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New nitrogenous constituents from the South African marine Ascidian *Pseudodistoma* sp.

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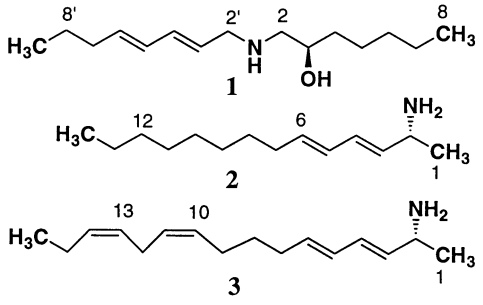
Abstract—Cytotoxicity-guided fractionation of an extract of the South African marine ascidian *Pseudodistoma* sp. provided pseudodistamine (**1**), a new bis alkyl amine, two new aliphatic amines **2** and **3**, and a new β-carboline alkaloid **4**. The structures of compounds **1**–**4** were elucidated by spectroscopic methods and by comparison with spectral data from structurally related compounds. The absolute stereochemistry of **1** was assigned by Mosher's ester analyses, while the stereochemistry of **2** and **3** was established by degradation and derivatization studies. Compound **2** demonstrated cytotoxic activity against four different human tumor cell lines, with an IC_{50} of approximately 6.0 μg/mL. Published by Elsevier Science Ltd.

Previous chemical investigations of ascidians in the genus *Pseudodistoma* (family Polyclinidae) have led to the isolation of piperidine,[1–4] indole,[5] and β-carboline[6] alkaloids as well as a series of aliphatic amines[7] and amino alcohols.[8,9] Some of these metabolites have been reported to exhibit antimicrobial,[7,8] cytotoxic,[1,7] or DNA damage-inducing activities.[4] The present study was initiated when an extract of *Pseudodistoma* sp. collected from Algoa Bay, South Africa exhibited cytotoxic activity in the US National Cancer Institute's (NCI) 60-cell antitumor screen.[10,11] Bioassay-guided fractionation of the extract provided a series of new nitrogenous constituents including pseudodistamine (**1**), two unsaturated aliphatic amines **2**–**3**, and a quaternary *N*-methylated β-carbolinium alkaloid **4**.



The cytotoxic CH_2Cl_2–MeOH extract (2.6 g) of the colonial ascidian *Pseudodistoma* sp. was initially separated by a solvent–solvent partitioning protocol.[12] The resulting aqueous partition fraction was purified by a combination of LH-20 chromatography and C_{18} HPLC to give compounds **1** (2 mg), **3** (1 mg), and **4** (2 mg). Fractionation of the $CHCl_3$ soluble partition fraction on LH-20 eluted with hexane–CH_2Cl_2–MeOH (2:5:1) provided pure compound **2** (13 mg).

The HRFABMS of pseudodistamine (**1**) gave a pseudomolecular ion $(M+H)^+$ at *m/z* 240.2325, which established its molecular formula as $C_{15}H_{29}NO$. The ^{13}C NMR spectrum displayed 15 carbon resonances, while data from DEPT experiments indicated the presence of 2 methyls, 4 olefinic methines, an oxygenated methine, and 8 methylene carbons. The carbon chemical shifts of two of the methylenes, which resonated at δ 46.1 and 42.3, indicated they were attached to nitrogen. The ^1H NMR spectrum of **1** contained signals appropriate for a conjugated diene (δ 6.40, 6.10, 5.85, and 5.60), and large vicinal couplings (J=15 Hz) between the olefinic protons established that the geometry of both double bonds was *trans*. COSY and TOCSY correlations revealed that the δ 5.60 olefin proton (H-3′) was coupled to the nitrogen substituted methylene protons at δ 3.55 (H_2-2′), while the δ 5.85 olefinic proton (H-6′) was adjacent to a $-CH_2CH_2CH_3$ group. Thus, one structural component of pseudodistamine (**1**) consisted of a nitrogen-substituted $\Delta^{2,4}$-(*E*,*E*)-octadiene moiety. COSY correlations within the remaining portion of **1** indicated that the protons on the other nitrogen-bearing methylene group (δ 3.01 and 2.75)

Keywords: β-carboline; ascidian; pseudodistamine; *Pseudodistoma*.

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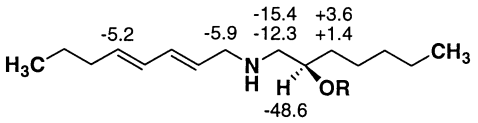


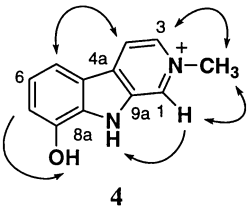
Figure 1. ^{1}H NMR $\Delta\delta$ ($\delta_S-\delta_R$) values (in Hz) obtained for the Mosher's esters of **1**.

were coupled with the oxymethine proton at δ 3.73, which in turn was coupled with a pair of methylene protons centered at δ 1.48. TOCSY data also established that there was a series of three contiguous methylenes and a terminal methyl group in **1**. This allowed assignment of the second substituent on nitrogen as a 2-hydroxyheptyl group. A comprehensive set of HMBC correlations verified the structure proposed for pseudodistamine (**1**). The configuration of the hydroxylated C-3 carbon was established by analysis of ^{1}H NMR data from the Mosher's MTPA ester derivatives of **1**.[13–15] The $\Delta\delta$ ($\delta_S-\delta_R$) values obtained with these derivatives (Fig. 1) allowed assignment of *R* absolute stereochemistry at C-3. While many examples of cyclic and acyclic alkyl amines and amino alcohols have been described from natural sources, pseudodistamine (**1**) represents a novel type of bis alkyl amine in which C_7 and C_8 aliphatic chains are joined via a secondary nitrogen atom.

Compound **2** was isolated as an optically active oil and its molecular formula was established as $C_{14}H_{27}N$ by HREIMS measurements. This molecular formula was isomeric with (3*E*,5*Z*)-tetradeca-3,5-diene-2-amine, a lipid constituent previously reported from a New Zealand collection of *Pseudodistoma novaezelandiae*.[7] The ^{1}H NMR spectral data of **2** confirmed that it was closely related to this *P. novaezelandiae* metabolite. However, coupling constant analysis of the olefinic protons in **2** (δ 6.34, 1H, dd, J=15.0, 10.0 Hz, H-4; δ 6.07, 1H, dd, J=15.0, 10.0 Hz, H-5; δ 5.83, 1H, dt, J=15.0, 7.0 Hz, H-6; δ 5.55, 1H, dd, J=15.0, 7.0 Hz, H-3) clearly indicated the presence of two *trans* double bonds. Data from ^{13}C NMR and DEPT experiments revealed the presence of 2 methyls, 7 methylenes, 4 olefinic methines and 1 methine bearing an amino constituent. Two deuterium-exchangeable NH protons were observed as a two proton broad singlet at δ 8.95 when the ^{1}H NMR spectrum of **2** was recorded in C_6D_6. The methine proton resonance at δ 3.89 showed an HSQC correlation with a carbon at δ 50.3, which was consistent with the presence of a nitrogen substituent at this position. COSY correlations established that the amino methine proton was coupled to a terminal methyl group and to the C-3 olefinic proton. We were thus able to assign the structure of **2** as (3*E*,5*E*)-tetradeca-3,5,-diene-2-amine. The absolute stereochemistry of the lone chiral center at C-2 was established by ozonolysis of **2**, derivatization of the resulting alanine with Marfey's reagent, *N*-(3-fluoro-4,6-dinitrophenyl)-L-alaninamide (FDAA),[16] and then LC–MS comparison with FDAA derivatized D- and L-alanine standards. The FDAA derivative of alanine from **2** co-eluted with the FDAA derivative of D-alanine, therefore the stereochemistry at C-2 was assigned as *R*.

The molecular formula of compound **3**, defined as $C_{16}H_{27}N$ by HRFABMS analysis, required four double bond equivalents in the molecule. The ^{1}H and ^{13}C NMR spectra of **3** contained signals which were nearly identical to resonances observed in the C-1 to C-6 portion of compound **2**, which indicated they shared this structural fragment. Two additional disubstituted olefins were evident from the NMR data for **3**, and a two-proton broad triplet at δ 2.76 indicated that these olefins were separated by a bis allylic methylene group. The location of these double bonds was established by COSY correlations between the C-16 terminal methyl protons at δ 0.96 and the C-15 allylic methylene protons, which in turn were coupled with the C-14 olefin proton at δ 5.38. HMBC correlations from H_3-16 to both C-15 (δ 21.5) and C-14 (δ 132.6) and from H_2-12 to the carbon resonances in both adjacent olefins supported the presence of $\Delta^{10,13}$ unsaturations in **3**. Assignment of *Z* geometries of these two double bonds was based on the upfield chemical shifts observed for C-15, C-12 (δ 26.3) and C-9 (27.6), and by analogy with similar allylic methylene carbons of *cis* olefins previously reported in the *Pseudodistoma crucigaster* metabolite crucigasterin 227.[9] Additional HMBC correlations from H_2-8 to C-10 (δ 130.4), C-9, and C-6 (δ 138.4) linked the two terminal portions of the molecule, and thus the structure of compound **3** was identified as (3*E*,5*E*,10*Z*,13*Z*)-hexadeca-3,5,10,13-tetraene-2-amine. The stereochemistry at C-2 was assigned as *R* based on the results from an ozonolysis and FDAA analysis of the degradation products, similar to that described above for **2**.

Compound **4** was optically inactive and HRFABMS analysis provided a $[M]^+$ ion which established its molecular formula as $C_{12}H_{11}N_2O$. This formula required the presence of a quaternary nitrogen atom and nine degrees of unsaturation in the molecule. Its UV spectrum with λ_{max} values at 389, 317, 268, and 228 nm was suggestive of a β-carboline moiety,[17,18] which would account for all of the unsaturation equivalents in **4**. The IR spectrum displayed a broad band (3400–3200 cm^{-1}) which suggested the presence of hydroxyl and NH functionalities. The ^{1}H NMR spectrum of compound **4** recorded in CD_3OD revealed two mutually coupled doublets (J=5.0 Hz) at δ 8.43 and 8.59 which could be assigned to H-3 and H-4, respectively, while a broad signal at δ 9.07 (1H) was attributed to H-1. A three-proton spin system provided signals at δ 7.15 (1H, d, J=8.0 Hz), 7.27 (1H, t, J=8.0 Hz) and 7.84 (1H, d, J=8.0 Hz) which were determined to be H-7, H-6, and H-5, respectively. Placement of the H-5 resonance was supported by its characteristic downfield chemical shift,[6,19] and by a strong NOE interaction that was observed with H-4. A three-proton singlet at δ 4.51 which had an HSQC correlation to a carbon at δ 48.5 was attributable to a quaternary *N*-Me group. H-1 and H-3 each exhibited significant NOE interactions with the *N*-methyl protons and they both had HMBC correlations with the *N*-methyl carbon resonance. The methyl could thus be placed at N-2, which must be a quaternary nitrogen. The chemical shift of C-8 (δ 145.8) indicated that it was attached to oxygen and it exhibited HMBC correlations with both H-6 and H-7. When the ^{1}H NMR spectrum of **4** was acquired in DMSO-d_6, OH-8 was observed at δ 10.70 and it showed an NOE interaction with H-7 which confirmed its location. NOE interactions were also observed between the NH-9 proton at δ 12.83 and H-1. Thus, compound **4** was characterized as 8-hydroxy-2-methyl-β-carbolinium alkaloid (Fig. 2). While quaternary β-carbolines have been reported from terrestrial plants of the genera



4

Figure 2. Arrows indicate key NOE interactions in **4**.

Desmodium,[20–23] *Hedyotis*,[24] and *Strychnos*,[25] the only other β-carbolinium alkaloid from a marine source was recently described from the soft coral *Lignopsis spongiosum*.[26]

Compounds **1**–**4** were evaluated for cytotoxic properties in a 2-day in vitro assay.[27] The IC_{50} value for **2** against the LOX (melanoma), A549 (non-small cell lung), SNB-19 (CNS), and OVCAR-3 (ovarian) human tumor cell lines was approximately 6.0 μg/mL. Compounds **1**, **3**, and **4** were inactive in this assay at a high-test concentration of 20 μg/mL.

1. Experimental

1.1. General experimental procedures

HPLC was performed on a Varian–Rainin system employing a Dynamax C_{18} column (1×25 cm), using a flow rate of 3 mL/min and UV detection at 220 nm. Optical rotations were measured with a Perkin–Elmer 241 polarimeter. Ultraviolet (UV) and infrared (IR) spectra were obtained on a Beckman DU-640 and Perkin–Elmer 1600 FTIR spectrometer, respectively. The ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded in CD_3OD, C_6D_6, or DMSO-d_6 on a Varian INOVA 500 spectrometer and the chemical shifts are reported in ppm relative to the residual non-deuterated solvents. Multiplicities for the ^{13}C resonances were established from DEPT experiments. Inverse detected heteronuclear correlations were measured using HSQC (optimized for $^1J_{CH}$=140 Hz) and HMBC (optimized for $^nJ_{CH}$=8.5 and 3.5 Hz) pulse sequences. High-resolution mass spectra were acquired on a JEOL SX102 mass spectrometer.

1.2. Animal materials

Samples of the ascidian *Pseudodistoma* sp. were collected from Algoa Bay, South Africa in November 1992 by the Coral Reef Research Foundation and subsequently identified by S. Parker-Nance. A voucher specimen (# 0CDN6128) for this collection is maintained at the Smithsonian Institution, Washington, DC.

1.3. Extraction and isolation

The frozen ascidian (78.8 g) was ground to a fine powder, extracted with H_2O to give an aqueous extract and then sequentially extracted with CH_2Cl_2–MeOH (1:1) followed by MeOH (100%). The combined organic extracts were evaporated in vacuo to provide 2.56 g of residue which was subjected to a four step solvent–solvent partitioning protocol.[12] Cytotoxic activity was concentrated into both the $CHCl_3$ soluble material (159 mg) and the aqueous soluble fraction (86 mg). The $CHCl_3$ soluble material was separated by gel permeation chromatography on a Sephadex LH-20 column (2.5×100 cm) eluted with *n*-hexane–CH_2Cl_2–MeOH (2:5:1). One of the fractions (23 mg) contained a 1:1 mixture of compounds **2** and **3**, as evident from its ^1H NMR spectrum, while a later eluting fraction afforded pure **2** (13.4 mg). The aqueous partition fraction was chromatographed on Sephadex LH-20 (1.5×100 cm) eluted with MeCN–MeOH (1:1) and finally purified by C_{18} HPLC using an isocratic 6:4 mixture of MeCN and 0.1% aqueous TFA to give pseudodistamine **1** (2.0 mg), compound **3** (1.0 mg) and β-carboline **4** (2.0 mg).

1.3.1. Pseudodistamine (1). Colorless gum; $[\alpha]_D$=−3.0° (*c*=0.1, MeOH); UV (MeOH) λ_{max} (log ε) 225 (3.72) nm; IR (film) ν_{max} 3400–3085, 3000–2850, 1636, 1507, 1440, 1194, 1141, 992 cm^{-1}; ^1H (500 MHz, CD_3OD) δ 6.40 (1H, dd, *J*=15.0, 10.0 Hz, H-4′), 6.10 (1H, dd, *J*=15.0, 10.0 Hz, H-5′), 5.85 (1H, dt, *J*=15.0, 7.0 Hz, H-6′), 5.60 (1H, dd, *J*=15.0, 7.0 Hz, H-3′), 3.73 (1H, m, H-3), 3.55 (2H, d, *J*=7.0 Hz, H-2′), 3.01 (1H, dd, *J*=13.0, 3.0 Hz, H-2a), 2.75 (1H, dd, *J*=13.0, 9.5 Hz, H-2b), 2.12 (2H, q, *J*=7.0 Hz, H-7′), 1.48 (2H, m, H-4), 1.41 (2H, m, H_2-5) 1.34 (4H, m, H_2-8′ and H_2-7), 1.31 (2H, m, H_2-6); 0.91 (6H, t, *J*=7.0 Hz, H_3-9′ and H_3-8); ^{13}C NMR (125 MHz, CD_3OD) δ 139.1 (d, C-6′), 138.5 (d, C-4′), 130.1 (d, C-5′), 121.9 (d, C-3′), 68.8 (d, C-3), 46.1 (t, C-2), 42.3 (t, C-2′), 36.0 (t, C-4), 33.6 (t, C-7′), 33.0 (t, C-6), 30.4 (t, C-8′), 26.4 (t, C-5), 23.7 (t, C-7), 14.4 (2C, q, C-9′ and C-8); HRFABMS obs. $[M+H]^+$ *m/z* 240.2325 (calcd 240.2327 for $C_{15}H_{30}NO$).

1.3.2. (3*E*,5*E*)-Tetradeca-3,5-diene-2*R*-amine (2). Colorless oil; $[\alpha]_D$=−1.2° (*c*=0.25, MeOH); UV (MeOH) λ_{max} (log ε) 229 (4.24) nm; IR (film) ν_{max} 3330, 3000–2800, 1606, 1508, 1461, 990 cm^{-1}; ^1H (500 MHz, CD_3OD) δ 6.34 (1H, dd, *J*=15.0, 10.0 Hz, H-4), 6.07 (1H, dd, *J*=15.0, 10.0 Hz, H-5), 5.83 (1H, dt, *J*=15.0, 7.0 Hz, H-6), 5.55 (1H, dd, *J*=15.0, 7.0 Hz, H-3), 3.89 (1H, pent, *J*=7.0 Hz, H-2), 2.10 (2H, q, *J*=7.0 Hz, H-7), 1.38 (3H, d, *J*=7.0 Hz, H-1), 1.29 (8H, m), 1.28 (2H, m, H-13), 1.27 (2H, m, H-12), 0.89 (3H, t, *J*=7.0 Hz, H-14); ^1H (500 MHz, C_6D_6) δ 8.95 (2H, bs, NH_2), 6.35 (1H, dd, *J*=15.0, 10.0 Hz, H-4), 5.98 (1H, dd, *J*=15.0, 10.0 Hz, H-5), 5.87 (1H, dd, *J*=15.0, 7.0 Hz, H-3), 5.71 (1H, dt, *J*=15.0, 7.0 Hz, H-6), 3.90 (1H, m, H-2), 2.01 (2H, q, *J*=7.0 Hz, H-7), 1.58 (3H, d, *J*=6.0 Hz, H-1), 1.28–1.32 (12H, m), 0.93 (3H, t, *J*=7.0 Hz, H-14); ^{13}C NMR (125 MHz, CD_3OD) δ 139.1 (d, C-6), 135.9 (d, C-4), 130.1 (d, C-5), 127.8 (d, C-3), 50.3 (d, C-2), 33.6 (t, C-7), 33.0 (t, C-12), 30.5 (t), 30.4 (t), 30.24 (t), 30.21 (t), 23.7 (t, C-13), 19.6 (q, C-1), 14.4 (q, C-14); HREIMS obs. $[M]^+$ *m/z* 209.2150 (calcd 209.2144 for $C_{14}H_{27}N$).

1.3.3. (3*E*,5*E*,10*Z*,13*Z*)-Hexadeca-3,5,10,13-tetraene-2*R*-amine (3). Colorless oil; $[\alpha]_D$=−6° (*c*=0.05, MeOH); UV (MeOH) λ_{max} (log ε) 229 (3.38) nm; IR (film) ν_{max} 3380, 3000–2850, 1560, 1442, 1208, 1139, 991 cm^{-1}; ^1H (500 MHz, CD_3OD) δ 6.35 (1H, dd, *J*=15.0, 10.0 Hz, H-4), 6.07 (1H, dd, *J*=15.0, 10.0 Hz, H-5), 5.84 (1H, dt, *J*=15.0, 7.0 Hz, H-6), 5.54 (1H, dd, *J*=15.0, 7.0 Hz, H-3), 5.38 (1H, m, H-14), 5.34 (3H, m, H-10, H-11, H-13), 3.87

(1H, pent, J=7.0 Hz, H-2), 2.76 (2H, bt, J=6.5 Hz, H-12), 2.05–2.12 (6H, m, H-7, H-9, H-15), 1.48 (2H, m, H-8), 1.37 (3H, d, J=7.0 Hz, H-1), 0.96 (3H, t, J=7.5 Hz, H-16); ^{13}C NMR (125 MHz, CD_3OD) δ 138.4 (d, C-6), 135.6 (d, C-4), 132.6 (d, C-14), 130.4 (3C, d, C-10, C-11, and C-13), 130.2 (d, C-5), 128.3 (d, C-3), 50.3 (d, C-2), 33.2 (t, C-7), 30.2 (t, C-8), 27.6 (t, C-9), 26.3 (t, C-12), 21.5 (t, C-15), 19.7 (q, C-1), 14.6 (q, C-16); CIMS $[M+H]^+$ m/z 234; HRFABMS obs. $[M+H]^+$ m/z 234.2217 (calcd 234.2222 for $C_{16}H_{28}N$).

1.3.4. 8-Hydroxy-2-methyl-β-carboline (4). Colorless gum; UV (MeOH) λ_{max} (log ε) 228 (3.63), 268 (3.56), 317 (3.23), 389 (2.70) nm; IR (film) ν_{max} 3400–3200, 1640, 1592, 1286, 841, 800 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD) δ 9.07 (1H, s, H-1), 8.59 (1H, d, J=5.0 Hz, H-4), 8.43 (1H, d, J=5.0 Hz, H-3), 7.84 (1H, d, J=8.0 Hz, H-5), 7.27 (1H, t, J=8.0 Hz, H-6), 7.15 (1H, d, J=8.0 Hz, H-7), 4.51 (3H, s, *N*-Me); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.83 (1H, bs, NH), 10.70 (1H, s, OH-8), 9.18, (1H, s, H-1), 8.72 (1H, d, J=6.0 Hz, H-4), 8.58 (1H, d, J=6.0 Hz, H-3), 7.89 (1H, d, J=8.0 Hz, H-5), 7.25 (1H, t, J=8.0 Hz, H-6), 7.17 (1H, d, J=8.0 Hz, H-7), 4.51 (3H, s, *N*-Me); ^{13}C NMR (125 MHz, CD_3OD) δ 145.8 (s, C-8), 136.7 (s, C-8a), 134.8 (s, C-9a), 133.9 (d, C-3), 131.1 (d, C-1), 127.7 (s, C-4a), 124.1 (d, C-6), 122.5 (s, C-4b), 118.8 (d, C-4), 116.1 (d, C-7), 114.5 (d, C-5), 48.5 (q, *N*-Me); ^{13}C NMR (125 MHz, DMSO-d_6) δ 144.3 (s, C-8), 134.7 (s, C-9a), 134.3 (s, C-8a), 133.0 (d, C-3), 132.3 (s, C-4a), 130.3 (d, C-1), 122.4 (d, C-6), 120.7 (s, C-4b), 117.6 (d, C-4), 114.7 (d, C-7), 113.5 (d, C-5), 47.3 (q, *N*-Me); HRFABMS m/z 199.0873 $[M]^+$ (calcd 199.0871 for $C_{12}H_{11}ON_2$).

1.4. Mosher's analysis of pseudodistamine (1)[13–15]

A solution of (*R*)-α-methoxy-α-(trifluoromethyl)phenylacetic acid chloride, *R*-(−)-MTPA–Cl, (1.0 mg in 10 μL of dry pyridine-d_5) was added to 0.5 mg of **1** dissolved in 170 μL of dry pyridine-d_5. The reaction mixture was poured into a dry NMR tube and the course of the reaction was monitored by recording ^{1}H NMR spectra at the initiation of the reaction and then after 1, 2, 3, 4 and 24 h. Proton resonances corresponding to the (*S*)-MTPA ester of **1** were clearly distinguished and assigned. The same procedure was repeated with *S*-(+)-MTPA–Cl to get the corresponding (*R*)-MTPA ester of **1**. In both cases, the reaction was completed in 24 h as evidenced by the absence of proton signals assignable to the starting material.

1.5. Stereochemical determination of compounds 2 and 3

A slow stream of O_3 was bubbled into a solution of compound **2** (0.5 mg) in 10 mL of CH_2Cl_2 at room temperature for 8 min. Solvent was removed under a stream of N_2 and the residue was treated with 10 μL 6% triethylamine dissolved in CH_3CN–H_2O (1:1) and 5 μL of a 1% solution of Marfey's reagent,[16] *N*-(3-fluoro-4,6-dinitrophenyl)-L-alaninamide (FDAA), in Me_2CO for 1 h at 40°C. The reaction mixture was diluted with 15 μL of H_2O and an aliquot was analyzed by C_{18} HPLC eluted with a linear gradient of CH_3CN in 5% aqueous CH_3COOH (from 5 to 50% CH_3CN over 25 min). The FDAA-derivatized alanine was detected by absorption at 340 nm and by MSD (200–400 Da) and compared with similarly derivatized D- and L-alanine standards. The same procedure was followed with the product obtained from ozonolysis of compound **3**. The retention times for the FDAA derivatized D- and L-alanine standards were 17.61 and 12.95 min, respectively. The derivatized alanine residues derived from compounds **2** and **3** eluted at 17.69 min.

1.5.1. Cytotoxicity evaluation. DMSO solutions of chromatography fractions and aliquots of the purified compounds **1**–**4** were assayed for cytotoxic properties in a 2-day in vitro assay, experimental details of which have been reported previously.[27]

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