Pyranosylmagellanicus a novel structural class of polyhalogenated acetogenins from *Ptilonia magellanica*

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Abstract—Three naturally occurring pyranosyl-like polyhalogenated metabolites **1–3** as well as their likely biogenetic precursor, the linear compound **4**, have been isolated from the red alga *Ptilonia magellanica*. They are the first compounds within the genus that incorporate chlorine in their network. Compound **3** have structural features reminiscent of the universal chemical signal AI-2 (autoinducer-2) for bacterial communication.

1. Introduction

The significant antimicrobial activity of the extract of several species of red alga of the Bonnemaisoniaceae family led to the discovery of fimbrolides from *Delisea pulchra*.[1–3] They are halogenated furanones structurally similar to bacterial *N*-acyl homoserine lactone (AHLs or autoinducer-I), a family of small diffusible intercellular signaling molecules found in nature as key regulators of the community behavior of a number of genera of Proteobacteria, in a process commonly termed quorum sensing (QS) and that gram-negative bacteria use to coordinate cell population density-dependent control of gene expression.[4] Additionally, furanones possess AHL-antagonistic activity, interfering with AHL-regulated processes by competition for the binding site on the receptor proteins,[5] inhibiting QS in biofilm bacteria,[6] swarming motility,[7] and defending *D. pulchra* against extensive bacterial colonization by regulating expression of the virulence factor required for infection.[8,9]

In spite of the significant research carried out with naturally occurring furanones from *D. pulchra* little additional work to find new related naturally occurring metabolites was pursued since 1997.[10,11] This prompted us to search for novel compounds from the scarcely studied genus *Ptilonia* within the Bonnemaisoniaceae family. While *P. australasica* was rich in polybrominated acyclic and cyclic γ-pyrone derivatives,[12] *Ptilonia magellanica* from the Kergelen Islands yielded solely a linear polybrominated acetate,[13] these two being the only studied species of the genus. *P. magellanica*, like *D. pulchra*, stave off colonization by common epiphytes, which colonize other alga in the immediate area. Because of our interest in benthic organisms from the Chilean coasts[14–18] we decided to study *P. magellanica* collected at the Strait of Magellan, and now we report on three polyhalogenated pyranosyl-like acetogenins and an acyclic polyhalogenated enone.

2. Results and discussion

Pyranosylmagellanicus A–C, **1–3**, Figure 1, are polyhalogenated pyranosyl-like hemiacetal that represent a new

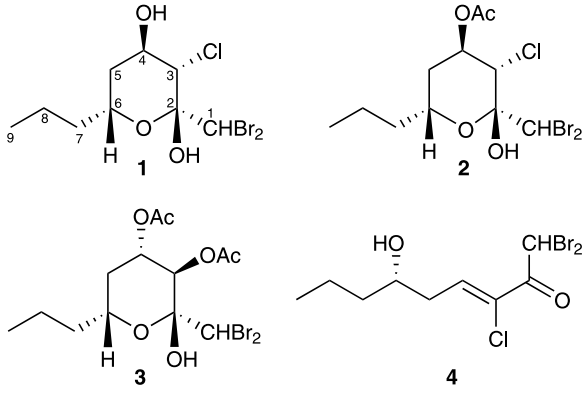

Figure 1. Pyranosylmagellanicus A–C, **1–3**, and **4**.

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structural class of acetogenins, being the first compounds within the genus that incorporate chlorine in their network.

From the crude extract of *P. magellanica* compounds **1–4** were obtained after flash chromatography followed by HPLC. Compound **1** was obtained as an oil whose EIMS spectrum showed peaks at *m/z* 347/349/351/353, with relative intensities suggestive of two bromine atoms and one chlorine, which correspond to the empirical formula $C_9H_{14}O_2ClBr_2$ $[M-OH]^+$ (HREIMS). Absorptions for hydroxyl groups at 3480 and 3154 cm^{-1} were observed in their IR spectrum.

The ^{13}C NMR and DEPT spectra of **1** (Table 1), showed the presence of nine carbon signals assigned to $1CH_3$, $3CH_2$, 4CH, and one quaternary carbon bearing oxygen. The following ^1H NMR signals: four protons geminal to heteroatom [δ 5.85 (s), δ 4.32 (d, $J=2.9$ Hz), δ 4.24 (br s) and δ 4.20 (m)]; six methylene protons at δ between 1.95–1.38; two hydroxylic protons [δ 4.47 (s), δ 3.63 (d, $J=7.9$ Hz)] interchangeable with D_2O and a terminal methyl group [δ 0.92 (t, 7.2)] coupled to an adjacent methylene account for all of the 15 protons of **1**.

A COSY experiment established the connectivities H-3–H_3-9. The chemical shifts of C-4 (δ 70.4) and C-6 (δ 66.8) indicate that these carbons bear oxygen. The acetylation of **1** gave a monoacetate derivative suggesting the remaining hydroxyl group was tertiary. The absence of other insaturations in the IR spectrum combined with the ^{13}C NMR signal at 96.8 ppm indicates that the remaining oxygen atom must form part of a hemiacetalic ring, which agrees with the degree of unsaturation given by the molecular formula. The remaining heteroatoms, a chlorine, and two bromine, were attached to C-3 (δ_H 4.32d, δ_C 55.2) and C-1 (δ_H 5.85s, δ_C 51.9), respectively.

The regiochemistry of the halogens was corroborated by the MS of **1**, which showed a peak at *m/z* 199/201/203 corresponding to the fragment $[O{\equiv}C{-}CHBr_2]^+$. The hemiacetalic carbon C-2 was confirmed by the HMBC correlations 2-*OH*/C-1, C-2, C-3, which also secured the linkage C-1–C-2. All these data support the structure proposed for **1**.

The ^1H NMR data for **2** are very similar to those of **1**. The most significant difference was the proton chemical shift of H-4. This was observed at δ 4.24 for **1** and appeared at δ 5.18 in compound **2**. This variation together with the methyl signal at δ 2.12 ppm indicates that **2** is the acetate derivative of **1**. Acetylation of **1** gave **2** both having identical NMR data.

Compound **3** was a colorless oil. Their ^1H and ^{13}C NMR spectra showed signals for two acetate groups. This compound contains the same C-4–C-9 fragment as **2**. H-3 and C-3 chemical shifts (δ_H 5.47 ppm, δ_C 70.0 ppm) indicate that the chlorine atom at C-3 in **2** has been replaced by an acetate group.

The relative stereochemistry of compounds **1–3** was assigned on the basis of a NOESY experiment, coupling constants, and molecular mechanics calculations, Figure 2. The NOEs observed between the respective H-6 of **1** and **2** with their corresponding hydroxylic protons established a syn periplanar relationship. This, crossed with the NOEs observed between the remaining methines bearing heteroatom, allowed the whole stereochemistry of both **1** and **2** to be established. The large coupling constants measured for H-3, H-4, and H-6 of **3** are compatible with the NOEs depicted in Figure 2. The minimized structures **1–3** (Fig. 2) are consistent with the observed NOEs. The comparison of the well-resolved *J*-values of the selected protons of these compounds with the theoretical coupling constants given by the program[19] proved to be in good agreement.

Compound **4** was isolated as a colorless oil. The EIMS spectrum showed peaks at *m/z* 346/348/350/352, with relative intensities suggestive of two bromine and one chlorine atoms, which correspond to the empirical formula

Table 1. ^1H and ^{13}C NMR Data of compounds **1–4** [500 MHz, δ ppm, (*J*) Hz, $CDCl_3$]

#	**1**		**2**		**3**		**4**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	5.85s	51.9	5.91 (s)	53.1	5.59s	49.3	6.66s	38.4
2	—	96.8	—	95.8	—	96.6	—	181.0
3	4.32 (d, 2.9)	55.2	4.34 (d, 2.6)	53.4	5.47 (dd, 1.7, 9.6)	70.0	—	127.9
4	4.24 br s	70.4	5.18, ddd (2.6, 2.9, 2.9)	71.0	5.27 (ddd, 5.0, 9.6, 11.5)	70.4	7.39 (dd, 7.0, 7.0)	142.5
5	*β*: 1.63m	31.6	*β*: 1.60m	29.0	*β*: 2.16 (ddd, 2.7, 5.0, 12.7)	36.1	*a*: 2.56m	37.8
	α: 1.95 (ddd, 3.1, 12.3, 12.3)		*α*: 1.98 (ddd, 3.3, 12.0, 12.0)		*α*: 1.48m		*b*: 2.64m	
6	4.20m	66.8	4.20m	67.1	4.03 (dddd, 2.2, 4.5, 10.0, 10.0)	69.1	3.89m	70.0
7	*a*: 1.49m	37.2	*a*: 1.40m	37.1	*a*: 1.38m	36.7	*a*: 1.50m	39.7
	b: 1.62m		*b*: 1.58m		*b*: 1.60m		*b*: 1.50m	
8	*a*: 1.38m	18.5	*a*: 1.30m	18.4	*a*: 1.40m	18.5	*a*: 1.39m	18.7
	b: 1.50m		*b*: 1.56m		*b*: 1.48m		*b*: 1.50m	
9	0.92 (t, 7.2)	13.9	0.91 (t, 7.2)	13.8	0.92 (t, 7.2)	13.9	0.95 (t, 7.1)	13.9
3 C=O	—	—	—	—	—	169.5	—	—
3 CH_3	—	—	—	—	2.10s	20.8	—	—
4 C=O	—	—	—	169.7	—	170.3	—	—
4 CH_3	—	—	2.12s	21.1	2.00s	20.9	—	—
2 OH	4.47s	—	3.54s	—	3.17 (d, 1.7)	—	—	—
4 OH	3.63 (d, 7.9)	—	—	—	—	—	—	—

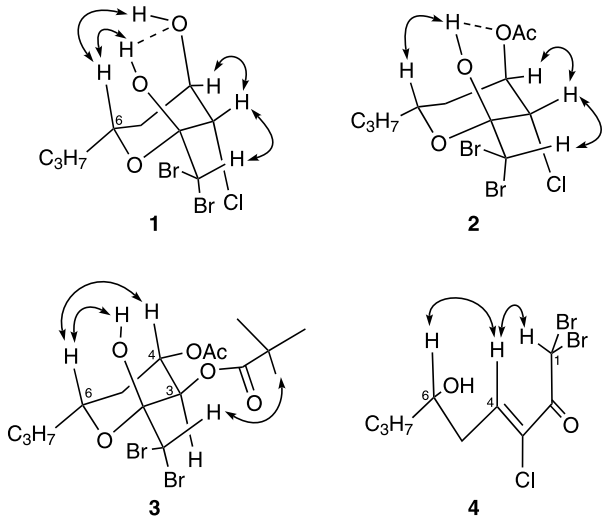

Figure 2. Selected NOEs of **1**–**4**.

$C_9H_{13}O_2ClBr_2$ $[M]^+$ (HREIMS). Absorption for hydroxyl and carbonyl groups at 3389 and 1701 cm^{-1}, respectively, were observed in the IR spectrum. Its ^{13}C NMR and DEPT spectra (Table 1) showed the presence of nine carbon signals assigned to $1CH_3$, $3CH_2$, 3CH (one olefinic and two geminal to heteroatom) and two quaternary carbons (one olefinic and one carbonyl). The two degrees of unsaturation given by the molecular formula indicate that **4** is acyclic.

Compound **4** has an identical carbon atom number and halogen pattern to **1**–**3** suggesting an open form of the pyrane ring for the backbone of **4**. A COSY experiment established the connectivities H-4–H3-9. The HMBC correlation H-4/C-2, C-3 allowed connecting C-3 bearing chlorine (δ 123.9) with C-2. Thus, the remaining carbon attached to two bromine atoms must be linked to C-2. The regiochemistry of the halogens was confirmed by the MS of **4**, which showed the base peak at *m/z* 175/177 corresponding to fragment $[O{\equiv}C{-}CCl{=}CHC_5H_{11}O]^+$.

The relative stereochemistry at C-6 and the geometry of the double bond were established by NOESY experiments. The NOE effect observed between H-4 and H-1, and H-6 established the *Z* geometry of the double bond, and an **S* configuration for C-6. This completes the structure and stereochemistry of the enone as depicted in **4**. On standing **4** converts to **5**, suggesting that **4** may be the biogenetic precursor of the cyclic acetogenins **1**–**3**, Figure 3. Attempts to determine the absolute configuration of **1** using the Mosher method were unsuccessful due to the lack of reactivity toward MTPA.

At least two types of signaling molecules are widespread and have been extensively studied: the *N*-acylhomoserine lactones (AHLs) commonly used by Gram-negative

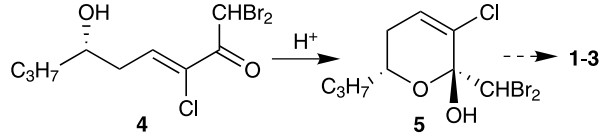

Figure 3. Possible biogenetic pathway for **1**–**3**.

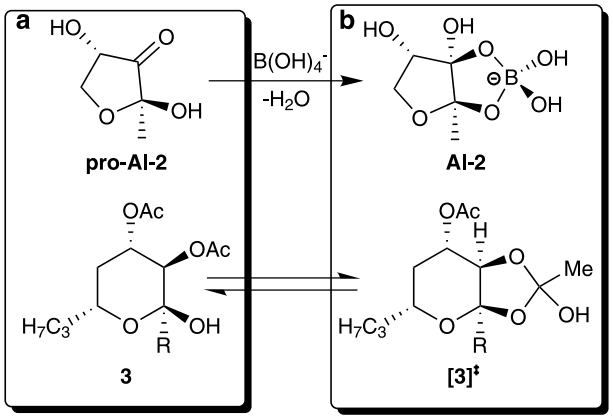

Figure 4. Structural and stereochemical analogy of the pairs pro-AI-2/**3** and AI-2/**3**‡.

bacteria, and peptide pheromones generally used by Gram-positive bacteria.[20]

Gram-positive bacteria, such as *V. harveyi*, have evolved an alternative quorum-sensing mechanism involving two independent autoinducers, one of which is the AHL (AI-1) and the other chemical signal, autoinducer-2 (AI-2), is a furanosyl borate diester molecule (Fig. 4). While AHLs are used for intraspecies communication, AI-2 has been proposed to act as a universal chemical signal for interspecies communication,[21] allowing this microorganism to sense and respond to cells of differing species.

Higher organisms have developed mechanisms that enable them to detect and respond to AHL messaging systems in order to prevent or limit infection. An example, as stated above, is the alga *D. pulchra*, which produces furane lactones to specifically interfere with AHL-mediated QS-systems.[5] Pyranosylmagellanicus, for instance **3**, Figure 4, that conserve the key stereocenters of pro-AI-2/AI-2, (box a), or even a likely intermediate **3**,‡ which is reminiscent of AI-2, (box b), encourage speculations about if these compounds may serve as autoinducer agonist or antagonist.

Naturally occurring metabolites that are structural and stereochemically related to AI-2 add attractiveness as potential interesting biological and biomedical compounds since QS has received much attention as a novel target for drug design.[22] We are now pursuing raw material for new tasks.

3. Experimental

3.1. General procedures

Optical rotations were measured on a Perkin-Elmer model 343 Plus polarimeter using a Na lamp at 25 °C. IR spectra were obtained with a Perkin-Elmer 1650/FTIR spectrometer. ^1H and ^{13}C NMR, HSQC, HMBC, COSY, and NOESY spectra were measured employing a Bruker AMX 500 instrument operating at 500 MHz for ^1H NMR and at 125 MHz for ^{13}C NMR. Two-dimensional NMR spectra were obtained with the standard Bruker software. EIMS and HRMS data were taken on a Micromass Autospec

spectrometer. HPLC separations were performed with a Hewlett Packard 1050 (Jaigel-Sil semipreparative column, 10 μ, 20×250 mm) with hexane–EtOAc mixtures. Merck Si gels 7734 and 7729 were used in column chromatography. The spray reagent for TLC was H_2SO_4–H_2O–AcOH (1/4/20).

3.2. Biological material

P. magellanica (Montagne) J Agardh 1852, was collected by SCUBA diving off Strait of Magellan (Chile).

3.3. Extraction and isolation

Air-dried samples (636 g) were extracted with petroleum ether at room temperature, and were concentrated to give a dark residue (1.4 g), which was chromatographed by flash chromatography on silica gel. The fraction eluted with hexane–EtOAc (92/8) (381 mg) was further separated on HPLC to give compounds **1** (4.2 mg), **2** (5.0 mg) and **4** (4.5 mg). From the fraction eluted with hexane–EtOAc (70/30) (381 mg) compound **3** (2.7 mg) was obtained after purification on HPLC.

3.3.1. Compound 1. Colorless oil; $[\alpha]_D^{25}$ +2 (*c* 1.5, CH_2Cl_2); IR ν_{max} (film) 3480; 3154; 2954; 1443; 1072; 861; 708 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 347/349/351/353 $[M-OH]^+$ (<1, <1, <1, <1), 321/323/325/237 $[M-C_3H_7]^+$ (<1, <1, <1, <1), 303/305/307/309 $[M-C_3H_7-H_2O]^+$ (<1, 2, 1, <1), 294/296/298/300 $[C_4H_5O_3Br_2Cl]^+$ (2, 4, 3, <1), 271/273/275 (6, 13, 6), 199/201/203 $[C_2HOBr_2]^+$ (4, 7, 3), 130/132 (100, 34); HREIMS 348.8993 (calcd for $C_9H_{14}O_2^{35}Cl^{79}Br^{81}Br$, 348.9019), 322.8459 (calcd for $C_6H_8O_3^{35}Cl^{79}Br^{81}Br$, 322.8499), 304.8348 (calcd for $C_6H_6O_2^{37}Cl^{79}Br_2$, 304.8393), 297.8233 (calcd for $C_4H_5O_3^{37}Cl^{79}Br^{81}Br$, 297.8243), 198.8467 (calcd for $C_2HO^{79}Br_2$, 198.8394).

3.3.2. Compound 2. Colorless oil; $[\alpha]_D^{25}$ +2 (*c* 2.0, CH_2Cl_2); IR ν_{max} (film) 3436; 2942; 1736; 1366; 1243; 1049 cm^{-1}; ^1H and ^{13}C NMR in $CDCl_3$, see Table 1; EIMS *m/z* 346/348/350/352 $[M-HOAc]^+$ (<1, 1, <1, <1), 329/331/333/335 $[M-HOAc-OH]^+$ (<1, <1, <1, <1), 199/201/203 $[C_2HOBr_2]^+$ (4, 7, 3), 130/131 $[C_7H_{11}Cl]^+$ (100, 31).; HREIMS 345.8914 (calcd for $C_9H_{13}O_2^{35}Cl^{79}Br_2$, 345.8971), 330.8923 (calcd for $C_9H_{12}O^{35}Cl^{79}Br^{81}Br$, 330.8875), 198.8428 (calcd for $C_2HO^{79}Br_2$, 198.8394), 130.0587 (calcd for $C_7H_{11}^{35}Cl$, 130.0549).

3.3.3. Compound 3. Colorless oil; $[\alpha]_D^{25}$ +6 (*c* 0.24, CH_2Cl_2); IR ν_{max} (film) 3344; 2954; 1738; 1368; 1252; 1055 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 413/415/417 $[M-OH]^+$ (<1, <1, <1), 353/355/357 $[M-AcOH-OH]^+$ (<1, <1, <1), 327/329/331 $[M-AcOH-C_3H_7]^+$ (<1, <1, <1), 267/269/271 $[M-2AcOH-C_3H_7]^+$ (<1, 1, <1), 259 (1), 217 (1), 154 (41), 112 (100); HREIMS 412.9546 (calcd for $C_{13}H_{15}O_5^{79}Br_2$, 412.9599), 352.9350 (calcd for $C_{11}H_{15}O_3^{79}Br_2$, 352.9388).

3.3.4. Compound 4. Colorless oil; $[\alpha]_D^{25}$ −8 (*c* 0.24, CH_2Cl_2); IR ν_{max} (film) 3389; 2931; 1701; 1607; 1261; 1114; 1014 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 346/348/350/352 $[M]^+$ (<1, <1, <1, <1), 329/331/333/335 $[M-OH]^+$ (<1, 1, 1, <1), 303/305/307/309 $[M-C_3H_7]^+$ (<1, 2, 2, <1), 274/276/278/280 (16, 36, 24, 5), 195/197/199 (68, 90, 2), 175/177 $[C_8H_{12}O_2Cl]^+$ (100, 32); HREIMS 349.8957 (calcd for $C_9H_{13}O_2^{37}Cl^{79}Br^{81}Br$, 349.8920), 328.9023 (calcd for $C_9H_{12}O^{35}Cl^{79}Br_2$, 328.8943), 302.8458 (calcd for $C_6H_6O_2^{35}Cl^{79}Br_2$ 302.8423), 177.0447 (calcd for $C_8H_{12}O_2^{37}Cl$, 177.0496).

3.3.5. Compound 5. ^1H ($CDCl_3$) δ 0.94 (t, 7.4), 1.40–1.70 (m), 2.18 (ddd, 2.9, 6.1, 9.3), 4.05 (m), 6.03 (s), 6.22 (dd, 3.2, 5.6); EIMS *m/z* 346/348/350/352 $[M]^+$ (<1, <1, <1, <1), 329/331/333/335 $[M-OH]^+$ (<1, 1, <1, <1), 303/305/307/309 $[M-C_3H_7]^+$ (<1, 2, 2, <1), 274/276/278/280 (9, 22, 15, 3), 195/197/199 (37, 47, 12), 175/177 $[C_8H_{12}O_2Cl]^+$ (100, 32); HREIMS 345.8939 (calcd for $C_9H_{13}O_2^{37}Cl^{79}Br_2$, 345.8971), 328.9037 (calcd for $C_9H_{12}O^{35}Cl^{79}Br_2$, 328.8943), 177.0447 (calcd for $C_8H_{12}O_2^{37}Cl$, 177.0496).

3.4. Acetylation of 1

A solution of compound **1** (2.0 mg) in dry C_5H_5N (0.4 mL) was treated with Ac_2O (0.5 mL), stirred at room temperature for 16 h, and then poured into 5% aqueous HCl and extracted with CH_2Cl_2 and brine, dried (Na_2SO_4) and concentrated affording **2** (1.2 mg).

Acknowledgements

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References and notes

1. Kazlauskas, R.; Murphy, P. T.; Quinn, R. J.; Wells, R. J. *Tetrahedron Lett.* **1977**, 37–40.
2. Pettus, J. A.; Wing, R. M.; Sims, J. J. *Tetrahedron Lett.* **1977**, 41–44.
3. De Nys, R.; Wright, A. D.; König, G. M.; Sticher, O. *Tetrahedron* **1993**, *49*, 11213–11220.
4. Fuqua, W. C.; Winans, S. C.; Greenberg, E. P. *J. Bacteriol.* **1994**, *176*, 269–275.
5. Manefield, M.; Rasmussen, T. B.; Henzter, M.; Andersen, J. B.; Steinberg, P.; Kjelleberg, S.; Givkov, M. *Microbiology* **2002**, *148*, 1119–1127.
6. Hentzer, M.; Riedel, K.; Rasmussen, T. B.; Heydorn, A.; Andersen, J. B.; Parsek, M. R.; Rice, S. A.; Eberl, L.; Molin, S.; Hoiby, N.; Kjelleberg, S.; Givskov, M. *Microbiology* **2002**, *148*, 87–102.
7. Rasmussen, T. B.; Manefield, M.; Andersen, J. B.; Eberl, L.; Anthoni, U.; Christophersen, C.; Steinberg, P.; Kjelleberg, S.; Givskov, M. *Microbiology* **2000**, *146*, 3237–3244.
8. Manefield, M.; de Nys, R.; Kumar, N.; Read, R.; Givkov, M.; Steinberg, P.; Kjelleberg, S. *Microbiology* **1999**, *145*, 283–291.

9. Manefield, M.; Welch, M.; Givkov, M.; Salmond, G. P. C.; Kjelleberg, S. *FEMS Microbiol. Lett.* **2001**, *205*, 131–138.
10. Cueto, M.; Darias, J.; San-Martín, A.; Rovirosa, J. *J. Nat. Prod.* **1997**, *60*, 279–281.
11. Ankisetty, S.; Nandiraju, S.; Win, H.; Park, Y. C.; Amsler, C. D.; McClintock, J. B.; Baker, J. A.; Diyabalanage, T. K.; Pasaribu, A.; Singh, M. P.; Maiese, W. M.; Walsh, R. D.; Zaworotko, M. J.; Baker, B. J. *J. Nat. Prod.* **2004**, *67*, 1295–1302.
12. Kazlauskas, R.; Lidgrd, R. O.; Wells, R. J. *Tetrahedron Lett.* **1978**, 3165–3168.
13. Nicod, F.; Tillequin, F.; Vaquette, J. *J. Nat. Prod.* **1987**, *50*, 259–260.
14. Díaz-Marrero, A. R.; Dorta, E.; Cueto, M.; Rovirosa, J.; San-Martín, A.; Darias, J. *Tetrahedron* **2004**, *60*, 5049–5052.
15. Díaz-Marrero, A. R.; Cueto, M.; Dorta, E.; Rovirosa, J.; San-Martín, A.; Darias, J. *Org. Lett.* **2002**, *4*, 2949–2952.
16. Díaz, A. R.; Rovirosa, J.; Darias, J.; San Martín, A.; Cueto, M. *J. Nat. Prod.* **2002**, *65*, 585–588.
17. Darias, J.; Rovirosa, J.; San Martín, A.; Díaz, A.; Dorta, E.; Cueto, M. *J. Nat. Prod.* **2001**, *11*, 1383–1387.
18. Díaz-Marrero, A. R.; Cueto, M.; Dorta, E.; Rovirosa, J.; San-Martín, A.; Darias, J. *Tetrahedron* **2002**, *58*, 8539–8542.
19. PCModel, version 7.0; Serena Software: Bloomington, IN.
20. Ji, G.; Beavis, R. C.; Novick, R. P. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 12055–12059.
21. Chen, X.; Schauder, S.; Potier, N.; Van Dorsselaer, A.; Pelczer, I.; Hughson, F. M.; Bassler, B. L. *Nature* **2002**, *415*, 545–549.
22. Hiroaki, S.; Smith, K. M. *Curr. Opin. Chem. Biol.* **2003**, *7*, 586–591.