NPC Natural Product Communications

2010
Vol. 5
No. 8
1187 - 1190

Polar Alkaloids from the Caribbean Marine Sponge *Niphates digitalis*

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Received: January 19th, 2010; Accepted: May 25th, 2010

A method involving flash chromatography, semi-preparative phenylhexyl RP HPLC-DAD-ELSD combined with analytic polar-RP HPLC-DAD, was applied to separate and purify six highly nitrogenated bases and a bicyclic amidine alkaloid, the major components of the marine sponge *Niphates digitalis*. Their structures were identified as 1,8-diazabicyclo[5.4.0]undec-7-ene (**1**), deoxycytidine (**2**), phenylalanine (**3**), adenosine (**4**), deoxyguanosine (**5**), adenine (**6**) and thymidine (**7**) on the basis of spectroscopic data analyses. This is the first report of these compounds in a marine sponge belonging to the *Niphates* genus and the first evidence of the presence of **1** from a natural source.

Keywords: Marine sponge, *Niphates digitalis*, nucleosides, nucleobases, antimalarial activity, cytotoxicity.

As part of our ongoing work on the secondary metabolites produced by marine sponges, we undertook the chemical study of the Caribbean species *Niphates digitalis* Lamarck, 1814 (phylum Porifera, class Demospongiae, order Haplosclerida, family Niphatidae) collected off the Cuban coasts. The order Haplosclerida is known to produce a large diversity of novel bioactive molecules [1a-1d]. In contrast to other sponge genera of Niphatidae family, chemical studies on genus *Niphates*, resulted the characterization of pyridine alkaloids [2,3a-3d]. Herein, we detail the results of our chemical studies on *N. digitalis*. To our knowledge; this is the first report on the isolation and structure identification of metabolites from this common Caribbean species even if the associated larvae were shown to exhibit ecological activities [3e].

The ultrasonic extraction of a freeze-dried portion of a *N. digitalis* specimen collected off the Cuban coast gave a brown syrup with 14.6% (w/w) yield; The column separation of this extract gave a fraction with antimalarial and cytotoxic activity (IC_{50} of 60 µg/mL and 55,8 µg/ml, respectively), which on repeated chromatographic purification resulted in isolation of seven metabolites (**1-7**).

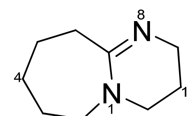


Figure 1: Structure of compound **1** (**DBU**) isolated from *N. digitalis*

Compound **1** (5.2 mg) was isolated as a yellowish liquid and its molecular formula was determined as $C_9H_{16}N_2$ by HRESI-MS. Analysis of the ^{13}C, and HSQC NMR data for **1** revealed the presence of eight methylene units and a quaternary carbon. These data accounted for all ^1H and ^{13}C resonances and required compound **1** to be bicyclic. The ^1H–^1H COSY NMR data evidenced two isolated proton spin-systems corresponding to the C-2/C-6 and C-9/C-11 subunits of **1**. HMBC correlations of the isolated methylene protons H_2-2, H_2-6, H_2-9 and H_2-11 with the deshielded quaternary carbon at δ_C 165.5, revealed the presence of an amidine group. Consequently, compound **1** was identified as 1,8-

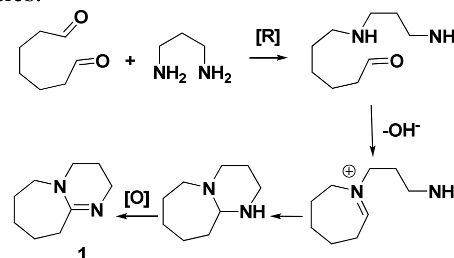
diazabicyclo[5.4.0]undec-7-ene (Figure 1). NMR data comparison with a commercial sample of DBU confirmed this assumption (see supporting information). Compounds (**2-7**) were identified as deoxycytidine (**2**, 1.0 mg), phenylalanine (**3**, 3.5 mg), adenosine (**4**, 4.1 mg), deoxyguanosine (**5**, 3.0 mg), adenine (**6**, 4.7 mg) and thymidine (**7**, 4.8 mg) by a combination of spectroscopic methods and comparison with data from commercial samples.

Although investigations on these polar compounds are scarce, some studies have reported the isolation and identification of DNA and RNA bases from the marine environment [4a-4d]. Nucleobases, such as uracil, cytosine and thymine, were isolated from *Ophiactis asperula* (Philippi, 1858), *Ophiacantha vivipara* (Ljungman, 1870) and *Gorgonocephalus chilensis* (Philippi, 1858) [4c]. In our case, *N. digitalis* was found to contain various known 2-deoxyribonucleosides. In the marine environment, this type of nucleosides have only been described from two starfish, *Acanthaster planci* (Linnaeus, 1758) and *Luidia maculata* (Müller & Troschel, 1842) [4d], from an ascidian [5], and from three ophiuroids [4c].

Many of these nucleosides and nucleobases have been described as precursors in the biosynthesis of more complex families of secondary metabolites. Novel bioactive analogues of these compounds have been discovered in marine organisms [6a-6b], including marine sponges [7a-7c]. The most famous examples of nucleosides are spongosine, spongothymidine and spongouridine, isolated in the early 1950s from the Caribbean sponge *Cryptotethya crypta* (de Laubenfels, 1949) [8a-8c]. The two pyrimidine nucleosides contained arabinose instead of the classical ribose, and spongosine combined ribose with a novel base, methoxyadenine. These nucleosides led to the first marine drugs already well-established on the market [9a], Ara-C cytarabine as an antitumor agent [9b] and Ara-A vidarabine as an antiviral [9c].

We consider that the nucleosides isolated from *N. digitalis* are not the result of either DNA or RNA degradation during work up because the resistance of DNA and RNA to selective hydrolysis is well known [9d]. Herein we have identified compounds **1-7** as the major secondary metabolites of *N. digitalis* and they could be part of the defense mechanism of this marine sponge.

Adenine and several modified ribonucleosides are known to induce cell death and the mode of action was apoptotic, so they are considered potential anticancerogenic agents [9e]. The non specific bioactivity found in *N. digitalis,* characterized by the presence of nucleosides and nucleobases could be explained in this sense. However, in another apicomplexa parasite, *Toxoplasma gondii,* the enzyme adenosine kinase (EC 2.7.1.20), involved in purine salvage, has been identified as a potential chemotherapeutic target inhibited by modified purine nucleosides [10]. This justifies the interest for seeking new antiparasitic compounds among this group of molecules.



Scheme 1. Hypothetical biosynthesis of **1**

1,8-Diazabicyclo-[5.4.0]-undec-7-ene (**1**), commonly known as DBU, is largely used as an organic base in chemical synthesis [11]. Its presence in *N. digitalis* as a natural compound was largely unexpected, but unequivocally confirmed by LC–MS analysis after repeating the collection and the same extraction process, The biosynthesis of this molecule could arise from the reductive amination of adipaldehyde with the well known propane-1,3-diamine (Scheme 1). The resulting diamino aldehyde could undergo a first cyclisation to lead to an azepane type Schiff base presumedly trapped in an intramolecular manner to afford the [5.4.0] bicycle core of **1**. A final oxidation step would give the natural compound. To the best of our knowledge, this is the first report on the isolation of compound **1** from a natural source and the first evidence for the presence of metabolites **2-7** in a specimen belonging to the *Niphates* genus.

Experimental

General experimental procedures: Flash chromatography was performed on an Armen Instrument Spot Liquid Chromatography system, with the detection wavelength set at 254 nm. The PrepPak® DeltaPak™ C18 cartridge (25 x100 mm, 15 µm, 100 Å) used for flash chromatography was obtained from Waters. HPLC purifications were carried out on a Waters 600 system equipped with a Waters 717 plus autosampler, a Waters 996 photodiode array detector, and a Sedex 55 evaporative light-scattering detector (Sedere, France). Detection wavelengths were set at 214, 254 and 280 nm. NMR experiments were performed on a Bruker Avance 500 MHz spectrometer in D_2O with the residual solvent peak (δ_H 4.79) as reference. Low resolution electrospray ionization (ESI) MS were obtained with a Bruker Esquire 3000 Plus spectrometer in either the positive or negative mode.

HRESIMS were conducted on a LTQ Orbitrap mass spectrometer (Thermo Finnigan). All organic solvents used for material extraction were of analytical grade and purchased from Merck (Darmstadt, Germany). Acetonitrile used for HPLC was of HPLC-grade and purchased from Fisher (USA). Formic acid of HPLC grade was purchased from Acros (USA).

Biological material: A specimen of the marine sponge *N. digitalis* was collected at a depth of about 10 m from “Boca de Calderas”, Havana, Cuba (23º 05’ 55” N 82º 28’ 30” W) in March 2008 and identified by Dr Pedro Alcolado (Institute of Oceanology, Havana, Cuba). A voucher sample (ANC.02.009) has been deposited in the sponge collection of the Cuban National Aquarium. The sponge was kept frozen from collection until the extraction process.

Extraction and isolation: A portion of *N. digitalis* was freeze-dried and ground to obtain a dry powder (15 g), which was exhaustively extracted with a mixture of $MeOH/CH_2Cl_2$ (1:1) to yield 2.3 g of the crude extract after concentration under reduced pressure. The crude extract was fractionated by RP-C18 flash chromatography (elution with a decreasing polarity gradient of H_2O/MeOH from 1:0 to 0:1, then $MeOH/CH_2Cl_2$ from 1:0 to 0:1) (flow: 10 $mL.min^{-1}$). The H_2O/MeOH (2:1) fraction (264 mg) was then subjected to semi-preparative HPLC-DAD (Phenomenex Luna C6-Pheny, 250 × 10 mm id, 5 µm) with an isocratic gradient of H_2O/CH_3CN/formic acid (98:2:0.1) (flow: 3.0 $mL.min^{-1}$, injection volume: 100 µL) and some subsequent mixtures were finally purified by analytical HPLC-DAD (Phenomenex Synergi Polar-RP, 250 × 4.60 mm, 4 µm), using the same mobile phase at a flow rate of 0.8 mL/min and 50 µL of injection volume to afford pure compounds **1-7**.

1.8-Diazabicyclo[5.4.0]undec-7-ene (1)

Yellowish liquid.

^{1}H NMR (500 MHz, D_2O): δ 3.60 (2H, t, *4.8*, H-2), 1.72 (2H, m , H-3), 1.75 (2H, m , H-4), 1.77 (2H, m , H-5), 2.66 (2H, t, 5.2 , H-6), 3.35 (2H, t, 5.9 , H-9), 2.05 (2H, q, 5.9 , H-10), 3.56 (2H, t, 5.9 , H-11).

^{13}C NMR: (125 MHz, D_2O): 53.7 (C-2), 25.4 (C-3), 22.9 (C-4), 28.0 (C-5), 32.4 (C-6), 166.5 (C-7), 37.5 (C-9), 18.4 (C-10), 47.8 (C-11).

HRESI-MS *m/z* 153.1298 $[M+H]^+$ (calcd for $C_9H_{17}N_2$, 153.1312).

Antimalarial assay: *In vitro* drug susceptibility was determined in standard short-term cultures of *Plasmodium berghei* ANKA blood stages, as described before [12]. Briefly, erythrocytes infected with parasites of *P. berghei* ring forms/young trophozoites are incubated at 2% parasitemia at a final cell concentration of 1% in complete culture medium (RPMI 1640 with 20% Fetal Calf Serum, Sigma) containing serial dilutions of samples from *N. digitalis*, each in duplicate wells of 96-well culture plates. These plates are incubated for a period of 24 h at 37°C under standardized *in vitro* culture conditions. The antimalarial activity was expressed as IC_{50}, which was determined according to reported methodology [13a] using data of inhibition of schizont maturation measured as described by Schlichtherle *et al* [13b], and adapting recommendations for *P. falciparum* isolates [13c]. Chloroquine phosphate and artemisinin (Sigma) were used as references.

Cytotoxicity assay: MRC-5, a human diploid lung fibroblast line, was grown (37°C, 5% CO_2) in 96-well culture plates, in minimal essential medium (EMEM; Gibco-BRL), supplemented with 10% fetal calf serum (Cellect gold). Confluent monolayers were incubated for 3 days with serial dilutions of *N. digitalis* sample. Each test was performed in duplicate. The effect of samples on cell viability was measured using the methyl tetrazolium assay (MTT, Sigma) described by Mossman [14] and modified by Sladowski *et al* [15]. The cytotoxicity was expressed as IC_{50}, which was determined according to reported methodology [13a].

Acknowledgments - Financial support was provided by the research grant No. 4500045956 of IOC/UNESCO 2008 (E. L. R and O. P. T). We are also grateful to Boehringer Ingelheim Fonds for financial support provided by a fellowship (E. L. R) in April 2008. The authors thank Dr Pedro M. Alcolado (Institute of Oceanology, Havana, Cuba) for careful taxonomical sponge identification and Dr Mohamed Mehiri for technical support in the automatic liquid chromatography Armen Spot Prep system.

Supporting information available: Separation details,; ^{1}H, ^{13}C, and 2D NMR spectra for **1-7**.

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