1) and ¹³C NMR, 1D TOCSY (Figure 1), TOCSY, DQFCOSY, gHSQC, and gHMBC spectra established the presence of three leucine residues, one tryptophan, one proline, one glycine, and one threonine moiety in compound **1** (Table 1 and Supporting Information).

¹H–¹³C-long-range correlations as well as *d* _{α N(i, i+1)} and *d*_{NN(i, i+1)} connectivities^{10,11} obtained by ROESY unambiguously allowed the sequence to be determined as cyclo(-Trp¹-Gly²-Leu³-Leu⁴-Leu⁵-Thr⁶-Pro⁷-) (Figure 2).

As mentioned above the ¹H NMR spectra of **1** in DMSO-*d*₆ displayed duplicate signals in the ratio 4:1. ROESY correlations between Pro⁷- α H at δ 4.78 and Thr⁶- α H (δ 4.32), Thr⁶- β H (δ 3.88), and Thr⁶- γ H (δ 1.05) together with ¹³C chemical shifts of Pro⁷- β C at δ 30.75 and Pro⁷- γ C at δ 21.53 indicated that the major



conformer (**1a**) exhibits a *cis*-Pro⁷ amide bond^{7,12} (Figure 3). ROEs between the protons Pro⁷- δ H (δ 3.68 and 3.45) and Thr⁶- α H (δ 4.64) and Thr⁶- β H (δ 4.15) as well as ¹³C chemical shifts of Pro⁷- β C at δ 29.23 and Pro⁷- γ C at δ 25.04 proved the presence of a *trans*-Pro⁷ amide bond in the minor conformer **1b** in DMSO-*d*₆^{7,12} (Figure 3). Using the above-mentioned arguments, the *trans*-configuration of the Pro⁷ amide bond could be deduced for the major conformer (92%) in the mixture of CDCl₃/DMSO-*d*₆ (Supporting Information).

Integerrimide B (**2**) obtained as colorless needles (CH₂Cl₂/MeOH), mp 196–199 °C, [α]_D²⁵ –66.6 (c 0.5, MeOH), showed its [M + H]⁺ and [M + Na]⁺ ions at *m/z* 767 and 789 in the ESIMS spectrum. Its molecular formula of C₃₉H₅₈N₈O₈ was established by HRFABMS (*m/z* 767.4484, [M + H]⁺ calcd for C₃₉H₅₉N₈O₈ 767.4456). As in the case of compound **1**, the ¹H NMR spectrum of **2** showed the presence of at least two or more stable conformers in solution depending on the solvents used at room temperature. Thus, two conformers (**2a** and **2b**) in the ratio 70:30 could be observed in DMSO-*d*₆ (Figure 4), and one major (~90%) together with at least four minor conformers could be detected in the case

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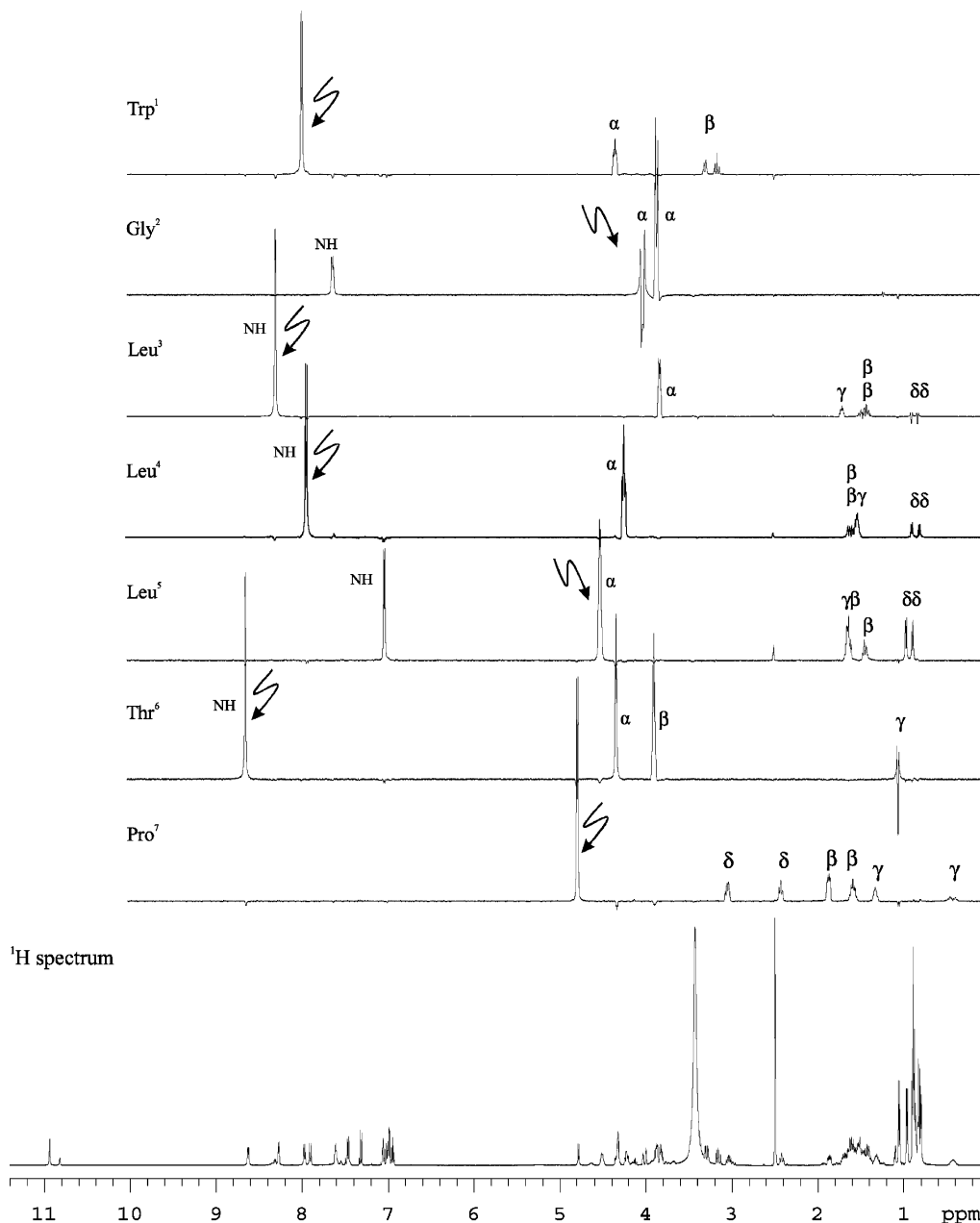


Figure 1. ^1H NMR spectrum of **1** in $\text{DMSO-}d_6$ (lower trace) and 1D TOCSY spectra of the individual amino acid residues (upper traces). Sites of selective excitation are shown by arrows.

of a mixture of $\text{CDCl}_3/\text{DMSO-}d_6$. In order to simplify and speed up the structure elucidation process, ^1H NMR spectra were recorded at different temperatures up to 80°C . However, the coalescence point could not be reached within this temperature range (Supporting Information, S3).

Therefore, the structure of **2** was elucidated by NMR in the same way as described for compound **1** and resulted in the sequence cyclo(-Trp¹-Ala²-Leu³-Leu⁴-Val⁵-Ser⁶-Pro⁷-) (Table 2 and Supporting Information). In $\text{DMSO-}d_6$ the major conformer (**2a**) contains a *cis*-Pro⁷ amide bond, as shown by ROESY correlations between Pro⁷- αH at δ 4.66 and Ser⁶- αH (δ 4.28), Ser⁶- βH (δ 3.54), and Ser⁶-OH (δ 5.19) together with ^{13}C chemical shifts of Pro⁷- βC at δ 30.96 and Pro⁷- γC at δ 21.69.^{7,12} ROEs between the protons Pro⁷- δH (δ 3.77 and 3.43) and Ser⁶- αH (δ 4.81), Ser⁶- βH (δ 4.12 and 3.72), and Ser⁶-OH (δ 6.01) as well as ^{13}C chemical shifts of Pro⁷- βC at δ 28.41 and Pro⁷- γC at δ 25.74 indicated a *trans*-Pro⁷ amide bond in the minor conformer **2b** in $\text{DMSO-}d_6$.^{7,12} Similarly, the *trans*-configuration of the Pro⁷ amide bond could be deduced for the major conformer ($\sim 90\%$) in the mixture of $\text{CDCl}_3/\text{DMSO-}d_6$

(Supporting Information). The L-configurations of the individual amino acids of **1** and **2** were unambiguously determined by comparative HPLC on a chiral column using the respective D and L standard amino acids after complete hydrolysis of each compound.

Both peptides were tested for HSV-1, antifungal, antimalarial, and cell biological activities including neurite outgrowth, cell proliferation, and cell migration. The latter three processes have been shown to strictly depend on intact cytoskeleton dynamics and to be easily blocked in the presence of known actin inhibitors such as cytochalasins or known microtubule inhibitors such as nocodazole or Taxol at submicromolar concentrations.¹³ The influence of **1** and **2** on neurite outgrowth was assessed by culturing E7 chicken spinal cord neurons in the presence of up to $50\ \mu\text{M}$ of the compounds for 2 days. Figure 5A shows that both peptides led to a significant inhibition of neurite outgrowth at $50\ \mu\text{M}$. Likewise, in another experiment we found that blebbistatin-induced neurite sprouting^{14–16} was completely abolished in the presence of $50\ \mu\text{M}$ of **1** (Supporting Information, S4 A). In both experiments, the

Table 1. ¹H (500 MHz) and ¹³C (125 MHz) NMR Data of Major Conformers of **1** in DMSO-*d*₆ (**1a**: *cis*- and **1b**: *trans*-oriented amide bond of proline⁷)

pos	1a			1b	
	δ_C	δ_H , mult. (<i>J</i> in Hz) ^a	δ_C	δ_H , mult. (<i>J</i> in Hz) ^a	
Trp ¹	NH			7.46, ov ^b	
	α	56.55	4.34, ddd (3.6, 7.6, 12.3)	4.32, ov ^b	
	β	27.95	3.29, dd (3.5, 14.0)	3.31, ov ^b	
			3.15, dd (12.4, 14.1)	2.99, dd (10.7, 14.2)	
	1' (NH)		10.94, d (2.1)	10.82, d (2.0)	
	2'	124.00	6.98, d (2.1)	7.06, ov ^b	
	3'	110.89		111.33	
	4'	118.51	7.46, d (7.9)	118.68	
	5'	119.38	6.95, dt (0.7, 7.5)	118.99	
	6'	121.78	7.06, dt (1, 7.9)	121.65	
	7'	112.07	7.31, d (8.1)	112.13	
	3a'	127.58		128.05	
	7a'	136.94		136.79	
CO	172.12		171.62		
Gly ²	NH			7.55, bt (5.7)	
	α	43.24	4.02, dd (3.5, 17.7) 3.85, dd (5.6, 17.6)	3.84, ov ^b 3.75, dd (4.5, 17.5)	
Leu ³	CO	170.11		169.24	
	NH		8.26, d (4.8)	7.60, ov ^b	
	α	54.48	3.82, dt (4.6, 9.2)	4.21, ov ^b	
	β	40.4, ov ^b	1.47, ov ^b 1.41, ov ^b	~40.2, ov ^b 1.45–1.53, ov ^b 1.45–1.53, ov ^b	
	γ	24.86	~1.7, ov ^b	~25.0 ov ^b	
	δ	22.07	0.82, d (6.7)	21.39 ^d	
	δ	23.45	0.90, d (6.8)	21.73 ^d	
Leu ⁴	CO	172.78		173.32	
	NH		7.91, d (9.3)	8.31, d (6.91)	
	α	52.35	4.23, ddd (4.1, 9.2, 11.5)	3.91, ov ^b	
	β	~40, ov ^b	1.59, ov ^b 1.45, ov ^b	~40.2, ov ^b 1.65, ov ^b 1.48, ov ^b	
	γ	25.19	~1.51, ov ^b	~25.0 ov ^b	
	δ	23.74, ov ^{b,c}	0.88, d (6.1) ^{b,c}	22.15 ^d	
	δ	21.74	0.80, d (6.1)	22.42 ^d	
Leu ⁵	CO	172.45		172.4 ^e	
	NH		7.01, d (7.1)	8.34, bs	
	α	51.58	4.51, q (7.2)	3.93, ov ^b	
	β	41.76	1.61, ov ^b 1.42, m	~40.2, ov ^b 1.77, m ~1.62, ov ^b	
	γ	24.80	1.63, ov ^b	~25.0 ov ^b	
	δ	23.74, ov ^{b,c}	0.88, d (6.1) ^{b,c}	23.57 ^d	
	δ	22.95	0.96, d (6.4)	23.90 ^d	
Thr ⁶	CO	172.49		172.21 ^e	
	NH		8.63, d (5.5)	7.64, ov ^b	
	α	58.34	4.32, t (4.9)	4.64, bs	
	β	67.20	3.88, quint. (5.3)	67.49	
	γ	20.15	1.05, d (6.4)	19.34	
Pro ⁷	CO	169.16		170.53 ^e	
	α	60.86	4.78, d (7.8)	61.53	
	β	30.75	1.88, bdd (6.7, 12.2) 1.57, m	29.23	
	γ	21.53	0.42, m 1.31, m	25.04	
	δ	46.28	3.04, ddd (8.2, 10.5, 11.7) 2.42, bt (9.9)	48.06	
	CO	171.27		171.57	

^a Coupling constants were not averaged and were extracted from the ¹H- and the 1D-TOCSY spectra. The positions of the leucine residues Leu³–Leu⁵ in **1b** could not be unambiguously assigned because of the low concentration of conformer **1b** and strong signal overlapping. ^bov = overlapped by other signals. ^{c–e}NMR values with same letters may be interchanged.

viability of the neurons (density of live cells) was not affected by either of the compounds (Figure 5B,C and Supporting Information, S4 B, C).

Human melanoma cells (IPC-298) were treated with up to 50 μ M of **1** and **2** to elucidate potential effects on cell proliferation. Figure 6A demonstrates that both peptides caused significantly lower (up to 40% at 50 μ M) cell densities after 2 days of culture. Since cell debris and apoptotic cell nuclei were obviously absent in the treated cell cultures (Figure 6B,C), these data suggest that both peptides have an antiproliferation but no cytotoxic activity.

As migration, which also represents a fundamental feature of cells besides proliferation, is involved in many biological processes

such as embryonic development, wound healing, immune response, or metastatic tumor invasion, it was of interest to prove potential activities of **1** and **2** on cell migration of confluent human Capan II pancreatic carcinoma cells. Standard cell migration assays (“scratch migration assay”) revealed that both **1** and **2** (50 μ M each) led to a significant inhibition of cell migration activity by about 30% and 20%, respectively (Supporting Information, S5 A). The viability of the cells, as far as could be judged from their morphology, was however not affected (Supporting Information, S5 B–G). Summarizing the above results with primary neuronal and human tumor cell cultures, it can be concluded that both peptides **1** and **2** showed an inhibition of basic cytoskeleton-

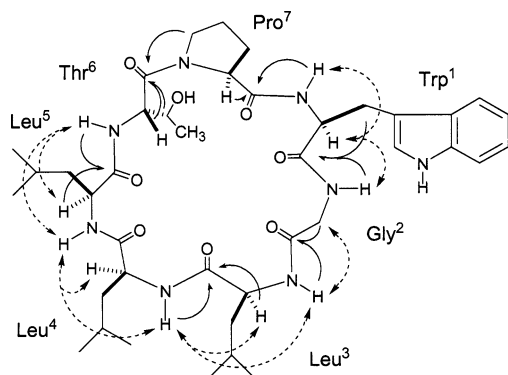


Figure 2. Selected ROESY (\leftrightarrow) and HMBC correlations (\rightarrow) of the major conformer **1a**.

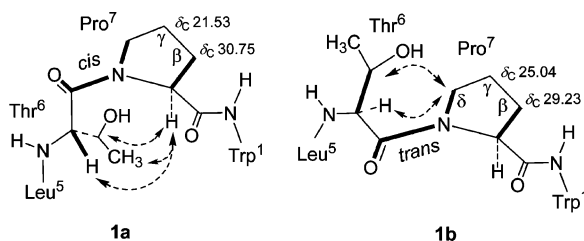


Figure 3. *cis/trans*-Orientation of the Pro⁷ amide bond in **1a** and **1b**. Arrows indicate important ROESY correlations.

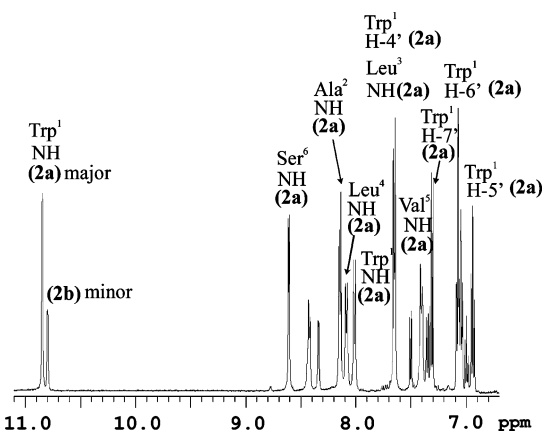


Figure 4. Part of the ¹H NMR spectrum of **2** in DMSO-*d*₆ displaying the amide and aromatic protons of the two conformers **2a** and **2b**.

dependent cellular processes such as neurite outgrowth, cell proliferation, and cell migration. The observed inhibition was, however, at least 100-fold less pronounced than those exerted by, for example, cytochalasins, nocodazole, or Taxol.¹³ Further testing on bioactivity revealed that both compounds were not active in HSV-1 (test concentration 50 μ g/mL), antifungal (test concentration 50 μ g/mL), and antimalarial assays (test concentration 10 μ g/mL).

Experimental Section

General Experimental Procedures. Melting points were measured by the Electrothermal melting point apparatus and are not corrected. Optical rotations were determined with a Perkin-Elmer Model 341 polarimeter. Ultraviolet spectra were recorded using a Varian Cary 4E UV-visible spectrophotometer. FT-IR spectra were obtained with a Perkin-Elmer Spectrum One FT-IR spectrophotometer. NMR spectra were recorded on a Varian Unity Inova 500 or 300 MHz instrument. The ¹H and ¹³C chemical shifts were referenced to residual solvent signals at $\delta_{\text{H/C}}$ 2.49/40.22 (DMSO-*d*₆), 7.27_{CDCl₃}/40.22_{DMSO-*d*₆} (mixture of CDCl₃ (650 μ L)/DMSO-*d*₆ (70 μ L)) relative to TMS. All 1D (¹H, ¹³C, 1D TOCSY) and 2D NMR (DQFCOSY, TOCSY, ROESY, GHSQC, GHMBC) measurements were performed using standard

Varian pulse sequences. Mass spectra were obtained on a Finnigan MAT TSQ 700 (ESIMS) and a JEOL JMS-700 (HRFABMS with NBA as matrix).

Analytical and semipreparative HPLC were performed on a Varian ProStar 230 HPLC equipped with a Varian ProStar 335 PDA detector and a Knauer K501 HPLC pump equipped with a TC931 column heater and a Bischoff Lambda 1000 UV detector.

For light microscopy and quantitative live imaging analysis a Zeiss Axiovert 35 microscope equipped with high-resolution epi-fluorescence, phase-contrast optics, an axio-cam MRm digital camera, and Axiovision 4.4 software was used.

Plant Material. The latex of *J. integerrima* was collected from the plants growing within the Ramkhamhaeng University area in May 2004. Botanical identification was achieved by comparison with a voucher specimen (No. SN 239681–239682) kept in the herbarium collections of the Sirindhorn Museum (Bangkok Herbarium), Botanical Section, Botany and Weed Section, Department of Agriculture, Ministry of Agriculture and Cooperatives. A voucher specimen (SSJI 1/04) is kept at the Department of Chemistry, Ramkhamhang University, Bangkok, Thailand.

Extraction and Isolation. The latex of *J. integerrima* was collected from the freshly cut leaf stalks using a pipet and kept in MeOH/H₂O. After concentration under reduced pressure, the crude residue was reconstituted in MeOH/H₂O and partitioned using CH₂Cl₂ (5 \times 20 mL). The combined CH₂Cl₂ layers were concentrated to yield the CH₂Cl₂ extract (294.1 mg). Column chromatography of the CH₂Cl₂ extract using silica gel (40–63 μ m; Merck) and a gradient of CH₂Cl₂/MeOH (98:2 to 85:15) afforded four major fractions. The third fraction was further purified using RP-18 silica gel (Lichrolut RP-18; 40–60 μ m; Merck) and MeOH/H₂O (50:50) to yield five subfractions (3.1–3.5). Subfractions 3.2 and 3.4 were purified by semipreparative HPLC on RP 18 (Merck LiChroCART 250 \times 10 mm Cartridge with LiChrospher 100 RP 18 material; 100 μ m) using a gradient of 32–72% CH₃CN in 0.01% TFA in 40 min and a flow rate of 5 mL/min to yield compound **1** (22 mg; *t*_R 24.9 min) and compound **2** (20.8 mg; *t*_R 18.3 min), respectively.

Integerrimide A (1): colorless solid (CH₂Cl₂/MeOH); mp 184–186 $^{\circ}$ C; $[\alpha]_{\text{D}}^{25}$ –76.6 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ϵ) 290 (0.19), 282 (0.21), and 221 (1.35) nm; IR (film ATR) ν_{max} 3298, 3060, 2957, 2872, 1634, 1517, 1434, 1368, 1340, 1249, 1201, 1130, 1026, 742 cm^{-1} ; ¹H and ¹³C NMR (Table 1 and Supporting Information); ESIMS *m/z* 781 [M + H]⁺, 803 [M + Na]⁺; HRFABMS *m/z* 781.4605 (calcd for C₄₀H₆₁N₈O₈, 781.4612) [M + H]⁺, 803.4382 (calcd for C₄₀H₆₀N₈O₈Na, 803.4432) [M + Na]⁺.

Integerrimide B (2): colorless needles (CH₂Cl₂/MeOH); mp 196–199 $^{\circ}$ C; $[\alpha]_{\text{D}}^{25}$ –66.6 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ϵ) 290 (0.24), 282 (0.27), 221 (1.76) nm; IR (film ATR) ν_{max} 3281, 3062, 2956, 2873, 2471, 2160, 2028, 1634, 1524, 1452, 1387, 1367, 1335, 1242, 1200, 1164, 1060, 922, 740, 685 cm^{-1} ; ¹H and ¹³C NMR (Table 2 and Supporting Information); ESIMS *m/z* 767 [M + H]⁺, 789 [M + Na]⁺; HRFABMS *m/z* 767.4484 (calcd for C₃₉H₅₉N₈O₈, 767.4456) [M + H]⁺, 789.4302 (calcd for C₃₉H₅₈N₈O₈Na, 789.4276) [M + Na]⁺.

Hydrolysis of the Peptides 1 and 2. Compounds **1** and **2** (1 mg each) were hydrolyzed with 6 N HCl (1 mL) in a sealed tube at 115 $^{\circ}$ C for 24 h. After hydrolysis, the reaction mixture was evaporated to dryness. The residue was dissolved in H₂O (100 μ L) and directly subjected to HPLC. For the determination of tryptophan, hydrolysis was conducted in 1 mL of 6 N HCl containing 0.4% (v/v) β -mercaptoethanol. This solution was frozen in liquid N₂ under vacuum and then gently warmed to melt. This procedure was repeated three times, and the evacuated tube was sealed. After 24 h at 115 $^{\circ}$ C the reaction mixture was adjusted to pH 6 with 2 N NaOH and extracted with 4 \times 5 mL of *n*-butanol. The combined organic layers were evaporated to dryness, redissolved in MeOH, filtered through an Adsorbex RP-18 extraction column (Merck, Darmstadt, Germany), and again evaporated to dryness. The residue was dissolved in 200 μ L of MeOH containing 4 μ L of 2 N HCl and subjected to HPLC.

HPLC Analysis of the Acid Hydrolysates. HPLC analysis was performed on a chiral Chirex 3126 (D)-penicillamine column, 5 μ m, 250 \times 4.6 mm (Phenomenex Inc., Torrance, CA); UV 254 nm. For complete analysis three different solvent systems were used and the configuration was determined by co-injection with commercial D- and L-amino acids. Retention times *t*_R (min) of authentic amino acids were as follows: (1) 1 mM Cu(OAc)₂, 50 $^{\circ}$ C, flow rate 1.0 mL/min: Gly (*t*_R 7.0), L-Thr (*t*_R 8.4), D-Thr (*t*_R 10.1), L-Ser (*t*_R 8.6), D-Ser (*t*_R 9.3),

Table 2. ¹H (500 MHz) and ¹³C (125 MHz) NMR Data of Major Conformers of **2** in DMSO-*d*₆ (**2a**: *cis*- and **2b**: *trans*-oriented amide bond of proline⁷)

pos	2a		2b		
	δ_C	δ_H , mult. (<i>J</i> in Hz) ^a	δ_C	δ_H , mult. (<i>J</i> in Hz) ^a	
Trp ¹	NH	8.02, d (7.9)		7.41, ov ^b	
	α	55.43	4.46, ddd (4.9, 7.9, 11.3)	54.79	4.33, ddd (3.9, 10.2, 12.0)
	β	27.99	3.32, ov ^b	27.92	3.35, dd (3.7, 15.0)
			3.28, ov ^b		2.74, dd (12.1, 14.6)
	1' (NH)		10.86, d (2.0)		10.81, d (2.0)
	2'	124.26	7.07, d (2.1)	123.92	7.09, d (2.2)
	3'	111.19		111.46	
	4'	119.10	7.65, d (7.8) ^c	118.78	7.50, d (7.7)
	5'	119.01	6.94, ddd (0.8, 7.0, 7.8)	119, ov ^b	7.00, dt (0.9, 7.8)
	6'	121.62	7.05, dt (0.9, 6.9)	121.56	7.05, ov ^b
	7'	111.92	7.31, d (7.8)	112.09	7.32, d (6.5)
	3a'	127.82		128.19	
	7a'	136.85		136.69	
	CO	171.64		170.88 ^d	
Ala ²	NH	8.16, d (6.9)		7.35, d (8.1)	
	α	49.13	4.32, dq (6.4, 7.1)	48.63	4.57, dq (6.9, 7.5)
	β	19.05	1.39, d (7.0)	19.49	1.21, d (6.6)
	CO	172.97		172.07	
Leu ³	NH		7.65, d, (7.8) ^c		8.37, bd (3.0)
	α	53.63	4.04, m	54.33	3.86, dt (3.2, 7.4)
	β	41.42	1.53, ov ^b	40.26	1.53, ov ^b
			1.42, ov ^b		1.42, ov ^b
	γ	24.86	1.60, m	24.78	1.60, m
	δ	23.42	0.90, d (6.8)	23.91 ^e	0.92, d (6.4)
	δ	22.67	0.87, d (6.8)	23.12 ^e	0.87, d (6.4)
CO	173.24		172.34		
Leu ⁴	NH		8.11, d (7.7)		8.46, ov ^b
	α	54.59	3.93, m	54.96	3.69, m
	β	40.1 ov ^b	1.75, m	39.46	2.03, m
			1.61, ov ^b		1.55, ov ^b
	γ	25.10	1.51, ov ^b	25.31	~1.53
	δ	23.59	0.87, d (6.4)	23.08 ^e	0.84, ov ^b
	δ	21.67	0.82, d (6.3)	21.61 ^e	0.84, ov ^b
CO	172.05		172.70		
Val ⁵	NH		7.42, d (8.7)		8.15, ov ^b
	α	57.39	4.25, t (8.1)	62.15	3.99, t (8.9)
	β	32.04	1.97, m	31.75	2.07, m
	γ	19.41	0.94, d (6.7)	20.11 ^f	0.87, ov ^b
	γ	19.11	0.90, d (6.6)	19.01 ^f	0.82, ov ^b
	CO	171.23		170.91 ^c	
	Ser ⁶	NH		8.63, d (4.7)	
α		55.19	4.28, m	54.21	4.81, dt (3.0, 6.5)
β		61.70	3.58, m	62.15	4.12, m
			3.54, m		3.72, m
OH			5.19, t (5.6)		6.01, bs
Pro ⁷	CO	170.53		169.93	
	α	60.71	4.66, bd (8.0)	62.32	3.98, t (8.6)
	β	30.96	1.81, m	28.41	1.83, m
			1.59, m		0.61, m
	γ	21.69	1.32, m	25.74	1.61, m
			0.47, m		1.54, m
	δ	46.07	3.11, dt (7.7, 11.9)	47.84	3.77, bt (8.5)
CO	171.51	2.62, bt (9.8)	170.82 ^d	3.43, m	

^a Coupling constants were not averaged and were extracted from the ¹H- and the 1D-TOCSY spectra. ^bov = overlapped by other signals. ^{c-f}NMR values with same letters may be interchanged.

L-Ala (*t_R* 9.3), D-Ala (*t_R* 12.6), L-Pro (*t_R* 18.2), D-Pro (*t_R* 40.5); (2) 12% (v/v) isopropanol in 1 mM CuSO₄, 50 °C, flow rate 1.0 mL/min: L-Val (*t_R* 5.5), D-Val (*t_R* 7.9), L-Leu (*t_R* 10.5), D-Leu (*t_R* 14.3); (3) 30% (v/v) MeOH in 2 mM CuSO₄, 25 °C, flow rate 0.8 mL/min: L-Trp (*t_R* 111), D-Trp (*t_R* 121).

Retention times *t_R* (min) of amino acids obtained from the hydrolysis of **1** and **2** were as follows: (1) 1 mM Cu(OAc)₂, 50 °C, flow rate 1.0 mL/min: Gly (*t_R* 7.0), L-Thr (*t_R* 8.4), and L-Pro (*t_R* 18.2) of **1**; L-Ser (*t_R* 8.6), L-Ala (*t_R* 9.3), L-Pro (*t_R* 18.2) of **2**; (2) 12% (v/v) isopropanol in 1 mM CuSO₄, 50 °C, flow rate 1.0 mL/min: L-Leu (*t_R* 10.5) of **1**; L-Val (*t_R* 5.5) and L-Leu (*t_R* 10.5) of **2**; (3) 30% (v/v) MeOH in 2 mM CuSO₄, 25 °C, flow rate 0.8 mL/min: L-Trp (*t_R* 112) of **1**; L-Trp (*t_R* 111) of **2**.

Inhibition of Neurite Outgrowth. Hindbrains (medulla) or the anterior parts of the spinal cords were dissected from 7-day-old

incubated chicken embryos, rinsed in ice-cold serum-free HEPES-buffered medium containing 4.5 g glucose/L, 100 units/mL penicillin, 100 μ g/mL streptomycin, 4.4 mM NaHCO₃, and 10 mM HEPES, pH 7 (HBM), and collected in 1 mL of dissociation buffer containing 140 mM NaCl, 26 mM KCl, 14 mM KH₂PO₄, 10 mM glucose, 0.5 mg/mL trypsin, and 50 μ g/mL DNaseI. The samples were triturated, incubated at 37 °C for 20 min, and triturated again. Cell dissociation was stopped by addition of 10 vol of ice-cold HBM. The cells were washed two times (400 g, 10 min) in cold HBM. For the assays, 50 000 cells suspended in 50 μ L of HBM were plated within an area of 1 cm² on polylysine/fibronectin-coated glass or plastic cell culture dishes (3.5 cm) and cultured in HBM supplemented with 10% fetal calf serum in an incubator without CO₂ supply in a water-saturated air atmosphere at 37 °C. In order to test potential effects of **1** and **2** on the viability of the neurons and the outgrowth of neuritic processes, freshly prepared

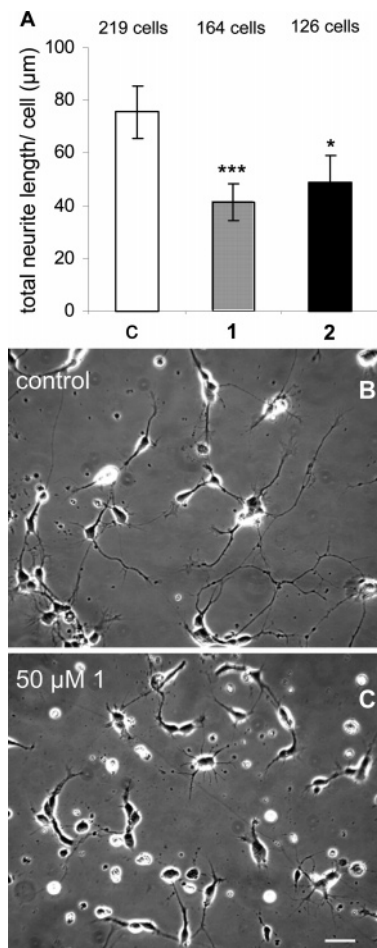


Figure 5. Inhibition of neurite outgrowth in E7 chicken spinal cord neurons treated with **1** and **2** (50 μM each) for 2 days (A); untreated neurons with well-developed neuritic processes (B); neurons cultured in the presence of **1** (50 μM) with no or only short neuritic processes (C); the bar in C corresponds to 30 μm ($*p = 0.0206$, $***p < 0.0001$).

spinal cord neurons (see above) were cultured in the presence or absence of **1** and **2** (10 or 50 μM) for 2 days (Figure 5). In a separate series, cultures of medulla neurons containing 50 μM **1** were treated with the actomyosin inhibitor blebbistatin (BBS, 50 μM), which was previously shown to induce a strikingly enhanced neurite outgrowth.^{14–16} Neurite length was estimated by quantitative live imaging and given as total neurite length (μm) per cell (Figure 5 and Supporting Information, S4).

Proliferation Assay. Human melanoma cells (IPC-298) were cultured in DMEM/Ham's F12 (Biochrom) with stabilized L-glutamine, supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 units/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. The cells were plated in a low density of 100 000 cells per cm^2 and cultured in the presence or absence of **1** and **2** (at 10 and 50 μM) for 2 days. Viable cells were counted on phase-contrast digital pictures and given as cells per 0.36 mm^2 (Figure 6).

Scratch Migration Assay (SMA). Confluent human pancreatic carcinoma cells (Capan II) were scratched by means of the tip of a small plastic pipet, resulting in a straight cell-free channel of 230–260 μm width. The samples were then cultured in the presence or absence of **1** or **2** (at 10 and 50 μM) for 20 h. The migration activities of the cells were determined as “resettled” cell–substrate area (μm^2)/20 h by digital live imaging (Zeiss, Axiovision 4.4) (Supporting Information, S5).

HSV-1 Activity. Determination of anti-herpes simplex virus type 1 (HSV-1) activity was evaluated against HSV-1 strain ATCC VR 260, using the colorimetric method¹⁷ and concentrations of 50 $\mu\text{g}/\text{mL}$ of each compound **1** and **2**. Acyclovir was used as a positive control (IC_{50} value of 0.5 ± 0.2 $\mu\text{g}/\text{mL}$).

Antimalarial Activity. Antimalarial activity was evaluated against *Plasmodium falciparum* (K1 multidrug-resistant strain) cultured con-

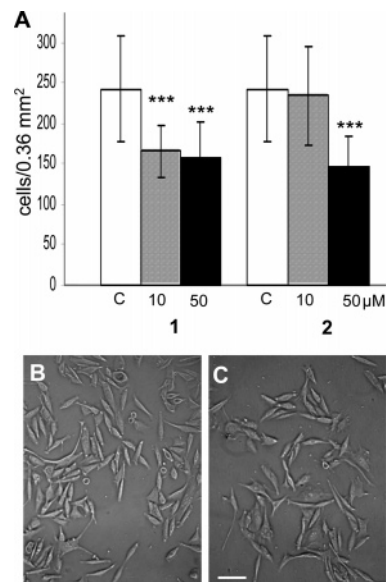


Figure 6. Effect of **1** and **2** on proliferation and survival of human IPC-298 melanoma cells: as compared to controls significant ($p < 0.0001$) lower cell densities of cultures that had been treated with **1** or **2** (10 and 50 μM) for 2 days (A); untreated controls (B); cells grown in the presence of **1** (50 μM) (C); the bar in C corresponds to 30 μm ($***p < 0.0001$).

tinuously according to Trager and Jensen.¹⁷ Quantitative determination of antimalarial activity *in vitro* was achieved using the microculture radioisotope technique based on the method of Desjardins et al.¹⁸ The compounds were tested at 10 $\mu\text{g}/\text{mL}$. The standard drugs, rifampicin, kanamycin, and isoniazide, used as positive controls for the antimycobacterial activity showed minimum inhibitory concentrations (MICs) of 0.0023, 2.5, and 0.1 $\mu\text{g}/\text{mL}$, respectively.

Antifungal Activity. Antifungal tests against *Candida albicans* (ATCC 90028) were performed according to the tetrazolium/formazan assay method.¹⁹ Compounds **1** and **2** were tested at 50 $\mu\text{g}/\text{mL}$. Amphotericin B and DMSO were used as a positive (IC_{50} value of 0.08 ± 0.012 $\mu\text{g}/\text{mL}$) and a negative control, respectively.

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Supporting Information Available: ¹H and ¹³C NMR data of **1** and **2** in CDCl_3 (650 μL)/DMSO-*d*₆ (70 μL), part of the ¹H NMR spectrum of **2** at different temperatures, inhibition of blebbistatin (BBS, 50 μM)-induced neurite outgrowth in E7 chicken medulla neurons by **1** (50 μM), and attenuation of migration activity of human Capan II pancreatic carcinoma cells by **1** and **2** (scratch migration assay, 20 h). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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