Homophymines B–E and A1–E1, a family of bioactive cyclodepsipeptides from the sponge *Homophymia* sp.†

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Nine new cyclodepsipeptides, homophymines B–E (**2–5**) and A1–E1 (**1a–5a**), were isolated from the polar extracts of the sponge *Homophymia* sp. The new structures, featuring new polyketide-derived end groups, were determined by interpretation of NMR and MS data. The configurations of the new end groups was secured by the application of *J*-based configurational analysis. Homophymines displayed very potent antiproliferative activity (IC_{50} in the nM range) against a panel of human cancer cell lines.

Introduction

Marine sponges have proven to be a significant source of biologically active cyclic peptides and depsipeptides.[1] Among these sponge depsipeptides, callipeltin A (from a New Caledonian sponge *Callipelta* sp.),[2] neamphamide A (from a Papua New Guinea sponge *Neamphius huxleyi*),[3] papuamides A–D[4] and theopapuamide[5] (from Papua New Guinea sponges *Theonella mirabilis* and *Theonella swinhoei*), mirabamides, (from the Micronesian sponge *Siliquariaspongia mirabilis*)[6] are well known for their potent HIV-inhibitory activity and their structurally unique features incorporating several non proteinogenic amino acid residues. The rarity of these atypical amino acid residues has inspired their chemical synthesis.[7]

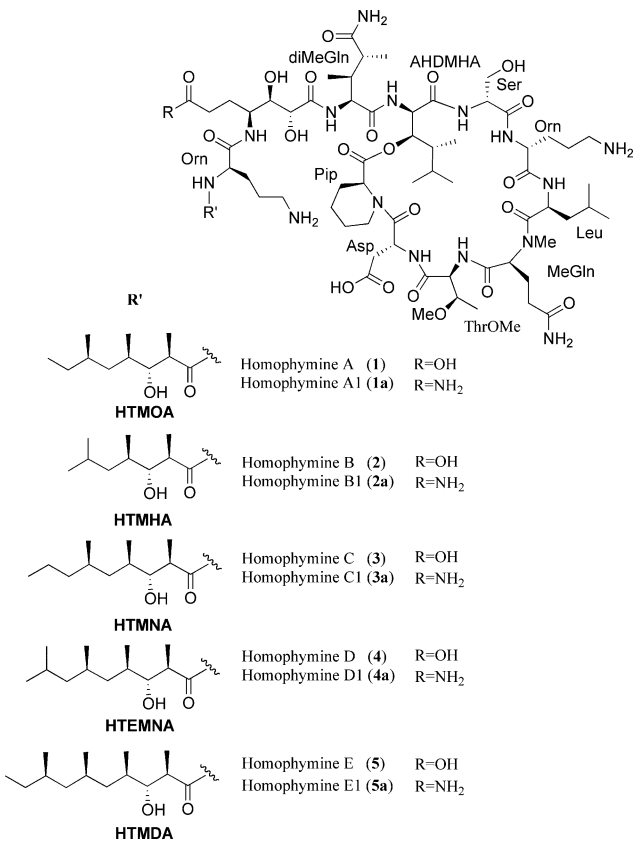
In the frame of our long-standing interest towards bioactive marine compounds we recently reported the isolation from the sponge *Homophymia* sp. (order Lithistida) of a related cyclodepsipeptide homophymine A (**1**).[8] This undecapeptide shares some residues with the abovementioned cyclodepsipeptides, such as the 2,3-dimethylglutamine residue common to all compounds; the pipecolic acid, the *N*-methylglutamine and leucine also found in neamphamide and theopapuamide; the 3-hydroxy-2,4,6-trimethyloctanoic acid (HTMOA) also found in theopapuamide. Homophymine A (**1**) also contains two unprecedented residues in natural sources, *i.e.* the 4-amino-2,3-dihydroxy-1,7-heptandioic acid (ADHA) and 2-amino-3-hydroxy-4,5-dimethylhexanoic acid (AHDMHA). As the related cyclodepsipeptides, homophymine A exhibited cytoprotective activity against the HIV-1 infection with an IC_{50} of 75 nM.

A careful examination and fractionation of the crude polar extracts of *Homophymia* sp. led to the isolation of nine new homophymines, which we named homophymines B–E (**2–5**) and A1–E1 (**1a–5a**).

In this paper, we describe the isolation and the structure determination of the new compounds, together with the analysis of their biological activity.

Results and discussion

The lyophilized sponge (300 g) was extracted with MeOH, and the combined extracts were fractionated according to the Kupchan partitioning procedure.[9] The bioactive chloroformic extract was



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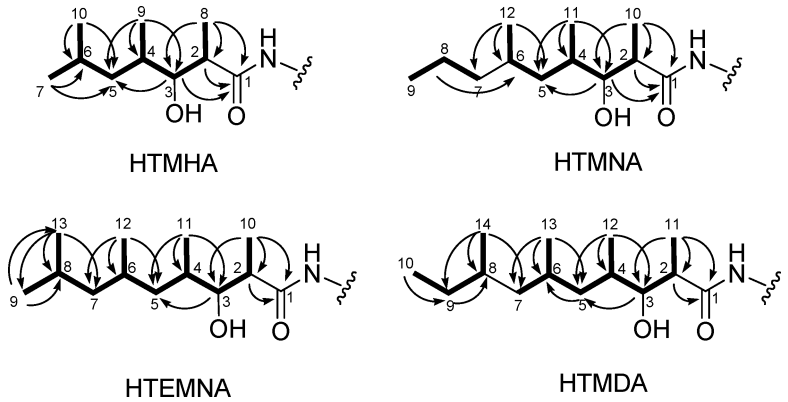

Fig. 1 New acyl units in homophymine B–E with COSY/TOCSY connectivities (bold bonds) and HMBC correlations (arrows).

purified by DCCC followed by reverse-phase HPLC (MeOH aqueous) to give homophymines B–E (**2–5**) and A1–E1 (**1a–5a**).

Homophymine B (**2**) was isolated as a colourless amorphous solid and showed the pseudomolecular ion peak at *m/z* 1584.9055 $(M + H)^+$ in the HRESIMS spectrum, corresponding to the molecular formula $C_{72}H_{125}N_{15}O_{24}$. The ^1H NMR spectrum indicated a close structural analogy with homophymine A (**1**).

Extensive analysis of 1D and 2D NMR spectra by comparison with those of homophymine A evidenced the same amino acid spin systems found in the parent compound whereas a different β-hydroxy acyl end group was observed. A 3-hydroxy-2,4,6-trimethylheptanoic acid (HTMHA) residue, also found as the end group in callipeltin A[2] and neamphamide A[4] was inferred from the analysis of COSY and HMBC data (Fig. 1). The connectivity between amino acid residues was established on the basis of careful analysis of HMBC and ROESY data acquired in CD_3OH (Table 1 in ESI†) and was proved to be the same found in homophymine A.

Further confirmation of the proposed sequence arose from MS/MS analysis performed on the intact peptide. In addition to a pseudomolecular ion corresponding to a linear peptide derived from ester cleavage with concomitant proton transfer from AHDMHA to a proline residue, the ESI Q/TOF spectrum provided several fragmentation ion peaks (b series) which were consistent with the amino acid sequence as shown in **2** (see ESI†).

The close resemblance of the ^1H and ^{13}C NMR chemical shifts of all the amino acid residues with the corresponding values of homophymine A suggest that they have the same configuration. To confirm this hypothesis, homophymine B was subjected to the same procedures described for the stereochemical characterization of the amino acid residues in homophymine A. Therefore the presence of L-Leu, two D-Orn, D-Asp, L-Pip, (3*S*,4*R*)-3,4-diMe-L-Gln, L-ThrOMe, (2*R*,3*R*,4*S*)-4-amino-2,3-dihydroxy-1,7-heptandioic acid (ADHA) and (2*R*,3*R*,4*R*)-2-amino-3-hydroxy-4,5-dimethylhexanoic acid (AHDMHA) was established by Marfey's analysis.[10] The D configuration of Asp and the L configuration of *N*-MeGln were determined by LC-MS analysis of GITC derivatives.[11] The D configuration of Ser residue was determined by chiral HPLC analysis of the hydrolysate mixture (see Experimental).

The ^1H and ^{13}C NMR chemical shifts and the homonuclear coupling constant pattern of the 3-hydroxy-2,4,6-trimethylheptanoic acid (HTMHA) residue match with those of the corresponding residue in callipeltin A for which the 2*R*,3*R*,4*R* absolute configuration was secured by synthesis.[7i,j] Further confirmation of the relative configuration arose from the results of *J*-based NMR configurational analysis[12,13] performed on intact homophymine B (see ESI†).

HRESIMS data indicated that homophymine B1 (**2a**) (*m/z* 1583.9304 $(M + H)^+$ corresponding to the molecular formula $C_{72}H_{126}N_{16}O_{23}$) differs from **2** for the presence of a NH_2 group in the place of a OH group, therefore one of the two carboxy-bearing amino acid residues (Asp or ADHA) in homophymine B is in amide form in homophymine B1. This hypothesis was confirmed by the ^{15}N HSQC (CD_3OH) experiment that evidenced the presence of three primary amide groups (δ_N 108.6, δ_H 6.90 s and 7.59 s; δ_N 109.0, δ_H 7.01 s and 7.69 s; δ_N 109.2, δ_H 6.83 s and 7.19 s). The amide proton pair at δ_H 6.90 and 7.59 showed HMBC correlation with an acyl group at δ_C 177.8 and with an allylic methylene carbon at δ_C 32.4, that on the basis of COSY and HMBC data were assigned as C6 and C7 of a 5-*acyl*-4-amino-2,3-dihydroxyheptanoic acid. Therefore the new 4-amino-6-carbamoyl-2,3-dihydroxyhexanoic acid was assigned and the structure of homophymine B1 as **2a** was secured by NMR data, which was almost superimposable with those of homophymine B (**2**).

The MS/MS fragmentation pattern of **2a** was consistent with the replacement of 4-amino-2,3-dihydroxy-1,7-heptandioic acid (ADHA) with the 4-amino-6-carbamoyl-2,3-dihydroxyhexanoic acid residue.

Homophymine A1 (**1a**) was analyzed for molecular formula $C_{73}H_{128}N_{16}O_{23}$ by HRESIMS data *m/z* $[M + H]^+$ 1597.9456. NMR data indicated that homophymine A1 differs from homophymine A for the replacement of 4-amino-2,3-dihydroxy-1,7-heptandioic acid (ADHA) with the 4-amino-6-carbamoyl-2,3-dihydroxyhexanoic acid residue.

The molecular formula $C_{74}H_{129}N_{15}O_{24}$ of homophymine C (**3**), deduced by HRESIMS, indicated the presence of one additional carbon atom with respect to homophymine A. A careful analysis of the NMR data, including COSY, HSQC, TOCSY, ^{15}N HSQC indicated the presence of the same amino acid residues found in homophymine A and a variation of the end group. Even if from ^1H NMR and HMQC data an additional methylene group with respect to homophymine A was easily detected (δ_C 21.3, δ_H 1.27, 1.42), the analysis of the proton spin system of the end group subunit was not straightforward due to the heavy overlap in the ^1H NMR high field region and to the absence of some

scalar coupling in the COSY spectrum (Fig. 1). The ^{1}H and ^{13}C NMR resonances of C-1/C-6 nuclei relative to this portion were almost superimposable with the corresponding values found for the 3-hydroxy-2,4,6-trimethyloctanoic acid (HTMOA) residue in homophymine A. Additionally, a *n*-propyl group was inferred by COSY data that indicated a sequence CH_2-CH_2-CH_3 (C7-C9). Even if any COSY correlation was observed between H6 and H_2-7 diagnostic HMBC correlation $H_3$12/C7 and $H_2$8/C6 secured the linkage of the *n*-propyl chain to C6. Therefore the new 3-hydroxy-2,4,6-trimethylnonanoic acid (HTMNA) end group was established (Fig. 1). The sequence of the amino acids, the location of nonanoic acid derivative and the macrocyclic structure of homophymine C (**3**) was established on the basis of careful analysis of HMBC and ROESY data acquired in CD_3OH (Table 2 in ESI†) and was proved to be the same as found in homophymine A. The stereochemistry of amino acid residues was established as previously described for homophymine B whereas similarity in ^{1}H and ^{13}C NMR shifts observed for HTMOA residue in **1** and HTMNA in **3** implied that the stereogenic centres in the β-hydroxyacid fragment had the same configurations.

As deduced from HRESIMS data and NMR data homophymine C1 (**3a**) (HRESIMS *m/z* 1611.9538 $[M + H]^+$) differs from the homophymine C for the replacement of 4-amino-2,3-dihydroxy-1,7-heptandioic acid (ADHA) with the 4-amino-6-carbamoyl-2,3-dihydroxyhexanoic acid residue.

Homophymine D (**4**) analyzed for $C_{75}H_{131}N_{15}O_{24}$ by HRESIMS. The molecular formula indicated further C1 homologation with respect to homophymine C, that also in this case was referred to the end group, as the combined analysis of 2D NMR spectra indicated the presence of the same amino acid spin systems found in homophymines B and C. The analysis of the ^{1}H NMR spectrum indicated the presence of two doublet methyl signals (0.87, d, J = 6.7 Hz and 0.91, d, J = 6.7 Hz) that replace the methyl triplet signal at 0.89 observed in the ^{1}H NMR spectrum of homophymine A, suggesting the presence of an isopropyl group as the terminal appendage of the end group. The presence of an isopropyl group was clearly inferred by COSY data that indicated the scalar coupling of both methyl doublets with a methine signal at δ 1.70, this latter in turn coupled with two diastereotopic methylene protons at δ 1.23 and 0.94. Starting from this methylene, COSY, HMQC and HMBC indicated a C-1/C-6 spin system identical to that present in homophymines A–C (Fig. 1). Therefore the new 3-hydroxy-2,4,6,8-tetramethylnonanoic acid (HTEMNA) end group was established. The stereochemistry of amino acid residues was established by Marfey's methods whereas *J*-based NMR configurational analysis allowed us to define the relative stereochemistry for HTEMEA (see ESI†).

As deduced from HRESIMS data and NMR data homophymine D1 (**4a**) (HRESIMS *m/z* 1625.9773 $[M + H]^+$) differs from the homophymine D for the replacement of 4-amino-2,3-dihydroxy-1,7-heptandioic acid (ADHA) with the 4-amino-6-carbamoyl-2,3-dihydroxyhexanoic acid residue.

The HRESIMS data of homophymine E (**5**) (*m/z* 1640.9782 $[M + H]^+$) indicated the presence of an additional carbon atom with respect to homophymine D. Also in this case the difference with respect to the other homophymines is the β-hydroxy acid that acylates the *N*-terminus. After subtracting the signals relative to the methyls ascribable to amino acid spin systems (Leu, ThrOMe, DiMe-Gln, AHDMHA) four methyl doublets and one methyl triplet appeared in the high field region of the ^{1}H NMR spectrum. A spin system ascribable to a sec-butyl subunit was evidenced from the COSY spectrum. Even if no scalar coupling was observed between H_2-7 and H-8, the new spin system as 3-hydroxy-2,4,6,8-tetramethyldecanoic acid (HTMDA) was easily inferred from analysis of the COSY and HMBC correlation depicted in Fig. 1.

The new end group has an additional stereogenic centre with respect with the other end groups found in homophymines. To assign the relative configuration of the five stereogenic centres, three contiguous and two methylene-spaced, we used Murata's method.[12,13] The pattern of homonuclear and heteronuclear coupling constants was almost superimposable to the corresponding values found for the 3-hydroxy-2,4,6-trimethyloctanoic acid (HTMOA) residue in homophymine A, indicating the same 2,3-*anti*-3,4-*anti*-4,6-*syn* relative configuration (Fig. 2). Stereochemistry at positions C6 and C8 were related through the intervening C7 diastereotopic methylene protons. The low-field proton at C7 (H7a) showed a large (9.9 Hz, anti) $^{3}J_{HH}$ to H6, whereas the high-field proton (H7b) showed a small (3.0 Hz, gauche) $^{3}J_{HH}$ to H6. Small $^{3}J_{HH}$ were observed between H7a/H8 and H7b/H8. Additionally, small (~1 Hz) $^{3}J_{CH}$ were observed from H7a to C5 and from H6 to C8, and a large $^{3}J_{CH}$ (6.2 Hz) from H7b to Me at C8 (C14) supported the relative orientation depicted (6,8-*syn*). Analysis of the NOESY spectra revealed a series of correlations that corroborate the proposed stereochemistry.

Absolute stereochemistry of amino acid residues was determined by Marfey, GITC and chiral HPLC analysis as described for homophymine B.

As deduced from HRESIMS and NMR data homophymine E1 (**5a**) (HRESIMS *m/z* 1639.9832 $[M + H]^+$) differs from the homophymine E (**5**) by the replacement of 4-amino-2,3-dihydroxy-1,7-heptandioic acid (ADHA) with the 4-amino-6-carbamoyl-2,3-dihydroxyhexanoic acid residue.

Biological evaluation

In a first attempt, the antiproliferative activity of homophymines was determined on a wide panel of cell lines that includes human and simian cancer and non-cancer cells. Table 1 reports the observed IC_{50} values, in the range 2–100 nM, that evidenced a potent cytotoxic activity. A comparison of the activity against different tumor cell lines showed a moderate selectivity toward human prostate (PC3) and ovarian (OV3) carcinoma. When sensitive and their resistant counterpart cell lines were compared (MCF7/MCF7R, HCT116/HCT15, HL60/HL60R), no significant difference was observed, indicating that overexpression of P-gp (MDR1, ABCB1) did not affect the intracellular accumulation of homophymines and therefore evidences that they are not substrates for the efflux pump.

Concerning the structure–activity relationship it is possible to point that homophymines A1–E1, featuring the 4-amino-6-carbamoyl-2,3-dihydroxyhexanoic acid residue, exert stronger potency, when compared with the corresponding A–E in which the same residue is in its carboxy form. This assay based on a 3-day exposure to the compounds is a mixed figure between antiproliferative and acute toxic activity. To discriminate between these two biological effects, we decided to use the quiescent EPC cell line and the IC_{50} values obtained (Table 1, last line) were in

Table 1 IC_{50} (nM) of homophymines A–E (**1–5**) and A1–E1 (**1a–5a**) against various cell lines[a]

	Compounds									
Cell line	**1**	**1a**	**2**	**2a**	**3**	**3a**	**4**	**4a**	**5**	**5a**
KB	7.3	7.1	18.0	16.4	8.5	6.8	12.7	10.6	6.0	12.5
MCF7	23.6	12.4	16.8	14.2	8.8	6.3	19.6	3.5	14.2	3.9
MCF7R	22.9	13.5	26.3	12.3	10.8	5.4	37.7	3.5	15.6	7.1
HCT116	6.0	6.1	13.8	11.4	4.9	2.7	19.8	1.8	5.5	2.3
HCT15	22.5	13.5	22.9	14.1	19.2	17.2	43.2	11.4	27.2	10.1
HT29	70.0	30.9	101.9	93.8	62.8	38.2	81.3	32.2	35.1	31.8
OVCAR8	5.4	5.1	8.0	6.5	4.3	2.6	8.1	1.6	4.6	4.0
OV3	7.5	5.5	9.9	8.0	3.7	2.4	10.6	1.4	4.2	2.7
PC3	4.2	3.7	6.2	4.7	3.0	2.6	6.3	1.4	3.9	3.5
Vero	5.0	6.1	8.6	6.1	4.2	3.1	10.9	1.8	7.0	4.4
MRC5	11.0	7.8	17.1	10.2	16.8	8.0	16.9	10.5	9.5	12.3
HL60	24.1	17.3	43.1	18.7	23.0	14.6	29.6	13.1	23.3	20.5
HL60R	22.4	11.1	36.7	25.8	23.5	17.1	24.9	21.9	21.4	23.2
K562	24.0	12.8	26.7	16.6	22.5	11.9	35.3	12.9	22.2	17.8
PaCa	31.4	19.2	62.0	22.2	25.9	14.4	37.4	17.6	18.1	10.6
SF268	9.9	6.3	17.2	11.7	13.6	7.1	17.9	7.9	8.1	10.1
A549	8.3	6.0	19.8	8.6	8.3	6.2	13.8	5.0	9.6	11.4
MDA231	10.9	8.4	17.0	18.2	16.2	15.8	18.9	11.1	13.3	20.0
MDA435	39.0	27.0	40.1	29.5	35.0	20.3	49.9	23.4	38.3	37.0
HepG2	68.6	91.4	99.0	100.3	72.1	58.6	78.7	80.4	60.5	62.8
EPC	5.0	7.8	8.0	6.6	9.3	12.2	11.1	7.7	9.5	29.0

[a] The values are the mean of duplicate experiments.

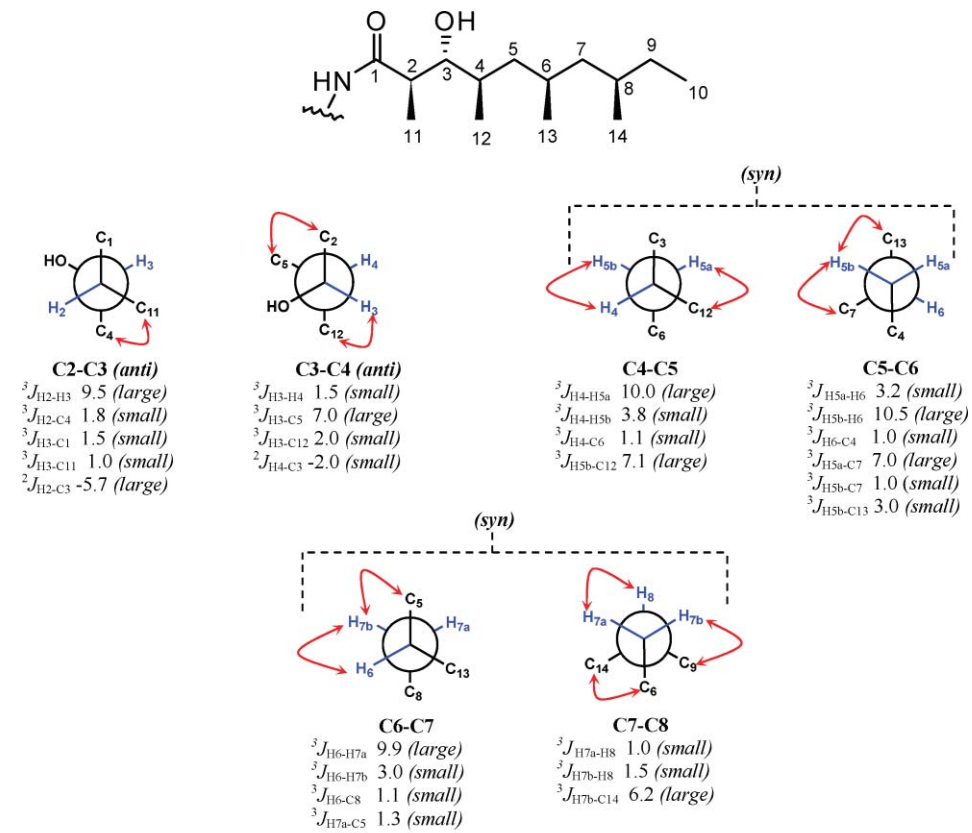

Fig. 2 Relative configuration for HTMDA residue in homophymine E (**5**).

the same low nanomolar range, indicating that homophymines display a toxic rather than an antiproliferative activity.

The cell viability/number was measured as MTS reduction and LDH release assays on HL60 cells.[14] After a 24 h exposure to 20 and 50 nM homophymines, the MTS assay indicated that the cells were only moderately affected by 20 nM homophymines, whereas a 50 nM dose caused a marked reduction in cell viability/number (panel A in Fig. 3).

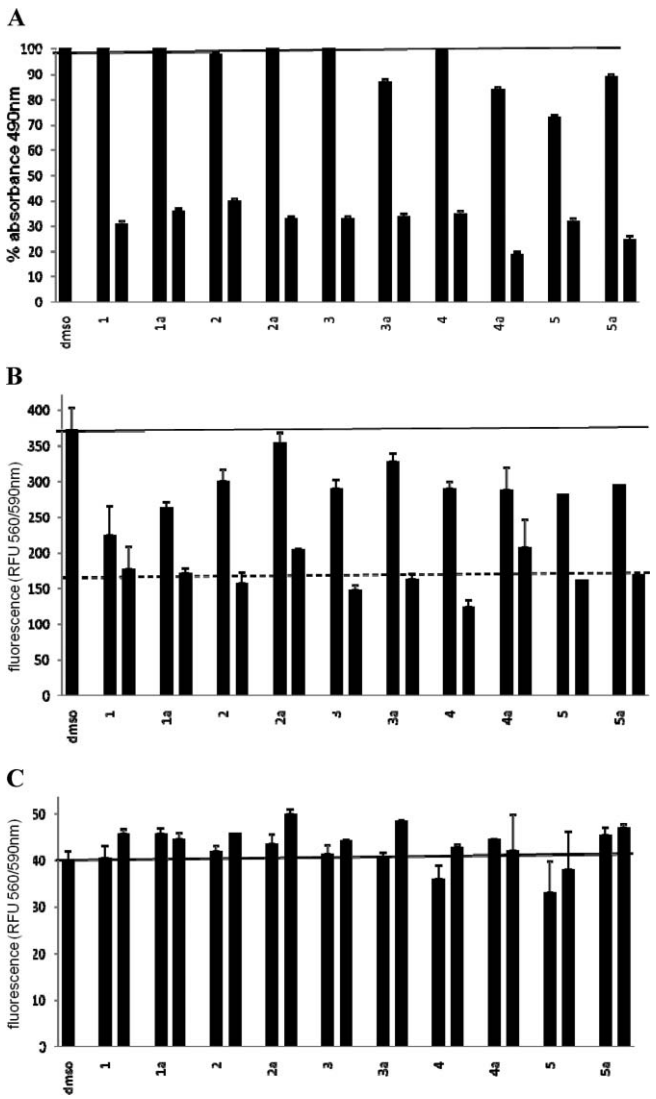

Fig. 3 Effect of homophymines on the proliferation/viability of HL60 cells treated for 24 h with 20 nM (left bars) and 50 nM (right bars) homophymines. **Panel A**: MTS reduction assayed in microplates. Results are expressed as the percentage of absorbance at 490 nm compared with cells treated with DMSO only. Values are the mean ± SE for three experiments. **Panel B**: release of LDH assayed in the culture medium after lysis of cells and the fluorescence units measured with em = 560 nm and ex = 590 nm. Results are expressed in fluorescence units and values are the mean ± SE for three experiments. **Panel C**: release of LDH assayed in the culture medium of non-lysed cells.

A similar behaviour was observed in the LDH release by lysed cells that was indicative of the total cells number in each well (panel B in Fig. 3). However when the LDH release was evaluated in non-lysed cells, no effect was observed (panel C in Fig. 3). A comparable figure was obtained when cells were exposed for 48 h to 50 or 100 nM homophymines (data not shown). This suggests that cell death occurred rapidly after the homophymine addition. Several cell death pathways can be explored with inhibitors: z-VAD-fmk is a potent inhibitor of caspases3/7 which totally prevents its activation and activity and thus irreversibly blocks the apoptotic pathway at a dose of 50 μg/mL (data not shown). When VAD alone was added to HL60 cells, proliferation and viability remained unchanged. When given in association with 25 nM homophymine C1 (**3a**), homophymine D1 (**4a**) or 100 nM homophymine B (**2**), VAD moderately increased the toxicity of homophymines, suggesting that the cell death pathway is caspase-independent (Fig. 4). Similarly, 2 mM 3-methyladenine has been reported to abrogate autophagy but did not reduce the toxic effect of homophymines. Lastly, necrostatin-1 is a selective blocker of necroptosis: 50 μM necrostatin-1 had no additive effect on the reduction of proliferation and viability elicited by homophymines in treated HL60 cells. To confirm the lack of effect of homophymines on caspases 3/7 activation, the catalytic cleavage of Ac-DEVD-AMC was assayed in HL60 and treated for 48 h with 100 nM, 1 μM and 10 μM homophymine C1, homophymine D1 and homophymine B. At doses 100 nM and 1 μM these three homophymines had no effect, whereas at 10 μM a moderate activation could be noticed (from 1.15 to 1.96-fold). In the same conditions, 1 μM doxorubicine elicited a 12.8-fold activation of caspases 3/7 activity.

All together the biological data strongly suggest that the toxic effect of homophymines likely results from an acute direct and non specific toxicity on various human cell lines.

Experimental

For general experimental procedures and sponge collection see Zampella *et al.*[8]

Extraction and isolation

The lyophilized material (300 g) was extracted with methanol (4 × 2.5 L) at room temperature and the crude methanolic extract (70 g) was subjected to a modified Kupchan's partitioning procedure as follows. The methanol extract was dissolved in a mixture of MeOH/H_2O containing 10% H_2O and partitioned against *n*-hexane. The water content of the MeOH extract was adjusted to 30% (v/v) and partitioned against $CHCl_3$. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-BuOH. Part of the chloroform-soluble material (*ca.* 2.7 g) was fractionated by DCCC (droplet counter current chromatography, $CHCl_3$:MeOH:H_2O 7:13:8, ascending mode) and fractions of 4 mL were collected and further purified by reverse phase chromatography. Fraction 11 was purified by HPLC on a Vydac C18 column (10 μ, 250 × 10 mm, 4 mL/min) eluting with 42% aqueous acetonitrile in 0.1% TFA to afford homophymine A1 (**1a**) (23.5 mg, t_R = 7.0 min), B1 (**2a**) (12.6 mg, t_R = 6.0 min), C1 (**3a**) (25.5 mg, t_R = 9.6 min), D1 (**4**) (54.5 mg, t_R = 13.0 min). Fraction 12 was purified using the same conditions to afford homophymines A1–D1 together A–D series: A (**1**) (41.8 mg, t_R = 7.4 min), B (**2**) (13.3 mg, t_R = 6.4 min), C (**3**) (19.1 mg, t_R = 10.6 min), D (**4**) (20.1 mg, t_R = 14.8 min).

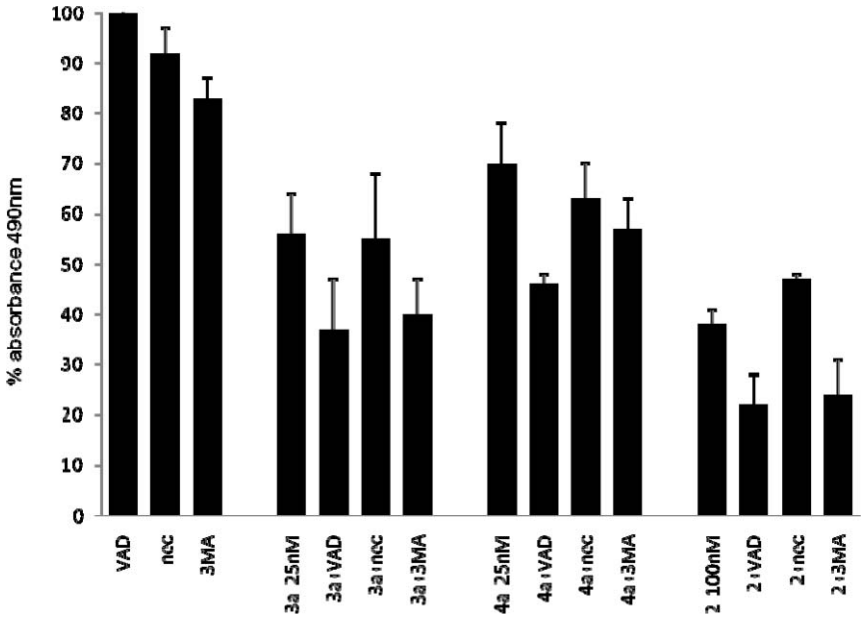

Fig. 4 Effect of VAD, necrostatin-1 and 3-methyladenine on the proliferation/viability of HL60 cells treated with homophymines. HL60 cells were plated and treated for 72 h with 25 nM homophymine C1 (**3a**), 25 nM homophymine D1 (**4a**) or 100 nM homophymine B (**2**) in the absence or the presence of 50 μM VAD, 50 μM necrostatin-1 or 2 mM 3-methyladenine. Results are expressed as the percentage of absorbance at 490 nm after addition of MTS reagent compared with cells treated with DMSO only. Values are the mean ± SE for three experiments.

Fraction 14 was purified by HPLC on a Vydac C18 column (10 μ, 250 × 10 mm, 4 mL/min) eluting with 46% aqueous acetonitrile in 0.1% TFA to afford homophymine D (**4**) (35.1 mg, $t_R = 6.0$ min) and E (**5**) (6.2 mg, $t_R = 10.4$ min).

Homophymine A1 (1a). White solid; $[\alpha]_D$ +5.21 (*c* 0.96, MeOH); tabulated NMR data in ESI†; ESI(+)MS *m/z* 1597.9 $[M + H]^+$; HRESI(+)MS: 1597.9456 $[M + H]^+$ ($C_{73}H_{129}N_{16}O_{23}$ requires 1597.9416), accurate mass error of 3.1 ppm.

Homophymine B (2). White solid; $[\alpha]_D = -1.28$ (*c* 0.57, MeOH); tabulated NMR data in ESI†; ESI(+)MS *m/z* 1584.9 $[M + H]^+$; HRESI(+)MS: 1584.9055 $[M + H]^+$ ($C_{72}H_{126}N_{15}O_{24}$ requires 1584.9100), accurate mass error of –2.84 ppm.

Homophymine B1 (2a). White solid; $[\alpha]_D = -1.48$ (*c* 0.50, MeOH); tabulated NMR data in ESI†; ESI(+)MS *m/z* 1583.9 $[M + H]^+$. HRESI(+)MS: 1583.9304 $[M + H]^+$ ($C_{72}H_{127}N_{16}O_{23}$ requires 1583.9260), accurate mass error of 2.77 ppm.

Homophymine C (3). White solid; $[\alpha]_D = +5.66$ (*c* 0.45, MeOH); tabulated NMR data in ESI†; ESI(+)MS *m/z* 1612.8 $[M + H]^+$; HRESI(+)MS: 1612.9465 $[M + H]^+$ ($C_{74}H_{130}N_{15}O_{24}$: requires 1612.9413), accurate mass error of 3.22 ppm.

Homophymine C1 (3a). White solid; $[\alpha]_D = +4.71$ (*c* 0.31, MeOH); tabulated NMR data in ESI†; ESI(+)MS *m/z* 1612.0 $[M + H]^+$. HRESI(+)MS: 1611.9538 $[M + H]^+$ ($C_{74}H_{131}N_{16}O_{23}$: requires 1611.9573), accurate mass error of –2.17 ppm.

Homophymine D (4). White solid; $[\alpha]_D = +4.19$ (*c* 0.36, MeOH); tabulated NMR data in ESI†; ESI(+)MS *m/z* 1627.0 $[M + H]^+$. HRESI(+)MS: 1626.9521 $[M + H]^+$ ($C_{75}H_{132}N_{15}O_{24}$: requires 1626.9570), accurate mass error of –3.01 ppm.

Homophymine D1 (4a). White solid; $[\alpha]_D = +1.94$ (*c* 0.65, MeOH); tabulated NMR data in ESI†; ESI(+)MS *m/z* 1626.0 $[M + H]^+$. HRESI(+)MS: 1625.9773 $[M + H]^+$ ($C_{75}H_{133}N_{16}O_{23}$: requires 1625.9729), accurate mass error of 2.70 ppm.

Homophymine E (5). White solid; $[\alpha]_D = +5.46$ (*c* 0.43, MeOH); tabulated NMR data in ESI†; ESI(+)MS *m/z* 1640.8 $[M + H]^+$. HRESI(+)MS: 1640.9782 $[M + H]^+$ ($C_{76}H_{134}N_{15}O_{24}$: requires 1640.9726), accurate mass error of 3.40 ppm.

Homophymine E1 (5a). White solid; $[\alpha]_D = +3.18$ (*c* 0.62, MeOH); tabulated NMR data in ESI†; ESI(+)MS *m/z* 1640.0 $[M + H]^+$. HRESI(+)MS: 1639.9832 $[M + H]^+$ ($C_{76}H_{135}N_{16}O_{23}$ requires 1639.9886), accurate mass error of –3.29 ppm.

Determination of the absolute configuration

General procedure for peptide hydrolysis. Peptide samples (200 μg) were dissolved in degassed 6 N HCl (0.5 mL) in an evacuated glass tube and heated at 160 °C for 16 h. The solvent was removed *in vacuo* and the resulting material was subjected to further derivatisation.

General procedures for LC-MS analysis of Marfey's (FDAA) derivatives. A portion of the hydrolysate mixture (800 μg) or the amino acid standard (500 μg) was dissolved in 80 μL of a 2:3 solution of TEA:MeCN and treated with 75 μL of 1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA) in 1:2 MeCN:acetone. The vials were heated at 70 °C for 1 h, and the contents were neutralised with 0.2 N HCl (50 μL) after cooling to room temperature. An aliquot of the L-FDAA derivative was dried under vacuum, diluted with MeCN–5% HCOOH in H_2O (1:1), and separated on a Vydac C18 (25 × 1.8 mm i.d.) column by means of a linear gradient from 10% to 50% aqueous acetonitrile containing 5% formic acid and 0.05% trifluoracetic acid, over 45 min at 1 mL/min. The RP-HPLC system was connected to the electrospray ion source by inserting a splitter valve and the

flow going into the mass spectrometer source was set at a value of 100 μL/min. Mass spectra were acquired in positive ion detection mode (*m/z* interval of 320–900) and the data were analyzed using the suite of programs Xcalibur (ThermoQuest, San José, California); all masses were reported as average values. Capillary temperature was set at 280 °C, capillary voltage at 37 V, tube lens offset at 50 V and ion spray voltage at 5 V.

Retention times (min) of FDAA-amino acids are given in parentheses: D-Pip (26.3), L-Pip (27.8), D-Leu (36.5), L-Leu (31.3), D-*allo*ThrOMe (23.1), L-*allo*ThrOMe (18.3), D-ThrOMe (24.8), L-ThrOMe (19.6), D-Orn (8.6), L-Orn (11.1).

To determine the absolute configuration of 3,4-diMeGln, 4-amino-2,3-dihydroxy-1,7-heptandioic acid (ADHA) or 4-amino-6-carbamoyl-2,3-dihydroxyhexanoic acid and 2-amino-3-hydroxy-4,5-dimethylhexanoic acid (AHDMHA), an authentic sample of homophymine A was used as a standard. The hydrolysate of homophymine A contained: (2*R*,3*R*,4*S*)-4-amino-2,3-dihydroxy-1,7-heptandioic acid (19.5), (2*S*,3*S*,4*R*)-3,4-diMeGlu (20.6) and (2*R*,3*R*,4*R*)-2-amino-3-hydroxy-4,5-dimethylhexanoic acid (33.5).

The hydrolysate of homophymines B–E (**2–5**) and of homophymines A1–E1 (**1a–5a**) contained: L-Pip, D-Orn, L-Leu, L-ThrOMe, (2*S*,3*S*,4*R*)-3,4-diMeGlu, (2*R*,3*R*,4*S*)-4-amino-2,3-dihydroxy-1,7-heptandioic acid and (2*R*,3*R*,4*R*)-2-amino-3-hydroxy-4,5-dimethylhexanoic acid as determined by co-elution with corresponding standards.

LC-MS analysis of GITC derivatives. Triethylamine (10 μL) and a GITC (2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl isothiocyanate) solution (50 μL, made with 3.9 mg/mL in MeCN) were added to the acid hydrolysate (100 μg) of homophymines B–E (**2–5**) and of homophymines A1–E1 (**1a–5a**) or homophymine A, or an authentic amino acid standard (100 μg). The reaction mixture was kept for 30 min at room temperature, and then the reaction was quenched by adding 40 μL of MeCN in 5% AcOH in H_2O (1:1). An aliquot was dried under vacuum and then diluted with the same solvent mixture and subjected to LC-MS analysis as described above, except for monitoring the absorption at 254 nm. Retention times (min): L-*N*MeGlu from homophymine A (25.3), L-Asp (16.9), D-Asp (17.9). The hydrolysate of **2–5** and **1a–5a** gave peaks for L-*N*MeGlu and D-Asp.

Chiral HPLC analysis. The acid hydrolysate of homophymines (aliquot of 10 μL) was analysed by chiral HPLC on a Phenomenex D-penicillamine column [Chirex phase 3126 (D) (150 × 4.6 mm)]. The identity of serine in the acid hydrolysate was confirmed by comparison of its retention times with those of authentic standards using HPLC under the following conditions: mobile phase, 2 mM $CuSO_4$, flow rate 1.0 mL/min; detection UV 254 nm; retention times of the standards (min): L-Ser (5.7), D-Ser (5.1). The hydrolysate of homophymines B–E (**2–5**) and of homophymines A1–E1 (**1a–5a**) contained D-serine.

Biological evaluation

Cell culture. Cell lines were purchased from ECACC (Salisbury, UK) or ATCC (LGC Standards, Molsheim, France) except when otherwise indicated. The human cell lines KB (nasopharyngeal epidermoid carcinoma), HepG2 (hepatocarcinoma), Vero (monkey kidney), MRC5 (fetal human lung) were grown in D-MEM medium supplemented with 10% fetal calf serum, in the presence of penicilline, streptomycine and fungizone in 75 cm^2 flask under 5% CO_2. HCT116, HCT15, HT29 (colon adenocarcinoma) and MCF7 (breast adenocarcinoma) were given by Dr Matthias Kassack (Bonn University, Germany), MDA435 and MDA231 (breast adenocarcinoma), HL60 (promyeocytic leukaemia), K562 (chronic myelogenous leukaemia) SK-OV3 (ovary adenocarcinoma from NCI), OVCAR-8 (ovary adenocarcinoma), PC-3 (prostate adenocarcinoma), A549 (lung carcinoma), MiaPaCa (pancreas carcinoma) and SF268 (glioblastoma from NCI) were grown in RPMI medium. Resistant MCF7 and resistant HL60 cells were obtained by prolonged treatment with doxorubicin. EPC cells (carp epithelium) provided by Dr Bremond (INRA France) were grown in Stocker-MacPherson MEM at room temperature.

Cell proliferation assay. Cells were plated in 96-well tissue culture plates in 200 μL medium and treated 24 h later with compounds dissolved in DMSO; compound concentrations ranged from 0.025 nM to 6 μM and were prepared by use of a Biomek 3000 (Beckman). Control cells received the same volume of DMSO (1% final volume). After 72 h exposure to the drug, MTS reagent (Cell titer Aqueous, Promega, Madison, WI) was added and incubated for 3 h at 37 °C: the absorbance was monitored at 490 nm and results are expressed as the inhibition of cell proliferation calculated as the ratio [1 – (OD_{490} treated/OD_{490} control)×100]. For IC_{50} determinations (50% inhibition of cell proliferation) experiments were performed in duplicate.

Caspase activity assay. Caspases3/7 activity was assayed in HL60 cell after treatment with chemicals for 48 h. HL60 cells (20 000 cells/well in 180 μL RPMI medium) were plated in black 96-well culture microplates and treated with homophymine D1, homophymine C1 and homophymine B at final concentrations of 100 nM, 1 μM and 10 μM. Plates were kept under 5% CO_2 for 48 h. Lysis buffer (20 μL of a 10× stock solution), consisting of 250 mM Hepes (pH7.5), 5 mM EDTA, 0.5% NP40, 0.1% SDS and 50 mM dithiothreitol, was added with DEVD-AMC at a final concentration of 50 μM. Plates were incubated at 37 °C and fluorescence was recorded (exc 360 nm, em 435 nm) after 0, 30, 60, 120, and 180 min. Reaction rates were calculated from the slope of the linear time-dependent reaction and are expressed as the fold-activation relative to the control (HL60 with DMSO alone). 10^{-6} M doxorubicin was used as a positive control.

LDH release assay. The release of cytoplasmic LDH into the culture medium was estimated with the Cytotox-ONE reagent from Promega (Madison, WI). HL60 cells (20 000 cells/well in 100 μL RPMI) were plated in 96-well culture microplates and treated with compounds at final concentrations of 20 and 50 nM. Plates were kept under 5% CO_2 for 24 and 48 h. One set of cells was lysed with 2 μL 9% Triton X-100 for 30 min at room temperature under orbital agitation. Lysed and non-lysed cells were spun down for 1 min at 500 g and 25 μL supernatant removed for LDH activity: 25 μL of reagent was added and incubated for 20 min at room temperature in the dark. The fluorescent signal was monitored with a Paradigm microplate reader (Beckman) with excitation set at 560 nm and emission at 590 nm.

Conclusion

Homophymines A–E and A1–E1 represent further examples of bioactive natural cyclodepsipeptides featuring a complex molecular architecture. As found in some lipopeptides of bacterial origin,[15] the main distinction between the members of this family is the length of the side chain. The PKS involved in the formation of the homophymine side chain could be an iteratively acting enzyme with the intrinsic capacity to produce polyketide chains of varying length.[16,17]

The rationale of this observed slight structural variability in the same sponge should be explained with the need of a modulation of the toxic activity. In particular the replacement of OH with an NH_2 group in the A1–E1 series invariably produces an improvement in cytotoxicity.

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