Tolyporphins J and K, Two Further Porphinoid Metabolites from the Cyanobacterium *Tolypothrix nodosa*

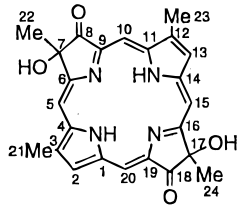
Michèle R. Prinsep,*,† Gregory M. L. Patterson,‡ Linda K. Larsen,‡ and Charles D. Smith§

Department of Chemistry, University of Waikato, Private Bag 3105, Hamilton, New Zealand, Department of Chemistry, 2545 The Mall, University of Hawaii, Hawaii 96822, and Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, Pennsylvania, 19111

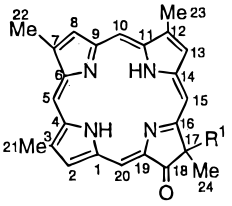
Received December 21, 1997

Two new porphinoids, tolyporphins J (**1**) and K (**2**), have been isolated from the terrestrial cyanobacterium, *Tolypothrix nodosa* (HT-58-2) and identified by NMR and mass spectral analysis. The activities of tolyporphins J and K in cell sensitization and drug accumulation assays for multidrug resistance (MDR) reversal were compared with those of tolyporphin A. Unusual NMR spectroscopic shifts were observed for tolyporphin J (**1**) in $CDCl_3$.

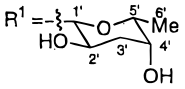
We have previously reported the structures of tolyporphin A[1] and eight analogues, tolyporphins B−I.[2] These multidrug resistance reversal agents were isolated from an extract of the cyanophyte *Tolypothrix nodosa* Bharadwaja (UH strain HT-58-2), which was in turn isolated from a soil sample collected at Nan Madol, Pohnpei. We have also reported the structure of tolypodiol, an antiinflammatory diterpenoid that was isolated from the same species.[3] In a continuing investigation of this cyanophyte, we report here the isolation, structural elucidation, and biological activity of two further compounds, tolyporphins J (**1**) and K (**2**).



1



2



Isolation and culture of the cyanobacterium was carried out by a general procedure.[4] The freeze-dried cyanophyte was extracted with CH_2Cl_2/2-propanol (1:1) and the extract subjected to reversed-phase and repeated normal-phase chromatography to give tolyporphins J (**1**) and K (**2**) in 0.0016 and 0.0011% yields, respectively.

The structure of tolyporphin J (**1**) was determined by the use of one- and two-dimensional NMR spectroscopic techniques. The ^{1}H NMR spectrum of **1** in $CDCl_3$ differed from those of all other tolyporphins in that the protons directly attached to nitrogens resonated at ca. −5 ppm instead of at ca. −3 ppm. All other protons in the structure also resonated at a somewhat higher field than comparable protons in tolyporphins A−I (Table 1). The spectrum consisted of 12 singlets, eight single proton resonances, and four methyl resonances, indicating that the macrocyclic ring skeleton was identical to that of tolyporphins A−I and that as in tolyporphins G−I there were no C-glycoside ring substituents. Tolyporphin J (**1**) was not particularly soluble in $CDCl_3$, so it was dissolved in acetone-d_6 for further NMR spectroscopy. In acetone-d_6, the protons directly attached to nitrogens resonated at positions similar to those of the chemical shifts of these protons in tolyporphins A−I, (ca. −3 ppm), and all other protons in the structure also reverted to chemical shifts comparable to those in other tolyporphins (Table 1). The hydroxyl protons were visible as broad singlets at 6.12 and 6.18 ppm. Reasons for the more upfield shifts of the protons of **1** in $CDCl_3$ are not clear. The ^{13}C NMR spectrum of **1** in acetone-d_6 (Table 1) contained 24 carbon resonances, and the chemical shifts of two quaternary carbon resonances at 78.03 and 77.72 ppm indicated that these carbons were oxygenated. Assignment of ^{1}H and ^{13}C NMR spectra was assisted by the use of HMBC, HMQC, and ROESY 2-D NMR experiments (Table 1). HREIMS established the molecular formula $C_{24}H_{22}N_4O_4$, confirming that tolyporphin J (**1**) contained two hydroxyl substituents.

The structure of tolyporphin K (**2**) was similarly deduced. The ^{1}H NMR spectrum of **2** in $CDCl_3$ (Table 2) was similar to that of tolyporphins A−I except that it contained an extra aromatic proton signal and one additional aromatic methyl signal. It also contained signals indicative of a 3,6-dideoxy-*xylo*-hexopyranose ring that was β-linked to C-17 as in tolyporphins B−D. The ^{13}C NMR spectrum of **2** (Table 2) contained 30 resonances, including one carbonyl signal and two aromatic signals in addition to those found in the spectra of tolyporphins A−J, implying that tolyporphin K contained a unique macrocyclic ring system with three fully aromatic pyrrole rings. As for tolyporphin J, assignment of ^{1}H and ^{13}C NMR spectra was assisted by the use of HMBC, HMQC, and ROESY 2-D NMR experiments (Table 2). HREIMS established the molecular formula $C_{30}H_{32}N_4O_4$.

Tolyporphins J and K were tested for biological activity in MDR reversal and [^{3}H]vinblastine accumula-

* To whom correspondence should be addressed. Tel.: (64)-7-856-2889 ext. 8902. Fax: (64)-7-838-4219. E-mail: michele@waikato.ac.nz.
† University of Waikato.
‡ University of Hawaii.
§ Fox Chase Cancer Center.

S0163-3864(97)00566-1 CCC: $15.00 © 1998 American Chemical Society and American Society of Pharmacognosy
Published on Web 08/07/1998

Table 1. ^{1}H and ^{13}C NMR Data (δ in ppm) for Tolyporphin J (**1**)

position	δ_H[a]	δ_H[b]	δ_C[a]	δ_C[b]	HMBC (H to C)	ROESY
1			137.5	134.7[c]		
2	9.23	8.32	126.5	124.8	C-4	H-21
3			136.4[c]	136.1		
4			137.9[c]	136.5[d]		
5	9.60	8.76	96.4	95.8	C-4	H-21, H-22
6			163.3	159.6		
7			77.7	77.2		
8			207.8	207.8		
9			144.4[d]	142.7		
10	9.62	8.86	95.1	94.1	C-8, C-11	H-23
11			138.0[e]	134.5[c]		
12			136.7[e]	136.0		
13	9.18	8.35	126.1	124.1	C-11	H-15, H-23
14			138.1	136.5[d]		
15	9.52	8.74	98.5	96.4		H-13, H-24
16			163.9	160.1		
17			78.0	77.8		
18			207.9	207.6		
19			145.6[d]	141.5		
20	9.57	8.85	96.9	94.3	C-18	
21	3.77	3.45	13.7[f]	13.3	C-2	H-2, H-5
22	2.08	1.72	25.0[g]	24.7[e]	C-6, C-8	H-5
23	3.76	3.33	13.6[f]	13.0	C-13	H-10, H-13
24	2.07	1.71	24.9[g]	24.7[e]	C-16, C-18	H-15
NH 1	−2.9 br	−5.1[c] br				
NH 2	−2.9 br	−5.2[c] br				
OH 1	6.27[c]					
OH 2	6.23[c]					

[a] Chemical shifts determined in acetone-d_6. [b] Chemical shifts determined in $CDCl_3$. [c] Values within a column with the same superscript may be interchanged.

Table 2. ^{1}H and ^{13}C NMR Data (δ in ppm, J in Hz) for Tolyporphin K (**2**) in $CDCl_3$

position	δ_H	δ_C	HMBC (H to C)	ROESY
1		136.6[a]		
2	8.97	124.8	C-3	H-20, H-21
3		134.9[b]		
4		137.3[b]		
5	10.01	100.8	C-4, C-7	H-21, H-22
6		152.6[c]		
7		143.7		
8	8.82 d (1.0)	131.3	C-9	H-10, H-22
9		152.8[c]		
10	9.82	101.9	C-8, C-11	H-8, H-23
11		138.4		
12		136.4[a]		
13	8.94	125.2	C-11	H-15, H-23
14		138.5		
15	9.48	96.2	C-13, C-14, C-17	H-13, H-24
16		163.7		
17		56.2		
18		207.8		
19		147.2		
20	9.75	95.1	C-2	H-2
21	3.70 d (0.6)	13.5	C-2, C-3	H-2, H-5
22	3.61 d (1.0)	13.5	C-6, C-7, C-8	H-5, H-8
23	3.69 d (0.7)	13.6	C-12	H-10, H-13
24	2.18	20.7	C-16, C-17, C-18, C-1′	H-15, H-1′
NH 1	−3.0[a] br			
NH 2	−2.9[a] br			
R^1 1′	4.31 d (9.6)	87.0	C-16, C-2′	H-24, H-3′$_{ax}$, H-5′
2′	3.05 br (9.6)	65.7		H-3′$_{ax}$, H-3′$_{eq}$, H-6′
3′$_{ax}$	1.59 obscured	41.0	C-4′, C-5′	H-1′, H-2′, H-3′$_{eq}$, H-4′
3′$_{eq}$	1.79 ddd (13.3, 5.0, 4.8)			H-2′, H-3′$_{ax}$, H-4′
4′	3.64 br d (5.8)	69.8		H-3′$_{ax}$, H-3′$_{eq}$, H-5′
5′	3.97 dq (6.4, 1.1)	77.2	C-4′	H-1′, H-4′, H-6′
6′	1.60 d (6.4)	17.1		H-2′, H-5′

[a] Values within a column with the same superscript may be interchanged.

tion assays along with tolyporphin A as a comparison. We have previously shown that the tolyporphins are reasonably potent inhibitors of drug transport by P-glycoprotein.[2,6] However, these compounds only become cytotoxic at doses 5−10-fold higher than those necessary for inhibition of P-glycoprotein, making the therapeutic index similar to that of verapamil and most other MDR reversing agents.[6] In the MDR reversal assay, tolyporphin J (**1**) exhibited virtually identical activity to tolyporphin A. Both compounds sensitized MCF-7/ADR cells to actinomycin D, reversing MDR and verifying their abilities to enhance drug accumulation. Tolyporphin K (**2**) exhibited little activity (Figure 1).

Doses of tolyporphins A and J that sensitized MCF-7/ADR cells to actinomycin D were approximately 2-fold lower than doses that began to demonstrate cytotoxicity. While this relatively narrow window of therapeutic efficacy is quite common for MDR modulators,[7] these porphyrin-based compounds pose a unique difficulty in that their cytotoxicity is mediated by light-dependent production of oxygen-free radicals[8] while the reversal of drug resistance is due to their binding to P-glycoprotein.[6] This results in underestimation of the potential utility of these compounds as MDR agents since the cytotoxicities of the porphyrins is negligible *in vivo* as no light will be available to activate the metal.

To more mechanistically characterize the biological properties of these compounds, we have also assessed the abilities of tolyporphins to inhibit P-glycoprotein function. This can be measured as an increase in the accumulation of [^{3}H]vinblastine by MCF-7/ADR cells. Both tolyporphin A and tolyporphin J (**1**) increased the accumulation of [^{3}H]vinblastine in dose-dependent manners, with tolyporphin A being approximately 2-fold more potent than tolyporphin J (Figure 2). It is notable that both of these compounds were at least as potent as verapamil and that the maximal amount of [^{3}H]-vinblastine accumulation was greater in cells treated with either tolyporphin A or tolyporphin J than in cells treated with verapamil. We have previously demonstrated[6] that increased accumulation of [^{3}H]vinblastine by MCF-7/ADR cells by tolyporphin A is associated with inhibition of drug efflux due to direct interaction of this compound with P-glycoprotein, and this seems likely to be the mechanism of the effects of tolyporphin J as well. The tolyporphins were not toxic to the cells in the time period of this assay (60 min), assessed as a lack of an increase in trypan blue-staining of the cells (data not shown). In contrast with tolyporphins A and J (**1**), tolyporphin K (**2**) promoted only modest increases in [^{3}H]vinblastine accumulation, consistent with its poor ability to sensitize these cells to cytotoxic drugs (Figure 2). It is possible that the marked lack of activity for tolyporphin K is due to its different macrocyclic ring structure compared with all other tolyporphins but scarcity precluded further investigation.

Experimental Section

General Experimental Procedures. NMR spectra were determined on a 9.4 T instrument, operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. ^{1}H NMR chemical shifts are referenced in $CDCl_3$ to residual $CHCl_3$ (7.26 ppm) and in acetone-d_6 to the solvent (2.20

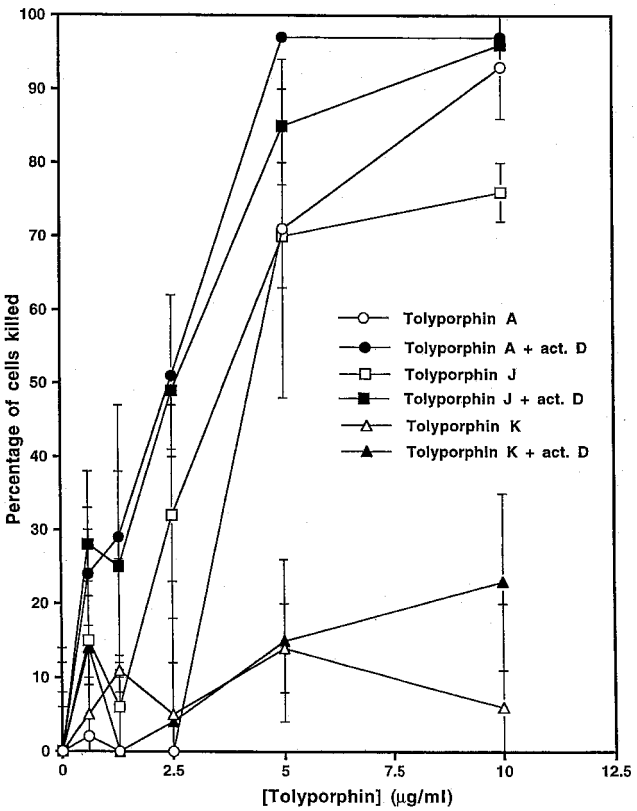


Figure 1. Results of MDR reversal assay (expressed as a percentage of cells killed) for tolyporphins A, J, and K, alone and when administered with actinomycin D (act. D).

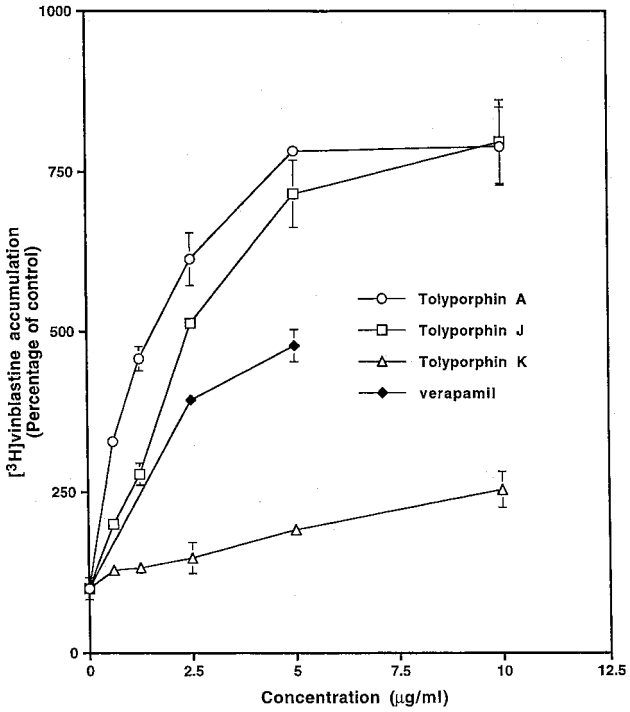


Figure 2. Results of [^{3}H]vinblastine accumulation assay (expressed as a percentage of control) for tolyporphins A, J, and K and for verapamil.

ppm). ^{13}C NMR chemical shifts are referenced to solvent peaks, 77.0 ppm in $CDCl_3$ and 30.2 ppm in acetone-d_6. Heteronuclear ^{1}H−^{13}C connectivities were determined by HMQC and HMBC experiments. Mass spectra were recorded on a VG 7070E mass spectrometer, operating at 70 eV. Optical rotations were measured in a JASCO J-20c automatic recording spectropolarimeter while UV spectra were obtained using an Hitachi 150−20 spectrophotometer.

Organism and Culture Conditions. *T. nodosa*, designated strain HT-58-2, was isolated from a soil sample collected at Nan Madol, Pohnpei. The culture is deposited in the culture collection at the Department of Chemistry, University of Hawaii. Clonal cultures were prepared by repeated subculture on solidified media. The cyanophyte was cultured in 20-L glass bottles containing a modification of inorganic medium BG-11.[5] Prior to autoclaving, the pH of the medium was adjusted to 7.0 with NaOH. Cultures were illuminated continuously at an incident intensity of 25 μmol photons PAR (photosynthetically active radiation) m^{-2} s^{-1} from banks of cool-white fluorescent tubes. Aeration was at a rate of 5 L/min with a mixture of 0.5% CO_2 in air and incubation at a temperature of 24 ± 1 °C. After 28 days, the cyanophyte was harvested by filtration and freeze-dried. Yields of lyophilized cells ranged from 0.17 to 0.33 g/L.

Isolation of Tolyporphin J (1). Freeze-dried cyanophyte (93 g) was extracted in a blender with 3 × 1 L portions of CH_2Cl_2/2-propanol (1:1). The combined extract was filtered and the solvent removed in vacuo. The crude extract (7.5 g) was fractionated by reversed-phase flash chromatography on a column of C-18 silica (80 g, YMC Gel ODS-A, 120 Å) using a steep-stepped gradient from H_2O to MeOH to CH_2Cl_2. Repeated normal-phase column chromatography on silica gel (Davisil 200−425 mesh, 60 Å) using a hexane/EtOAc gradient yielded a mixture of tolyporphins (6.8 mg). Gel permeation on Sephadex LH-20 (10 g, Pharmacia) in MeOH yielded **1** (1.5 mg) as an amorphous red-purple solid, pure by TLC analysis (silica gel, hexane/EtOAc 1:2, R_f = 0.8), $[\alpha]^{25}_D$ +130° (*c* 0.0001, MeOH); UV (MeOH) λ_{max} (log ϵ) 287 (3.66), 306 (sh) (3.64), 396 (3.90), 480 (sh) (2.44), 486 (2.43), 503 (2.54), 544 (2.52), 580 (2.14), 607 (sh) (2.11), 635 (2.64), 669 (2.83), 688 (3.19) nm; for ^{1}H and ^{13}C NMR see Tables 1-2; HREIMS *m/z* 430.1651 $[M]^+$ (calcd for $C_{24}H_{22}N_4O_4$, 430.1641).

Isolation of Tolyporphin K (2). Tolyporphin K was isolated in a manner similar to that for tolyporphin J. After reversed-phase flash column chromatography and repeated column chromatography on silica gel, gel permeation on Sephadex LH-20 (10 g, Pharmacia) in MeOH yielded **2** (1 mg) as an amorphous red-purple solid, pure by TLC analysis (silica gel, hexane:EtOAc 1:2, R_f = 0.5): $[\alpha]^{25}_D$ −70° (*c* 0.0001, MeOH); UV (MeOH) λ_{max} (log ϵ) 298 (2.82), 397 (3.54), 502 (2.33), 539 (2.24), 581 (2.09), 635 (2.58), 663 (2.09), 682 (1.86) nm; for ^{1}H and ^{13}C NMR see Tables 1-2; HREIMS *m/z* 512.2450 $[M]^+$ (calcd for $C_{30}H_{32}N_4O_4$, 512.2424).

Biological Assays and Cell Culture. Tolyporphins were dissolved in ethanol and stored at 4 °C. [^{3}H]-Vinblastine sulfate was obtained from the Amersham Corp., and all other drugs and reagents were purchased from the Sigma Chemical Co. MCF-7/ADR cells were grown as previously described.[6]

MDR Reversal Assay. To test the effects of drugs on growth, MCF-7/ADR cells were seeded in 96-well tissue culture dishes at approximately 10% of confluency in Basal Medium containing Earl's salts (BME) plus

10% fetal bovine serum (FBS) and were allowed to attach and recover for at least 24 h. Varying concentrations of tolyporphins A, J, and K and verapamil were then added to each well, either alone or combined with actinomycin D. The plates were incubated for an additional 48 h, and the number of surviving cells was then determined by staining with sulforhodamine B (SRB).[9] The percentage of cells killed was calculated as the percentage decrease in SRB binding compared with control cultures. Control cultures included equivalent amounts of dimethylformamide, which does not modulate the growth or drug sensitivity of these cells. Reversal of MDR is defined as the ability of the compound, i.e., verapamil or a tolyporphin, to potentiate the cytotoxicity of P-glycoprotein-transported drugs.

[^{3}H]Vinblastine Accumulation Assay. Following methods previously described,[2,6] MCF-7/ADR cells were plated into 24-well tissue culture dishes and allowed to grow to 90% confluency. The cells were washed with phosphate buffer saline (PBS) and then incubated in medium (0.5 mL) containing tolyporphins A, J, or K and 10−20 nM [^{3}H]vinblastine sulfate (10−15 Ci/mmol) for 60 min at 37 °C. The cultures were rapidly washed three times with ice-cold PBS. Intracellular [^{3}H]vinblastine was solubilized with 0.3 mL of 1% sodium dodecyl sulfate (SDS) in PBS and quantified by liquid scintillation counting.

Acknowledgment. This research was supported by a University of Waikato Research Commitee grant and a New Zealand Lotteries Science Research grant. The culture of the cyanophyte was supported by NIH Grant No. CA 12623. We gratefully acknowledge the assistance of Dr. Dick Moore (University of Hawaii) for helpful discussions and Mr. Michael Walker (University of Auckland) for determining the mass spectra.

References and Notes

(1) Prinsep, M. R.; Caplan, F. R.; Moore, R. E.; Patterson, G. M. L.; Smith, C. D. *J. Am. Chem. Soc.* **1992**, *114*, 385−387.
(2) Prinsep, M. R.; Patterson, G. M. L.; Larsen, L. K.; Smith, C. D. *Tetrahedron* **1995**, *51*, 10523−10530.
(3) Prinsep, M. R.; Thomson, R. A.; West, M. L.; Wylie, B. L. *J. Nat. Prod.* **1996**, *59*, 786−788.
(4) Patterson, G. M. L.; Baldwin, C. L.; Bolis, C. M.; Caplan, F. R.; Karuso, H.; Larsen, L. K.; Levine, I. A.; Moore, R. E.; Nelson, C. C.; Tshappat, K. D.; Tuang, G. D.; Furusawa, E.; Furusawa, S.; Norton, T. R.; Raybourne, R. B. *J. Phycol.* **1991**, *27*, 530−536.
(5) Patterson, G. M. L.; Baker, K. K.; Baldwin, C. L.; Bolis, C. M.; Caplan, F. R.; Larsen, L. K.; Levine, I. A.; Moore, R. E.; Nelson, C. S.; Tshappat, K. D.; Tuang, G. D.; Boyd, M. R.; Cardellina, J. H., II; Collins, R. P.; Gustafson, K. R.; Snader, K. M.; Weislow, O. S.; Lewin, R. A. *J. Phycol.* **1993**, *29*, 125−130.
(6) Smith, C. D.; Prinsep, M. R.; Caplan, F. R.; Moore, R. E.; Patterson, G. M. L. *Oncology Res.* **1994**, *6*, 211−218.
(7) Ford, J. M.; Hait, W. N. *Pharmacol. Rev.* **1990**, *42*, 155−199.
(8) Moliere, P.; Maziere, J.-C.; Santus, R.; Smith, C. D.; Prinsep, M. R.; Stobbe, C. C.; Fenning, M. C.; Golberg, J. L.; Chapman, J. D. *Cancer Res.*, submitted.
(9) Skehan, P.; Stoneng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107−1112.

NP970566+