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(+)-Echinobetaine B: isolation, structure elucidation, synthesis and preliminary SAR studies on a new nematocidal betaine from a southern Australian marine sponge, *Echinodictyum* sp.

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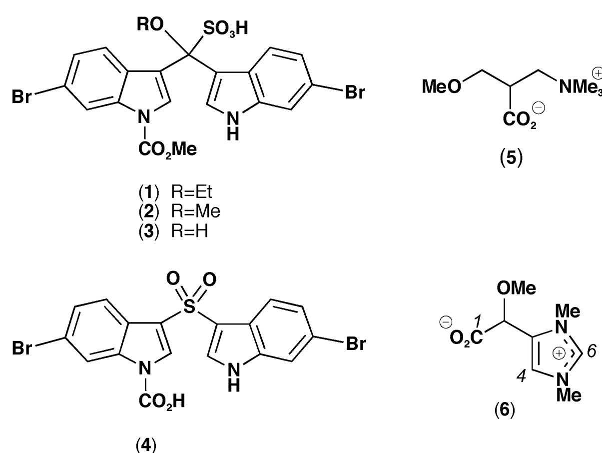
The principle nematocidal agent present in a southern Australian marine sponge of the genus *Echinodictyum* has been isolated and identified as the novel betaine (+)-echinobetaine B (**6**), and the structure assigned by spectroscopic analysis has been confirmed by total synthesis. Preliminary SAR conclusions are drawn from analysis of synthetic intermediates and the known marine metabolites zooanemonin (**12**) and norzooanemonin (**13**), and the new sponge metabolite norzooanemonin methyl ester (**14**). The latter compound is reported for the first time from a selection of Australian sponges, including an *Axinyssa* sp., a *Niphates* sp., an *Axinella* sp. and a *Ptilocaulis* sp.

Introduction

During earlier investigations into new agrochemical agents from the Australian marine sponge *Echinodictyum* sp. we reported a series of novel antibacterial metabolites, echinosulfonic acids A–C (**1–3**) and echinosulfone (**4**),[1] along with the nematocidal betaine (−)-echinobetaine A (**5**).[2] Although initial interest in this *Echinodictyum* sp. was prompted by promising nematocidal bioassay data against the endo parasite *Haemonchus contortus*, it is noteworthy that **1–4** displayed no nematocidal activity while the nematocidal properties of (−)-echinobetaine A (**5**) were insufficient (LD_{99} 83 µg mL^{-1}) to account for the level of activity observed in the crude EtOH extract (LD_{99} 22 µg mL^{-1}). The identity of the more potent *Echinodictyum* sp. nematocidal agent can now be revealed as the novel betaine (+)-echinobetaine B (**6**) (LD_{99} 8.3 µg mL^{-1}).



Results and discussion

The EtOH extract of the *Echinodictyum* specimen was decanted, concentrated *in vacuo* and triturated with DCM after which the residual solid was partitioned between *n*-BuOH and H_2O. Whereas the *n*-BuOH solubles displayed antibacterial activity and ultimately led to the isolation and identification of the echinosulfonic acids A–C (**1–3**) and echinosulfone (**4**),[1] the crude H_2O solubles displayed significant nematocidal activity.

Table 1 NMR (400 MHz; D_2O) data for (+)-echinobetaine B (**6**)

Number	^{13}C (ppm)	^{1}H δ (multiplicity)	gHMBC (^{1}H–^{13}C)
1	172.5		
2	73.3	4.78 (s)	C-1, C-3, C-4, 2-OMe
2-OMe	57.1	3.22 (s)	C-2
3	130.7		
4	123.6	7.31 (s)	N5–Me, C-3, C-6,
N5-Me	35.7	3.78 (s)	C-6, C-4
6	138.1	8.52 (s)	C-3, C-4
N7-Me	33.9	3.63 (s)	C-6, C-3

The H_2O solubles were initially further fractionated by elution through Sephadex G-10 (H_2O) followed by isocratic C_{18} HPLC (0.1% TFA in H_2O) to yield nematocide (−)-echinobetaine A (**5**).[2] More extensive HPLC studies on nematocidal active fractions have since yielded the principle nematocidal agent as (+)-echinobetaine B (**6**).

High resolution ESI(+)MS analysis of **6** revealed a highest mass ion at *m*/*z* 185 consistent with a molecular formula ($C_8H_{12}N_2O_3$, Δ 0.5 mmu) requiring four double bond equivalents. Examination of the NMR data for **6** (see Table 1) revealed resonances consistent with an OMe (^{1}H: δ 3.22 (s); ^{13}C: 57.1 ppm), two NMe's (^{1}H: δ 3.78 and 3.63; ^{13}C: 35.7 and 33.9 ppm), two sp^2 methines (^{1}H: δ 8.52 and 7.31; ^{13}C: 138.1 and 123.6 ppm), one deshielded sp^3 oxymethine (^{1}H: δ 4.78; ^{13}C: 73.3 ppm), one carboxylic acid or amide carbonyl (^{13}C: δ 172.5 ppm), and a quaternary sp^2 hybridized carbon (^{13}C: 130.7 ppm). The presence of the carbonyl functionality was further supported by a characteristic IR absorbance (1689 cm^{-1}), while a measurable $[\alpha]_D$ (+30) confirmed the presence of a chiral centre. With a total of four sp^2 carbons, one attributed to a carboxylic acid moiety, **6** was determined to contain an N-heterocycle. Analysis of the gHMBC NMR data (Table 1) confirmed that the chiral tertiary carbon (C-2) was substituted by both OMe and carbonyl moieties. Further gHMBC NMR correlations from N5-Me to C-4 and C-6, from N7-Me to C-3 and C-6, and from H-2 to C-3 and C-4, suggested that (+)-echinobetaine B was the substituted imidazole betaine as shown. To confirm the assigned structure a total synthesis of (±)-echinobetaine B (**11**) was undertaken following the synthetic pathway outlined in Scheme 1.

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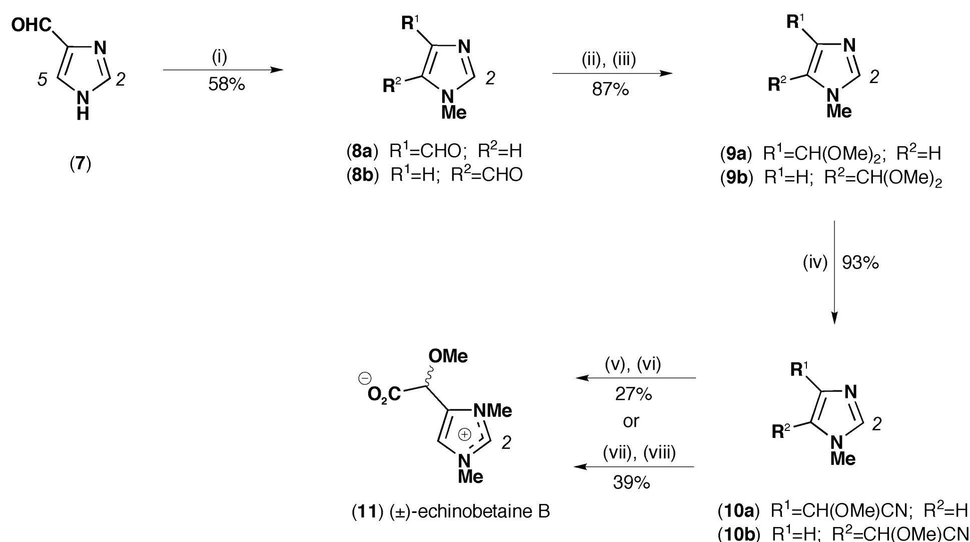
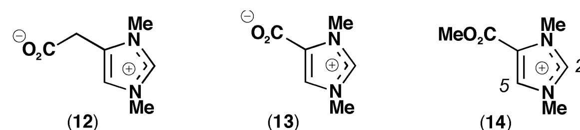


Scheme 1 Synthesis of (±)-echinobetaine B (**11**). *Reagents and conditions*: (i) NaH, MeI, DMF, N_2, 0 °C to RT, 3.5 h; (ii) 10% $HCl_{(aq)}$, RT; (iii) *p*-TsOH, MeOH, N_2, 64 °C, 12 h; (iv) TMSCN, $SnCl_2$, CH_3CN, N_2, RT, 96 h; (v) MeI, acetone, N_2, 56 °C, 15 h; (vi) 10 M $HCl_{(aq)}$, 100 °C, 36 h; (vii) 10 M $HCl_{(aq)}$, 100 °C, 15 h; (viii) MeI, DMF, N_2, 70 °C, 14 h.

Commercially available imidazole carboxaldehyde (**7**) was viewed as a suitable starting material for the synthesis of (±)-echinobetaine B (**11**). As initially conceived, treatment of **7** with cyanide might be expected to return the cyanohydrin, which on per-methylation about oxygen (2-OMe) and nitrogen (N5-Me and N7-Me) could yield a suitable organonitrile precursor. This precursor would in turn be a potential substrate for commercially available stereoselective nitrilases[3,4] leading to an asymmetric synthetic outcome. In practice, the proposed cyanohydrin derived from treatment of **7** with either cyanide salts or HCN proved unstable and difficult to handle, necessitating a modification (see Scheme 1) to the strategy as detailed above. Monomethylation of **7** yielded the mixture **8a** and **8b**, which was readily converted in good yield to the mixed acetals **9a** and **9b**. Subsequent treatment with trimethylsilylcyanide and the Lewis acid $SnCl_2$[5,6] successfully converted the acetals in high yield to the unstable organonitrile mixture **10a** and **10b**. The noted instability of cyanohydrin and organonitrile precursors precluded stereoselective enzymatic hydrolysis, and necessitated a more direct chemical approach. Acid hydrolysis of the organonitrile moiety followed by N-methylation proceeded in low yield (27%), with the alternative strategy of N-methylation followed by acid hydrolysis returning a slightly improved yield (39%). The product obtained by either route, (±)-echinobetaine B (**11**), was identical by NMR, MS, UV and HPLC retention time comparison to the natural product (+)-echinobetaine B (**6**), differing only in the lack of a measurable optical rotation, as befits a racemate. Attempts to resolve the racemate by chiral HPLC proved unsuccessful.

Of particular note, the nematocidal properties for (±)-echinobetaine B (**11**) (LD_{99} 65 μg mL^{-1}) were considerably lower than those for the natural product (+)-echinobetaine B (**6**) (LD_{99} 8.3 μg mL^{-1}), implicating stereochemistry as a critical factor in the echinobetaine B pharmacophore. A similar observation had been made regarding the pharmacophore of the nematocidal co-metabolite (−)-echinobetaine A (**5**).[2]

(+)-Echinobetaine B (**6**) is a new example of a rare class of naturally occurring imidazole betaine. The first example of this structure class was zooanemonin (**12**) reported by Ackermann and Menssen in 1960 from the "horse sponge", *Hippospongia equina*.[7] Although the structure of **12** was revised shortly thereafter (following communication on its synthesis by Woodward and List),[8] few subsequent references have appeared in the scientific literature. The most recent reference to **12** was in 2001 by Hattori *et al.*, in which **12** was assessed as an antifouling constituent of the sponge *Protophilaspongia aga*.[9] As with zooanemonin (**12**), the closely related homologue norzooanemonin (**13**) is known but remains equally elusive in the scientific literature. First reported in 1973 by Weinheimer *et al.* from the gorgonian *Pseudopterogorgia americana*,[10] **13** was also described in 1977 by Gupta *et al.* from the hydroid *Tubularia larynx*.[11]



During our earlier investigation into Australian marine sponges we isolated norzooanemonin (**13**) from an *Axinyssa* and a *Niphates* sp. The latter sponge, together with an *Axinella* sp. and a *Ptilocaulis* sp., also yielded the previously undescribed norzooanemonin methyl ester **14**. The molecular formula assigned to **14** by high resolution mass spectrometry ($C_7H_{11}N_2O_2$, Δmmu −0.3) was consistent with a salt possessing 4 double bond equivalents. Analysis of the ^1H NMR (400 MHz, D_2O) data for **14** revealed resonances diagnostic for a deshielded sp^2 methine (H_δ 8.27, H-5) that proved to be D_2O exchangeable (see below), as well as an isolated deshielded sp^2 methine (H_δ 7.07, H-3), a CO_2Me (H_δ 3.46) and two quaternary ammonium methyl moieties (H_δ 3.56 and 3.66, N6-Me and N4-Me respectively). The data presented above supported a doubly *N*-methylated imidazole heterocycle substituted by a methyl ester moiety (as shown). Both ^{13}C and 2D NMR correlations supported this determination and facilitated assignment of the NMR data. In this way **14** was confirmed to be the methyl ester of norzooanemonin as indicated. As all sponge samples were extracted with EtOH, and were stored for several years in EtOH prior to investigation, we are confident that the methyl ester **14** is not an esterification artifact. Curiously, during acquisition of ^1H NMR data in neutral deuterated protic solvents (CD_3OD or D_2O) the imidazole methine H-5 in both **13** and **14** underwent slow but complete deuterium exchange. While such exchange processes have been described for a range of heterocycles, they are typically pH or metal-ion activated.[12,13] Such an exchange was not observed for **6** or **12** suggesting that the conjugated carbonyl present in **13** and **14** may play a pivotal role in this process, and revealing a measure of specificity not previously recorded.

It is worthwhile noting that the nematocidal activity displayed against *Haemonchus contortus* by the natural product (+)-echinobetaine B (**6**) (LD_{99} 8.3 μg mL^{-1}) in our assay system

is comparable to that of the two commercial synthetic anthelmintics closantel and levamisole (LD_{99} 5–10 µg mL^{-1}). By contrast, none of **12–14** displayed nematocidal activity, and the synthetic racemate **11** was considerably less active (LD_{99} 65 µg mL^{-1}). Thus it would appear that the 2-OMe and stereochemistry are critical components in the echinobetaine B nematocidal pharmacophore. Ongoing studies are directed at confirming enantiopurity and assignment of absolute stereochemistry for **6**, and towards further SAR investigations.

Experimental

General procedures

Solvents were dried according to standard techniques. Anhydrous $SnCl_2$ was prepared by heating commercial $SnCl_2$ dihydrate to 200 °C under an atmosphere of N_2 for 1 hour. Column chromatography (flash) was performed using Merck silica gel 60. Thin layer chromatography (TLC) was performed on Merck Silica 60 F_{254} sheets. Compounds were visualised with 254 nm UV light and/or heating of the TLC plate that had been dipped in *p*-anisaldehyde, phosphomolybdic acid or potassium permanganate reagent solutions. High performance liquid chromatography (HPLC) was performed using either a Waters 2790 Separations Module equipped with a Waters 996 Photodiode Array Detector, Alltech 500 Evaporative Light Scattering Detector and a Waters Fraction Collector II, running Waters Millennium software or an Agilent 1100 Series Separations Module equipped with a six column switching capability, Agilent 1100 Series Diode Array and/or Multiple Wavelength Detectors, Polymer Laboratories PL-ELS1000 Evaporative Light Scattering Detector (ELSD) and an Agilent 1100 Series Fraction Collector and running ChemStation Rev.9.03A and Purify version A.1.2 software. Unless otherwise specified, a constant level of 0.01% TFA was used in all HPLC separations. Chiroptical measurements ($[\alpha]_D$), given in 10^{-1} deg cm^2 g^{-1}, were obtained on either a Jasco Dip-1000 digital polarimeter or a Jasco P-1010 Intelligent Remote Module type polarimeter, at ambient temperature, in a 100 × 3 mm cell. Ultraviolet (UV) absorption spectra were obtained using either a Shimadzu UV-1650PC or a CARY3 UV-visible Spectrophotometer. Infrared (IR) spectra were acquired using either a Shimadzu FTIR-8400 or a Jasco FT/IR-460*Plus* Spectrometer, with samples examined as films on NaCl discs. ^1H and ^{13}C NMR spectra were acquired on a Varian Inova 400, a Varian Unity 400 plus, a Bruker Avance 500 or a Bruker Avance 600 Spectrometer, in the solvents indicated and referenced to residual ^1H and ^{13}C signals in the deuterated solvents. Electrospray Ionisation Mass Spectra (ESI-MS) and Atmospheric Pressure Chemical Ionisation Mass Spectra (APCI-MS) were acquired using either a Waters 2790 Separations Module equipped with a Micromass ZMD mass detector or an Agilent 1100 Series Separations Module equipped with an Agilent 1100 Series LC/MSD mass detector. High resolution (HR) ESI-MS measurements were obtained on either a Bruker BioApex 47E FT mass spectrometer at a cone voltage of 100 kV or a Finnigan MAT 900 XL-Trap instrument with a Finnigan API III source, while EI-MS measurements were obtained on a Kratos MS25RFA mass spectrometer at 70 eV.

Parasitic nematode larval development assay. Nematode eggs were applied in duplicate to the surface of an agar matrix containing the "test solution" (extract, chromatographic fraction and/or pure compound at a concentration range of ∼100 to 0.5 ppm). The eggs were allowed to hatch and develop through to the L3 infective stage (6 days). The extent of larval development was then determined quantitatively by counting the number of eggs, L1s, L2s and L3 larvae to establish the proportion of undeveloped larvae (eggs, L1s and L2s) at each concentration. The resulting data were fitted to a log-concentration–logit model to determine the LD_{99} value.

Collection, extraction and isolation

The marine sponge *Echinodictyum* sp. (Museum of Victoria Registry Number MVF88741) was collected by beam trawl from the Great Australian Bight at a depth of 85 m at position 32°58′92″S : 128°00′84″E. A taxonomic description has been reported.[1] The sponge was diced, steeped in EtOH and kept at −20 °C prior to extraction. The EtOH extract was decanted and concentrated *in vacuo* to yield a bright orange solid (4.08 g). Trituration with DCM returned insoluble soluble material that was partitioned between *n*-BuOH and H_2O, with the nematocidal activity of the crude extract concentrated into the H_2O solubles (2.96 g, 73%). The *n*-BuOH solubles yielded (in prior studies) echinosulfonic acids A-C (**1–3**) and echinosulfone (**4**).[1] This nematocidal H_2O soluble material was concentrated *in vacuo* and fractionated by Sephadex G-10 (H_2O) chromatography, followed by isocratic C_{18} HPLC (2 mL min^{-1} H_2O + 0.1% TFA, Zorbax C_{18} 10 µm 250 × 10 mm column), to yield the nematocidal agent (−)-echinobetaine A (**5**) (as documented in prior studies).[2] Further bioassay directed HPLC fractionation, with careful peak shaving, has yielded the dominant nematocidal agent as (+)-echinobetaine B (**6**) (25 mg, 0.044%) (*H. contortus*, LD_{99} 8.3 µg mL^{-1}) as the trifluoroacetate salt. Note that percentage yields of natural products are calculated against the total dry weight mass of the crude EtOH extract.

The marine sponge *Axinyssa* sp. (Museum of Victoria Registry Number MVF83453) was collected by hand (SCUBA) off Beware Reef, Cape Conran, Victoria, 38°12′S : 148°47′E. The sponge was diced, steeped in EtOH and kept at −20 °C prior to extraction. The crude EtOH extract was processed as described above for the *Echinodictyum* sp. to return an H_2O soluble fraction that was fractionated by Sephadex G-10 (H_2O) chromatography, followed by isocratic C_{18} HPLC (2 mL min^{-1} H_2O + 0.1% TFA, Zorbax C_{18} 10 µm 250 × 10 mm column), to yield trigonelline[14] (0.4 mg, 0.037%) together with norzooanemonin (**13**) (1.1 mg, 0.10%). Norzooanemonin possessed MS and ^1H NMR data consistent with those reported in the literature.[15]

The marine sponge *Axinella* sp. (Northern Territory Museum Registry Number NTMZ3725) was collected by trawling in the Great Australian Bight. The sponge was diced, steeped in EtOH and kept at −20 °C prior to extraction. The crude EtOH extract was processed as described above for the *Axinyssa* sp. to return an H_2O soluble fraction that was fractionated by Sephadex G-10 (H_2O) chromatography, followed by isocratic C_{18} HPLC (1 mL min^{-1} H_2O + 0.1% TFA, Phenomenex Aqua C_{18} 5 µm 250 × 10 mm column), to yield norzooanemonin methyl ester (**14**) (1.1 mg, 1.45%). λ_{max} (MeOH)/nm 264; δ_H (400 MHz; D_2O) 8.27 (1 H, s), 7.07 (1 H, s), 3.66 (3 H, s), 3.56 (3 H, s) and 3.46 (3 H, s); δ_C (100 MHz; D_2O) 175.0 (C-1), 131.0 (C-2), 35 (N4-Me), 33.0 (N6-Me) and 32.0 (CO_2Me); ESI(+)MS *m/z* 177 (M − H + Na), 155 (M); HRESI(+)MS *m/z* 177.0639 ($C_7H_{10}N_2O_2Na$ requires 177.0640) and 155.0817 ($C_7H_{11}N_2O_2$ requires 155.0820).

The marine sponge *Niphates* sp. (Museum of Victoria Registry Number MVF83541) was collected by trawling off the east coast of Tasmania. The sponge was diced, steeped in EtOH and kept at −20 °C prior to extraction. The crude EtOH extract was processed as described above for the *Axinyssa* sp. to return norzooanemonin (**13**) (1.6 mg, 0.36%) and the methyl ester **14** (27.0 mg, 6.14%).

The marine sponge *Ptilocaulis* sp. (Museum of Victoria Registry Number MVF83529) was collected by scientific trawling in the Great Australian Bight, 38°02′S : 128°28′E. The sponge was diced, steeped in EtOH and kept at −20 °C prior to extraction. The crude EtOH extract was processed as described above for the *Axinyssa* sp. to return norzooanemonin methyl ester (**14**) (1.7 mg, 0.7%).

(+)-Echinobetaine B (6). $[\alpha]_D$ +30.0 (*c* 0.59 in MeOH); λ_{max} (MeOH)/nm (ε/dm^3 mol^{-1} cm^{-1}) 214 (2344); ν_{max} (film)/cm^{-1} 1689; ^1H and ^{13}C NMR data (400 MHz; D_2O) see Table 1;

HRESI(+)MS m/z 185.0921 (M + H, $C_8H_{13}N_2O_3$ requires 185.0926).

1-Methyl-4-imidazolecarboxaldehyde[16] (8a) and 1-methyl-5-imidazolecarboxaldehyde[16] (8b). A solution of 4(5)-imidazolecarboxaldehyde (**7**) (1.05 g, 10.90 mmol) in anhydrous DMF (20 mL) was added dropwise to a suspension of NaH (95%) (0.27 g, 11.25 mmol) in anhydrous DMF (15 mL) at 0 °C under an atmosphere of N_2. The effervescent mixture was stirred for 30 min at 0 °C after which MeI (0.80 mL, 12.85 mmol) was slowly added and the reaction mixture stirred for a further 3 h at RT. At this point the reaction product was concentrated *in vacuo*, and the residue dissolved in DCM (90 mL), washed with water (3 × 50 mL), and the combined aqueous fractions extracted with DCM (2 × 70 mL). The combined organic extracts were dried ($MgSO_4$), filtered, concentrated *in vacuo* and the residue purified by silica gel column chromatography (EtOAc : hexane (1 : 1) to EtOAc then from MeOH : DCM (1:49) to MeOH : DCM (1 : 1), *via* a stepwise gradient elution) to afford a mixture of *mono-N-methylated imidazoles*[16] **8a** and **8b** (0.44 g, 36%) as a colourless oil which solidified to white crystals on cooling to −30 °C. The above combined aqueous fractions were concentrated *in vacuo*, MeOH added and the mixture filtered through a pad of celite. The filtrate was concentrated *in vacuo* and a solution of the residue in H_2O (5 mL) eluted through an Alltech C_{18} Solid Phase Extraction (SPE) cartridge (2 g) with water (15 mL). The eluent was concentrated *in vacuo* (0.44 g) and subjected to silica gel column chromatography under identical conditions as above to provide a further yield of recovered pure *mono-N-methylated imidazoles* **8a** and **8b** (0.26 g, 22%) as a colourless oil: ν_{max} (film)/cm^{-1} 3111, 2956, 2833 and 1673; δ_H (**8a**) (500 MHz; $CDCl_3$) 3.70 (3 H, s, N1-Me), 7.45 (1 H, s, 5-H), 7.53 (1 H, s, 2-H) and 9.78 (1 H, s, CHO); δ_C (**8a**) (125 MHz; $CDCl_3$) 34.0 (q, N1-Me); 125.5 (d, C-5), 139.5 (d, C-2), 142.7 (s, C-4) and 186.1 (s, *C*HO); δ_H (**8b**) (500 MHz; $CDCl_3$) 3.86 (3 H, s, N1-Me), 7.55 (1 H, s, 4-H), 7.70 (1 H, s, 2-H) and 9.69 (1 H, s, CHO); δ_C (**8b**) (125 MHz; $CDCl_3$) 34.1 (q, N1-Me), 131.7 (d, C-4), 143.3 (d, C-2), 144.2 (s, C-5) and 179.5 (s, *C*HO); ESI(+)MS (30V) m/z 111.1 (M + H$^+$).

1-Methyl-4-(dimethoxymethyl)-imidazole (9a) and 1-methyl-5-(dimethoxymethyl)-imidazole (9b). 10% HCl_{aq} (10 mL) was added to a mixture of imidazoles **8a** and **8b** (0.41 g, 3.70 mmol) and the solution concentrated *in vacuo*. A solution of the resultant oily residue and *p*-toluenesulfonic acid monohydrate (0.07 g, 0.37 mmol) in anhydrous methanol (20 mL) was refluxed for 12 h under an atmosphere of N_2, after which it was cooled to RT and quenched with saturated aqueous $NaHCO_3$ (15 mL) and the organic solvent removed *in vacuo*. The resulting aqueous solution was extracted with ethyl acetate (3 × 20 mL) and the combined organic fractions dried ($MgSO_4$), filtered and concentrated *in vacuo* to provide a quantitative yield of a 7 : 3 mixture of *dimethoxy acetals* **9a** and **9b** (0.51 g, 87%) as a colourless oil. The ratio of 7 : 3 for the mixture **9a** and **9b** was determined by integration of the two singlets at δ_H 3.31 (**9a**) and 3.27 (**9b**), respectively, in the ^{1}H NMR spectrum: ν_{max} (film)/cm^{-1} 3126, 2939, 1507 and 1102; δ_H (**9a**) (500 MHz; $CDCl_3$) 3.31 (6 H, s, 2 × OMe), 3.60 (3 H, s, N1-Me), 5.37 (1 H, s, 6-H), 6.87 (1 H, s, 5-H) and 7.33 (1 H, s, 2-H); δ_H (**9b**) (500 MHz; $CDCl_3$) 3.27 (6 H, s, 2 × OMe), 3.59 (3H, s, N1-Me), 5.32 (1 H, s, 6-H), 7.02 (1 H, s, 4-H) and 7.35 (1 H, s, 2-H); δ_C (**9a** and **9b**) (125 MHz; $CDCl_3$) 32.3, 33.5, 53.0, 53.3, 98.3, 100.4, 118.5, 128.2, 129.8, 137.7, 139.5 and 140.5; HR(EI)MS m/z 156.0897 (M, $C_7H_{12}N_2O_2$ requires 156.0899).

1-Methyl-4-[cyano(methoxy)methyl]imidazole (10a) and 1-methyl-5-[cyano(methoxy)methyl]imidazole (10b). Trimethylsilyl cyanide (0.40 mL, 3.00 mmol) and anhydrous $SnCl_2$ (0.19 g, 1.00 mmol) were successively added to a solution of acetals **9a** and **9b** (0.16 g, 1.00 mmol) in dry MeCN (7 mL), under an atmosphere of N_2, and the mixture stirred at RT for 96 h. The reaction mixture was quenched with H_2O (30 mL), extracted with DCM (3 × 40 ml) and the combined organic fractions washed with H_2O (20 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification of the resultant residue by silica gel column chromatography (a stepwise gradient elution from MeOH : DCM (1 : 49) to MeOH : DCM (1 : 1)) afforded a mixture of *methylated cyanohydrins* **10a** and **10b** (0.14 g, 93%) as a colourless oil: ν_{max} (film)/cm^{-1} 3066, 2954, 1504 and 1081; δ_H (**10a**) (500 MHz; $CDCl_3$) 3.47 (3 H, s, OMe), 3.65 (3 H, s, N1-Me), 5.17 (1 H, s, 6-H), 7.07 (1 H, s, 5-H) and 7.39 (1 H, s, 2-H); δ_H (**10b**) (500 MHz; $CDCl_3$) 3.41 (3 H, s, OMe), 3.68 (3H, s, N1-Me), 5.26 (1 H, s, 6-H), 7.19 (1 H, s, 4-H) and 7.61 (1 H, s, 2-H); δ_C (**10a** and **10b**) (125 MHz; $CDCl_3$) 32.2, 33.8, 56.5, 57.1, 63.2, 67.0, 115.3, 116.9, 119.8, 123.7, 131.5, 135.7, 138.6 and 141.1; HR(EI)MS m/z 151.0745 (M, $C_7H_9N_3O$ requires 151.0746).

(±)-Echinobetaine B (11)—methylation followed by hydrolysis. A solution of methylated cyanohydrins **10a** and **10b** (0.06 g, 0.37 mmol), and MeI (0.23 mL, 3.70 mmol) in anhydrous acetone (8 mL) was refluxed for 15 h under an atmosphere of N_2 after which the solution was concentrated *in vacuo* to an orange solid (0.12 g) tentatively identified by NMR as the desired methylated product. δ_H (500 MHz, CD_3OD) 3.58 (3-H, s, CO_2CH_3), 3.92 (6-H, s, N5-Me and N7-Me), 6.01 (1-H, s, 2-H), 7.93 (1-H, s, 4-H) and 9.08 (1-H, s, 6-H). This intermediate product (0.12 g) was refluxed in 10 M HCl_{aq} (10 mL) for 36 h, after which the reaction mixture was cooled to RT and the solvent removed *in vacuo* to give a yellow oil (0.10 g) which was purified by C_{18} HPLC (4.2 mL min^{-1} H_2O + 0.1% TFA, Agilent Zorbax SB-Aq 5 μm 150 × 9.4 mm column) to yield (±)-echinobetaine B (**11**) (27%) as a colourless oil with ESI(+)MS, and ^{1}H and ^{13}C NMR data in agreement with data for the natural product (+)-echinobetaine B (**6**).

(±)-Echinobetaine B (11)—hydrolysis followed by methylation. A solution of methylated cyanohydrins **10a** and **10b** (0.10 g, 0.66 mmol) was refluxed in 10 M HCl_{aq} (20 mL) for 15 h, after which the reaction mixture was cooled to RT and the solvent removed *in vacuo* to give a white solid (0.218 g), which was added to H_2O and the pH adjusted to 8.0 prior to concentration *in vacuo*. The resultant residue was dissolved in anhydrous DMF (4 mL) and MeI (0.30 mL, 4.80 mmol) and heated at 70 °C under N_2 for 14 h before cooling to RT and concentrating to dryness *in vacuo*. Purification by C_{18} HPLC (4.2 mL min^{-1} H_2O + 0.1% TFA, Agilent Zorbax SB-Aq 5 μm 150 × 9.4 mm column) to yield (±)-echinobetaine B (**11**) (39%) as a colourless oil with ESI(+)MS, and ^{1}H and ^{13}C NMR data in agreement with data for the natural product (+)-echinobetaine B (**6**).

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