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Crucigasterins A–E, antimicrobial amino alcohols from the Mediterranean colonial ascidian *Pseudodistoma crucigaster*

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ABSTRACT

Five new unsaturated 2-amino-3-alcohols, crucigasterins A–E (**2**–**6**), were isolated together with known related compound **7** from the Mediterranean ascidian *Pseudodistoma crucigaster* and characterised as diacetyl derivatives (**2a**–**6a**) by spectroscopic methods. The *threo*-relative configuration of the amino alcohol portion was inferred by NOE analysis of the oxazolidinone derivative of crucigasterin A (**2**) as well as by ^{13}C NMR comparison with synthetic *threo* and *erythro* model compounds. The co-occurring metabolites were assumed to have the same relative configuration as **2** by comparison of the diagnostic carbon value of C-1. The absolute stereochemistry of compound **7** that had not been previously reported was determined by applying the modified Mosher's method on the corresponding *N*-acetyl derivative. The same absolute configuration was suggested for the other co-occurring crucigasterins by biogenetic considerations. Antibacterial and antifungal activities of selected crucigasterins were also evaluated.

1. Introduction

Ascidians (tunicates) represent a prolific source of structurally novel compounds, especially rich in nitrogen. Chemical studies on ascidians have led to a smaller number of molecules with respect to the multitude of structures from sponges.[1] In spite of this, many compounds isolated from tunicates have been demonstrated to possess strong pharmacological properties and some of these (i.e., Yondelis®) are currently used in medical practice for the treatment of soft-tissue sarcoma, whereas others (didemnin B, aplydine) are in Phase II anticancer preclinical trials.[2]

Among tunicates, the genus *Pseudodistoma*, including several species that are widely distributed in tropical as well as in temperate waters, has been found to be rich in cytotoxic alkaloids,[3] linear[4–6] and cyclic[7–11] amino alcohols, tryptophan-related compounds,[12,13] alkyl amines[14,15] and nucleosides.[16,17] In the course of our continuing study on bioactive compounds from marine organisms, we have analysed specimens of Mediterranean *Pseudodistoma crucigaster* Gaill, 1972, collected off the coasts of Sardinia. A previous chemical investigation on the same Mediterranean species[4] resulted in the finding of linear *erythro* 2-amino-alken-3-ols, crucigasterins (i.e., crucigasterin 277, **1**). Related linear 2-amino-3-ol alkyl compounds have been encountered in *Pseudodistoma obscurum*,[6] in *Pseudodistoma* sp.,[5] and also in ascidians of genus *Clavelina*,[18,19a,b] in sponges[20a,b,21] and in the mollusc *Mactromeris polynyma*.[22] These molecules are closely related to sphingolipids and show interesting bioactivities as antibiotics,[4,5,14,21] antimycotics[4,18,20a,b,21] and as antitumour agents.[4,15,19,23]

In this paper, we describe the structural elucidation of five novel unsaturated amino alcohols, crucigasterins A–E (**2**–**6**), isolated along with related known compound **7**, previously reported from South African *Pseudodistoma* sp.[5] These molecules, which exhibit different carbon chain length and oxidation degree and are all characterised by the *threo*-configuration of the amino alcohol moiety, in contrast with the *erythro*-stereochemistry of previously reported crucigasterins,[4] have been characterised as diacetyl derivatives **2a**–**6a**. In addition, a complete NMR assignment of the diacetyl derivative of known compound **7a**,[5] which has not been previously fully characterised, is reported here.

2. Results and discussion

The diethyl ether soluble portion of the acetone extract of *P. crucigaster* was analysed by TLC chromatography showing the presence of a series of polar compounds at R_f 0.2 ($CHCl_3/CH_3OH$ 9:1). This extract was fractionated by silica gel column chromatography using a gradient eluent system with increasing polarity (see Experimental). A preliminary NMR analysis revealed that the

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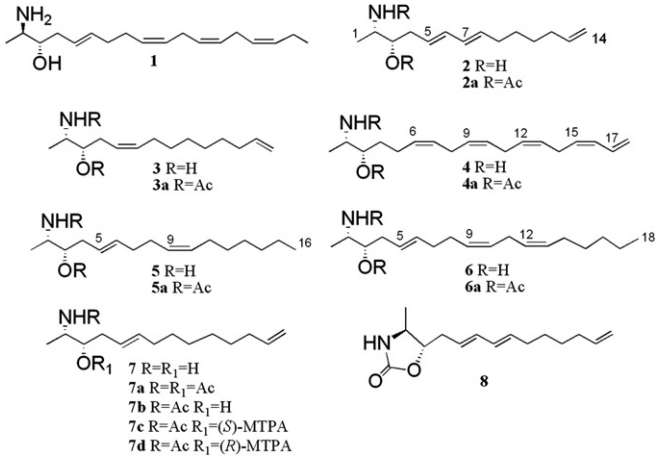
most polar fraction was constituted by a complex mixture of unsaturated amino alcohols. Every effort to separate this mixture was unsuccessful. In addition, a significant degradation of the components of the fraction was observed to occur in the purification conditions used. Thus, with the aim of finding suitable chromatographic conditions and also of preventing degradation we decided to treat a portion of the fraction with acetic anhydride and pyridine. The acetylated mixture was easily fractionated by reverse-phase HPLC (MeOH/H_2O 8:2) to give six pure metabolites, the diacetyl derivatives **2a**–**6a** of five novel molecules, named crucigasterins A–E (**2**–**6**), and the diacetyl derivative **7a** of known related compound **7**.[5] The esters **2a**–**7a** appeared to be more stable than the corresponding native compounds, even though some degradation by-products were observed to be formed in $CHCl_3$ and MeOH solutions.

Compound **7a** was identified by comparison of spectroscopic data with the literature[5] even though only a partial NMR assignment had been reported. The proton and carbon resonances of **7a** were fully assigned in this work by interpretation of 2D-NMR spectra (Tables 1 and 2).



Analysis of the NMR spectra of compounds **2a**–**6a** revealed a close structural relationship with compound **7a** indicating the presence in all molecules of the same 2-amino-3-hydroxy-alkyl residue (Tables 1 and 2). The structural differences among compounds **2a**–**6a** were in either the alkyl chain length or in the number and/or geometry of the double bonds. Diacetyl crucigasterins **2a**–**6a** were characterised as described below.

Diacetyl crucigasterin A (**2a**) was isolated as an optically active colourless oil, and had the molecular formula $C_{18}H_{29}NO_3Na$, as suggested by the sodiated molecular peak at *m*/*z* 330.2042 in the HRESIMS spectrum. The ^{13}C NMR spectrum contained signals at δ 114.3 (t, C-14), 125.1 (d, C-5), 129.9 (d, C-7), 134.0 (d, C-6), 133.9 (d, C-8) and 138.9 (d, C-13) (Table 2) that indicated the presence of three double bonds, one of which had to be in the terminal position.

Accordingly, the ^1H NMR spectrum displayed seven olefinic signals at δ 6.03 (1H, dd, J=14.6 and 10.5 Hz, H-6), 5.99 (1H, dd, J=14.6 and 10.5 Hz, H-7), 5.80 (1H, ddt, J=16.8, 10.1 and 6.7 Hz, H-13), 5.60 (1H, dt, J=14.6 and 7.0 Hz, H-8), 5.46 (1H, m, H-5), 4.99 (1H, br d, J=16.8 Hz, H-14a) and 4.93 (1H, br d, J=10.1 Hz, H-14b) (Table 1), which were assigned to a conjugated diene system and to the terminal vinyl moiety by analysis of the ^1H–^1H COSY and HSQC spectra.

The 2-amino-3-hydroxy-alkyl residue located on the other side of the molecule was easily recognised by sequentially correlated proton signals at δ 1.10 (3H, d, J=6.7 Hz, H_3-1), due to the terminal methyl, and at δ 4.25 (1H, dq, J=3.8 and 6.7 Hz, H-2) and 4.85 (1H, dt, J=3.8 and 6.7 Hz, H-3) that were attributed to two methines linked to the amino and the hydroxyl groups, respectively. In the ^{13}C NMR spectrum, carbon signals at δ 18.5 (q, C-1), 46.7 (d, C-2) and 75.9 (d, C-3) confirmed this assignment. The expected signals due to two acetyl residues esterifying both amino and hydroxyl groups were observed in the carbon and proton spectra [δ_C 20.9 (q, *O*–$COCH_3$), 23.4 (q, *N*–$COCH_3$), 169.2 (s, *N*–$COCH_3$) and 170.6 (s, *O*–$COCH_3$); δ_H 1.99 (s, *N*–$COCH_3$) and 2.07 (s, *O*–$COCH_3$)]. A single spin system from H_3-1 to H_2-14 including five sp^3 methylene groups was readily identified by interpretation of the ^1H–^1H COSY spectrum (Table 1). The methylene signal resonating at δ 2.25–2.35 (2H, m, H_2-4) was correlated to both the carbinol proton at δ 4.85

Table 1
^1H NMR data[a,b,c] for crucigasterins A–E (**2a**–**6a**) and compound **7a**

Position	δ_H, mult.					
	2a	**3a**	**7a**	**4a**	**5a**	**6a**
1	1.10 (d, 6.7)	1.11 (d, 7.0)	1.10 (d, 6.7)	1.10 (d, 6.7)	1.11 (d. 6.7)	1.11 (d, 6.7)
2	4.25 (dq, 3.8, 6.7)	4.24 (dq, 4.0,7.0)	4.23 (dq, 4.2, 6.7)	4.22 (m)	4.24 (m)	4.23 (m)
3	4.85 (dt, 3.8, 6.7)	4.85 (ddd, 4.0, 7.6, 6.5)	4.83 (dt, 4.2, 6.7)	4.88 (dt, 3.8, 6.7)	4.84 (dt, 4.1, 6.7)	4.84 (dt, 4.1,6.7)
4	2.30 (m)	2.25 (ddd, 14.7, 6.5, 7.6) 2.35 (ddd, 14.7, 7.6, 7.6)	2.23 (m)	1.61 (m)	2.23 (m)	2.24 (m)
5	5.46 (m)	5.30 (ddd, 10.0, 7.6, 7.6)	5.31 (dt, 14.9,6.9)	2.02 (m)	5.41–5.28 (m)	5.41–5.28 (m)
6	6.03 (dd. 14.6. 10.5)	5.51 (dt, 10.0, 7.6)	5.49 (m)	5.42–5.33 (m)	5.51 (dt, 14.0, 7.0)	5.51 (dt, 14.0, 7.0)
7	5.99 (dd, 14.6, 10.5)	2.01 (m)	1.98 (m)	5.42–5.33 (m)	2.06 (m)	2.06 (m)
8	5.60 (dt, 14.6, 7.0)	1.32 (m)	1.26 (m)	2.84 (app. t, 5.6)[c]	2.05 (m)	2.10 (m)
9	2.10–1.98 (m)	1.24–1.34 (m)	1.24–1.38 (m)	5.42–5.33 (m)	5.41–5.28 (m)	5.41–5.28 (m)
10	1.42–1.36 (m)	1.24–1.34 (m)	124–1.38 (m)	5.42–5.33 (m)	5.41–5.28 (m)	5.41–5.28 (m)
11	1.42–1.36 (m)	1.38 (m)	1.38 (m)	2.79 (app. t, 5.6)[c]	2.02 (m)	2.76 (app. t, 5.8)
12	2.10–1.98 (m)	2.06 (m)	2.02 (m)	5.42–5.33 (m)	1.35–1.23 (m)	5.41–5.28 (m)
13	5.80 (ddt, 16.8, 10.1.6.7)	5.81 (ddt, 16.4, 10.0, 7.0)	5.80 (ddt, 17.2, 10.2, 7.0)	5.42–5.33 (m)	1.35–1.23 (m)	5.41–5.28 (m)
14	4.99 (br d, 16.8) 4.93 (br d, 10.1)	4.99 (br d, 16.4) 4.93 (br d, 10.0)	4.99 (br d, 17.2) 4.93 (br d, 10.2)	2.97 (app. t, 5.6)	1.35–1.23 (m)	2.02 (m)
15				5.42 (m)	1.29 (m)	1.38–1.25 (m)
16				6.02 (dd, 11.3, 10.8)	0.88 (t, 6.7)	1.38–1.25 (m)
17				6.67 (ddd, 16.9, 10.8, 10.2)		1.38–1.25 (m)
18				5.21 (br d, 16.9), 5.12 (br d, 10.2)		0.89 (t, 6.7)
2-NH	5.51 (br d, 9.0)	5.53 (br d, 9.0)	5.51 (overlapped)	5.51 (br d, 9.0)	5.50 (br d, 8.8)	5.50 (br d, 9.0)
NHAc	1.99 (s)	1.99 (s)	1.99 (s)	1.99 (s)	1.99 (s)	2.00 (s)
OAc	2.07 (s)	2.08 (s)	2.07 (s)	2.07 (s)	2.08 (s)	2.08 (s)

[a] Bruker 400 and 600 MHz.
[b] Assignments made by COSY, HSQC and HMBC (J=10 Hz experiments).
[c] Values with the same superscript in the same column may be interchanged.

Table 2
^{13}C NMR data[a,b,c] for crucigasterins A–E (**2a**–**6a**) and compound **7a**

No.	δ_C, m					
	2a	**3a**	**7a**	**4a**	**5a**	**6a**
1	18.5 q	18.5 q	18.5 q	18.5 q	18.5 q	18.5 q
2	46.7 d	46.9 d	46.7 d	46.9 d	46.8 d	46.8 d
3	75.9 d	76.1 d	76.1 d	75.9 d	76.0 d	76.0 d
4	35.0 t	29.5 t	35.0 t	31.5 t	35.0 t	35.0 t
5	125.1 d	123.2 d	123.9 d	27.0 t	124.4 d	124.5 d
6	134.0 d	133.5 d	134.8 d	128.8[c] d	134.1 d	134.0 d
7	129.9 d	27.3 t	32.5 t	128.7[c] d	32.7 t	32.6 t
8	133.9 d	29.0[c] t	29.0[c] t	25.3 t	27.l t	27.0 t
9	32.3 t	29.5[c] t	29.3[c] t	128.6[c] d	130.5 d	127.8 d
10	28.7[c] t	29.4[c] t	29.0[c] t	128.8[c] d	128.2 d	129.1[c] d
11	28.4[c] t	29.1[c] t	29.0[c] t	25.3 t	27.3 t	25.7 t
12	33.6 t	33.7 t	33.8 t	128.7[c] d	29.7[c] t	128.5[c]d
13	138.9 d	138.9 d	139.2 d	128.7[c] d	29.0[c] t	130.3 d
14	114.3 t	114.1 t	114.1 t	25.6 t	31.8 t	27.2 t
15				128.4[c] d	22.6 t	29.3 t
16				129.2 d	14.1 q	31.5 t
17				131.9 d		22.6 t
18				117.5 t		14.0 q
NHAc	23.4 q	23.4q	23.5 q	23.2 q	23.4 q	23.4 q
	169.2 s	169.3 s	169.3 s	169.3 s	169.3 s	169.3 s
OAc	20.9 q	21.0 q	21.0 q	21.1 q	21.0 q	21.0 q
	170.6 s	170.6	170.6 s	170.7 s	170.6 s	170.6 s

[a] Bruker 300 MHz.
[b] Multiplicity deduced by DEPT.
[c] Assignments may be interchanged.

(H-3) and the olefinic proton at δ 5.46 (H-5) clearly inferring the position of the diene system at C-5/C-8. The sequence of the remaining portion of the molecule was easily determined, leading to structure **2a**.

The geometries of Δ^5 and Δ^7double bonds were suggested to be *E*,*E* by the coupling constants ($J_{5,6}$=$J_{7,8}$=14.6 Hz) and further supported by the ^{13}C chemical shift values of the allylic methylene carbons C-4 (δ 35.0) and C-9 (δ 32.3) (Table 2). The relative configuration of the chiral centres C-2 and C-3 was deduced by analysing a series of NOE difference experiments recorded on the corresponding oxazolidinone derivative **8**, which was obtained by the reaction of **2** with 1,1′-carbonyldiimidazole, according to the procedure applied for related molecules.[5,6] The NOE effects observed between H-3 and H_3-1 and between H-2 and H_2-4 suggested that the substituents at both C-2 and C-3 were on the opposite face of oxazolidinone ring. Consequently, H-2 and H-3 were suggested to be in a *threo* relationship in the acyclic starting compound **2**. This suggestion was definitively confirmed by a comparative analysis of NMR data of diacetyl crucigasterin A (**2a**) with those reported in the literature for synthetic *threo* and *erythro* 2-acetamido-3-acetoxy-5*E*,7*E*-tetradecadiene models.[24] A good agreement of carbon NMR resonances was observed between **2a** and the *threo*-isomer, in particular with regards to diagnostic ^{13}C chemical shift value of C-1 (δ_{threo} 18.49; $\delta_{erythro}$ 14.93; $\delta_{\mathbf{2a}}$ 18.5). On this basis, the relative configuration of **2a**, and consequently of **2**, was assigned to be *threo* (2*R**,3*R** or 2*S**,3*S**) analogously with co-occurring known **7a**. Crucigasterin A (**2**) differed from known **7** in the presence of an additional *E* double bond at C-7.

Diacetyl crucigasterin B (**3a**) had the molecular formula $C_{18}H_{31}NO_3Na$ the same as compound **7a**. Analysis of the ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) revealed strong similarities with **7a** and, in particular, indicated the presence of an identical structural sequence. Differences were observed in the multiplicity of the ^{1}H NMR signals as well as in the corresponding ^{13}C NMR carbons of allylic C-4 and C-7, and vinyl C-5 and C-6 (Tables 1 and 2). This implied that crucigasterin B and compound **7a** differed only in the geometry of Δ^5 double bond. In particular, the proton coupling constant J_{5-6}=10.0 Hz and the carbon values of C-4 (δ 29.5) and C-7 (δ 27.3) clearly indicated that **3a** was the 5*Z*-isomer of **7a**. The *threo* stereochemistry of the 2-amino-3-hydroxy moiety of **3a** was assigned by the diagnostic ^{13}C NMR value of the terminal methyl C-1 (δ 18.5).

Diacetyl crucigasterin C (**4a**) had the molecular formula $C_{22}H_{33}NO_3$ indicating the presence of a highly unsaturated C18 alkyl chain. The ^{1}H NMR spectrum (Table 1) contained signals accounting for 11 olefinic protons [δ 6.67 (1H, ddd, J=16.9, 10.8 and 10.2 Hz, H-17), 6.02 (1H, dd, J=11.3 and 10.8 Hz, H-16), δ 5.42 (1H, m, H-15), δ 5.42–5.33 (6H, m, H-6, H-7, H-9, H-10, H-12, and H-13), δ 5.21 (1H, br d, J=16.9 Hz, H-18a), δ 5.12 (1H, br d, J=10.2 Hz, H-18b)] according to the presence of five vinyl unsaturations (four disubstituted and one terminal double bond) as required by the molecular formula. These data also indicated that the terminal vinyl moiety was conjugated with one of the disubstituted double bonds, whereas the remaining three were isolated. Analysis of the ^{1}H–^{1}H COSY spectrum revealed that the 6H signal at δ 5.42–5.33 was coupled with three bis-allylic methylene groups at δ 2.97 (2H, app. t, J=5.6 Hz, H_2-14), 2.84 (2H, app. t, J=5.6 Hz, H_2-8 or H_2-11) and 2.79 (2H, app. t, J=5.6 Hz, H_2-11 or H_2-8), and to an allylic methylene at δ 2.02 (2H, m, H_2-5). This latter signal was additionally correlated with the methylene multiplet at δ 1.61 (2H, m, H_2-4), which in turn showed cross-peaks with the carbinol proton of 2-amino-3-hydroxy moiety thus inferring the sequence as depicted in formula **4a**.

The *Z* geometry was assigned to all disubstituted double bonds of **4a** by the ^{13}C NMR values of bis-allylic and allylic methylenes resonating at δ 25.3 (2C, C-8 and C-11), 25.6 (C-14), and 27.0 (C-5) (Table 2). The similarity of the carbon value of the terminal methyl C-1 (δ 18.5) with those of already described **2a**, **3a** and **7a** indicated also for crucigasterin C (**4a**) the *threo* stereochemistry of 2-amino-3-hydroxy moiety. Crucigasterin C was closely related to obscuraminol A[6] from which it differs in the presence of an additional terminal double bond and in the stereochemistry of the 2-amino-3-hydroxy moiety.

The molecular formula of diacetyl crucigasterin D (**5a**) $C_{20}H_{35}NO_3Na$ was consistent with the presence of two additional CH_2 units in the alkyl chain with respect to **3a** and **7a**. The ^{1}H and ^{13}C NMR spectra exhibited signals due to two disubstituted double bonds [δ_C 134.1 (d, C-6), 130.5 (d, C-9), 128.2 (d, C-10), 124.4 (d, C-5); δ_H 5.51 (1H, dt, J=14.0 and 7.0 Hz, H-6), 5.41–5.28 (3H, m, H-5, H-9 and H-10)], in addition to the signals due to the 2-amino-3-hydroxy moiety (Tables 1 and 2). The ^{1}H–^{1}H COSY spectrum of **5a** showed diagnostic correlations between the methylene signal centred at δ 2.23 (H_2-4) and both the olefinic proton at δ 5.41–5.28 (H-5) and the carbinol proton at δ 4.84 (H-3) clearly inferring the position of the first double bond at C-5. The geometry of this double bond was assigned as *E* by the proton coupling constant J_{5-6}=14.0 Hz and the ^{13}C NMR chemical shift values of the allylic methylene groups [δ 35.0 (C-4) and 32.7 (C-7)], analogously with **2a** and **7a**. The second double bond, the geometry of which was suggested to be *Z* by the chemical shift values of the remaining allylic methylenes [δ 27.1 (C-8) and 27.3 (C-11)], was positioned at C-9 by interpretation of the ^{1}H–^{1}H COSY spectrum. In fact, the allylic methylene resonating at δ 2.02 (H_2-11) showed cross-peak correlations with the signals at δ 1.35–1.23 whereas those resonating at δ 2.05 (H_2-8) and 2.06 (H_2-7) were only coupled to each other. This implied a sequence exhibiting adjacent C-7 and C-8 according to the location of the second double bond at C-9. Finally, analogously with the already described co-occurring metabolites, the stereochemistry of the 2-amino-3-hydroxy moiety was assigned to be *threo* by the ^{13}C NMR value of C-1 (Table 1).

Diacetyl crucigasterin E (**6a**) had the molecular formula $C_{22}H_{37}NO_3$ that was consistent with a C18 alkyl chain exhibiting three double bonds. The ^{1}H NMR spectrum of **6a** showed olefinic signals at δ 5.51 (1H, dt, J=14.0 and 7.0 Hz) and 5.41–5.28 (5H, m),

a signal at δ 2.76 (2H, app. t, 5.8 Hz), which was due to a bis-allylic methylene group, and multiplets centred at δ 2.24 (2H, m, H_2-4), 2.10 (2H, m, H_2-8), 2.06 (2H, m, H_2-7) and 2.02 (2H, m, H_2-14) attributed to four allylic methylene groups. The $^1H-^1H$ COSY experiment helped us define the spin–spin coupling sequence from H_3-1 to H_3-18 (Table 1) and to locate the three double bonds as depicted in formula **6a**. In particular, the correlation between the allylic methylene resonating at δ 2.24 (H_2-4) and the carbinol proton of the 2-amino-3-hydroxy terminal moiety implied the location of a double bond at C-5. The bis-allylic methylene (H_2-11) connecting two other double bonds showed homoallylic couplings with both methylenes at δ 2.02 (H_2-14) and 2.10 (H_2-8), the latter of which was correlated to the remaining methylene at δ 2.06 (H_2-7) inferring the positioning of the diene system at C-9/C-12. The geometry of the three double bonds was suggested by the ^{13}C NMR values (Table 2) of bis-allylic and allylic methylenes that resonated at δ 25.7 (C-11), 27.0 (C-8), 27.2 (C-14), 32.6 (C-7), and 35.0 (C-4) indicating the *Z* stereochemistry for Δ^9 and Δ^{12} double bonds and the *E* stereochemistry for Δ^5 double bond. The stereochemistry of the 2-amino-3-hydroxy moiety of diacetyl crucigasterin E (**6a**) was assigned to be *threo*, the same as the other crucigasterins, by the ^{13}C NMR value of C-1 (Table 1). Crucigasterin E showed structural analogies with crucigasterin 277 (**1**), previously isolated from the same ascidian[4] and exhibiting an additional double bond and the *erythro*-stereochemistry of 2-amino-3-hydroxy moiety.

With the aim of determining the absolute stereochemistry of crucigasterins by application of the Mosher's method on suitable hydroxyl substrates, we tried to obtain the *O*-deacetyl derivatives of compounds **2a**–**6a** by methanolysis. Unfortunately, a significant degradation was observed to occur under the reaction conditions for most compounds preventing the formation of the desired hydroxyl substrates. However, the reaction was successfully applied on known co-occurring compound **7a**, the absolute stereochemistry of which had not been reported in the previous paper.[5] The *O*-deacetyl derivative **7b** obtained by methanolysis of **7a** was quite stable. The (*S*)- and (*R*)-MTPA esters **7c** and **7d** were obtained by treating **7b** with (*R*)- and (*S*)-MTPA chlorides, respectively, and characterised by 2D-NMR experiments [$\Delta\delta$ ($\delta_{S\text{ester}}-\delta_{R\text{ester}}$) are reported in Fig. 1]. The $\Delta\delta$ values observed for the signals of protons close to the hydroxyl group at C-3 indicated the *S* configuration as depicted in formula **7b**. Consequently, the absolute stereochemistry of naturally occurring metabolite **7** was assigned as 2*S*,3*S*.

The same configuration was suggested for the other crucigasterins based on biogenetic considerations. This suggestion was supported by the optical rotation values of diacetyl derivatives of crucigasterins (**2a**–**7a**), which were all laevorotatory according to the 2*S* absolute configuration.[25] Particularly diagnostic was the comparison of the $[\alpha]_D$ value of diacetyl derivative of crucigasterin A (**2a**) {$[\alpha]_D$ −20.3 (*c* 0.03, CH_3OH)} with those reported for synthetic (2*R*,3*R*)- {$[\alpha]_D$ +20.7 (*c* 1.66, CH_3OH)} and (2*S*,3*S*)- {$[\alpha]_D$ −20.6 (*c* 0.32, CH_3OH)} 2-acetamido-3-acetoxy-5*E*,7*E*-tetradecadiene enantiomers[24] further confirming the 2*S*,3*S* absolute configuration for crucigasterin A and for all co-occurring metabolites.

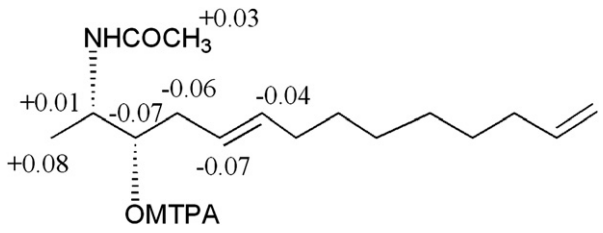


Figure 1. Chemical shifts differences ($\Delta\delta=\delta_{S\text{ ester}}-\delta_{R\text{ ester}}$) between (*S*)- and (*R*)-MTPA derivatives of compound **7a**.

Selected crucigasterins isolated in this work have been tested for antibacterial and antifungal activities. In particular, crucigasterin B (**3**) was active at 50 μg/mL against both *Escherichia coli* and *Candida albicans*, whereas crucigasterin E (**6**) was active at 100 μg/mL against *E. coli*.

3. Conclusion

In conclusion, the chemical investigation of the secondary metabolite pattern of Mediterranean *P. crucigaster* resulted in the isolation of novel crucigasterins A–E belonging to the *threo* series of 2-amino-3-alcohols in contrast with the previous study on the same species reporting *erythro* crucigasterins.[4] An overview of the literature on this group of natural products[4–6,18–22] provided evidence that the *threo*-relative stereochemistry in linear 2-amino-3-alcohols is quite unusual.[5,20a] The finding of both (2*S*) and (2*R*) series in these compounds strongly suggests[4,9,20a] that they are biosynthesised from fatty acids and either L- or D-alanine. The absolute configuration of crucigasterins A–E was established to be 2*S*,3*S* thus implying that they should derive from L-alanine.

4. Experimental section

4.1. General experimental procedures

Optical rotations were measured on a Jasco DIP 370 digital polarimeter; IR spectra were measured on a Biorad FTS 155 FTIR spectrophotometer; 1D- and 2D-NMR spectra were recorded on a Bruker Avance-400 (400.13 MHz) and on a Bruker DRX-600 equipped with TXI CryoProbe™ in $CDCl_3$ (δ values are reported referred to $CHCl_3$ at 7.26 ppm) and ^{13}C NMR were recorded on a Bruker DPX-300 (75.47 MHz) (δ values are reported to $CDCl_3$, 77.0 ppm); HRESIMS were carried out on a Micromass Q-TOF micro; HPLC Waters 501 pumps with a refractometer detector was used equipped with a reverse-phase column Kromasil C-18, 5 μ (250×4.60 mm, Phenomenex); TLC plates (KieselGel 60 F_{254}) were from Merck (Darmstadt, Germany), silica gel powder (Kieselgel 60 0.063–0.200 mm) was from Merck (Darmstadt, Germany).

4.2. Biological material

The colonial ascidian *P. crucigaster* Gaill, 1972 was collected in October 2007 off Cala Sgombro (inside part) along the Sardinia coasts by G.V. at a depth of 10 m, immediately frozen and transferred to ICB. The ascidian was classified by Dr. Flavia Greco of Zoologic Department of University of Bari. A voucher specimen is stored at ICB under the code ATC.

4.3. Extraction and isolation procedures

The frozen ascidian *P. crucigaster* (dry weight, 5.2 g) was chopped and then extracted exhaustively with Me_2CO (400 mL×4) using ultrasound. After filtration and evaporation in vacuo of the organic solvent, the residue was subsequently extracted with Et_2O (200 mL×4) and BuOH (100 mL×4). The evaporation of Et_2O and BuOH extracts gave two gummy residues (0.783 g and 0.976 g, respectively). The ethereal extract was subjected to a silica gel column chromatography using as eluent a gradient of light petroleum ether and Et_2O, $CHCl_3$ and finally MeOH to give eight fractions. Preliminary ^1H NMR analysis of these fractions evidenced that the last fraction (0.207 g) contained a mixture of some interesting compounds. Part of this fraction (0.120 g) was first acetylated with acetic anhydride in pyridine (2 h, room temperature) to prevent degradation of the compounds and then subjected to reverse-phase HPLC column chromatography (MeOH/H_2O, 8:2; flow 1 mL/min) to afford in order of decreasing polarity diacetyl crucigasterin A (**2a**)

(t_R 9.6 min, 1.4 mg), B (**3a**) (t_R 11.2 min, 1.9 mg), **7a** (t_R 12.0 min, 2.1 mg), C (**4a**) (t_R 15.2 min, 5.4 mg), D (**5a**) (t_R 25.0 min,3.3 mg), E (**6a**) (t_R 34.0 min, 2.1 mg).

*4.3.1. Diacetyl crucigasterin A (**2a**).* Colourless oil; $[\alpha]_D$ −24.7 (*c* 0.1, $CHCl_3$); $[\alpha]_D$ −20.3 (*c* 0.03, CH_3OH); UV (MeOH) λ_{max} (ε) 230 (10,216); IR (KBr) ν_{max} 3418, 2925, 2830, 1715 cm^{-1}; ^1H and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m*/*z* 330.2042 (M+Na), calcd for $C_{18}H_{29}NO_3Na$ 330.2045.

*4.3.2. Diacetyl crucigasterin B (**3a**).* Colourless oil, $[\alpha]_D$ −16.8 (*c* 0.2, $CHCl_3$); IR (KBr) ν_{max} 3425, 2926, 2837, 1741, 1718, 1651 cm^{-1}; ^1H and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m*/*z* 332.2204 (M+Na), calcd for $C_{18}H_{31}NO_3Na$ 332.2202.

*4.3.3. Diacetyl crucigasterin C (**4a**).* Colourless oil, $[\alpha]_D$ −28.0 (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} 3425, 2926, 2837, 1744, 1718, 1643 cm^{-1}; ^1H and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m*/*z* 382.2341 (M+Na), calcd for $C_{22}H_{33}NO_3Na$ 382.2358.

*4.3.4. Diacetyl crucigasterin D (**5a**).* Colourless oil; $[\alpha]_D$ −12.1 (*c* 0.3, $CHCl_3$); IR (KBr) ν_{max} 3425, 2926, 2854, 1744, 1718 cm^{-1}; ^1H and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m*/*z* 360.2518 (M+Na), calcd for $C_{20}H_{35}NO_3Na$ 360.2515.

*4.3.5. Diacetyl crucigasterin E (**6a**).* Colourless oil; $[\alpha]_D$ −15.2 (*c* 0.2, $CHCl_3$); IR (KBr) ν_{max} 3423, 2936, 2861, 1744, 1718 cm^{-1}; ^1H and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m*/*z* 386.2686 (M+Na), calcd for $C_{22}H_{37}NO_3Na$ 386.2671.

*4.3.6. Compound **7a**.* Colourless oil, $[\alpha]_D$ −19.1 (*c* 0.12, $CHCl_3$); IR (KBr) ν_{max} 3425, 2926, 2837, 1740, 1718 cm^{-1}; ^1H and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m*/*z* 332.2204 (M+Na), calcd for $C_{18}H_{31}NO_3Na$ 332.2202.

4.4. Preparation of oxazolidinone derivative (8)

In order to prepare the oxazolidinone derivative of crucigasterin A (**2**), the diacetyl derivative **2a** (1 mg) was treated with NaOH (4 M) at 70 °C for 20 h. The free aminoalcohol (0.8 mg, **2**) obtained from this reaction was then reacted with 1,1′-carbonyldiimidazole (3 mg) in 1 mL of CH_2Cl_2 and 100 μL of DMF at 0 °C for 19 h under argon atmosphere. The solution was extracted with water and the organic layer was dried under nitrogen yielding 0.5 mg of the oxazolidinone **8**.

*4.4.1. Oxazolidinone (**8**).* ^1H NMR ($CDCl_3$) δ 6.12 (dd, *J*=15.3, 10.5 Hz, H-6), 6.00 (dd, *J*=15.3, 10.5 Hz, H-7), 5.80 (ddt, *J*=16.4, 10, 6.5 Hz, H-13), 5.65 (ddd, *J*=15.3, 7.0, 7.0 Hz, H-5), 5.50 (ddd, *J*=15.3, 7.0, 7.0 Hz, H-8), 5.0 (br d, *J*=17 Hz, H-14a), 4.95 (br d, *J*=10 Hz, H-14b), 4.17 (m, H-3), 3.62 (m, H-2), 2.48 (m, H_2-4), 2.05 (m, H_2-9 and H_2-12), 1.38 (m, H_2-10 and H_2-11), 1.28 (d, *J*=6 Hz, H_3-1); ESIMS *m*/*z* 272 $(M+Na)^+$; HRESIMS *m*/*z* 272.1628 (M+Na), calcd for $C_{15}H_{23}NO_2Na$ 272.1621.

4.5. Preparation of MTPA esters

*4.5.1. Methanolysis of **7a**.* Diacetyl crucigasterin A (**7a**, 2.1 mg) was treated with Na_2CO_3 in anhydrous MeOH and stirred at room temperature overnight. After filtration and evaporation of the solvent, the reaction product (**7b**) was divided into two parts, which in turn were reacted with *R*-(−)-MTPA chloride and *S*-(+)-MTPA chloride, to give the *S*-(**7c**) and *R*-MTPA-(**7d**)-esters, respectively.

*4.5.2. Compound **7c** (S-MTPA ester).* *S*-MTPA ester was prepared by treating 1 mg of **7b** with 0.005 mL of *R*-(−)-MTPA chloride in dry CH_2Cl_2 (0.5 mL) with catalytic amount of DMAP under stirring for 16 h at room temperature. The ester was purified by chromatography in a Pasteur pipette (SiO_2, $CHCl_3$). Selected ^1H NMR values ($CDCl_3$): δ 5.81 (H-13), 5.49 (H-6), 5.25 (H-5), 5.00 (H-3), 4.98 (H-14a), 4.96 (H-14b), 4.33 (H-2), 2.28 (H_2-4), 2.00 (H_2-7 and H_2-12), 1.95 ($NHCOCH_3$), 1.30 (H_2-8, H_2-9, H_2-10 and H_2-11), 1.12 (H_3-1).

*4.5.3. Compound **7d** (R-MTPA ester).* *R*-MTPA ester was prepared by treating 1 mg of **7b** with 0.005 mL of *S*-(+)-MTPA chloride in dry CH_2Cl_2 (0.5 mL) with catalytic amount of DMAP under stirring for 16 h at room temperature. The ester was purified by chromatography in a Pasteur pipette (SiO_2, $CHCl_3$). Selected ^1H NMR values ($CDCl_3$): δ 5.81 (H-13), 5.53 (H-6), 5.32 (H-5), 5.07 (H-3), 4.98 (H-14a), 4.96 (H-14b), 4.32 (H-2), 2.34 (H_2-4), 1.92 ($NHCOCH_3$), 1.30 (H_2-8, H_2-9, H_2-10 and H_2-11), 1.04 (H_3-1).

4.6. Biological assays

Antifungal assay was performed by the broth macrodilution method following the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) document M27-P.[26,27] The medium used to prepare the 10× drug dilutions and the inoculum suspension was liquid RPMI 1640 with L-glutamine (Sigma Aldrich), 0.165 M morpholinopropanesulfonic acid (MOPS) and 2% glucose (pH 7.0).[28,29] The yeast suspension was adjusted with the aid of a spectrophotometer to a cell density of 0.5 McFarland (2×108 CFU/mL) standard at 530 nm and diluted to 1:4000 (50,000 CFU/mL) in RPMI 1640 broth medium. The yeast inoculum (0.9 mL) was added to each test tube that contained 0.1 mL of 10 twofold dilutions (256–0.05 mg/mL final) of each compound. Broth macrodilution MICs were determined after 48 h of incubation at 35 °C. MIC was defined as the lowest concentration of the compound that completely inhibited the growth of the test organism. The antibacterial assay was performed by using the same method as the antifungal test, only differing in the assay medium (Luria Bertani medium: 10 g/L bactotryptone, 5 g/L bactoyeast, and 10 g/L NaCl, pH 7.5) and in the incubation temperature (37 °C for 24 h). Crucigasterin B (**3**) and E (**6**) were tested for antifungal and antibacterial activities, exhibiting moderate activity against *E. coli* and *C. albicans*, respectively.

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tet.2010.07.056.

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