

## Synthesis and biological evaluation of pyridinebetaine A and B

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The synthesis of the marine natural products pyridinebetaine A and B is reported. The biological evaluation of pyridinebetaine A and B and several analogues as cytotoxic, antifungal and antiviral agents is also described. Unfortunately, none of the compounds tested showed relevant antifungal or cytotoxic activity. Only pyridinebetaine B reduced the *Herpes simplex virus* type 1 virus replication, though only weakly.

**Keywords:** marine natural products; pyridinebetaine; antibacterial; antiviral

### 1. Introduction

Ammonium alkanocarboxylates are zwitterions (or inner salts, or betaines) because they possess formally charged ammonium and carboxylate groups separated by one or more carbon atoms. The chemistry of betaines has become a subject of particular interest due to their applications in biological research, especially with regard to their metabolic roles in the living organism (Myers & Jibril, 1957). Alkylbetaines nowadays constitute a group of products of increasing importance in cosmetic, household and industrial applications. They have a variety of uses in medicine, pharmacy, biology and other scientific fields (Domingo, 1996). A number of alkylbetaines have been isolated from marine sources and it has been suggested that these quaternary ammonium compounds play an important role in the cellular osmotic activities of marine invertebrates. Their occurrence appears to be quite widespread, suggesting them to be primary, not secondary metabolites (Jahn, König, Wright, Wörheide, & Reitner, 1997).

In the course of the search for biologically active compounds from marine sources, Cafieri et al. reported the isolation and structure of three novel betaine alkaloids: aminozooanemonin (**1**), pyridinebetaine A (**2**), and pyridinebetaine B (**3**) (Figure 1), from the methanolic extract of the marine sponge *Agelas dispar* (Cafieri, Fattorusso, & Taglialatela-Scafati, 1998). Aminozooanemonin (**1**) and pyridinebetaine A (**2**) showed moderate antibacterial activity against Gram-positive bacteria with minimal inhibitory concentrations (MICs) ranging from 2.5 to 8  $\mu\text{g mL}^{-1}$ . Pyridinebetaine B (**3**) has also been isolated from the sponge *Aaptos* sp., a taxonomically distant marine sponge from *Agelas dispar*, which confirms previous assumptions that such betaines should be regarded as

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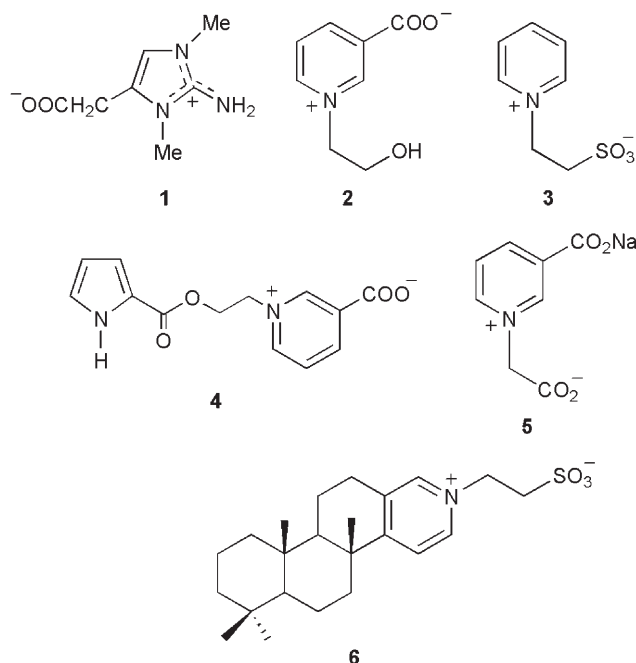


Figure 1. Chemical structures of natural betaines.

primary metabolites (Granato et al., 2000). The extract of the sponge *Aaptos* sp. showed inhibition of the development of sea-urchin eggs, and the bioassay-guided study led to the isolation of pyridinebetaine B (**3**) (Rangel et al., 2001). This compound was first described in 1961 and 1964 as a synthetic product (Barnhurst, 1961; Klass, 1964).

Other betaines isolated from marine sponges possess similar structures to pyridinebetaines A and B, and have shown interesting biological properties. For example, daminin (**4**) (Figure 1) is a pyrrole alkaloid isolated from the marine sponge *Axinella damicornis* and has showed significant neuroprotective properties (Aiello et al., 2005). 1-Carboxymethylnicotinic acid (**5**) (Figure 1) is a cysteine protease inhibitor and has been isolated from the marine sponge *Anthosigmella* cf. *raromicrosclera* (Matsunaga, Kamimura, & Fusetani, 1998). Spongidine D (**6**) (Figure 1) is a pyridinium alkaloid isolated from a marine sponge of the genus *Spongia*, which inhibits human synovial phospholipase A<sub>2</sub> (De Marino et al., 2000).

To date, the investigation of the biological activities of pyridinebetaines A and B has been very limited. The occurrence of the same chemical moieties in nature presenting interesting biological activities has prompted us to study these molecules further.

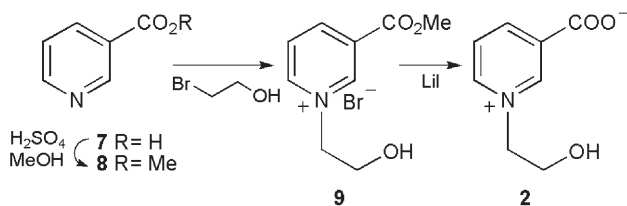
In this article, we describe the synthesis of pyridinebetaines A and B and several analogues, as well as the study of their pharmacological properties in a number of biological assays. In particular, we have tested the antifungal activity following the standard microdilution protocols AFST-EUCAST for *Candida krusei*, *C. parasilopsis*, *C. tropicalis* and *C. albicans*, and CLSI-M38-A for *Aspergillus fumigates*, *A. flavus*, *A. niger* and *A. terreus*. Their antitumour and cytotoxic activity on the cancer cell lines HeLa and Vero, respectively, and antiviral activity against *Herpes simplex virus* type 1 (HSV-1) *in vitro*, was also tested.

## 2. Results and discussion

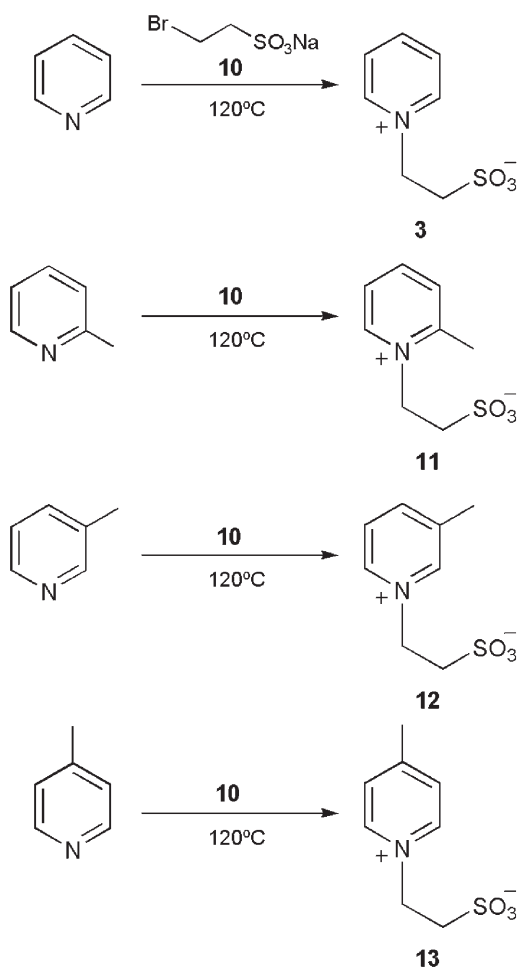
The synthesis of pyridinebetaine **2** began with the esterification of nicotinic acid (**7**) with methanol in acidic media, which gave methyl nicotinate **8** in 50% yield (Scheme 1). The treatment of ester **8** with bromoethanol in refluxing toluene gave the ammonium salt **9**, which was subjected to reaction with lithium iodide in pyridine at 100°C to afford the desired betaine **2** in 45% overall yield. The NMR data of synthetic **2** were in complete agreement with the values reported in the literature (Cafieri et al., 1998).

Our synthesis of pyridinebetaine **3** was different and more efficient than the reported syntheses in the literature in several steps (Barnhurst, 1961; Klass, 1964). We thought that the synthesis of **3** would be straightforward if we treated pyridine with an appropriate alkylating agent. Thus, neat pyridine was refluxed in the presence of the sodium salt of 2-bromoethanesulfonic acid (**10**), which gave the betaine **3** in 87% yield (Scheme 2). The NMR data of synthetic **3** were in complete agreement with the values reported in the literature (Cafieri et al., 1998). With the betaine **3** in hand, we envisaged the use of this new method to prepare several analogues, using as starting material different commercially available methyl pyridines. Therefore, on using 2-methylpyridine (2-picoline) we obtained the betaine **11** (Scheme 2), and similarly with 3-methylpyridine (3-picoline) and 4-methylpyridine (4-picoline) we obtained the betaines **12** and **13**, respectively.

With the pyridinebetaines **2**, **3**, and **11–13** in hand, we carried out the biological study. None of the pyridinebetaines showed antimycotic activity against *C. parasilopsis*, *C. krusei*, *C. tropicalis*, *C. albicans*, *A. fumigates*, *A. flavus*, *A. niger* and *A. terreus* in concentrations below 100 µg mL<sup>-1</sup>. MIC values for the two reference antifungal drugs, amphotericine B and itraconazole (Sigma, New Jersey, USA), used as positive controls, were within the established values for the AFST-EUCAST and CLSI-M38-A protocols. Unfortunately, none of the compounds showed cytotoxic activity below 60 µg mL<sup>-1</sup> on tumour cells HeLa and non-tumour cells Vero, except pyridinebetaine **2**, which showed, on HeLa values, 50% cytotoxic concentration evaluated in 48 h of 50 ± 3.0 µg mL<sup>-1</sup>. The antiviral activity of the pyridinebetaines against HSV-1 was determined using a modified end-point titration technique (EPPT) (Betancur-Galvis, Zuluaga, Arnó, González, & Zaragozá, 2002). None of the compounds except pyridinebetaine **3** reduced virus replication with values below 100 µg mL<sup>-1</sup>. According to Vlietinck et al. (1995), only the compounds with reduction factor of the viral titer over 1 × 10<sup>3</sup> (*Rf*: ratio of the virus titer in the absence of the tested compound over virus titer in the presence of the tested compound) show relevant antiviral activity. Pyridinebetaine **3** was found to be slightly active against HSV-1 both over infected confluent monolayers of HeLa and Vero cells, with an *Rf* value of 1 × 10<sup>1</sup> at concentration of 25 µg mL<sup>-1</sup>. These results encourage us to continue our research of this series by synthesising additional pyridinebetaine-type derivatives with the aim of obtaining compounds that are more biologically potent.



Scheme 1. Synthesis of pyridinebetaine **2**.

Scheme 2. Syntheses of pyridinebetaines **3**, and **11–13**.

### 3. Experimental

#### 3.1. Pyridinebetaine A (2)

A suspension of nicotinic acid (3.0 g, 25 mmol) in methanol was treated dropwise with concentrated sulfuric acid (2 mL, 36 mmol) and refluxed for 4 h. The solvent was removed under vacuum and the residue poured into 15% aqueous sodium hydroxide solution. The resulting mixture was extracted with ethyl acetate. The organic extract was washed with saturated  $\text{NaHCO}_3$  and brine, dried and concentrated to afford 1.7 g (50%) of known methyl nicotinate **8** as a white solid, which was used in the next step without further purification.

A solution of methyl nicotinate **8** (580 mg, 4.2 mmol) and 2-bromoethanol (0.6 mL, 8.4 mmol) in toluene (5 mL) was refluxed for 1 day. Then, the solvent was removed under vacuum and the orange residue was washed with diethyl ether ( $3 \times 3$  mL). The resulting orange residue was dissolved in hot pyridine (10 mL) and lithium iodide (2.6 g, 19.4 mmol)

was added. The mixture was heated at 100°C for 4 h. The solvent was removed under vacuum and the residue was chromatographed on silica eluting with methanol:ethyl acetate 8:2 to give 315 mg (45%) of piridinebetaine A (**2**) as a colourless amorphous solid (Cafieri et al., 1998).  $^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{OD}$ ) 9.26 (1H, s), 8.94 (1H, d,  $J=7.5$ ), 8.91 (1H, d,  $J=7.5$ ), 8.09 (1H, dd,  $J=7.5, 7.5$ ), 4.76 (2H, t,  $J=5.0$ ), 4.02 (2H, t,  $J=5.0$ ); HRMS (FAB) calcd for  $\text{C}_8\text{H}_{10}\text{NO}_3$  168.0583  $[\text{M} + \text{H}]^+$ , found 168.0572.

### 3.2. Pyridinebetaine B (**3**)

A suspension of sodium 2-bromoethanesulfonic acid (**10**) (130 mg, 0.61 mmol) in pyridine (1 mL, 12.2 mmol) was refluxed for 24 h. Then, the solvent was removed under vacuum and the resulting white solid was dissolved in  $\text{CD}_3\text{OD}$  to take the NMR data. Upon concentration, 100 mg (87%) of betaine **3** was obtained as an amorphous solid (Cafieri et al., 1998).  $^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{OD}$ ) 9.25 (2H, d,  $J=6.0$ ), 8.77 (1H, t,  $J=7.5$ ), 8.27 (2H, t,  $J=7.0$ ), 5.22 (2H, t,  $J=6.0$ ), 3.68 (2H, t,  $J=6.0$ );  $^{13}\text{C}$  NMR (75 MHz,  $\text{CD}_3\text{OD}$ ) 147.1 (d), 146.7 (d), 146.7 (d), 129.0 (d), 129.0 (d), 59.1 (t), 51.7 (t); HRMS (FAB) calcd for  $\text{C}_7\text{H}_{10}\text{NO}_3\text{S}$  188.0376  $[\text{M} + \text{H}]^+$ , found 188.0365.

### 3.3. General procedure for the synthesis of pyridinebetaines 11–13

A suspension of sodium 2-bromoethanesulfonic acid (**10**) (0.5 g, 2.37 mmol) in the corresponding methyl-pyridine (4.6 mL, 47.4 mmol) was heated at 120°C for 24 h. Then, the brown solid that appeared was removed by filtration and the solvent was removed under vacuum. The resulting brown solid was washed with ethyl acetate and then triturated in methanol and the solid filtered off. Upon concentration of the filtrate, 375–450 mg (80–90%) of betaines **11–13** were obtained as amorphous solids (King, Hillhouse, & Skonieczny, 1984). Compound **11**:  $^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{OD}$ ) 8.98 (1H, d,  $J=6.0$ ), 8.42–8.51 (1H, m), 7.90–8.02 (1H, m), 7.43–7.53 (1H, m), 5.01 (2H, t,  $J=6.5$ ), 3.47 (2H, t,  $J=6.5$ ), 2.99 (3H, s);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CD}_3\text{OD}$ ) 147.3 (d), 146.7 (d), 141.4 (s), 131.2 (d), 126.5 (d), 55.3 (t), 50.7 (t), 20.7 (q); HRMS (FAB) calcd for  $\text{C}_8\text{H}_{12}\text{NO}_3\text{S}$  202.0532  $[\text{M} + \text{H}]^+$ , found 202.0542. Compound **12**:  $^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{OD}$ ) 9.04 (1H, s), 8.97 (1H, d,  $J=6.0$ ), 8.48 (1H, d,  $J=8.0$ ), 8.02 (1H, t,  $J=6.5$ ), 5.06 (2H, t,  $J=6.5$ ), 3.58 (2H, t,  $J=6.5$ ), 2.59 (3H, s);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CD}_3\text{OD}$ ) 147.4 (d), 146.2 (d), 143.9 (s), 140.4 (d), 128.3 (d), 58.8 (t), 51.8 (t), 18.6 (q); HRMS (FAB) calcd for  $\text{C}_8\text{H}_{12}\text{NO}_3\text{S}$  202.0532  $[\text{M} + \text{H}]^+$ , found 202.0522. Compound **13**:  $^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{OD}$ ) 8.98 (1H, d,  $J=6.0$ ), 7.99 (1H, d,  $J=6.0$ ), 5.06 (2H, t,  $J=6.0$ ), 3.58 (2H, t,  $J=6.0$ ), 2.70 (3H, s);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CD}_3\text{OD}$ ) 161.1 (s), 145.5 (d), 145.5 (d), 129.4 (d), 129.4 (d), 58.0 (t), 51.7 (t), 22.2 (q); HRMS (FAB) calcd for  $\text{C}_8\text{H}_{12}\text{NO}_3\text{S}$  202.0532  $[\text{M} + \text{H}]^+$ , found 202.0546.

### 3.4. Antifungal assay

The antifungal activity of piridinebetaines **2**, **3** and **11–13** was evaluated following the Clinical and Laboratory Standards Institute M38-A protocol (CLSI) (2002) for filamentous fungi and the standard method proposed by the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST) (Cuenca-Estrella et al., 2003) for fermentative yeasts. *Candida parapsilosis* (ATCC 22019), *C. krusei* (ATCC 6258), *C. tropicalis* (CECT

11901), *C. albicans* (ATCC 10231), *A. flavus* (ATCC 204304) *A. fumigatus* (ATCC 204305), *A. terreus* (CDC 317) and *A. niger* (ATCC 10124) were used to evaluate antifungal activity. Briefly, seven serial dilutions of the pyridinebetaines were dispensed into 96-well flat-bottom microdilution plates in duplicate at final concentrations between 100 and  $2\text{ }\mu\text{g mL}^{-1}$ . Amphotericin B (Sigma Chemical Co, MO, USA) and itraconazole (Sigma Chemical Co, MO, USA) were used as positive controls at a range of  $0.031\text{--}16\text{ }\mu\text{g mL}^{-1}$ . Tween 80 was included at a final concentration of 0.001% (v/v) to enhance the solubility of the pyridinebetaines. The plates were frozen at  $-70^{\circ}\text{C}$  until required. The inoculum size for microdilution plates were  $0.5\text{--}2.5 \times 10^5$  and  $0.4\text{--}5 \times 10^4$  CFU  $\text{mL}^{-1}$  for yeast and filamentous fungi, respectively. For the AFST-EUCAST method, the MICs were determined after 24 h of incubation and were defined as the lowest concentration that resulted in a 90% reduction of growth. For the CLSI M38-A method, the MICs were determined after 48 h of incubation and were defined as the lowest dilution that resulted in total inhibition of visible growth. MICs results were expressed as a range and a geometric mean (GM) of triplicates of each compound tested three times against each of the fungi species in different assays.

### 3.5. Antitumour, cytotoxicity and antiviral assays

The cell lines used were human cervix epitheloid carcinoma cells (HeLa cell line ATCC CCL-2) and *Cercopithecus aethiops* African green monkey kidney cells (Vero cell line ATCC CCL-81). HSV-1 was obtained from the Center for Disease Control (Atlanta, GA). The virus stock was prepared from HSV-1-infected HeLa cell cultures. Cells were grown in MEM supplemented with 10% FBS, 100 units per mL of penicillin,  $100\text{ mg mL}^{-1}$  of streptomycin,  $20\text{ mg mL}^{-1}$  of l-glutamine, 0.14%  $\text{NaHCO}_3$ , and 1% each of nonessential amino acids and vitamin solution. The cultures were maintained at  $37^{\circ}\text{C}$  in humidified 5%  $\text{CO}_2$  atmosphere. The antitumour activity on HeLa cells and cytotoxic activity on Vero cells have been carried out using *in vitro* assay on cell growth and tetrazolium dye (MTT) cytotoxicity assay, according to the protocol reported by us (Arnó, Betancur-Galvis, González, Sierra, & Zaragoza, 2003). The antiviral activity against HSV-1 have been carried out both in HeLa and Vero cells using an end-point titration technique, according to the protocol reported by us (Betancur-Galvis et al., 2002), which was used with a few modifications: two-fold dilutions of the compounds and viral suspension were mixed and incubated for 0.5 h at  $37^{\circ}\text{C}$  before they were added on confluent monolayer cells.

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