Niphatoxin C, a Cytotoxic Tripyridine Alkaloid from *Callyspongia* sp.

Malcolm S. Buchanan,[†] Anthony R. Carroll,[†] Rama Addepalli,[†] Vicky M. Avery,[†] John N. A. Hooper,[‡] and Ronald J. Quinn*[,†]

Eskitis Institute, Griffith University, Brisbane, Queensland 4111, Australia, and Queensland Centre for Biodiversity, Queensland Museum, South Brisbane, Queensland 4101, Australia

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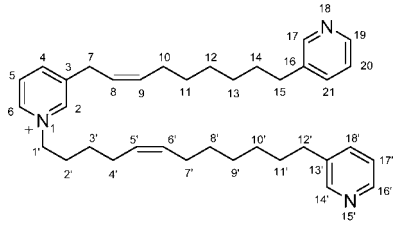
As part of our studies to discover $P2X_7$ receptor antagonists, the sponge *Callyspongia* sp. was investigated. A new tripyridine alkaloid niphatoxin C (**1**) was isolated and had $P2X_7$ receptor antagonism; however, cytotoxicity of THP-1 cells was the predominant biological effect at higher concentrations. Its structure was determined by 1- and 2-D NMR spectroscopy.

Previously we reported the isolation of the tetrameric pyrrole-imidazole alkaloids stylissadines A and B, from *Stylissa flabellata,* as specific $P2X_7$ antagonists.[1] During our $P2X_7$ campaign (62 749 extracts) another extract that showed $P2X_7$ inhibition was the Australian marine sponge *Callyspongia* sp. (Callyspongiidae). Bioassay-guided purification of this extract yielded the tripyridine alkaloid niphatoxin C (**1**) as the bioactive constituent. The aim was to discover selective $P2X_7$ receptor antagonists for the treatment of the inflammatory diseases osteoarthritis, rheumatoid arthritis, and chronic obstructive pulmonary disease (COPD). Niphatoxin C (**1**) belongs to the 3-alkylpyridinium class of alkaloids, which are a well-known family of marine natural products.[2] The known compounds niphatoxins A and B, which have ichthyo- and cytotoxic activity, are also tripyridine alkaloids and have similar structures to niphatoxin C (**1**).[3] Sponges of the genus *Callyspongia* are known to produce polymeric pyridinium alkaloids,[4,5] but no small molecular weight pyridinium compounds have been reported. The isolation, structure elucidation, and biological activity of niphatoxin C (**1**) will be discussed here.



(1)

Niphatoxin C (**1**) had a molecular formula determined to be $C_{36}H_{50}N_3^+$ by HRESIMS (*m/z* 262.702930 $[C_{36}H_{50}N_3+H]^{2+}$, calcd 262.703601). The ^{1}H NMR data analysis (Table 1) showed resonances for a 1,3-disubstituted pyridinium ring, including four aromatic hydrogens at δ 8.97 (s, H2), 8.94 (d, 6.0 Hz, H6), 8.41 (d, 8.4 Hz, H4), and 8.08 (dd, 8.4, 6.0 Hz, H5) as well as methylene resonances at δ 4.57 (t, 7.5 Hz, H1′) and 3.59 (d, 7.2 Hz, H7). There were also resonances for two 3-alkylpyridine units with isochronous chemical shifts, including eight aromatic hydrogens at δ 8.60 (brs, H17/14′), 8.57 (brd, 6.0 Hz, H19/16′), 8.00 (brd, 8.4 Hz, H21/18′), and 7.62 (brdd, 8.4, 6.0 Hz, H20/17′) as well as a methylene resonance at δ 2.67 (m, H15/12′). The ^{1}H NMR spectrum contained a further four olefinic resonances [δ 5.61 (brdt, 10.8, 7.2 Hz, H9); 5.56 (brdt, 10.8, 7.2 Hz, H8); 5.35 (brdt, 10.8, 7.2 Hz, H6′); 5.30 (brdt, 10.8, 7.2 Hz, H5′)], and the remaining resonances were for upfield aliphatic protons that resonated between δ 1.28 and 2.12, many of which were overlapping. A thorough study of the 2D NMR data (Table 1) revealed the partial structures shown in Figure 1. The *Z*-geometry of the double bonds was obtained on the basis of the coupling constant of the H8−H9 (10.8 Hz) and H5′−H6′ (10.8 Hz) double bonds as well as the ^{13}C chemical shift values of the allylic methylenes (δ_C 29.5, 26.7, 26.0, 26.5).[6] With one unit of partial structure A and two units of partial structure B, there remained four methylenes (δ 1.28, 8H; δ_C28.3, 4C) to incorporate into the structure and complete the molecular formula. These were assigned C12–C13 and C9′−C10′ and joined partial structure A to the two units of partial structure B. ESIMS data strongly supported this structure. Thus, under high-energy

Table 1. ^{1}H (600 MHz), ^{13}C (125 MHz), gCOSY, and gHMBC NMR Data for Niphatoxin C (**1**) in DMSO-d_6

position	δ_C	δ_H (mult, *J* Hz)	COSY (H no.)	$^{2,3}J_{CH}$ HMBC (C no.)
2	143.7 CH[a]	8.97 (s)		3, 4, 6, 7, 1′
3	141.8 qC			
4	144.9 CH	8.41 (d, 8.4)	5	6, 7
5	127.7 CH	8.08 (dd, 8.4, 6.0)	4, 6	3
6	142.4 CH	8.94 (d, 6.0)	5	4, 5, 1′
7	29.5 CH_2	3.59 (d, 7.2, 2H)	8	3, 4, 8, 9
8	125.0 CH	5.56 (brdt, 10.8, 7.2)	7	7, 10
9	133.0 CH	5.61 (brdt, 10.8, 7.2)	10	7, 10
10	26.7 CH_2	2.12 (q, 7.0, 2H)	9, 11	8, 9, 11
11	28.7 CH_2	1.34 (m, 2H)	10	
12	28.3 CH_2[b]	1.28 (m, 2H)[c]		
13	28.3 CH_2[b]	1.28 (m, 2H)[c]		
14	30.2 CH_2	1.58 (m, 2H)[d]	15	15, 16
15	31.8 CH_2	2.67 (q, 7.0, 2H)	14	14, 16, 17, 21
16	139.5 qC			
17	145.8 CH	8.60 (brs)		15, 16, 21
19	143.7 CH[a]	8.57 (brd, 6.0)	20	17, 20, 21
20	124.8 CH	7.62 (brdd, 8.4, 6.0)	19, 21	16
21	140.1 CH	8.00 (brd, 8.4)	20	17
1′	60.6 CH_2	4.57 (t, 7.5, 2H)	2′	2, 6, 2′, 3′
2′	30.3 CH_2	1.90 (quin., 7.5, 2H)	1′	1′, 3′
3′	25.5 CH_2	1.29 (m, 2H)[c]		
4′	26.0 CH_2	2.01 (q, 7.5, 2H)	5′	2′, 3′, 5′, 6′
5′	128.7 CH	5.30 (brdt, 10.8, 7.2)	4′	
6′	130.2 CH	5.35 (brdt, 10.8, 7.2)	7′	
7′	26.5 CH_2	1.96 (q, 7.5, 2H)	6′	5′, 6′, 8′
8′	28.9 CH_2	1.28 (m, 2H)[c]		
9′	28.3 CH_2[b]	1.28 (m, 2H)[c]		
10′	28.3 CH_2[b]	1.28 (m, 2H)[c]		
11′	30.2 CH_2	1.58 (m, 2H)[d]	12′	12′, 13′
12′	31.8 CH_2	2.67 (q, 7.0, 2H)	11′	11′, 13′, 14′, 18′
13′	139.5 qC			
14′	145.8 CH	8.60 (brs)		12′, 13′, 18′
16′	143.7 CH[a]	8.57 (brd, 6.0)	17′	14′, 17′, 18′
17′	124.8 CH	7.62 (dd, 8.4, 6.0)	16′, 18′	13′
18′	140.1 CH	8.00 (brd, 8.4)	17′	14′

[a–d] Assignments with the same superscript are interchangeable.

* To whom correspondence should be addressed. Tel: +61 7 3735 6006.Fax: +61 7 3735 6001. E-mail: r.quinn@griffith.edu.au.
[†] Griffith University.
[‡] Queensland Museum.

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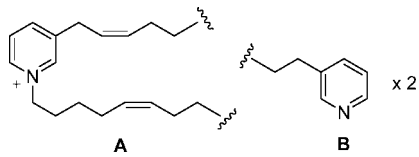

Figure 1. Partial structures of compound **1**.

source conditions the fragments at *m/z* 281 and 244 were produced from cleavage of the N1−C1′ bond. Niphatoxin C was therefore the tripyridine alkaloid with structure **1**.

The $P2X_7$ biological screen, described in detail previously,[1] was set up to identify specific antagonists of the $P2X_7$ receptor (ligand gated ion channel). Receptor inhibition was determined from the reduction of plasma membrane pore formation by the synthetic ATP analogue benzoylbenzoyl adenosine triphosphate (BzATP). Upon pore formation by BzATP, the membrane-impermeable nucleic acid stain Sytox Orange enters the cells through the pores and binds to nucleic acids, forming a fluorescent complex. Antagonists of the $P2X_7$ receptor inhibit opening of these pores, stopping Sytox Orange from entering the cells, and this leads to reduced fluorescence. When niphatoxin C (**1**) was screened at 10 μM in the $P2X_7$ assay (90 min incubation), it showed 41% reduction in fluorescence compared to DMSO, suggesting that it was antagonizing the $P2X_7$ receptor. At higher concentrations the cytotoxicity predominates (0% at 30 μM; 99% at 100 μM), and so it was not possible to determine an IC_{50} for $P2X_7$ antagonism. When **1** was incubated for 21 h, it had cytotoxicity IC_{50} of 11.5 μM. To determine nonspecific compound inhibition, a counterassay was used where BzATP was replaced with the nonspecific pore-forming compound hemolysin. Hemolysin is a bacterial protein toxin that can form nonspecific transmembrane channels, which allow uncontrolled permeation of water, ions, and small organic molecules across the membrane. When **1** was screened at 10 μM using the hemolysin counterscreen, it showed an increased fluorescence (101%) relative to DMSO, and when the dose was increased to 100 μM, **1** showed a 185% increase in fluorescence relative to DMSO. This indicated that the compounds were making the cells even more leaky than the hemolysin alone. Interestingly, it has been suggested that the halitoxins (1,3-alkylpyridinium salts) insert into biological and artificial lipid membranes to form ion-permeable pores.[5] We could therefore speculate that the unusual activity values in the $P2X_7$ and hemolysin assays could be partly due to **1** forming ion-permeable pores in the membrane of the THP-1 cells. However, the main biological effect of **1** was cytotoxicity against the premonocytic cell line THP-1, on which the $P2X_7$ receptor was expressed.

In conclusion a new 3-alkylpyridinium alkaloid has been isolated and its structure determined (**1**). Niphatoxin C (**1**) demonstrates cytotoxicity against THP-1 cells and possible ion permeable pore forming ability.

Experimental Section

General Experimental Procedures. Water was Millipore Milli-Q PF filtered, while all other solvents used were Laboratory-Scan HPLC grade. TFA was Fluka spectroscopic grade. A Betasil C_{18} 5 μm (21.2 mm × 150 mm i.d.) was used for semipreparative HPLC. A Waters 600 pump fitted with a 996 photodiode array detector and 717 plus autosampler was used for the semipreparative separations. C_{18} was 04K-4348 Sepra C_{18} end-capped silica. NMR spectra were recorded at 30 °C on Varian Inova 500 and 600 MHz NMR spectrometers. Samples were dissolved in DMSO-d_6 (residual ^{1}H δ 2.50 and ^{13}C δ 39.5 ppm). Multiplicity was determined by DEPT (s = C, d = CH, t = CH_2, q = CH_3). Standard parameters were used for the 2D experiments, which included gradient COSY, HSQC ($^1J_{CH}$ = 140 Hz) and HMBC ($^nJ_{CH}$ = 8.3 Hz). FTIR and UV spectra were recorded on a Bruker Tensor 27 FTIR spectrophotometer and an Agilent 8453 UV/vis spectrophotometer, respectively. HRESIMS were measured on a Bruker Daltonics Apex III 4.7e Fourier transform mass spectrometer, fitted with an Apollo API source. A WALLAC Victor II was used as the spectrophotometric reader. Assay materials: EDTA, glucose, and BzATP were obtained from Sigma Aldrich. KCl and Hepes were obtained from BDH. Sytox Orange was purchased from Molecular Devices. RPMI media with glutamax was purchased from Invitrogen. White 384 optiplates were obtained from Perkin-Elmer Life Sciences. THP-1 cells were from AstraZeneca.

Animal Material. The sponge sample *Callyspongia* sp. 1379 (phylum Porifera, class Demospongiae, order Haplosclerina, family Callyspongiidae) was collected by scuba diving at a depth of 14 m at North East Erskine Island, Capricorn-Bunker Group, Queensland, Australia, in August 1996. A voucher sample, QMG307571, was lodged at the Queensland Museum, South Brisbane, Queensland, Australia.

Extraction and Isolation. The material was ground (19.3 g) and extracted sequentially with *n*-hexane, CH_2Cl_2/MeOH (4:1), and finally MeOH. The CH_2Cl_2/MeOH (4:1) and MeOH extracts were combined (502 mg). The sample was preadsorbed on C_{18} and loaded into a refillable preparative guard column (30 mm × 10 mm i.d.) in line with the semipreparative C_{18} HPLC column. The following solvent conditions were used: H_2O/1% TFA to MeOH/1% TFA in 90 min, then isocratic for 30 min (flow 5 mL/min); 60 fractions were collected. Fraction 33 (4.1 mg) was purified further by C_{18} HPLC: H_2O/1% TFA to H_2O/1% TFA:MeOH/1% TFA (2:3) in 5 min, followed by isocratic for 15 min, then to MeOH/1% TFA in 5 min, and finally isocratic for 5 min. Niphatoxin C (**1**) (2.6 mg, 0.013% dry wt) was collected as its TFA salt, with a retention time of 13.2 min.

Bioassays. The principle, procedure, and method for the $P2X_7$, hemolysin specificity and Alamar Blue cytotoxicity assays have been described in detail previously.[1]

Niphatoxin C (1), 1-[(5*Z*)-12-pyridin-3-yldodec-5-en-1-yl]-3-[(2*Z*)-9-pyridin-3-ylnon-2-en-1-yl]pyridinium. Amorphous solid; UV (MeOH) λ_{max} (log ε) 203 (4.14), 263 (3.84) nm; IR ν_{max} (film) 3442, 1681, 1205, 1135, 723 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; positive-ion HRESIMS *m/z* 262.7029 $[C_{36}H_{51}N_3+H]^{2+}$ (calcd 262.7036), 281.2006 $[C_{19}H_{25}N_2]^+$ (calcd 281.2012), 244.2054 $[C_{17}H_{26}N]^+$ (calcd 244.2060).

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References and Notes

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