Bioorganic & Medicinal Chemistry Letters 20 (2010) 5905–5908

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Neopetrosiamine A, biologically active bis-piperidine alkaloid from the Caribbean sea sponge *Neopetrosia proxima*

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ARTICLE INFO

Article history:
Received 28 June 2010
Revised 19 July 2010
Accepted 20 July 2010
Available online 24 July 2010

Keywords:
Neopetrosia proxima
Bis-piperidine alkaloid
Cytotoxicity
Caribbean sponge
Tuberculosis
Malaria

ABSTRACT

A new tetracyclic bis-piperidine alkaloid, neopetrosiamine A (**1**), has been extracted from the marine sponge *Neopetrosia proxima* collected off the west coast of Puerto Rico. The structure of compound **1** was elucidated by analysis of spectroscopic data coupled with careful comparisons of its ^{1}H and ^{13}C NMR data with those of a well-known 3-alkylbis-piperidine alkaloid model. The new alkaloid displayed strong in vitro cytotoxic activity against a panel of cancer cell lines as well as in vitro inhibitory activity against the pathogenic microbes *Mycobacterium tuberculosis* and *Plasmodium falciparum*.

Marine sponges belonging to the order Haplosclerida constitute an abundant source of 3-alkylpyridine and 3-alkylpiperidine alkaloids, and thus far, well over 100 such alkaloids have been identified from this taxonomically complex group of marine organisms.[1,2] The occurrence of these biosynthetically intriguing families of alkaloids in sponges other than the Haplosclerida has also been described, albeit far less frequently.[3] Thus far, over 30 distinct 3-alkylpiperidine-based carbon frameworks have been discovered, including the haliclamine/cyclostellettamine, ircinal, ingenamine, petrosin, mandangamine, manzamine, halicyclamine, sarain, xestospongin/araguspongine, and aragupetrosine skeletons.[2b,4] Not surprisingly, several of these compounds have been the subject of recent total syntheses, thanks in no small part to their interesting biological activities and the observation that they usually possess symmetry relationships that challenge the most basic hypotheses as to the cyclization processes responsible for their biosynthesis.[5–7] The most recently discovered class of tetracyclic bis-piperidine alkaloids from marine sponges is perhaps best exemplified by the halicyclamines/haliclonacyclamines (isolated from *Haliclona* sponge specimens),[8,9] the arenosclerins (isolated from *Arenosclera brasiliensis* and *Pachychalina alcaloidifera*)[10] and halichondramine (isolated from a *Halichondria* species).[11]

A small specimen of freeze-dried *Neopetrosia proxima* (Duchassaing and Michelotti, 1864)[12] collected at a depth of 35 m from Mona Island off the west coast of Puerto Rico, was homogenized with chloroform–acetone (1:2) and filtered in vacuo. Soon thereafter, the dried sponge cake left behind was re-extracted with chloroform–methanol (1:1) and the resulting extract was concentrated in vacuo.[13] Preliminary in vitro antituberculosis activity assays indicated that the crude chloroform–acetone extract, together with the chloroform–methanol partition, showed anti-TB activities with MIC values at 121 and 30 μg/mL, respectively. As only the latter sponge extract was considered active, a small portion of it was subjected to fractionation using normal-phase flash column chromatography on silica gel with hexane–acetone–triethylamine (90:10:0.5) as eluant to afford neopetrosiamine A (**1**, 10.0 mg) as a thick colorless oil.[14] With the NMR and MS spectroscopic data of **1** in hand, we quickly identified the title metabolite as a new 3-alkylpiperidine alkaloid.[9–11]

The EI mass spectrum of **1** exhibited a molecular ion $[M]^{+}$ at *m/z* 440.4. The ^{13}C NMR experiment in CD_3OD (Table 1) revealed the presence of 30 carbons, and a DEPT NMR experiment showed that they could be divided into four sp^2 methine carbons, four sp^3 methine carbons, and 22 methylenes. Six of the methylenes were positioned downfield (δ_C 45.8–60.3), suggesting their proximity to nitrogen atoms. On the basis of the analyses outlined above, the molecular formula was determined to be $C_{30}H_{52}N_2$ with six degrees of unsaturation. This contention was further corroborated by the HREIMS and HRESIMS data. The presence of two double bonds indicated that the molecule was tetracyclic. The interpretation of the ^{1}H NMR spectrum was somewhat hampered by the severe overlapping of resonances in the high-field region, thus rendering interpretation of COSY and TOCSY experiments essentially impossible for the high-field protons. Complete assignment of the carbons and protons of **1**, as disclosed in Table 1, was accomplished by the combined

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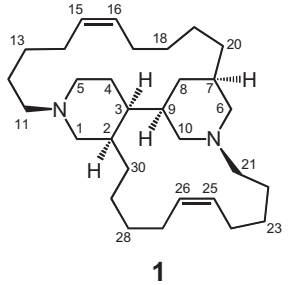
doi:10.1016/j.bmcl.2010.07.084

Table 1
^{13}C (125 MHz), ^{1}H NMR (500 MHz), and long-range correlation data for neopetrosiamine A (**1**)[a]

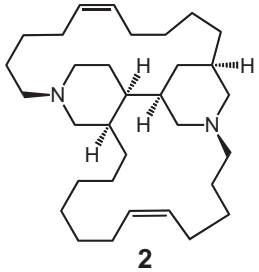
Position	δ_C[b] (mult.)	δ_H[c] (mult., *J* in Hz)	^{1}H–^{1}H COSY	HSQC-TOCSY	HMBC[d]
1a	52.7 (CH_2)	2.91 (br t, 12.5)	H1b, H2	H2, H3, H30ab	H2, H3, H5b, H11, H30b
1b		2.37 (dd, 12.5, 4.0)	H1a, H2		
2	40.7 (CH)	1.82 (br t, 12.5)	H1ab, H3, H30ab	H1ab, H3, H4ab, H30ab	H1ab, H3, H4b, H9, H30ab
3	34.2 (CH)	1.89 (m)	H2, H4ab, H9	H1ab, H2, H5ab, H9, H30ab	H1ab, H2, H5b, H9, H29
4a	35.8 (CH_2)	1.90 (m)	H3, H4b, H5ab	H2, H3, H5ab, H9	H2, H3, H5b, H9
4b		1.78 (br t, 12.5)	H3, H4a, H5ab		
5a	45.8 (CH_2)	3.12 (br t, 12.5)	H4ab, H5b	H4ab	H1b, H4a, H11
5b		2.59 (m)	H4ab, H5a		
6a	60.3 (CH_2)	2.72 (m)	H6b, H7	H7, H8ab, H20ab	H8ab, H10ab, H20a, H21a
6b		1.78 (br t, 12.5)	H6a, H7		
7	37.5 (CH)	1.50 (m)	H6ab, H8b, H20ab	H6ab, H8ab, H10ab, H20ab	H6ab, H8ab, H9, H10ab, H20a
8a	37.7 (CH_2)	2.28 (br d, 12.5)	H8b, H9	H6ab, H7, H9, H10ab, H20ab	H3, H6ab, H9, H10ab, H20ab
8b		0.92 (br q, 12.5)	H7, H8a, H9		
9	44.3 (CH)	1.78 (br t, 12.5)	H3, H8ab, H10ab	H6ab, H7, H8ab, H10ab	H2, H3, H4b, H8ab, H10ab
10a	59.8 (CH_2)	2.90 (m)	H9, H10b	H6ab, H8ab, H9	H6ab, H8ab, H9, H21ab
10b		2.16 (m)	H9, H10a		
11	57.0 (CH_2)	2.74 (m)	H12ab	H12ab, H13, H14a	H1a, H5b, H12ab, H13
12a	20.4 (CH_2)	1.60 (m)	H11ab, H12b, H13	H11, H13, H14ab, H15	H11
12b		1.45 (m)	H11ab, H12a, H13		
13	27.2 (CH_2)	1.38 (m)	H12ab, H14ab	H11, H12ab, H14ab, H15	H11, H15
14a	27.0 (CH_2)	2.18 (m)	H13, H14b, H15	H11, H12ab, H15, H17ab	H12ab, H13, H15, H16
14b		2.06 (m)	H13, H14a, H15		
15	131.1 (CH)	5.29 (m)	H14ab	H16	H14ab
16	130.3 (CH)	5.29 (m)	H17ab	H15	H17ab, H18b
17a	27.5 (CH_2)	2.12 (m)	H16, H18ab	H16	H15, H16, H18ab, H19
17b		2.04 (m)	H16, H18ab		
18a	30.0 (CH_2)	1.56 (m)		H7, H16, H17ab, H20ab	H17ab, H19, H20ab
18b		1.18 (m)			
19	26.9 (CH_2)	1.39 (m)	H20ab	H6ab, H7, H16, H17ab, H20ab	H7, H17ab, H18ab, H20ab
20a	33.9 (CH_2)	1.43 (m)	H7, H19, H20b	H6ab, H7, H8ab, H17ab	H6b, H8ab, H18b, H19
20b		0.89 (m)	H7, H19, H20a		
21a	57.5 (CH_2)	2.61 (m)	H21b, H22ab	H22ab, H23, H24ab	H6ab, H10b, H22ab
21b		2.44 (br t, 10.0)	H21a, H22ab		
22a	20.0 (CH_2)	1.60 (m)	H21ab, H23	H21ab, H23, H24ab, H25	H21ab
22b		1.45 (m)	H21ab, H23		
23	24.9 (CH_2)	1.45 (m)		H21ab, H24ab, H25, H26	H21ab, H22ab, H24ab, H25
24a	25.1 (CH_2)	2.12 (m)		H21ab, H23, H25, H26	H22ab, H23, H25, H26
24b		2.03 (m)			
25	130.4 (CH)	5.48 (m)	H24ab, H26	H26, H24ab	H24ab, H26, H27ab
26	130.8 (CH)	5.29 (m)	H25, H27ab	H24ab, H27	H24ab, H25, H27ab, H28a
27a	26.6 (CH_2)	2.18 (m)	H26, H28ab	H25, H26, H28ab, H29	H25, H26, H29
27b		2.04 (m)	H26, H28ab		
28a	30.7 (CH_2)	1.68 (m)	H27ab, H28b, H29	H25, H26, H27ab, H29	H27ab, H26, H29, H30ab
28b		1.57 (m)	H27ab, H28a, H29		
29	25.3 (CH_2)	1.45 (m)	H28ab	H1ab, H2, H27ab	H27ab, H30ab
30a	32.8 (CH_2)	1.43 (m)	H2, H30b	H1ab, H2, H3, H27ab, H28a	H29
30b		1.35 (m)	H2, H30a		

[a] Spectra recorded in CD_3OD at 25 °C. The letters a and b denote downfield and upfield resonances of a geminal pair, respectively.
[b] Chemical shifts refer to CD_3OD (δ_C = 49.0). Multiplicities were determined by DEPT NMR experiments.
[c] Chemical shifts refer to CD_3OD (δ_H = 3.30).
[d] HMBC experiment with a delay of 50 ms.

analysis of HSQC, COSY, TOCSY, HMBC, and HSQC-TOCSY NMR experiments.



1



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The two partial structures **a** and **b** depicted in Figure 1 were deduced from COSY, TOCSY, HSQC, and HMBC correlations. We began the elucidation of partial structure **a** from the methylene groups α to the N atom, namely C-1, C-5, and C-11. Correlations detected in the HMBC spectrum between C-1 and H-11 and between C-5 and H-11, established the presence of a trisubstituted amine moiety in **1**. Additional HMBC correlations between C-2 and H-1ab, and H-4b, and between C-3 and H-1ab, as well as sequential COSY

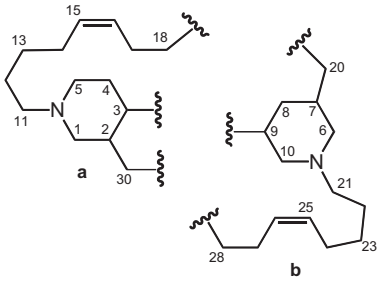

Figure 1. Partial structures **a** and **b** of neopetrosiamine A (**1**).

correlations between H-1ab and H-2, H-3, and H-4ab, between H-4ab and H-5ab, and also between H-3 and H-5ab, allowed the assignment of the piperidine ring in partial structure **a**. The COSY correlations between H-11 (δ_H 2.74) and H-12ab combined with sequential HSQC-TOCSY correlations between H-12ab through H-15, unequivocally established the position of the double bond. The locus of the $\Delta^{15,16}$ double bond in partial structure **a** was further supported by the conspicuous absence in the HMBC spectrum of $^2J_{CH}$ and $^3J_{CH}$ couplings between H-15 (δ_H 5.29) and either of C-11 (δ_C 57.0) or C-12 (δ_C 20.4). Following a similar strategy, a second piperidine system was suggested for partial structure **b** from HMBC correlations between C-21 and H-6ab and between C-6 and H-10ab. This conclusion was also deduced from sequential COSY correlations observed for protons H-6 through H-10. Another set of sequential COSY correlations, observed for protons H-21 through H-27, allowed the assignment of partial structure **b**, which was further corroborated by HMBC and HSQC-TOCSY correlations (Table 1). Worth mentioning was the lack of significant $^2J_{CH}$ or $^3J_{CH}$ couplings between H-25 (δ_H 5.48) and either of C-21 (δ_C 57.5) and C-22 (δ_C 20.0) in the HMBC spectrum of **1**.

The connectivities between the two partial structures **a** and **b** were determined from HMBC and HSQC-TOCSY correlations. C-20 (δ_C 33.9) showed HMBC correlations with protons at δ 1.78 (H-6b), 2.28 (H-8a) and 0.92 (H-8b), C-19 showed HMBC correlations with both CH_2-20 and CH_2-18, and C-17 showed HMBC correlations with CH-15, CH-16, CH_2-18, and CH_2-19. These correlations enabled us to complete the assignment of the 10-carbon bridge connecting C-11 of partial structure **a** and C-7 of partial structure **b** (Fig. 1). Additional correlations in the HMBC spectrum, between C-4 and H-9, C-2 and H-9, as well as between C-8 and H-3, allowed us to link the two piperidine rings through C-3/C-9 as shown in structure **1**. On the basis of the molecular mass of **1**, it was concluded that the remaining portion of neopetrosiamine A is comprised of another C10 bridge connecting C-21 and C-30. In the HMBC spectrum, correlations were observed between C-28 and both H-26 and H-29, and between C-29 and H-30ab. Additional HSQC-TOCSY correlations between C-30 and protons at δ 2.91 (H-1a), 2.37 (H-1b), 1.82 (H-2), and 1.89 (H-3) completed the assignments of the C and H atoms of this segment.

The relative configuration of the bis-piperidine system of neopetrosiamine A (**1**) was established from the multiplicity and coupling constants of several piperidine ring protons as well as from analysis of NOESY spectra in combination with molecular modeling studies. The configurational analysis of **1** using NOESY experiments was made possible only after the assignment of all hydrogen resonances by detailed analyses of the ^{1}H–^{1}H COSY, TOCSY, HSQC, HMBC, and HSQC-TOCSY spectra in comparison with data reported for haliclonacyclamine A (**2**), a two-carbon-higher-homologue previously isolated from a *Haliclona* sponge species whose absolute stereostructure was determined by single-crystal X-ray diffraction crystallography.[9]

Interestingly, no significant differences were observed for the ^{13}C and ^{1}H assignments of the bis-piperidine spin system of neopetrosiamine A (**1**) upon comparison with those for haliclonacyclamine A (**2**).[9b] Therefore, from the onset, it appeared obvious to us that the relative configuration of **1** was identical to that of **2**. Our contention was confirmed by ^{1}H coupling constants analysis and NOESY NMR data. The signal of H-8b was a well-defined quartet, displaying the same 12.5 Hz coupling constant as H-7, H-8a, and H-9. Therefore, the relative configuration between H-8b and H-7 must be axial–axial, as well as between H-8b and H-9, establishing the C-7 and C-9 relative configuration as the same as haliclonacyclamine A (**2**). Analysis of NOE dipolar couplings observed in the NOESY spectrum of neopetrosiamine A (**1**) (indicated in Fig. 2) clearly showed cross-peaks between H-8b and H-10b, between H-8b and H-6b, and between H-10b and H-6b, indicating that these

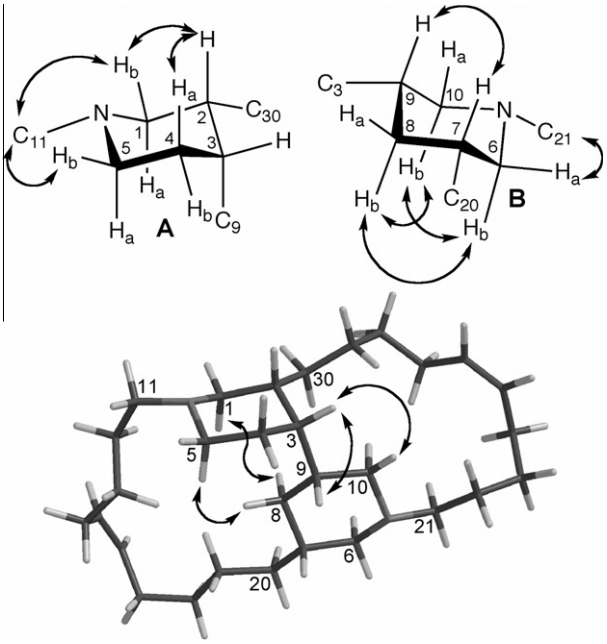

Figure 2. Relative configuration of the bis-piperidine ring system of neopetrosiamine A (**1**) showing intra- and inter-ring NOEs. The arrows indicate dipolar couplings observed in NOESY spectra.

protons were located on the same face of piperidine ring B, having axial orientations in a chair-like conformation. In ring A, the triplet signal at δ_H 2.91 for H-1a showed a 12.5 Hz coupling to its geminal partner H-1b and also to H-2, therefore H-1a and H-2 were *trans*-diaxial. In the NOESY spectrum, there were no correlations observed between H-3 and either H-1a or H-5a. The 1D-TOCSY data revealed that H-3 was a multiplet showing small couplings and suggested that this proton was equatorial rather than axial. Dipolar couplings observed between H_2-11 and protons H-1b and H-5b, as well as between H-2 and H-4a, were a clear evidence that piperidine ring A also adopted a chair conformation in which H-1a, H-2, H-4a, and H-5a were axial. When adjoined, the two piperidine rings adopt a nearly perpendicular orientation with a vicinal dihedral angle between H-3 and H-9 close to 90° (calculated θ = 78.9°) as suggested by the ^{1}H–^{1}H COSY data. Intra-rings NOESY couplings were observed between H-1a/H-8b, H-5a/H-8a, H-3/H-10a, and H-3/H9, which established that the relative configuration of the neopetrosiamine A bis-piperidine system is identical to that of haliclonacyclamine A (**2**), namely 2R^*, 3R^*, 7R^*, 9R^*. Thus, with the relative configuration of neopetrosiamine A (**1**) as shown in Figure 2 the piperidine rings are each found in a chair conformation with the two *N*-alkyl chains oriented equatorially and each nitrogen lone pair pointing toward the more favorable axial position. Furthermore, the configuration of the double bonds of neopetrosiamine A (**1**) was established by analysis of the ^{13}C NMR, NOESY and IR spectra. The chemical shifts of C-14 (δ 27.0) and C-17 (δ 27.5) indicated a *Z* configuration of the $\Delta^{15,16}$ double bond. The $\Delta^{25,26}$ double bond was also *Z* given the ^{13}C chemical shifts of C-24 (δ 25.1) and C-27 (δ 26.6). The NOESY spectrum indicated NOEs between H-25/H-26, which also supported the proposed configuration of $\Delta^{25,26}$. In addition, the presence of a medium to strong absorption peak at 752 cm^{-1} in the IR spectrum (C–H out-of-plane bending vibration) indicated that both C=C's in **1** are *cis*.

Preliminary chiral HPLC studies, together with the $[\alpha]_D$ value of −10.0 (*c* 1.0, $CHCl_3$), suggest that neopetrosiamine A (**1**) is present in the sponge in a single enantiomeric form. Unfortunately, after submission for biological testing, there was insufficient material of **1** to derivatize and conduct an X-ray crystallographic study

using Cu radiation to determine its absolute configuration. Furthermore, additional bioactive alkaloid-containing fractions were obtained from the chloroform–methanol extract of *N. proxima* during the course of this investigation, but the availability of only small amounts (each <2.0 mg) made their isolation and identification extremely difficult. Efforts to re-isolate larger quantities of these interesting 3-alkylpiperidine metabolites, including recollection of a larger sponge specimen, are in progress in our laboratory.

Upon screening in the NCI's in vitro antitumor assay consisting of 60 human tumor cell lines neopetrosiamine A (**1**) exhibited strong inhibitory activity against MALME-3M melanoma cancer, CCRF-CEM leukemia, and MCF7 breast cancer with IC_{50} values of 1.5, 2.0, and 3.5 μM, respectively. Compound **1** was also tested in vitro against a pathogenic strain of *Mycobacterium tuberculosis* ($H_{37}Rv$) in a microplate Alamar Blue assay (MABA) exhibiting an MIC value of 7.5 μg/mL, and notably, it did not exhibit significant cytotoxicity against VERO cells (IC_{50} = 42.4 μg/mL). Neopetrosamine A (**1**) also showed antiplasmodial activity against *Plasmodium falciparum* with an IC_{50} value of 2.3 μM.[15]

Acknowledgments

We thank J. Vicente, J. Marrero, J. C. Asencio, R. Rios, J. J. La Clair, M. J. Lear, and the crew of the R/V Pez Mar for providing logistic support during the sponge collection. The National Cancer Institute (NCI), the Institute for Tuberculosis Research at the University of Illinois at Chicago, and the Institute for Tropical Medicine and Health Sciences (Panama) provided in vitro cytotoxicity, antituberculosis, and antimalarial activity data, respectively. Mass spectral determinations were provided by the Mass Spectrometry Laboratory of the University of Illinois at Urbana-Champaign. The expert assistance of Mr. Jan Vicente for the sponge taxonomy determination and N.A. Avilés and Z. Pérez for diligent laboratory work are gratefully acknowledged. Financial support to K. Nieves was provided by the UPR-RISE Fellowship Program. This work was supported by a grant from the NIH-SC1 Program (Grant 1SC1GM086271-01A1) awarded to A.D.R.

Supplementary data

Supplementary data (underwater photograph of *Neopetrosia proxima*, copies of the ^{1}H and ^{13}C NMR spectra as well as 2D NMR spectra of neopetrosiamine A) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.07.084.

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12. A small specimen of *Neopetrosia proxima* (phylum Porifera, class Demospongiae, order Haplosclerida, family Petrosiidae) was collected on July 11, 2006 off Mona Island, Puerto Rico using scuba at depths of 9–13 m. A voucher specimen has been deposited at the Department of Chemistry, University of Puerto Rico, Río Piedras, Puerto Rico (deposit number IM06-14). This sponge, previously placed under the genus *Xestospongia*, was also known as *Densa araminta* (de Laubenfels, 1934); see: Campos, M.; Mothes, B.; Eckert, R.; Van Soest, R. W. M. *Zootaxa* **2005**, *963*, 1. The sponge specimen analyzed was a dark brown to tan hard mat incrusted in the substratum with scattered oscules. It was sticky to the touch and infested with *Parazoanthus* on the surface.
13. The sponge specimen was freeze-dried and the dry animal (535 g) was cut into small pieces and blended in 1:2 $CHCl_3$–acetone (11 × 1 L). After filtration in vacuo the combined organic extracts were concentrated and the gummy greenish residue obtained (11.1 g) was stored under refrigeration. The dry sponge cake left behind was immersed in 1:1 $CHCl_3$–MeOH (2 L) and allowed to soak overnight. Evaporation of the organic solvents yielded 51.2 g of a brownish gum whose TLC analysis (Si gel plates; *n*-hexane–acetone 90:10:0.5 Et_3N, sprayed with Dragendorff's reagent) indicated the presence of a single major alkaloid and traces of a complex mixture of alkaloids. A small portion (7.0 g) of the latter extract was chromatographed over silica gel (200 g) with a mixture of 10% acetone in *n*-hexane spiked with 0.5% Et_3N. This separation resulted in four fractions, the least polar of which (31 mg) was subsequently chromatographed over silica gel (10 g) using 10% acetone in *n*-hexane + 0.5% Et_3N to afford pure neopetrosiamine A (**1**) (10 mg; 0.01% yield).
14. *Neopetrosiamine A* (**1**): Thick colorless oil; $[\alpha]_D^{20}$ − 10.0 (*c* 1.0, $CHCl_3$); IR (film) ν_{max} 2997, 2929, 2855, 1651, 1460, 1369, 1261, 1133, 1098, 867, 804, 752, 696 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) (see Table 1); EIMS *m/z* $[M]^+$ 440 (35), 272 (8), 229 (10), 163 (23), 149 (31), 131 (11), 105 (66), 95 (12), 91 (12), 88 (16), 86 (68), 85 (51), 84 (100), 83 (76), 77 (33); HREIMS *m/z* $[M]^+$ 440.4099 (calcd for $C_{30}H_{52}N_2$, 440.4104); HRESIMS *m/z* $[M+H]^+$ 441.4213 (calcd for $C_{30}H_{53}N_2$, 441.4209).
15. Bioactivity tests were performed as reported previously; see: Wei, X.; Rodríguez, A. D.; Baran, P.; Raptis, R. G. *J. Nat. Prod.* **2010**, *73*, 925.