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Four diterpenoid inhibitors of Cdc25B phosphatase from a marine anemone

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Dedicated to Professor Iwao Ojima for his outstanding contributions to bioorganic and medicinal chemistry on the occasion of his 60th birthday.

Abstract—Three new diterpenoids and one known diterpenoid have been isolated from a sea anemone of the order Actiniara, and the structures of the new compounds, actiniarins A–C (**1**–**3**) were established on the basis of extensive 1D and 2D NMR spectroscopic data interpretation. Compound **1** has a six-membered ring hemiacetal ring, and the equilibrium of this ring is discussed. All the isolates were evaluated for their inhibition of Cdc25B and for cytotoxicity against the A2780 ovarian cancer cell line.

1. Introduction

The protein-tyrosine phosphatase Cdc25 is a key regulator of the cell cycle. Of the three homologs Cdc25A, B, and C, Cdc25B appears to play a role in G2 by regulating CDK2/cyclin A and CDK1/cyclinA. Cdc25 is thus an attractive target for the development of novel antimitotic natural products,[1] and several compounds have been reported as Cdc25 inhibitors.[2–8]

In continuation of our search for natural products with potential anticancer activity, we screened a collection of marine natural products from the National Cancer Institute for activity against Cdc25B, and found that an extract of an anemone designated as C010505 (Phylum: Cnidaria/Colenterata; Class: Zoantharia; Order: Actiniaria) showed reproducible activity in this assay. Bioassay-guided fractionation led to the isolation of the new bioactive cleaved xenicane derivative **2**, together with the new but weakly active xenicanes **1** and **3** and the known xenicane diterpenoid **4**.[9] We hereby report the isolation, structure elucidation, and bioactivity of this new class of xenicane diterpenoids.

Keywords: Diterpenoids; Cytotoxicity; Xenicanes; Hemiacetal; Actiniara; Anemone.
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Since the discovery of xenicin[10] from the soft coral *Xenia elongata*, many xenicane diterpenoids have been isolated from marine algae and soft corals. Most of the xenicane diterpenoids are nine membered,[11–27] but there are a few reports of degraded diterpenoids derived from xenicane-type precursors.[9,11,18] Xenicane-type diterpenoids are of considerable interest due to their cytotoxicity,[15,16,19–23] antimicrobial and antibacterial activities,[24,25] ability to inhibit the growth of the alga *Ceramium codii* (a common benthic fouling organism)[26] and inhibition of the production of superoxide in both rabbit and human cell neutrophils.[27]

2. Results and discussion

Extract C010505 was suspended in H_2O and centrifuged. The precipitate was suspended in 90% MeOH/H_2O, and extracted with hexanes. The H_2O solution was loaded onto a C_{18} column which was eluted with H_2O and then MeOH.

The MeOH eluent was combined with the aqueous MeOH fraction. This combined fraction was first separated on a reversed phase C_{18} HPLC column, furnishing 10 fractions (I–X). Compounds **1**–**3** were purified by phenyl HPLC from fraction III, while compound **4** was obtained using phenyl HPLC from fraction IX. The structure of compound **4** was identified by compar-

ison of its spectral data with previously published values.[9]

The molecular formula of compound **1** was established as $C_{20}H_{28}O_6$ by HRFABMS, indicating seven degrees of unsaturation, which was confirmed by ^{13}C, ^{1}H, and HSQC spectra. The structural assignment of **1** was complicated by its existence as a set of equilibrating isomers. Thus, signals in the ^{1}H and ^{13}C NMR spectra of compound **1** measured in MeOH-d_4 indicated it to be a mixture of two molecules in the approximate proportion of 1:1, but its spectra obtained in C_6D_6, acetone-d_6, or C_5D_5N showed that it was (or at least appeared to be) a single compound. This finding suggested that an equilibrium system might exist in MeOH-d_4 solution.

Inspection of the 1D and 2D NMR spectra of **1** in MeOH-d_4 (Table 1) indicated that the compound consisted of two isomeric hemiacetals, designated **1b** and **1c**, each of which also contained an additional aldehyde group. The ^{1}H NMR spectrum of **1** in C_5D_5N (or acetone-d_6 or C_6D_6), on the other hand, showed two strong aldehyde proton signals at δ_H 9.72 and 9.58 for an isomeric structure designated **1a**. It was clear that the NMR solvent had a crucial effect on the tautomeric form present, favoring a mixture of α- and β-hemiacetals (**1b** and **1c**) in MeOH-d_4, and favoring the open form dialdehyde **1a** in C_5D_5N, acetone-d_6, or C_6D_6.

The ^{1}H and ^{13}C NMR spectra of **1b/1c** in MeOH-d_4 showed the presence of three methyls, four methylenes, three methines, five olefinic protons, one aldehyde, one ketone, one carboxylic acid group, one quaternary olefinic carbon, and one oxygenated quaternary carbon. These data (Table 2) suggested that **1b/1c** belonged to a cleaved xenicane-type diterpenoid. Three spin systems, H-11a–H-4a–H_2-5–H-6 (fragment I), H_2-8–H_2-9–H_2-10 (fragment II), and H-12–H-13–H-14 (fragment III), were observed in the COSY spectrum of **1b/1c**. In the HMBC spectrum of **1b/1c**, H-3 and H_3-16/H_3-17 showed correlations with C-4/C-4a/C-12 and C-14/C-15, respectively, and ^{3}J and ^{2}J correlations between H_2-19 and C-10/C-11a, and between H_3-18 and C-7 were also observed. The structures of **1b/1c** were thus assigned as shown (Fig. 1). The only major difference in the ^{1}H NMR spectra of **1a** and **1b/1c** was that the hemiacetal group in **1b/1c** was replaced by an aldehyde group in **1a**.

The absolute stereochemistry at the 4a- and 11a-positions of **1** was not determined, but it is most likely the same as that of compound **4** and xenicin[9,10] since they have a common biosynthetic origin (Fig. 2). Stereochemical assignment about the $\Delta^{13,14}$ double bond was determined as *E* on the basis of $J_{13,14}$ = 14.9 Hz. The configuration of the double bond at 4(12)-position was also assigned as *E* based on the ROESY correlation between H-3 and H-12.

Table 2. ^{13}C NMR data (δ) for compounds **1**–**3**

No.	**1**[a]	**1**[b]	**2**[b]	**3**[b]
1	176.2/176.5	174.7	174.6	174.9
3	197.3	195.4	195.5	188.9
4	141.2	139.9	140.0	114.0
4a	37.1	33.8	35.7	32.4
5	36.7	44.5	42.8	45.1
6	98.7/97.3	201.3	174.6	202.3
7	211.8	207.7	207.5	207.7
8	43.7	42.8	43.0	42.9
9	22.6/22.7	21.9	22.0	21.9
10	34.8	34.5	34.4	34.5
11	146.4	146.2	146.3	146.8
11a	55.1	54.9	54.7	55.0
12	154.5/154.2	156.0	155.2	175.0
13	123.8	122.5	123	119.7
14	155.7	156.0	155.2	150.0
15	71.7/71.6	70.3	70.3	94.0
16	29.5	30.0	30.2	25.4
17	29.8	30.2	30.2	25.6
18	29.7	29.6	29.6	29.6
19	115.0/114.9	114.3	114.3	114.1

[a] In MeOH-d_4.
[b] In C_5D_5N.

Table 1. ^{1}H NMR data (δ) for compounds **1**–**3**

No.	**1**[a]	**1**[b]	**2**[b]	**3**[b]
3	9.34/9.35 s	9.58 s	9.68 s	10.11 s
4a	3.42 m	4.36 m	4.49 m	4.52 ddd (5.0, 11, 10.9)
5	1.70 m 1.95 m	2.83 dd (2.5, 17.2) 3.30 m	3.02 dd (3.0, 15.2) 3.36 m	2.84 dd (5.0, 15.8) 3.09 dd (11, 15.8)
6	4.10 dd (2.5, 9.2) 4.19 dd (3.5, 8.5)	9.72 br s		9.88 br s
8	2.52 m	2.28 m	2.28 m	2.30 m
9	2.14 m	1.83 m	1.87 m	1.88 m
10	1.76 m	2.30 m	2.34 m	2.35 m
11				
11a	3.71 m	4.24 d (11.2)	4.39 m	4.36 d (10.9)
12	7.06/7.09 d (10.3)	7.10 d (10.1)	7.20 d (10.1)	
13	6.93 dd (10.3, 14.6)	7.70 dd (10.1, 14.9)	7.84 dd (10.1, 14.9)	6.96 d (5.7)
14	6.43/6.46 d (14.6)	6.61 d (14.9)	6.59 d (14.9)	6.72 d (5.7)
16	1.35/1.36 s	1.50 s	1.48 s	1.40 s
17	1.36/1.37 s	1.51 s	1.49 s	1.43 s
18	2.14 s	1.95 s	1.95 s	1.95 s
19	5.06/5.07 br s	5.09 br s	5.14 br s	5.10 br s
	5.19/5.21 br s	5.38 br s	5.49 br s	5.48 br s

[a] In MeOH-d_4.
[b] In C_5D_5N.

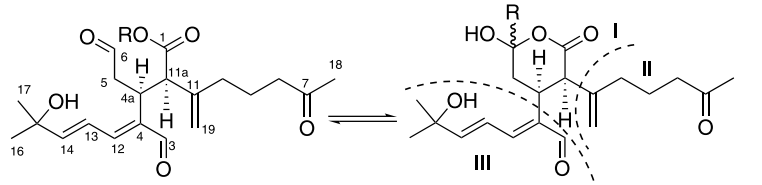

Figure 1. Equilibrating structures **1a**–**1c**.

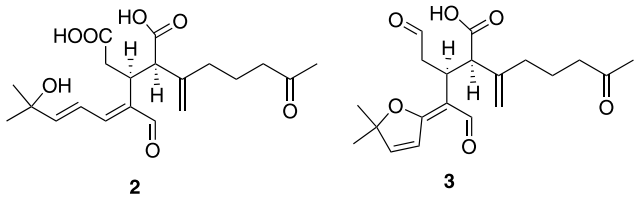

Figure 2. Structures of compounds **2** and **3**.

The molecular formula of compound **2** was deduced as $C_{20}H_{28}O_7$ by HRFABMS and ^{13}C NMR spectrometry. The spectral data for this compound were very similar to those of compound **1**, and the ^1H and ^{13}C NMR data of compound **2** in pyridine-d_5 revealed that the difference between **1a** and **2** was in the replacement of the aldehyde at the 6-position in compound **1a** by a –COOH group in compound **2**. Hence, the structure of **2** was also defined as a diterpenoid of the cleaved xenicane class.

The molecular formula of **3** was deduced as $C_{20}H_{26}O_6$ by HRFABMS and ^{13}C NMR methods. The NMR data (C_5D_5N) for this compound were also similar to those obtained for **1a**. The most noticeable differences in the ^{13}C NMR data were the shifts of the C-3, C-4, C-12, C-13, C-14, C-15, C-16, and C-17 signals compared to those of **1a** (Table 2). The corresponding change was also observed at the diene region in the ^1H NMR spectrum of **3** in that two olefinic protons appeared at δ_H 6.72 (d, $J = 5.7$ Hz, H-14) and 6.96 (d, $J = 5.7$ Hz, H-13) rather than three olefinic protons of **1a** at δ_H 6.61 (d, $J = 14.9$ Hz, H-14), 7.70 (dd, $J = 10.1$ and 14.9 Hz, H-13), and 7.10 (d, $J = 10.1$ Hz, H-12) in fragment III. It could be deduced that a dihydrofuran ring in **3** was formed through the cyclization of the hydroxyl group at the 15-position of **1a**. Compound **3** also had an *E* configuration at the 4(12)-position because H-3 correlated to H-13 in the ROESY spectrum. It is probable that compound **3** also exists in MeOH as two isomeric hemiacetals in the same way as compound **1**, but the available sample was too small to test this hypothesis.

Compounds **1**–**4** were tested in the Cdc25B assay. Among the four natural products compound **2**, which unlike compounds **1** and **3** cannot undergo cyclization to a hemiacetal, was the most active (Table 3). On the basis of the above results, it was concluded that the open form (**1a**) of **1** should be more active than its cyclized ones (**1b** and **1c**), which were the dominant forms in

Table 3. IC_{50} (μg/mL) values of compounds **1**–**5** against recombinant human Cdc25B[a] and A2780[b]

Compound	1	2	3	4	5
Cdc25B	53	1.6	40	33	4.2
A2780	>20	>20	>20	7.6	>20

[a] See Ref. 28.

[b] Concentration of each compound that inhibited 50% (IC_{50}) of the growth of the A2780 human ovarian cell line.[29] Actinomycin D (IC_{50} 1–3 ng/mL) was the positive control for the A2780 assay.

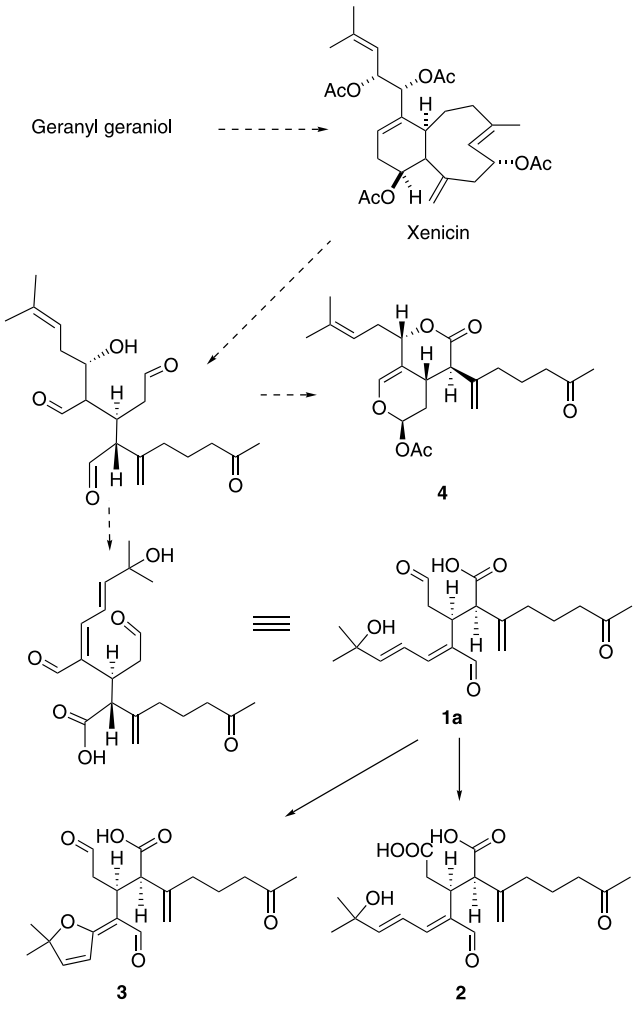

Figure 3. Proposed biosynthesis of compounds **1**–**4**.

protic solvent. To confirm the assumption, compound **1** was reacted with excess diazomethane to furnish **5**, the methyl ester of **1**. Compound **5**, which cannot form a hemiacetal ring via the 1- and 6-positions, was much more active than the parent molecule **1**. Compounds **1**–**5** were also tested in the A2780 assay, but only compound **4** was active against the A2780 cell line with an IC_{50} of 7.6 μg/mL.

Biogenetically, compound **4** could be regarded as being derived from a xenicin-type precursor by oxidative cleavage followed by cyclization of the resulting intermediate (Fig. 3).[9] To the best of our knowledge, there are no literature reports about intermediate-type xenicane diterpenoids such as compounds **1**–**3** from natural sources.

3. Experimental section

3.1. General procedures

Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. IR and UV spectra were measured on MIDAC M-series FTIR and Shimadzu UV-1201 spectrophotometers, respectively. NMR spectra were obtained on a JEOL Eclipse 500 and a Unity 400 spectrometer in CD_3OD and DMSO-d_6. Mass spectra were obtained on a JEOL JMS-HX-110 instrument. The chemical shifts are given in δ (ppm), and coupling constants are reported in Hertz (Hz). A Horizon^{TM} Flash Chromatograph from BioTage Inc. was used for flash column chromatography. HPLC was performed on a Shimadzu LC-10AT instrument with a semi-preparative C_{18}, a phenyl Varian Dynamax (5 μm, 250 × 10 mm), and a preparative C_{18} Varian Dynamax column (8 μm, 250 × 21.4 mm).

3.2. In vitro phosphatase assays

Bioassays were conducted as previously described[28] with an epitope-tagged (histidine$_6$) catalytic domain of human recombinant Cdc25B, which contained amino acids 275–539 of the full length protein.

3.3. Marine sample

The anemone sample used in this work was collected for the National Cancer Institute by Ernani Menez (Smithsonian Institution), and was assigned the collector number 0ALQ 0192. A photograph of the sample (EM89-10K) is available as supporting data. The deep frozen sample was pulverized at the National Cancer Institute in dry ice by use of a worm-fed grinder (hamburger mill), the powder produced was allowed to stand at −30 °C until the CO_2 sublimed, and the mass was then extracted at 4 °C with de-ionized water (1 L) by stirring (30 rpm) for 30 min. The mixture was centrifuged at room temperature (rt) and the supernatant was lyophilized to give the aqueous extract. The insoluble portion from the centrifugation was lyophilized and then statically extracted overnight at rt with 1 L of a 1:1 ratio of MeOH/CH_2Cl_2. The organic phase was filtered off, the pellet washed with a 10% volume of fresh MeOH, and the combined organic phases reduced to dryness at <35 °C by rotary evaporation and then finally dried under high vacuum at rt to give the organic extract as a gum. A portion of this extract was received from the National Cancer Institute as sample number C010505 (0.8 g).

3.4. Extract fractionation

Extract C010505 (0.8 g) was suspended in H_2O, and then centrifuged. The precipitate was suspended in aqueous MeOH (MeOH/H_2O, 9:1, 200 mL) and extracted with hexanes (3 × 200 mL portions, 127 mg after removal of the solvents). The clear H_2O solution was loaded on a C18 column, eluated with H_2O (33 mg after removal of H_2O), and then MeOH. The MeOH eluent was combined with the 90% MeOH/H_2O portion (640 mg after removal of the solvents). This 640 mg fraction was fractionated by column chromatography on a Shimadzu prep-HPLC over C18 Si gel using H_2O/MeOH (90:10 to 50:50 in 30 min, 50:50 to 0:100 in 20 min followed by 100% MeOH) to furnish 10 fractions (I–X). Fraction IX yielded compound **4** (44 mg, t_R: 49 min). The use of phenyl HPLC (30% MeOH) provided compounds **1** (3.0 mg, t_R: 17 min), **2** (0.8 mg, t_R: 12 min), and **3** (1.2 mg, t_R: 22 min) from fraction III (70 mg).

3.5. Actiniarin A (1)

Colorless oil; $[\alpha]_D^{22}$ −13° (*c* 0.23, EtOH); λ_{max} (EtOH)/nm (log ε) 277 (4.27); ν_{max} (film)/cm^{-1} 3444, 2960, 2932, 1713, 1674, 1633, 1408, 1365, 1260, 1200, 1159, 1066, 971, 910, and 797; *m/z* (HR FAB MS). Found: $[M-OH]^+$, 347.1838 ($C_{20}H_{27}O_5$ requires 347.1858.); ^{1}H NMR (C_6D_6) 1.30 (3H, s, H_3-17), 1.31 (3H, s, H_3-16), 1.56 (2H, m, H_2-9), 1.62 (3H, s, H_3-18), 1.81 (2H, m, H_2-8), 1.94 (2H, m, H_2-10), 2.42 (1H, br d *J* = 18.3 Hz, Ha-5), 2.84 (1H, m, Hb-5), 3.98 (2H, m, H-11a and H-4a), 4.88 (1H, br s, Ha-19), 5.16 (1H, br s, Hb-19), 6.02 (1H, d, *J* = 15.1 Hz, H-14), 6.46 (1H, d, *J* = 11.5 Hz, H-12), 7.31 (1H, dd, *J* = 11.5, 15.1 Hz, H-13), 9.16 (1H, br s, H-6), 9.17 (1H, s, H-3); ^{1}H NMR (acetone-d_6) 1.34 (3H, s, H_3-16), 1.35 (3H, s, H_3-17), 1.70 (2H, m, H_2-9), 2.08 (3H, s, H_3-18), 2.12 (2H, m, H_2-10), 2.48 (2H, m, H_2-8), 2.60 (1H, dd *J* = 4.1 and 16.9 Hz, Ha-5), 2.75 (1H, m, Hb-5), 3.69 (1H, d, *J* = 11.5 Hz, H-11a), 3.88 (1H, m, H-4a), 5.07 (1H, br s, Ha-19), 5.22 (1H, br s, Hb-19), 6.47 (1H, d, *J* = 15.1 Hz, H-14), 7.04 (1H, d, *J* = 12.1 Hz, H-12), 7.08 (1H, dd, *J* = 12.1, 15.1 Hz, H-13), 9.33 (1H, br s, H-3), 9.54 (1H, s, H-6); ^{13}C NMR (acetone-d_6) 22.1 (C-9), 30.0 (C-18), 30.0 (C-16), 30.0 (C-17), 33.8 (C-4a), 34.7 (C-10), 43.0 (C-8), 44.4 (C-5), 54.4 (C-11a), 70.7 (C-15), 114.5 (C-19), 122.8 (C-13), 140.0 (C-4), 146.3 (C-11), 155.1 (C-14), 155.1 (C-12), 173.4 (C-1), 195.4 (C-3), 201.4 (C-6), and 207.8 (C-7); ^{1}H and ^{13}C NMR data (C_5D_5N and MeOH-d_4) are listed in Tables 1 and 2.

3.6. Actiniarin B (2)

Colorless oil; $[\alpha]_D^{22}$ −18° (*c* 0.08, EtOH); λ_{max} (EtOH)/nm (log ε) 277 (4.05); ν_{max} (film)/cm^{-1} 3444, 2957, 2930, 1732, 1729, 1463, 1375, 1364,1271, 1115, and 1071; *m/z*

(HR FAB MS). Found: $[M-OH]^+$, 363.1839 ($C_{20}H_{27}O_6$ requires 363.1808.); ^1H and ^{13}C NMR data are listed in Tables 1 and 2.

3.7. Actiniarin C (3)

Colorless oil; $[\alpha]_D^{22}$ −27° (*c* 0.07, EtOH); λ_{max} (EtOH)/nm (log ε) 299 (4.28); ν_{max} (film)/cm^{-1} 2959, 2931, 1727, 1601, 1459, 1430, 1368, 1260, 1125, 1071, 1021, 845, and 799; *m/z* [Finnigan LC (-)ESI MS]. Found: $[M-H]^-$, 361; *m/z* (HR FAB MS). Found: $[M + H]^+$, 363.1796 ($C_{20}H_{27}O_6$ requires 363.1808); ^1H and ^{13}C NMR data are listed in Tables 1 and 2.

3.8. Methylation of compound 1

Compound **1** (1 mg) was methylated with diazomethane, followed by purification with TLC (CH_2Cl_2/MeOH, 20:1) to give compound 5 (1 mg). Compound **5**: colorless oil; $[\alpha]_D^{21}$ −30° (*c* 0.08, EtOH); λ_{max} (EtOH)/nm (log ε) 278 (3.86); ν_{max} (film)/cm^{-1} 3444, 2959, 2927, 2855, 1714, 1673, 1632, 1595, 1435, 1362, 1259, 1198, 1158, 1122, 1072, 1020, 972, 909, 846, and 801; ^1H NMR (500 MHz, C_6D_6) 1.16 (3H, s, CH_3-16), 1.16 (3H, s, CH_3-17), 1.58 (2H, m, CH_2-9), 1.59 (3H, s, CH_3-18), 1.80 (2H, t, *J* = 6.9 Hz, CH_2-8), 1.98 (2H, m, CH_2-10), 2.45 (1H, dd, *J* = 2.8 and 16.3 Hz, Ha-5), 2.93 (1H, m, Hb-5), 3.20 (3H, s, –CO–OCH_3), 4.07 (1H, m, CH-11a), 4.07 (1H, m, CH-4a), 4.90 (1H, br s, Ha-19), 5.17 (1H, br s, Hb-19), 5.95 (1H, d, *J* = 15.2 Hz, CH-14), 6.45 (1H, d, *J* = 11.0 Hz, CH-12), 7.20 (1H, dd, *J* = 11.0 and 15.2 Hz, CH-13), 9.20/9.21 (1H, br s, CH-6), 9.21/9.20 (1H, s, CH-3); *m/z* [Finnigan LC (+)ESI MS]. Found: $[M+Na]^+$, 401/$[M + H_2O]^+$, 396/$[M + H]^+$, 379.

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Supplementary data

A photograph of the organism, identified by its code EM89-10K, is available as a jpg file at http://www.sciencedirect.com. Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmc.2005.05.057.

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