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Amphiasterins: a new family of cytotoxic metabolites from the marine sponge *Plakortis quasiamphiaster*

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Abstract—A new family of marine metabolites, named amphiasterins (**1**–**17**), was isolated from the marine sponge *Plakortis quasiamphiaster*. They can be divided in five structurally homogeneous groups, whose components differ only in the length and/or in the unsaturation degree of the alkyl side chain. The structures of these compounds were elucidated by spectroscopic data. © 2000 Elsevier Science Ltd. All rights reserved.

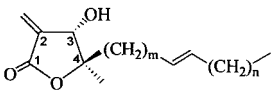
Marine sponges of the genus *Plakortis* are prominent members of both Caribbean and Indo-Pacific coral reefs.[1,2] They are known to be a prolific source of oxygenated polyketides, cyclic peroxides and related metabolites, formed from the combination of acetyl- propionyl- and/or butyrate units.[3]

During our search for biologically active metabolites from marine sponges from South Pacific waters, we have examined the sponge *Plakortis quasiamphiaster*, whose ethanolic extracts exhibited 91% of inhibition on Kb cancer cells at a concentration of 10 μg/mL. From the cytotoxic carbon tetrachloride extract (IC_{50}<6 μM on NSCLC-N6 cancer cells) we isolated several oxygenated long-chain derivatives (**1**–**17**), named amphiasterins, which constitute an unprecedent class of marine secondary metabolites. From a structural point of view, amphiasterins can be divided in five groups, whose components differ from each other only in the length and/or in the unsaturation degree of the alkyl side chain. The components of the first group, amphiasterins A1–A4 (**1**–**4**) contain a 2-exomethylene-3-hydroxy-4-methyl-γ-lactone moiety with a very long-chain alkyl side-chain at C-4 position. The amphiasterins B1–B5 (**5**–**9**) feature a hydroxymethyl group at the C-2 position which replaces the exomethylene funtionality, and a shorter side chain at the C-4 position. The other three groups are composed of a series of diastereoisomeric mixed-biogenesis metabolites, amphiasterins C1–C4 (**10**–**13**), D1–D3 (**14**–**16**) and E1 (**17**) which contain an *N*-alkylated pyroglutamic acid unit.

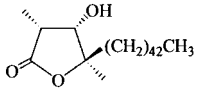
In this paper we report the isolation, structure elucidation, and relative stereochemistries of these metabolites.

1. Results and discussion

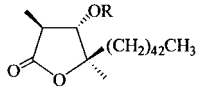
Fractionation of the bioactive carbon tetrachloride extract, obtained from a solvent partitioning Kupchan procedure of the crude methanolic extract, by MPLC (silica gel, eluent 0–10% MeOH/CH_2Cl_2) followed by reversed-phase HPLC



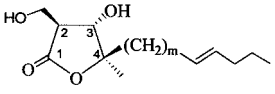
1 m=31, n=9
2 m=35, n=9
3 m=31, n=9 saturated
4 m=35, n=9 saturated



3a



3b R=H
3c-3d R=(*S*)-MTPA



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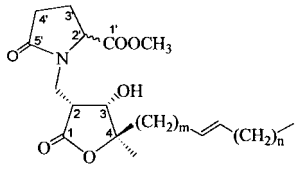
6 m=12
7 m=16
8 m=10 saturated
9 m=12 saturated



10 m=3, n=9
11 m=5, n=9
12 m=3, n=9 saturated
13 m=5, n=9 saturated

Keywords: marine metabolites; sponges.
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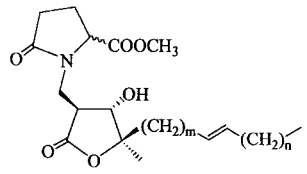
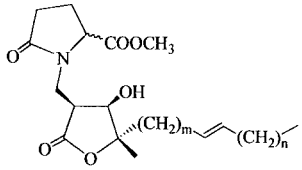


14 m=3, n=9
15 m=5, n=9
16 m=5, n=9 saturated



17 m=3, n=9

(C_{18} μ-Bondapack, 86% aqueous methanol) afforded the compounds **1**–**17**.

Amphiasterin A1 (**1**) has the molecular formula $C_{49}H_{92}O_3$ [HRFABMS (positive ion) *m/z* 729.7213] which requires four degrees of unsaturation. The ^{1}H NMR spectrum exhibited signals for a conjugated exomethylene functionality (δ_H 5.90, d, *J*=2.0 Hz; and 6.40, d, *J*=2.4 Hz), for a hydroxymethine group (δ_H 4.59, ddd, *J*=7.1, 2.4, 2.0 Hz; δ_H 2.05, d, *J*=7.1 Hz, exchangeable), for a methyl singlet (δ_H 1.33, s) and for a long unsaturated hydrocarbon chain (δ_H 5.37, m; 1.26, bs and 0.89, t, *J*=6.9 Hz). The ^{13}C NMR spectra indicated the presence of an ester carbonyl function (δ_C 166.7), of two oxygenated carbons (δ_C 73.0, d, and δ_C 87.2, s) and confirmed the presence of an exomethylene function (δ_C 138.2, s and 123.5, t) and of a disubstituted double bond (δ_C 130.2, d's). In the HMBC spectrum the hydroxymethine proton at δ_H 4.59 showed cross peak correlations with both exomethylene carbons, with the oxygenated quaternary carbon at δ_C 87.2 and with the ester carbonyl group at δ_C 166.7. The localization of the methyl singlet group and of the straight unsaturated side chain on the oxygenated quaternary carbon follows from the HMBC correlations between the above carbon and methyl protons at δ 1.33 and the first methylene proton group (δ_H 1.67) of the alkyl side chain. The closure of the γ-lactone moiety as in **1** was inferred by HRFABMS data, that indicated the presence of one ring system in the molecule, IR absorption at ν 1760 cm^{-1} and UV data (λ_{max}=230 nm). Having subtracted from the molecular formula $C_{49}H_{92}O_3$ the contribution of the γ-lactone subunit, the length of the monounsaturated side chain in amphiasterin A1 (**1**) was defined as $C_{43}H_{85}$. The position of the internal double bond in this side chain was determined by permanganate/periodate oxidative cleavage of the double bond, followed by methylation of the resulting carboxylic acid with diazomethane and GC–MS analysis of the resulting methyl ester. The *E* geometry of the aforementioned double bond was inferred form the ^{13}C NMR resonances of the vinylic methylene at δ_C 29.4.[4] The relative stereochemistry of amphiasterin A1 (**1**) was secured by a 1,3 dipolar effect observed in the 1D NOE spectrum between H-3 and H_2-5.

Amphiasterin A2 (**2**) contains four more methylene groups in the side chain than **1**, as evidenced by HRFABMS [*m/z* 785.7127 $(M+H)^+$, $C_{53}H_{101}O_3$] and NMR data. The localization of the internal double bond in the side chain was determined by using the same procedure described for amphiasterin A1 (**1**).

HRFABMS analysis allowed us to establish that amphiasterins A3 (**3**) [*m/z* 731.7331 $(M+H)^+$, $C_{49}H_{95}O_3$], and A4 (**4**) [*m/z* 787.7820 $(M+H)^+$, $C_{53}H_{103}O_3$] are the corresponding saturated derivatives of amphiasterins **1** and **2**, respectively.

All amphiasterins A showed a high chemical lability and reactivity toward nucleophiles and undergo heavy decomposition during manipulation and storage even at low temperature. Attempts to obtain the MTPA ester derivatives at the C3 carbinol centre of the major amphiasterin A3 (**3**), in order to determine its absolute stereochemistry resulted in the decomposition of the compound. Because the observed instability could be ascribed to the exocyclic conjugated double bond we reduced amphiasterin A3 (**3**) under catalytic hydrogenation conditions (H_2/Pd(OH)$_2$, 3 atm, rt, 12 h) to obtain two diastereomeric reduced derivatives **3a** and **3b**. The structure of these reduced derivatives was secured by ^{1}H NMR and NOE data. Reaction of the major **3b** with (*R*)-α-methoxy-α-(trifluoromethyl)-phenylacetic acid chloride (MTPACl) afforded a mixture of diastereoisomers (**3c** and **3d**) of the *S*-MTPA ester, indicating that **3** was a racemic mixture.

The molecular formula $C_{21}H_{38}O_4$ of amphiasterin B1 (**5**), isolated as a colorless oil, was detemined by HRFABMS [*m/z* 355.2875, $(M+H)^+$]. The ^{13}C NMR spectrum of amphiasterin B1 (**5**) showed signals ascribable to an ester carbonyl group (δ_C 174.6), to three oxygenated carbons (δ_C 86.8, s; 74.8, d; 59.6, t), to an unsaturated alkyl long chain (clusters of methylene signals around δ 32.5, two sp^2 carbons at δ_C 130.2 and a methyl group at δ_C 13.6), and to a methyl group a δ_C 18.6 (Table 1). In the COSY spectrum the protons assigned to an hydroxymethyl group (ABX system δ_H 4.03, dd, *J*=11.2 and 4.8 Hz; 3.93, dd, *J*=11.2 and 4.8 Hz) were found to correlate with a methine at δ_H 2.80 (dt, *J*=9.6 and 4.8 Hz), which, in turn, was coupled to an hydroxy methine signal at δ_H 4.28 (d, *J*=9.6 Hz). The HMBC correlations shown in the Table 1 allowed us to define the presence of a 2-hydroxymethyl-3-hydroxy-4-methyl-γ-lactone moiety in amphiasterin B1 (**5**). Even if at the first sight amphiasterin B1 might seem to be an artifact arising from conjugated addition of water to the α–β unsaturated double bond present in amphiasterins A1–A4 (**1**–**4**), it should be noted that amphiasterins B1–B5 feature shorter side chains. On the basis of MS data a monounsaturated C15 side chain was calculated for amphiasterin B1 (**5**). The position of the internal double bond was deduced from the $^3J_{H-C}$ HMBC correlation observed between the terminal methyl group at δ 0.86 and the C17 assigned to the vinylic position. The *E*-geometry of the double bond derived from the upfield chemical shift of C-14. The intense NOE effects H2/Me-4 and H3/CH_2-5 were indicative of the relative stereochemistry of amphiasterin B1 as showed in **5**.

Table 1. NMR data (500 MHz, $CDCl_3$) of compounds **1** and **5**

Amphiasterin A1 (**1**)				Amphiasterin B1 (**5**)			
No.	δ_H (*J* Hz)	δ_C	HMBC	No.	δ_H (*J* Hz)	δ_C	HMBC
1	–	166.7		1	–	174.6	
2	–	138.2		2	2.80 dt (9.6, 4.8)	49.0	C1, C3, CH_2-2
3	4.59 ddd (7.1, 2.0, 2.4)	73.0	C1, C2, C4, C5, C48, Me-4	3	4.28 d (9.6)	74.8	C2, C4, C5, CH_2-2, Me-4
4	–	87.2		4	–	86.8	
5	1.67 m	38.7	C4, C3	5	1.67–1.71 m	40.0	C4, C3, Me-4
35	1.96 m	29.4		14	1.96 m	32.5	
36	5.37 m	130.2		15	5.37 m	130.2	
37	5.37 m	130.2		16	5.37 m	130.2	
38	1.96 m	29.4		17	1.96 m	32.5	
39	1.30 m	23.0		18	1.26 m	22.5	
47	0.89 t (6.9)	13.9		19	0.86 t (6.9)	13.6	C17, C18
48	6.40 d (2.4) 5.90 d (2.0)	123.5	C1, C2, C3	$HOCH_2$-2	4.03 dd (11.2, 4.8) 3.93 dd (11.2, 4.8)	59.6	C1, C2, C3
Me-4	1.33 s	18.6	C3, C4, C5	Me-4	1.33 s	18.6	C3, C4, C5
OH-3	2.05 d (7.1)	–	C3, C2, C4				

NMR data clearly indicated that amphiasterins B2 (**6**) and B3 (**7**) are homologoues of amphiasterin B1 (**5**). In particular they were determined as C2 and C6 higher homologs of **5** (see Experimental). As in amphiasterin B1 (**5**), the localization of the isolated double bond in the side chain follows from HMBC analysis.

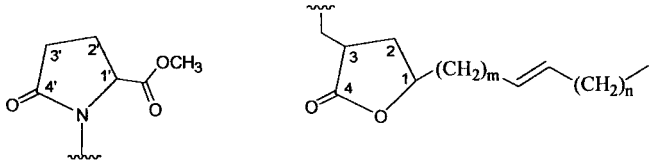
The molecular formulae of amphiasterin B4 (**8**), $C_{21}H_{40}O_4$ *m/z* 357.3187 $(M+H)^+$, and B5 (**9**), $C_{23}H_{44}O_4$ *m/z* 385.3382 $(M+H)^+$, in combination with NMR spectral data indicated that they represent the saturated derivatives of amphiasterin B1 (**5**) and B2 (**6**), respectively.

Amphiasterin C1(**10**) analyzed for $C_{27}H_{45}NO_6$ by HRFABMS [*m/z* 480.3276, $(M+H)^+$]. ^{13}C NMR spectra revealed the presence of three acyl carbonyls at δ_C 178.0, 175.3 and 172.7 and of one disubstituted double bond (130.5, d; 130.0, d), thus implying the presence of two cycles in the molecule. Even if some structural analogies with previous amphiasterins could be recognized, NMR data (Table 2) indicated a more complex structure for amphiasterin C1 (**10**). Combined analysis of NMR spectral data, including COSY, HMQC and HMBC, allowed us to deduce structural units A and B. In particular the NMR data for subunit A were similar to those of amphiasterins B except for the chemical shifts of the methylene attached to C2 (δ_c 40.0, δ_H 4.00 and 3.30). The remaining atoms were accommodated by the substructure B derived from COSY relationships and HMBC correlations. In the COSY spectrum the H2′ proton resonating at δ_H 4.28 was found to correlate to the diasterotopic methylene protons at δ_H 2.10 and 2.46 which, in turn, were coupled with a second diasterotopic methylene group (δ_H 2.53 and 2.44). The ^{13}C chemical shift of the C2′ carbon (δ_C 63.1), as derived from the HMQC spectrum, strongly suggested its α aminoacidic nature, also taking into account mass spectral data which indicated the presence of one nitrogen atom in the molecule. In the HMBC spectrum the H2′ and $H_2$3′ were found to correlate with the acyl carbonyl at δ_C 172.7, whereas H2′, $H_2$3′ and $H_2$4′ showed long-range coupling

Table 2. NMR data (500 MHz, $CDCl_3$) of compounds **10** and **14**

Amphiasterin C1 (**10**)				Amphiasterin D1 (**14**)			
No.	δ_H (*J* Hz)	δ_C	HMBC	δ_H (*J* Hz)	δ_C	HMBC
1	–	175.3		–	173.8	
2	3.26 ddd (11.0, 4.4, 2.9)	46.7	C1, C3, C4, CH_2-2	2.72 dt (10.4, 4.5)	47.7	C1, C3, C4, CH_2-2
3	4.11 dd (4.4, 2.2)	72.0	C1, C2, C4, C5, CH_2-2, Me-4	4.18 d (10.4)	76.1	C1, C2, C4, C5, CH_2-2, Me-4
4	–	89.3		–	86.4	
5	1.59 m 1.51 m	38.5	C4, C3, Me-4	1.78 m 1.74 m	39.9	C4, C3, Me-4
7	1.95 m	32.5		1.95 m	32.5	
8	5.37 m	130.5		5.37 m	130.2	
9	5.37 m	130.0		5.37 m	130.2	
10	1.95 m	32.5		1.95 m	32.5	
19	0.89 t (6.8)	13.9		0.89 t (6.8)	13.9	
Me-4	1.45 s	18.9	C3, C4, C5	1.30 s	18.2	C3, C4, C5
CH_2-2	4.00 dd (14.8, 11.0) 3.30 dd (14.8, 2.9)	40.0	C1, C2, C3, C1′, C5′	3.76 dd (14.5, 4.5) 3.55 dd (14.5, 4.5)	41.2	C1, C2, C3, C2′, C5′
OH-3	5.47 d (2.2)			Not observed		
1′	–	172.7		–	172.2	
2′	4.28 dd (9.0, 4.2)	63.1	C5′, *C1′*, CH_2-2	4.30 dd (7.2, 2.2)	62.0	C5′, *C1′*, CH_2-2
3′	2.46 m 2.10 m	24.0	C5′, *C1′*	2.40 m 2.10 m	23.9	C5′, *C1′*
4′	2.53 m 2.44 m	30.0	C5′	2.59 m 2.37 m	29.1	C5′
5′	–	178.0		–	178.1	
OCH_3	3.68 s	52.8	*C1′*	3.68 s	52.7	*C1′*

with the acyl carbonyl at δ_C 178.0. The carbonyl at δ_C 172.7 was assigned as a methyl ester on the basis of its HMBC correlation with the methoxy protons at δ_H 3.68. All these data were consistent with the presence of a *N*-substituted pyroglutamic methyl ester unit, as also confirmed by comparison with spectral data of *N*-methyl pyroglutamic acid, available in our laboratories.[5] The linkage of subunit B to subunit A through the methylene group at C2 was inferred by HMBC correlation between both diastereotopic methylene protons with Cα and Cδ of the pyroglutamic methyl ester unit.



Subunit A Subunit B

As in amphiasterin B1 (**5**), a monounsaturated C15 alkyl chain was deduced for **10** from MS data. Oxidative cleavage of **10** followed by treatment with CH_2N_2, resulted in the formation of the methyl ester of decanoic acid, thus defining the location of the isolated double bond. The *E* geometry of this latter was assigned on the basis of the diagnostic ^{13}C NMR resonance of the adjacent vinylic methylenes at δ_C 32.5. Strong NOEs observed between $H_2$5 and both H2 and H3, as well as between the methyl singlet at δ_H 1.45 (Me-4) and the two methylene protons at C2 revealed the relative stereochemistry around the γ-lactone ring with a *syn* relationship between H2 and H3 and the alkyl side chain.

Amphiasterins C2–C4 (**11**–**13**) were determined as homologues and/or saturated derivatives of the major amphiasterin C1 (see Experimental).

Amphiasterin D1 (**14**) is isomeric with amphiasterin C1 (*m/z* 480.3496, HRFABMS, positive ion). NMR analysis including 2D-COSY, HMQC and HMBC, indicated that both compounds share the same gross structure, even if significant differences in the chemical shifts and ^1H–^1H coupling constant pattern were observed for all nuclei belonging to the γ-lactone subunit (Table 2). Thus, amphiasterin C1 and D1 were suspected to be stereoisomers. The relative stereochemistry of the γ-lactone ring in **14** was established by 1D NOE experiment, which showed dipolar coupling between H_2-5 and H-3 and between methyl group on C4 and H-2. Therefore, **14** and **10** are epimeric at the C-2 position.

Amphiasterins D2 (**15**) and D3 (**16**) represent the corresponding C-2 epimers of amphiasterins C2 and C4, respectively.

Amphiasterin E1 (**17**) is isomeric with amphiasterins C1 (**10**) and D1 (**14**). Also in this case the same gross structure as in **10** was inferred by 2D NMR analysis. However, significant shifts for the resonances of H2 and H3 were observed (Table 3). NOE data indicated the same relative stereochemistry as amphiasterin C1 around the γ-lactone subunit. The observed differences could be explained supposing an enantiomeric configuration at the Cα chiral center of the pyroglutamic acid unit or an enantiomeric

Table 3. Selected NMR data of amphiasterin E1 (**17**)

No.	δ_H (*J* Hz)	δ_C
1	–	174.6
2	2.90 ddd (11.0, 4.4, 2.7)	46.2
3	3.98 dd (4.4, 2.0)	71.8
4	–	89.2
5	1.58 m	38.5
	1.51 m	
Me-4	1.45 s	18.7
CH_2-2	4.00 dd (14.7, 11.0)	39.3
	3.30 dd (14.7, 2.7)	
OH	5.22 d (2.0)	
1′	–	172.2
2′	4.31 dd (7.2, 2.2)	60.7
3′	2.42 m	23.4
	2.18 m	
4′	2.57 m	30.0
	2.44 m	
5′	–	178.0
OMe	3.68 s	52.9

configuration of the stereogenic centers of the γ-lactone subunit. Owing to the presence in the same sponge of an enantiomeric mixture of amphiasterins A we suppose that it is probable that amphiasterins C and E were enantiomeric with respect to the γ-lactone subunit.

Attempts to prepare the MTPA derivatives of amphiasterin C1 and E1 in order to confirm the above hypothesis failed, probably due to the steric hindrance around the C3 carbinol centre. It is noteworthy that there is a unifying trend in the chemical shifts of all nuclei belonging to the γ-lactone ring dependent on the relative stereochemistry of H2/H3. Particularly diagnostic are the ^{13}C resonances of C3 and C4, as well as the ^1H chemical shift of methyl group at C4 (ca. 1.30 ppm in compounds with the *anti* H2/H3 relationship, ca. 1.45 in compounds with the *syn* H2/H3 stereochemistry). The same behavior was observed in amphiasterin B1, in fact the chemical shift values for the γ-lactone ring were fully consistent with the relative stereochemistry assigned by means of dipolar effects.

Even though the α-methylene-γ-butyrolactone ring is an integral building block of many natural products with interesting biological properties,[6] to the best of our knowledge, amphiasterins represent a new class of natural products. Although it was not possible to subject all compounds to bioassay analysis, representative compounds **3**, **6** and **11** were tested and showed moderate cytotoxic activity against human carcinoma NSCLC-N6 cells in vitro with IC_{50} values of 3.5 μM for amphiasterin A3 (**3**), 26 μM for amphiasterin B2 (**6**), and 8.6 μM for amphiasterin C2 (**11**).

2. Experimental

2.1. General methods

NMR spectra were measured at 500 MHz (^1H) and 125 MHz (^{13}C). ^1H NMR and ^{13}C NMR are referenced to $CDCl_3$ solvent signals at 7.26 and 77.0 ppm, respectively. Multiplicities of ^{13}C spectra were assigned by DEPT

experiments. Standard pulse sequences were employed for DEPT and magnitude COSY. HMQC and HMBC were optimised for $^{1}J_{C-H}$=135 Hz and $^{2,3}J_{C-H}$=10 Hz, respectively. FAB-MS spectra were performed in a glycerol matrix on a VG Prospec-Autospec (Fisons) mass spectrometer. Optical rotations were measured at 589 nm on a Perkin–Elmer 141 polarimeter. UV spectra were recorded on a Beckman DU70 spectrophotometer. IR spectroscopy was performed on an IFS 48 Bruker instrument. HPLC was achieved on a Waters model 6000 A pump equipped with a U6K injector and a differential refractometer, model 401.

2.2. Isolation

The sponge was collected at Emae and Epi (Cook reef) in June 1996 and identified as *Plakortis quasiamphiaster* (family Plakinidae, order Homosclerophorida) by John Hooper (Queensland Museum, Brisbane, Australia). The voucher specimen R1621 was deposited at the ORSTOM Centre of Nouméa. Lyophilised animals (488 g) were extracted with MeOH to obtain 163 g of a brown amorphous solid successively extracted using a modified Kupchan[7] partition. The CCl_4 (16 g) extract was fractionated by silica gel MPLC (Merck Kiesegel 60, 230–400 mesh, 320 g) eluting with MeOH/CH_2Cl_2 0–0.2% followed by reversed-phase C_{18} μ-Bondapak HPLC with 86% aqueous MeOH. Amphiasterins A1–A4 (**1**–**4**) were obtained from the MPLC fractions (3.8 g) eluted with CH_2Cl_2. Reversed-phase HPLC chromatography of a ca. 100 mg aliquot of this fraction [C_{18} μ-Bondapak, 7.8 mm i.d.×30 cm, flow rate 5 mL/min, 86% aqueous MeOH], afforded amphiasterin A1 (**1**, 18.4 mg, t_R 10.4 min), amphiasterin A2 (**2**, 15.6 mg, t_R 23 min), amphiasterin A3 (**3**, 40.8 mg, t_R 14.2 min), amphiasterin A4 (**4**, 5.6 mg, t_R 26.4 min).

Amphiasterins E1 (**17**), C1–C4 (**10**–**13**), D1–D3 (**14**–**16**), B1–B5 (**5**–**9**) were eluted in that order from MPLC column (eluent CH_2Cl_2/MeOH 995:5). Enriched fractions were further purified by HPLC in the same condition used for amphiasterins A1–A4 (except for amphiasterins B1–B5 eluted on an analytic C_{18} μ-Bondapak column, flow rate 2 mL/min, 88% aqueous MeOH) to afford amphiasterin B1, (**5**, 3.0 mg, t_R 3.6 min), amphiasterin B2, (**6**, 9.6 mg, t_R 5.6 min), amphiasterin B3, (**7**, 2.5 mg, t_R 10.8 min), amphiasterin B4, (**8**, 2.4 mg, t_R 4.8 min), amphiasterin B5, (**9**, 2.6 mg, t_R 8.0 min), amphiasterin C1 (**10**, 2.3 mg, t_R 8.4 min), amphiasterin C2 (**11**, 16.6 mg, t_R 13.2 min), amphiasterin C3 (**12**, 8.6 mg, t_R 10.8 min), amphiasterin C4 (**13**, 4.8 mg, t_R 18.4 min), amphiasterin D1 (**14**, 1.7 mg, t_R 6.6 min), amphiasterin D2 (**15**, 6.7 mg, t_R 10.8 min), amphiasterin D3 (**16**, 2.1 mg, t_R 15.6 min), amphiasterin E1 (**17**, 6.1 mg, t_R 19.6 min).

2.2.1. Amphiasterin A1 (1). $C_{49}H_{92}O_3$, colorless oil; UV (MeOH) λ_{max} (log ϵ) 230 (2.99) nm; IR (KBr) ν_{max} 3420, 1760, 1682 cm^{-1}; ^{1}H and ^{13}C NMR data in Table 1; HRMS (FAB positive): *m/z* $(M+H)^+$, found 729.7213, $C_{49}H_{93}O_3$ requires 729.7125.

2.2.2. Amphiasterin A2 (2). $C_{53}H_{100}O_3$, colorless oil; IR (KBr) ν_{max} 3380, 1760, 1682 cm^{-1}; ^{1}H and ^{13}C NMR data superimposable with those of amphiasterin A1 (**1**); HRMS (FAB positive): *m/z* $(M+H)^+$, found 785.7627, $C_{53}H_{101}O_3$ requires 785.7751.

2.2.3. Amphiasterin A3 (3). $C_{49}H_{94}O_3$, colorless oil; IR (KBr) ν_{max} 3420, 1760 cm^{-1}; ^{1}H and ^{13}C NMR data superimposable with those of amphiasterin A1 (**1**), except for the absence of signals ascribable to the internal double bond; HRMS (FAB positive): *m/z* $(M+H)^+$, found 731.7331, $C_{49}H_{95}O_3$ requires 731.7281.

2.2.4. Amphiasterin A4 (4). $C_{53}H_{102}O_3$, colorless oil; IR (KBr) ν_{max} 3420, 1760 cm^{-1}; ^{1}H and ^{13}C NMR data superimposable with those of amphiasterin A1 (**1**) except for the absence of signals ascribable to the internal double bond; HRMS (FAB positive): *m/z* $(M+H)^+$, found 787.7820, $C_{53}H_{103}O_3$ requires 787.7907.

2.2.5. Amphiasterin B1 (5). $C_{21}H_{38}O_4$, colorless oil; $[\alpha]_D^{20}=-3.3$ (*c* 0.15, $CHCl_3$); IR (KBr) ν_{max} 3400, 1750, 1680 cm^{-1}; ^{1}H and ^{13}C NMR data in Table 1; HRMS (FAB positive): *m/z* $(M+H)^+$, found 355.2875, $C_{21}H_{39}O_4$ requires 355.2848.

2.2.6. Amphiasterin B2 (6). $C_{23}H_{42}O_4$, colorless oil; $[\alpha]_D^{20}=-0.63$ (*c* 0.96, $CHCl_3$); IR (KBr) ν_{max} 3400, 1750, 1680 cm^{-1}; ^{1}H and ^{13}C NMR data superimposable with those of amphiasterin B1 (**5**); HRMS (FAB positive): *m/z* $(M+H)^+$, found 383.3112, $C_{23}H_{43}O_4$ requires 383.3161.

2.2.7. Amphiasterin B3 (7). $C_{27}H_{50}O_4$, colorless oil; $[\alpha]_D^{20}=+3.1$ (*c* 0.13, $CHCl_3$); IR (KBr) ν_{max} 3400, 1750, 1680 cm^{-1}; ^{1}H and ^{13}C NMR data superimposable with those of amphiasterin B1 (**5**); HRMS (FAB positive): *m/z* $(M+H)^+$, found 439.3820, $C_{27}H_{51}O_4$ requires 439.3787.

2.2.8. Amphiasterin B4 (8). $C_{21}H_{40}O_4$, colorless oil; $[\alpha]_D^{20}=-3.3$ (*c* 0.3, $CHCl_3$); IR (KBr) ν_{max} 3400, 1750 cm^{-1}; ^{1}H and ^{13}C NMR data superimposable with those of amphiasterin B1 (**5**) except for the absence of signals ascribable to the internal double bond; HRMS (FAB positive): *m/z* $(M+H)^+$, found 357.3187, $C_{21}H_{41}O_4$ requires 357.3005.

2.2.9. Amphiasterin B5 (9). $C_{23}H_{44}O_4$, colorless oil; $[\alpha]_D^{20}=-30.0$ (*c* 0.18, $CHCl_3$); IR (KBr) ν_{max} 3400, 1750 cm^{-1};^{1}H and ^{13}C NMR data superimposable with those of amphiasterin B1 (**5**) except for the absence of signals ascribable to the internal double bond; HRMS (FAB positive): *m/z* $(M+H)^+$, found 385.3382, $C_{23}H_{45}O_4$ requires 385.3318 vv.

2.2.10. Amphiasterin C1 (10). $C_{27}H_{45}NO_6$, colorless oil; $[\alpha]_D^{20}=-19.3$ (*c* 0.14, $CHCl_3$); IR (KBr) ν_{max} 3310, 1775, 1700, 1680 cm^{-1}; ^{1}H and ^{13}C NMR data in Table 2; HRMS (FAB positive): *m/z* $(M+H)^+$, found 480.3276, $C_{27}H_{46}NO_6$ requires 480.3325.

2.2.11. Amphiasterin C2 (11). $C_{29}H_{49}NO_6$, colorless oil; $[\alpha]_D^{20}=-12.3$ (*c* 0.65, $CHCl_3$); IR (KBr) ν_{max} 3310, 1775, 1700, 1680 cm^{-1}; ^{1}H and ^{13}C NMR data superimposable with those of amphiasterin C1 (**10**); HRMS (FAB positive): *m/z* $(M+H)^+$, found 508.3498, $C_{29}H_{50}NO_6$ requires 508.3638.

2.2.12. Amphiasterin C3 (12). $C_{27}H_{47}NO_6$, colorless oil; $[\alpha]_D^{20}=-8.0$ (*c* 0.4, $CHCl_3$), IR (KBr) ν_{max} 3310, 1775, 1700 cm^{-1}; ^1H and ^{13}C NMR data superimposable with those of amphiasterin C1 (**10**) except for the absence of signals ascribable to the internal double bond; HRMS (FAB positive): *m/z* $(M+H)^+$, found 482.3537, $C_{27}H_{48}NO_6$ requires 482.3482.

2.2.13. Amphiasterin C4 (13). $C_{29}H_{51}NO_6$, colorless oil; $[\alpha]_D^{20}=-11.5$ (*c* 0.13, $CHCl_3$); IR (KBr) ν_{max} 3310, 1775, 1700 cm^{-1}; ^1H and ^{13}C NMR data superimposable with those of amphiasterin C1 (**10**) except for the absence of signals ascribable to the internal double bond; HRMS (FAB positive): *m/z* $(M+H)^+$, found 510.3674, $C_{29}H_{52}NO_6$ requires 510.3795.

2.2.14. Amphiasterin D1 (14). $C_{27}H_{45}NO_6$, colorless oil; $[\alpha]_D^{20}=-16.0$ (*c* 0.06, $CHCl_3$); IR (KBr) ν_{max} 3310, 1775, 1700, 1680 cm^{-1}; ^1H and ^{13}C NMR data in Table 2; HRMS (FAB positive): *m/z* $(M+H)^+$, found 480.3496, $C_{27}H_{46}NO_6$ requires 480.3325.

2.2.15. Amphiasterin D2 (15). $C_{29}H_{49}NO_6$, colorless oil; $[\alpha]_D^{20}=-2.9$ (*c* 0.24, $CHCl_3$); IR (KBr) ν_{max} 3310, 1775, 1700, 1680 cm^{-1}; ^1H and ^{13}C NMR data superimposable with those of amphiasterin D1 (**14**); HRMS (FAB positive): *m/z* $(M+H)^+$, found 508.3595, $C_{29}H_{50}NO_6$ requires 508.3638.

2.2.16. Amphiasterin D3 (16). $C_{27}H_{47}NO_6$, colorless oil; $[\alpha]_D^{20}=-1.5$ (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} 3310, 1775, 1700 cm^{-1};^1H and ^{13}C NMR data superimposable with those of amphiasterin D2 (**15**) except for the absence of signals ascribable to the internal double bond; HRMS (FAB positive): *m/z* $(M+H)^+$, found 508.3590, $C_{27}H_{48}NO_6$ requires 508.3638.

2.2.17. Amphiasterin E1 (17). $C_{27}H_{45}NO_6$, colorless oil; $[\alpha]_D^{20}=-3.8$ (*c* 0.11, $CHCl_3$); IR (KBr) ν_{max} 3310, 1775, 1700, 1680 cm^{-1}; ^1H and ^{13}C NMR data in Table 3; HRMS (FAB positive): *m/z* $(M+H)^+$, found 480.3408, $C_{27}H_{46}NO_6$ requires 480.3325.

2.3. Determination of the position of the internal double bond in amphiasterins

To a solution of an aliquot (0.5–3 mg) of amphiasterins A1–A2, C1–C3, D1–D2, E1 in acetone were added 0.5 mL of a 0.04 M solution of K_2CO_3 and 3 mL of an aqueous solution 0.025 M in $KMnO_4$ and 0.09 M in $NaIO_4$. The reaction was allowed to proceed at 37°C for 18 h. After acidification with 5N H_2SO_4, the solution was decolorized with a 1 M solution of oxalic acid and extracted with Et_2O (3×7 mL). The combined organic extracts were dried over Na_2SO_4, filtered and concentrated. The obtained carboxylic acid was methylated with CH_2N_2 and analyzed by GC–MS: EIMS: *m/z* 200 (M^+, 2), 169 (M−31, 5), 143 (M−57, 12), 74 (CH_3COOCH_3, 100).

2.4. Catalytic hydrogenation of amphiasterin A3 (3)

A solution of 20 mg of amphiasterin A3 in 1:1 EtOH/EtOAc was hydrogenated under catalytic conditions in a Parr apparatus ($H_2/Pd(OH)_2$, 3 atm, rt, 12 h). The mixture was filtered through Celite washed with EtOAc, concentrated, and separated by HPLC [Macherey–Nagel Nucleosil 100–5, 2% isopropanol/*n*-hexane] to give two diastereomeric hydrogenated derivatives **3a** and **3b**.

2.4.1. Compound 3a. $C_{49}H_{96}O_3$, colorless oil (2.6 mg): ^1H NMR data ($CDCl_3$) δ: 4.09 (1H, d, *J*=3.7 Hz, H-3), 2.93 (1H, dq, *J*=7.3 and 3.7 Hz, H-2), 1.60 (1H, m, H-5), 1.50 (1H, m, H-5), 1.41 (3H, s, Me-4), 1.27 (3H, d, *J*=7.3 Hz, Me-2), 1.26 (82 H, m, CH_2), 0.87 (3H, t, *J*=6.5 Hz, H-47); HRMS (FAB positive): *m/z* $(M+H)^+$, found 733.7321, $C_{49}H_{97}O_3$ requires 733.7438.

2.4.2. Compound 3b. $C_{49}H_{96}O_3$, colorless oil (4.6 mg): ^1H NMR data ($CDCl_3$) δ: 3.86 (1H, d, *J*=9.6 Hz, H-3), 2.65 (1H, dq, *J*=9.6 and 7.2 Hz, H-2), 1.67 (1H, m, H-5), 1.40 (1H, m, H-5), 1.33 (3H, s, Me-4), 1.31 (3H, d, *J*=7.2 Hz, Me-2), 1.26 (82 H, m, CH_2), 0.87 (3H, t, *J*=6.5 Hz, H-47); HRMS (FAB positive): *m/z* $(M+H)^+$, found 733.7340, $C_{49}H_{97}O_3$ requires 733.7438.

2.4.3. (*S*)-MTPA ester of 3b. Hydrogenated derivative **3b** (2.0 mg) was dissolved in fresh distilled CH_2Cl_2 and treated with triethylamine (10 μL), (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) (5 μL) and a catalytic amount of 4-(dimethylamino)pyridine. The mixture was left to stand at room temperature for 12 h, with the resulting mixture purified by silica gel column (eluent CH_2Cl_2) to give a diastereomeric mixture of the (*S*)-MTPA esters (2.0 mg) **3c** and **3d**.

2.4.4. Compound 3c. $C_{59}H_{103}F_3O_5$ colorless oil: ^1H NMR ($CDCl_3$) δ: 5.23 (1H, d, *J*=8.8 Hz, H-3), 2.84 (1H, dq, *J*=8.8 and 7.5 Hz, H-2), 1.36 (3H, d, *J*=7.5 Hz, Me-2), 1.13 (3H, s, Me-4). **3d**: colorless oil, ^1H NMR ($CDCl_3$) δ: 5.20 (1H, d, *J*=8.8 Hz, H-3), 2.76 (1H, dq, *J*=8.8 and 7.5 Hz, H-2), 1.32 (3H, d, *J*=7.5 Hz, Me-2), 1.24 (3H, s, Me-4); HRMS (FAB positive): *m/z* $(M+H)^+$, found 949.7752, $C_{59}H_{104}F_3O_5$ requires 949.7836.

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