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Homophymine A, an Anti-HIV Cyclodepsipeptide from the Sponge *Homophymia* sp.

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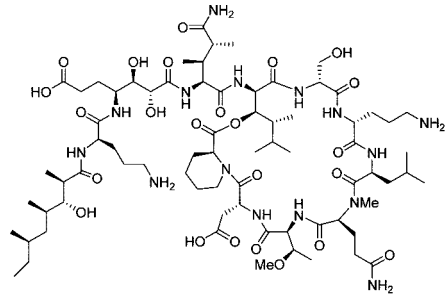

Homophymine A

A new anti-HIV cyclodepsipeptide, homophymine A, was isolated from a New Caledonian collection of the marine sponge *Homophymia* sp. The structure of homophymine A was determined by interpretation of spectroscopic data, acid hydrolysis, and LC-MS analysis. Homophymine A contains 11 amino acid residues and an amide-linked 3-hydroxy-2,4,6-trimethyloctanoic acid moiety. Along with four D-, two L-, and one *N*-methyl amino acids, it also contains four unusual amino acid residues: (2*S*,3*S*,4*R*)-3,4-diMe-Gln, (2*R*,3*R*,4*S*)-4-amino-2,3-dihydroxy-1,7-heptandioic acid, L-ThrOMe, and (2*R*,3*R*,4*R*)-2-amino-3-hydroxy-4,5-dimethylhexanoic acid. In a cell-based XTT assay, homophymine A exhibited cytoprotective activity against HIV-1 infection with a IC_{50} of 75 nM.

Introduction

Sponges in the order Lithistida have been a prolific source of new peptides with unusual structures and remarkable biological activity.[1] Within them is a growing family of cyclic depsipeptides, all displaying potent antiviral activity. The first to be identified was callipeltin A (Figure 1),[2] isolated, along with other inactive congeners,[3] in 1996 from the New Caledonian sponge *Callipelta* sp. Papuamides A−D[4] and theopapuamide[5] were obtained from the Papua New Guinea collections of *Theonella* species, while the Papua New Guinea sponge *Neamphius huxleyi* provided neamphamide A (Figure 1). Mirabamides, isolated from the Micronesian sponge *Siliquariaspongia mirabilis* represent the latest, very recent example of this class.[6] The distinguishable feature of these metabolites is the presence of previously unknown amino acid residues, such as (2*S*,3*S*,4*R*)-3,4-dimethylglutamine (diMeGln), common to all

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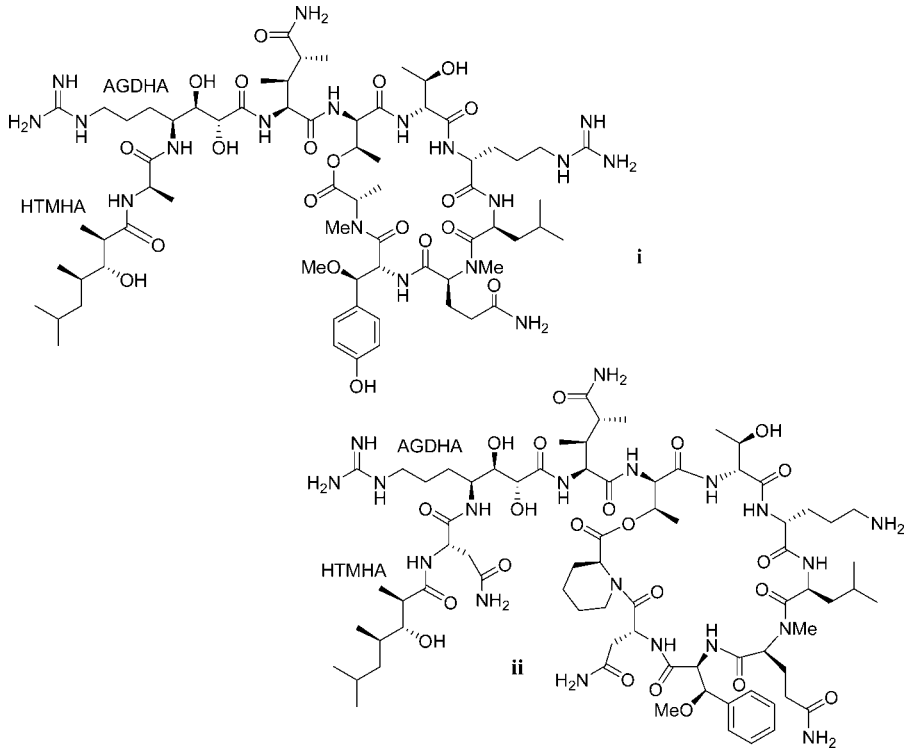


FIGURE 1. Antiviral marine cyclodepsipeptides: **i**, callipeltin A; **ii**, neamphamide A.

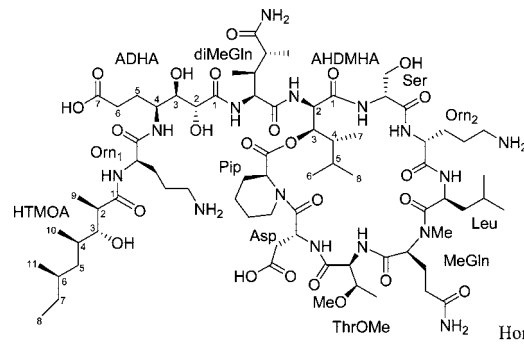
metabolites, and β-methoxytyrosine (β-OMeTyr), a unit of callipeltins A−C, papuamides, and neamphamide A,[7] and (2*R*,3*R*,4*S*)-4-amino-7-guanidino-2,3-dihydroxyheptanoic acid (AGDHA), present in callipeltin A and in neamphamide A. Also noteworthy is the acylation of the N-terminal unit with unique polyketide-derived β-hydroxyacid moieties. All the nonproteinogenic units in callipeltins and papuamides were obtained through stereoselective synthesis, which afforded suitable building blocks for the total synthesis and useful standards for stereochemical assignments.[8–22] The total syntheses of callipeltins B, D, and E, smaller analogues of callipeltin A, were recently achieved.[23]

Results and Discussion

In routine prescreening of sponge extracts we found that different extracts obtained from a new species of *Homophymia* collected in New Caledonia had moderate cytotoxicity or anti-HIV activity.

There was only one prior chemical study of *Homophymia* in the literature, and it described a tetramic acid acyl glycoside.[24] Anti-HIV bioassay-guided fractionation of the organic extracts of *Homophymia* sp. resulted in the isolation of a new depsiundecapeptide, designated homophymine A (**1**),[25] as the principal active constituent.

The freeze-dried sponge was extracted with H_2O. After lyophilization the residue was extracted with methanol and the methanol soluble material was subjected to gel-permeation on Sephadex LH-20 followed by HPLC, using a reversed-phase column to furnish 155 mg of **1**.



Homophymine A (**1**)

High-resolution LSIMS of homophymine A (**1**) gave an $[M + H]^+$ peak at *m/z* 1598.9307, corresponding to a molecular formula of $C_{73}H_{127}N_{15}O_{24}$. The peptidic nature of homophymine A was evident from an abundance of amide NH protons (δ 6.45−9.28) in the ^1H and carbonyl carbons (δ 170.2−178.8) in

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the ^{13}C NMR spectra obtained in CD_3OH. The presence of one *N*-methylated and one methoxy-bearing amino acid residue was suggested on the basis of the characteristic ^{1}H and ^{13}C chemical shifts of an *N*-methyl group at δ_H 3.03 (δ_C 31.2) and an *O*-methyl group at δ_H 3.27 (δ_C 56.9). A $^{15}N-^{1}H$ HSQC experiment showed two pairs of signals for two primary amide groups. Extensive interpretation of 2D NMR data obtained by gradient enhanced versions of COSY45, TOCSY, HSQC, HMBC, and ROESY experiments (Table 1) established the presence of leucine, serine, aspartic acid and two ornithine residues. Four uncommon amino acids were also elucidated: 3,4-dimethylglutamine (3,4-diMeGln), *N*-methylglutamine (*N*MeGln), pipecolic acid (Pip), and *O*-methylthreonine (ThrOMe). This latter unusual amino acid residue was found in perthamide B, a cyclic peptide from the Lithistida sponge *Theonella* sp.[26]

An amide-linked 3-hydroxy-2,4,6-trimethyloctanoic acid (HTMOA) was also elucidated. The complete proton spin system was deduced from COSY and TOCSY data (Figure 2). Key HMBC correlations corroborated the proposed structure. The HTMOA moiety, which is simply a C-1 homologue of the β-hydroxy acid end group in callipeltin A and neamphamide A (3-hydroxy-2,4,6-trimethylheptanoic acid, HTMHA), has already been found as the end group of the related theopapuamide.[5]

Further analysis of the 2D NMR data revealed the presence of a second spin system closely related to the AGDHA residue present in callipeltin A and in neamphamide A. As in AGDHA, a 2,3-dihydroxy-4 amino acid subunit was evidenced by the presence of three contiguous methines substituted by two oxygen and one nitrogen functionality. A carbonyl group was placed at C1 on the basis of HMBC cross peaks (Figure 2) between the hydroxymethine protons at C2 and C3 (δ_H 3.96 and 3.68, respectively) with a carbonyl at δ 176.1 ppm. COSY and TOCSY (Figure 2) analysis indicated that the 4-amide-linked proton (δ_H 4.11, δ_C 50.7) is adjacent to two consecutive methylene groups at δ_H 1.83−1.92 and 2.32−2.38. In the HMBC spectrum both methylene H_2-5 and H_2-6 showed cross-peak correlations with an acyl signal at δ 177.0, disclosing a 4-amino-2,3-dihydroxy-1,7-heptandioic acid (ADHA) residue.

The last residue in homophymine A (**1**) was identified as 2-amino-3-hydroxy-4,5-dimethylhexanoic acid (AHDMHA), which is unprecedented in natural peptides. Analysis of the proton spin system of this residue was complicated by the absence of scalar coupling between H2 and H3 (Figure 2) and by the fortuitous coincidence of the chemical shifts of H4 and H5. HMBC data (Figure 2) substantiated the proposed structure. In particular the linkage between the α amino acid carbon (δ_H 5.31, δ_C 55.2) and an hydroxy methine carbon (δ_H 5.61, δ_C 77.3) was inferred from the H2/C3 and H3/C2 cross peaks. The involvement of the β-hydroxy group of this residue in an ester linkage was suggested by a typical downfield shift of a β-hydroxymethine proton (δ_H 5.61).

The placement of the acyl moiety and the alignment of amino acid residues from an Orn *N*-terminus to ThrOMe was secured from the inter-residue NOE NH/CHα and NCH_3/CHα interactions and HMBC correlations acquired in CD_3OH (Table 1 and Figure 3).

The diagnostic HMBC cross peak between the β-proton of the AHDMHA residue and a carbonyl resonating at δ_C 170.2 confirmed the presence of a macrocycle lactone in homophymine A. However, the fortuitous coincidence in ^{1}H (δ_H 5.25 for Hα) and ^{13}C (δ_C 170.2, C1) resonances in the remaining two residues, pipecolic acid (Pip) and aspartic acid, left the assignment of the *C*-terminus not straightforward. An intense NOE between the amidic proton belonging to the Asp spin system and Hα of the ThrOMe residue established the linkage of Asp to ThrOMe and indirectly revealed the participation of the pipecolic acid carbonyl group in the lactone formation with the β-hydroxyl group of the AHDMHA residue.

Definitive confirmation of the homophymine A gross structure was derived from MS/MS analysis of its acyclic methyl ester, obtained by treatment of the parent peptide with sodium methoxide in dry methanol. In addition to the pseudomolecular ion at *m*/*z* 1630.74 $[M + H]^+$, corresponding to the introduction of 32 mass units (MeOH) in the molecule, the ESI Q-TOF spectrum provided several fragment ion peaks which were consistent with the amino acid sequence of homophymine A (Figure 4). The major peaks correspond to *N*-terminus fragments due to the cleavage of the amide bond, referred to as the b series in Roepstorff and Fohlman nomenclature.[27]

The detailed interpretation of the fragmentation pattern of the acyclic methyl ester confirmed both the sequence of amino acids and the identity of the amidic-bearing amino acid residues. In particular, the loss of 115.02 amu from the b_{10} fragment was indicative of the presence of an aspartic acid, the loss of 142.07 amu from b_8 was consistent with an *N*-methylglutamine residue, and the presence of a y_{10} fragmentation peak at 1332.70 supported the presence of a 4-amino-2,3-dihydroxy-1,7-heptandioic acid residue.

The absolute stereochemistry of Leu, Orn, and Pip residues was determined by complete acid hydrolysis of homophymine A (6 N HCl, 110 °C, 12 h) and Marfey's analysis.[28] The acid hydrolysate was derivatized with (1-fluoro-2,4-dinitrophenyl)-5-L-alaninamide (L-FDAA), and then LC-MS comparison of the derivatives from the parent peptide with the FDAA derivatives of appropriate standards established the presence of L-Leu, two D-Orn, and L-Pip. Under Marfey's conditions D- and L-serine showed the same retention time. Therefore the D configuration of this residue was determined by chiral HPLC analysis of the hydrolysate mixture.

To determine the absolute configuration of 3,4-diMeGln an authentic sample of callipeltin A was used as a standard. The L-FDAA derivative of 3,4-diMeGlu from homophymine A coeluted during LC-MS analyses with the equivalent standard

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TABLE 1. ^{1}H and ^{13}C NMR (700 MHz, CD_3OH) of Homophymine A (1)[a]

residue	δ_H	δ_C, (m)	δ_N	HMBC	NOE[b]
HTMOA					
1		178.8 (C)			
2	2.66, m	44.5 (CH)		1, 3, 4, 9	Orn_1-NH
3	3.54, dd (*9.3, 2.3*)	79.3 (CH)		1, 2, 4, 9, 10	
4	1.77, ovl	33.1 (CH)		2, 3, 5, 10	
5	1.09−1.32, ovl	36.9 (CH_2)		3, 4, 6, 10	
6	1.47, ovl	32.4 (CH)		4, 5, 7, 11	
7	1.01, 1.49, ovl	28.3 (CH_2)		5, 6, 8	
8	0.89, t (*7.0*)	11.0 (CH_3)		6, 7	
9	1.07, d (*6.7*)	14.5 (CH_3)		1, 2, 3	
10	1.00, d (*6.2*)	17.5 (CH_3)		3, 4, 5	
11	0.92, d (*6.7*)	20.5 (CH_3)		5, 6, 7	
Orn_1					
1		174.3 (C)			
2	4.33, m	54.6 (CH)		1, 3, 4	NH, ADHA-NH
3	1.78−1.91, ovl	29.0 (CH_2)		2, 4, 5	
4	1.78, ovl	25.0 (CH_2)		2, 3, 5	
5	3.03, ovl	40.4 (CH_2)		3, 4	
NH	8.31, d (*6.7*)		124.3	1, 2, HTMOA-1	2, HTMOA-2
NH_2	n.o.		n.o.		
ADHA					
1		176.1 (C)			
2	3.96, ovl	73.0 (CH)		1, 3, 4	DiMeGln-NH
3	3.68, ovl	74.6 (CH)		1, 2, 5	
4	4.11, ovl	50.7 (CH)		5, 6	NH
5	1.83−1.92, ovl	27.5 (CH_2)		4, 6, 7	
6	2.32−2.38, ovl	30.9 (CH_2)		5, 7	
7		177.0 (C)			
NH	7.83, br s		118.5	Orn_1-1	4, Orn_1-2
3,4-DiMeGln					
1		174.2 (C)			
2	4.41, m	58.7 (CH)		1, 3, 6, ADHA-1	AHDMHA-NH
3	2.22, m	38.0 (CH)		1, 2, 4, 6, 7	
4	2.79, ovl	41.1 (CH)		2, 3, 5, 6	
5		179.2 (C)			
6	1.08, d (*6.8*)	13.2 (CH_3)		2, 3, 4	
7	1.20, d (*7.0*)	14.9 (CH_3)		4, 5	
NH	8.97, br s		125.1		ADHA-2
NH_2	6.98, 7.55 (s)		109.3	5, 4	
AHDMHA					
1		173.4 (C)			
2	5.31, br s	55.2 (CH)		1, 3, 4	7, Ser-NH
3	5.61, br d (*10.0*)	77.3 (CH)		2, 4, Pip-1	8
4	1.95, ovl	38.5 (CH)		5, 7	
5	1.95, ovl	27.4 (CH)		3, 7, 8	
6	0.93 d (*6.8*)	21.1 (CH_3)		5, 8	
7	0.76 d (*6.8*)	8.7 (CH_3)		3, 4, 5	2
8	0.73 d (*6.8*)	14.9 (CH_3)		5, 6	3
NH	9.28, br s		116.2		DiMeGln-2
Ser					
1		172.5 (C)			
2	3.92, ovl	60.6 (CH)		1, 3	Orn_2-NH
3	3.99−4.06, ovl	62.0 (CH_2)		1, 2	
NH	8.34, br s		115.2	1	AHDMHA-2
Orn_2					
1		172.2 (C)			
2	4.53, m	52.5 (CH)		1	Leu-NH
3	1.82−2.11, ovl	27.5 (CH_2)		2, 4	
4	1.62−1.73, ovl	24.7 (CH_2)		3, 5	
5	2.91, m	40.1 (CH_2)		3, 4	
NH	8.36, br s		116.7	Ser-1	Ser-2
NH_2	n.o.		n.o.		
Leu					
1		176.2 (C)		MeGln-NMe	
2	4.60, m	50.9 (CH)		1, 3	MeGln-NMe
3	1.40−2.36, ovl	39.2 (CH_2)		2, 4, 5	
4	2.02, ovl	25.7 (CH)		3, 5, 6	
5	1.09, d (*6.7*)	23.9 (CH_3)		3, 4	
6	1.00, d (*6.3*)	21.0 (CH_3)		4, 5	
NH	7.60, br s		120.3	2, 3	Orn_2-2
N-MeGln					
1		172.0 (C)			
2	5.45, br s	57.4 (CH)		1	

TABLE 1. Continued

residue	δ_H	δ_C, (m)	δ_N	HMBC	NOE[b]
3	1.92−2.45, ovl	23.0 (CH_2)		1, 2, 4	
4	2.15−2.25, ovl	31.6 (CH_2)		2, 3, 5	
5		177.2 (C)			
NMe	3.03, s	31.2 (CH_3)		2, Leu-1	Leu-2
NH_2	6.78−7.19 (s)		109.0	5, 4	
ThrOMe					
1		170.6 (C)			
2	4.35, d (*9.6*)	58.7 (CH)		1, 3, NMeGln-1	Asp-NH
3	4.11, ovl	76.3 (CH)		1, 4, OMe	
4	1.20, ovl	16.1, (CH_3)		2, 3	
OMe	3.27, s	56.9 (CH_3)		3	
NH	6.45, d (*8.8*)		103.2	NMeGln-1	
Asp					
1		170.2 (C)			
2	5.25, ovl	46.9 (CH)		1, 3	
3	2.49−2.84, ovl	36.1 (CH_2)		1, 2, 4	
4		174.8 (C)			
NH	8.26 br s		118.2	2, ThrOMe-1	ThrOMe-2
Pip					
1		170.2 (C)			
2	5.25, ovl	53.6 (CH)		1, 3, 4, 6	
3	1.63−2.18, ovl	27.1 (CH_2)		2, 4, 5	
4	1.22−1.74, ovl	22.3 (CH_2)		3, 5	
5	1.50−1.64, ovl	26.0 (CH_2)		3, 4, 6	
6	3.00−3.72, ovl	44.4 (CH_2)		2, 4, 5	

[a] Coupling constants extracted from 2D E.COSY spectra are in parentheses and given in Hz. Ovl: overlapped; n.o.: not observed in the ^{1}H NMR and in ^{15}N−^{1}H HSQC experiments. [b] Noe correlations were obtained by ROESY with a 500 ms mixing time.

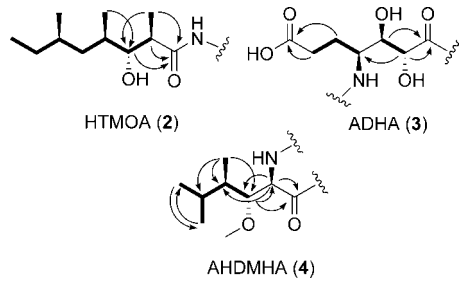

FIGURE 2. New subunits of homophymine A (**1**) with COSY/TOCSY connectivities (bold bonds) and key HMBC correlations (arrows).

derived from callipeltin A, which supported the presence of (2*S*,3*S*,4*R*)-3,4-diMe-Gln. Both diastereoisomers of ThrOMe were prepared starting from *N*-Boc L-Thr methyl ester and *N*-Boc L-*allo*Thr methyl ester by etherification with methyl triflate in 2,6-di-*tert*-butylpyridine. The so obtained standards were used in Marfey's analysis as described below, which revealed the L-configuration for ThrOMe.

The D configuration of Asp and the L configuration of *N*-MeGln were determined by LC-MS analysis of GITC derivatives[29] (see the Experimental Section).

J-based NMR configurational analysis[30,31] and chemical derivatization methods, as follow, were applied to define the absolute stereochemistry of 2-amino-3-hydroxy-4,5-dimethylhexanoic acid (AHDMHA), 4-amino-2,3-dihydroxy-1,7-heptandioic acid (ADHA), and 3-hydroxy-2,4,6-trimethyloctanoic acid (HTMOA). The $^3J_{H,H}$ coupling constants were extracted from a combination of 2D *J* resolved spectra, whereas the $^{2\text{-}3}J_{CH}$ values were measured by either HSQC-TOCSY or PFG-PS-HMBC experiments.

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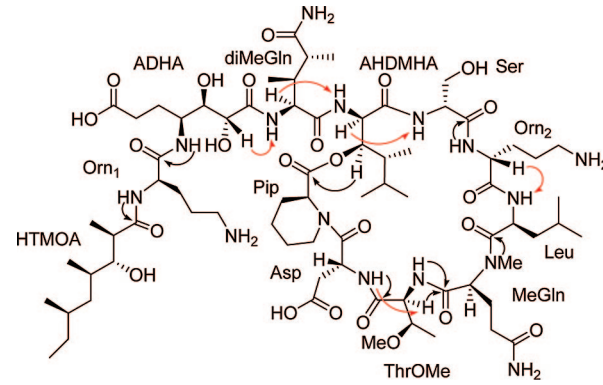

FIGURE 3. Selected NOE (red) and HMBC (black) correlations for **1**.

Absolute Configuration of 3-Hydroxy-2,4,6-trimethyloctanoic Acid (HTMOA). As depicted in Figure 5, the relative configuration of the 4 chiral centers in this unit (three contiguous and one methylene−spaced) was determined as 2,3-*anti*-3,4-*anti*-4,6-*syn*. The $^3J_{H\text{-}2,H\text{-}3}$ constant (9.3 Hz) indicated an anti orientation between these two protons, as supported by the pattern of heteronuclear couplings. Even if in this case the analysis of *J* values does not allow a discrimination between the two possible relative configurations, the NOE observed between H3 and Me-2 points to the C2−C3 *erythro* configuration. Assignment of an *anti* relationship between the adjacent hydroxyl and methyl substituents at C-3 and C-4 was determined by the small couplings observed from H-3 to Me-4, H-3 to H-4, and H-4 to C-3 and by the large couplings between H-3 and C-5, and confirmed by the pattern of the dipolar couplings.

Connectivity between the 1,3-related methyl-bearing stereocenters at C-4 and C-6 relied on an assignment of the diastereotopic methylene protons at C-5. Large scalar couplings, H-5a to H-4 and H-5b to H-6, and small couplings, H-4 to H-5b and H-5a to H-6, together with the reported heteronuclear

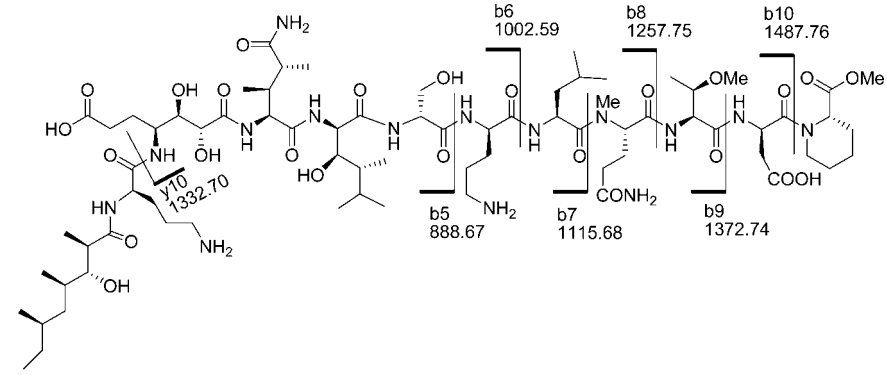

FIGURE 4. Opened methyl ester with ESI-QTOF fragmentations.

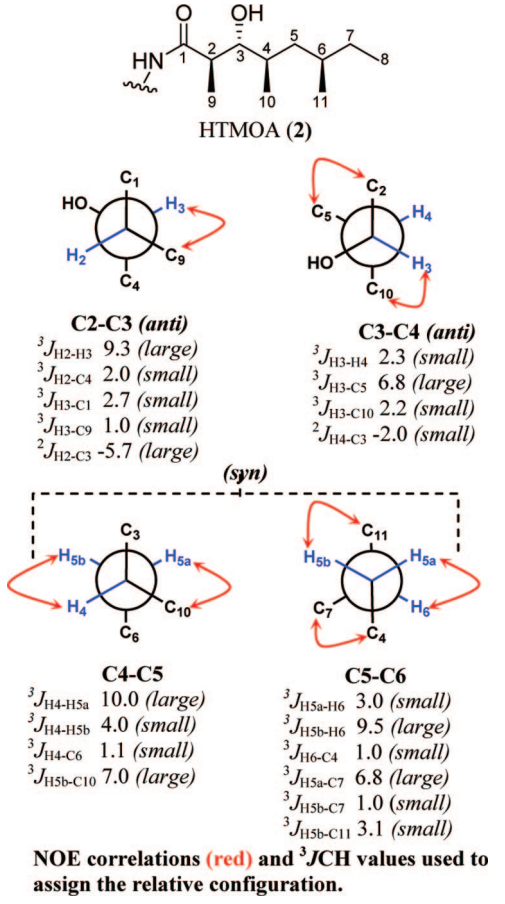

FIGURE 5. Relative configuration for the HTMOA (**2**) residue.

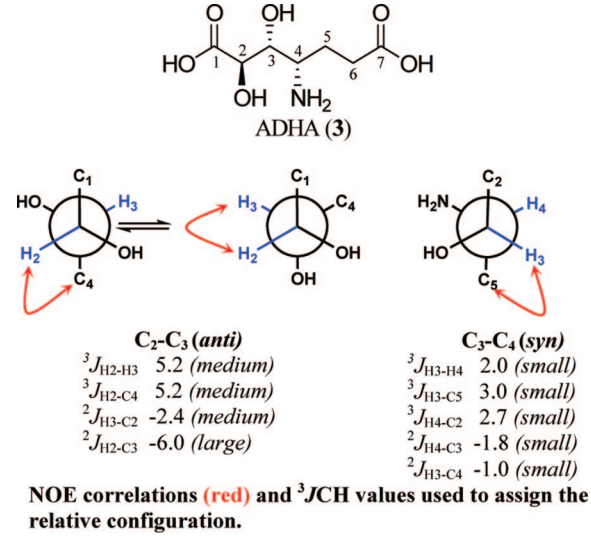

FIGURE 6. Relative configuration for the ADHA (**3**) residue.

couplings supported the relative orientation depicted (4,6-*syn*). Analysis of the NOESY spectra supported the assignment.

The dichloromethane extract of the acid hydrolysate of homophymine A (**1**) contained free HTMOA (**2**) as the major constituent. Chemical shifts and coupling constant values with respect to the C-2/C-4 portion of the molecule were almost superimposable with the corresponding values observed for the HTMHA residue in callipeltin A,[2,15–18] giving further support to the proposed relative configuration.

The absolute stereochemistry at C-3 of **2** was assigned as *R* by the modified Mosher method (see the Experimental Section), consequently a 2*R*,3*R*,4*R*,6*R* configuration for the 3-hydroxy-2,4,6-trimethyloctanoic acid was determined.

Absolute Configuration of 4-Amino-2,3-dihydroxy-1,7-heptandioic Acid (ADHA). The protons belonging to the ADHA (**3**) spin system resonated in a very crowded spectral region, hampering a reliable measurement of the homo- and heteronuclear coupling constants. Therefore, the analysis was performed on the isolated amino acid. A 30 mg sample of homophymine A was subjected to acidic hydrolysis and the crude hydrolysate was fractionated by HPLC eluting with 98% water/methanol to give the pure ADHA residue (**3**) (see the Experimental Section). The intermediate magnitude of the homonuclear coupling constant ($^3J_{H2-H3}$ = 5.2 Hz) for the C2/C3 subunit suggested a mixture of *gauche* and *anti* equilibrating rotamers at the two vicinal stereocenters. The pattern of the proton−carbon coupling constants is consistent for the *gauche*$^-$/*anti* pair of the *erythro* series (Figure 6). Key NOESY correlations between H2−H4 and H2−H3 confirmed the assignment. Assignment of a *syn* relationship between the adjacent hydroxy and amino substituents at C-3 and C-4 was determined by the small homonuclear coupling H-3 to H-4 and the small heteronuclear couplings H-3 to C-5, H-4 to C-2, H-4 to C-3, and H-3 to C-4. The *S* configuration at C-4 of the ADHA residue was determined by cleavage of the C-2, C-3 diol in **1** with sodium periodate, followed by oxidative workup and hydrolysis with 6 N hydrochloric acid and GITC derivatization, giving L-Glu.

Absolute Configuration of the 2-Amino-3-hydroxy-4,5-dimethylhexanoic Acid (AHDMHA) Residue. As for the ADHA residue the J-based configurational analysis of this

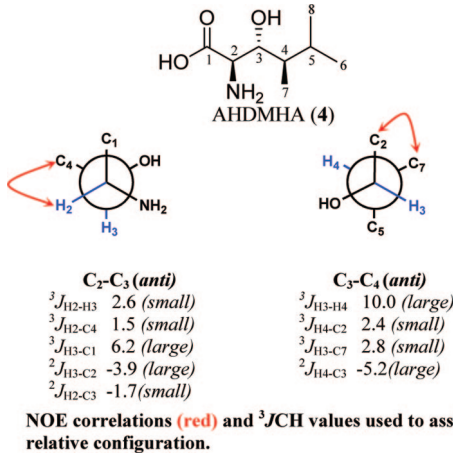

FIGURE 7. Relative configuration for the AHDMHA (**4**) residue.

residue was performed on the isolated amino acid (**4**) obtained after acid hydrolysis and HPLC fractionation of the crude hydrolysate.

The pattern of the homonuclear and heteronuclear coupling constants of the C2−C3 subunit of this unprecedented amino acid allowed the identification of a gauche$^-$ rotamer with the *erythro* arrangement (Figure 7). The 10 Hz value of the vicinal $^3J_{H3–H4}$ constant indicated an *anti* orientation between these two protons, again a case in which the two possible diasteroisomers could not be discriminated on the basis of the sole $^{2,3}J_{HX}$ values. An *erythro* arrangement between the substituents on C3 and C4 was established on the basis of a diagnostic NOE effect between H2 and Me-4.

The absolute configuration was assigned by the application of the nonempirical LC/MS advanced Marfey's method.[32] The method is based on the observation that an L-amino acid FDAA derivative is eluted from a C-18 column before its corresponding D-isomer.[32] Thus a small sample of homophymine A was hydrolyzed and derivatized with both enantiomers of the Marfey's reagent. By monitoring for FDAA-AHDMHA at *m*/*z* 418, a 2*R* configuration for the 2-amino-3-hydroxy-4,5-dimethylhexanoic acid residue was tentatively proposed. Definitive confirmation of the above stereochemical assignment could arise from the stereoselective synthesis of all possible stereoisomers of the 2-amino-3-hydroxy-4,5-dimethylhexanoic acid unit, a task currently in progress in our laboratories.

The antiviral activity of homophymine A (**1**) was measured in PBMC cell lines infected with HIV-1 (III B strain). At seven day postinfection, homophymine A (**1**) effectively inhibited the production of HIV-1 infection with an IC_{50} of 75 nM. Direct cytotoxicity of **1** against the host cells was observed with a TC_{50} (toxic concentration) of 1.19 μM. It has been proposed[6] that the antiviral activity of callipeltin A (EC_{50} 0.01 μg/mL),[2] papuamides, and neamphamide A could be related to the presence of the β-methoxytyrosine residue, conserved in all above-mentioned anti-HIV peptides but absent in the inactive theopapuamide. The retained, even if slightly attenuated, antiviral activity found in homophymine A, in which the β-methoxytyrosine is replaced by an *O*-methyl threonine, ruled out this hypothesis. The role of β-methoxytyrosine in directing the biological activities of callipeltin A and congeners was also investigated after the synthesis of desmethoxycallipeltin B, a derivative that was proved to display comparable cytotoxicity with respect to the parent compound.[33]

Conclusion

Homophymine A is a new member of a growing class of marine cyclodepsipeptides, all sharing a high degree of structural homology. Analysis of the structures of the derivatives so far isolated indicates a close relationship with respect to the type of amino acid introduced to selected positions. Despite the structural variability of the metabolites, it should be noted that the position in the peptide framework of the basic amino acids, as well as of the *N*-acylated ones, of the γ-amino acids and of the β-methoxy amino acids remains unaltered. Assuming the role of the bacterial NRPS modular multienzimatic complexes in the synthesis of these metabolites, the observed variability could arise from the presence of several isoforms of the NRPS complex, or, alternatively, to a lack of selectivity of the adenylation domains responsible for the loading and activation of the proper amino acid unit in each module.

Experimental Section

Isolation of Homophymine A (1). *Homophymia* sp. (Demospongiae, Lithistida, Corallistidae) was collected in 1992 in the shallow waters of the east coast of New Caledonia. Taxonomic identification was performed by Professor Claude Lévi, Muséum National d'Histoire Naturelle, Paris, France, where a voucher specimen is deposited under the accession number ORSTOM, R1531. Preliminary bioactivity tests on crude extracts showed antifungal activity against *Fusarium oxysporum*, *Helminthosporium sativum*, and *Phytophtora hevea* and antiyeast activity for *Candida albicans*, using disk diffusion assays. The aqueous extract also displayed cytotoxic activity against KB and P388 cells (80% inhibition at a dose of 10 μg/mL), and anti-HIV activity. The organism was freeze-dried, and the lyophilized material (287.5 g) was extracted with water for 3 h at 4 °C. After lyophilization, the aqueous residue (71.72 g) was extracted with methanol for 1 h and 30 min at 4 °C and the methanol soluble material was filtered and concentrated under reduced pressure to give 38.57 g of a brown amorphous solid. The methanolic extract was subjected to gel-permeation on Sephadex LH-20 eluting with methanol to obtain five fractions. Fraction 3 (4.24 g) containing homophymine A was further purified by HPLC on a reversed-phase Symmetry column (19 mm i.d. × 30 cm) by means of a linear gradient from 35% to 65% aqueous acetonitrile containing 0.1% trifluoroacetic acid, over 55 min at 17 mL/min to obtain 155 mg of homophymine A (R_t 16.8 min).

Homophymine A (1). $[\alpha]_D$ + 9.30 (*c* 0.48, MeOH); IR (neat) 3323, 2962, 1663 (broad), 1528, 1202 cm^{-1}; ^{1}H and ^{13}C NMR spectra in Table 1; ESI/MS *m*/*z* (%) 1598.8 [M + H]$^+$. HRMS (LSIMS) calcd for $C_{73}H_{128}N_{15}O_{24}$ 1598.9257, found 1598.9307 [M + H]$^+$, accurate mass error of 3.31 ppm.

Amino Acid Analysis of Homophymine A (1). For a large-scale hydrolysis, a 30 mg sample of homophymine A (**1**) was dissolved in 6 N HCl (5 mL) and heated at 130 °C for 12 h. The crude hydrolysate was extracted with CH_2Cl_2 (2 × 1 mL). The CH_2Cl_2 layer afforded 1.0 mg of 3-hydroxy-2,4,6-trimethyloctanoic acid (**2**). ESI/MS *m*/*z* 201.2 [M − H]$^-$; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.48 (1H, t, J = 6.8 Hz, H-3), 2.74 (1H, quintet, J = 7.1 Hz, H-2), 1.72 (1H, m, H-4), 1.46 (1H, m, H-7), 1.40 (1H, m, H-6), 1.29 (3H, d, J = 7.2 Hz, H-9), 1.06−1.07 (2H, m, H-5, H-7′), 1.03 (1H, m, H-5′), 0.97 (3H, d, J = 6.8 Hz, H-10), 0.91 (3H, d, J = 6.1 Hz, H-11), 0.87 (3H, t, J = 7.4 Hz, H-8).

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The aqueous layer was fractionated by HPLC on the reversed-phase Phenomenex Hydro (4 μ, 250 × 4.6 mm) column eluting with MeOH:H_2O 2:98 (flow rate 0.5 mL/min) to give 2.2 mg of pure 2-amino-3-hydroxy-4,5-dimethylhexanoic acid (**4**) (R_t 40.1 min). The additional peak at R_t 3.7 min was further purified by HPLC on the same column eluting with H_2O 100% containing 0.05% TFA (flow rate 0.5 mL/min) to give 0.7 mg of pure 4-amino-2,3-dihydroxyheptandioic acid (**3**) (R_t 34.8 min).

4-Amino-2,3-dihydroxyheptandioic Acid (3). ESI/MS *m/z* 208.1 [M + H$^+$]; ^{1}H NMR (500 MHz, CD_3OD) δ 4.29 (1H, d, J = 5.2 Hz, H-2), 3.93 (1H, dd, J = 5.2, 2.0 Hz, H-3), 3.37 (1H, m, H-4), 2.35 (1H, m, H-6), 2.42 (1H, m, H-6′), 1.91 (2H, m, H-5); ^{13}C NMR (125 MHz, CD_3OD) δ 179.4, 171.5, 74.3, 70.5, 53.2, 33.5, 27.3.

2-Amino-3-hydroxy-4,5-dimethylhexanoic Acid (4). ESI/MS *m/z* 176.1 [M + H$^+$]; ^{1}H NMR (500 MHz, CD_3OD) δ 3.70 (1H, d, J = 2.6 Hz, H-2), 3.65 (1H, dd, J = 10.0, 2.6 Hz, H-3), 2.22 (1H, m, H-5), 1.84 (1H, m, H-4), 0.94 (3H, d, J = 7.0 Hz, H-6), 0.85 (3H, d, J = 6.9 Hz, H-8), 0.80 (3H, d, J = 7.0 Hz, H-7); ^{13}C NMR (125 MHz, CD_3OD) δ 171.5, 74.0, 59.0, 41.2, 27.2, 21.4, 14.9, 9.5.

Determination of the Absolute Configuration: Peptide Hydrolysis. A peptide sample (2 mg) was 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 derivatization.

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 this solution was then 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 neutralized 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% trifluoroacetic 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 by using the suite of programs Xcalibur; 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), and L-Orn (11.1).

To determine the absolute configuration of 3,4-diMeGln an authentic sample of callipeltin A was used as standard. The hydrolysate of callipeltin A contained (2*S*,3*S*,4*R*)-3,4-diMe-Glu (20.5).

The hydrolysate of homophymine A (**1**) contained L-Pip (28.3), D-Orn (8.9), L-Leu (31.5), L-ThrOMe (19.6), and (2*S*,3*S*,4*R*)-3,4-diMe-Glu (20.6).

To determine the absolute configuration of 2-amino-3-hydroxy-4,5-dimethylhexanoic acid (AHDMHA, **4**), two aliquots of the hydrolysate mixture were derivatized with L- and D-FDAA, respectively, and then they were subjected to LC-MS as described above. Retention times (min): L-FDAA-AHDMHA (33.5) and D-FDAA-AHDMHA (28.1).

Chiral HPLC Analysis. The acid hydrolysate of **1** (aliquot of 10 μL) was analyzed 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) and D-Ser (5.1). The hydrolysate of homophymine A (**1**) contained D-serine.

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 **1** or callipeltin 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−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 callipeltin A (25.3), L-Asp (16.9), D-Asp (17.9), L-Glu (15.6), and D-Glu (14.5). The hydrolysate of **1** gave peaks for L-*N*MeGlu (25.5) and D-Asp (17.9).

Stereochemistry of the 4-Amino-2,3-dihydroxyheptandioic Acid Residue (ADHA, 3). A 1 mg portion of **1** was stirred in a mixture of EtOH (300 μL) and 1% (w/w) $NaIO_4$ in water (750 μL) for 18 h at ambient temperature. The reaction solution was concentrated and then dissolved in 700 μL of 50% AcOH/H_2O and boiled for 2 h at 102 °C in the presence of 30% H_2O_2 (200 μL) and concentrated HCl (100 μL). The residue was hydrolyzed, derivatized with GITC as described above, and analyzed by LC-MS, which revealed the presence of L-Glu (15.4).

Mosher Analysis of 2. Freshly distilled (+)-α-methoxy-α-(trifluoromethyl)phenylacetic (MTPA) chloride (6 μL) was added to a solution of **2** (0.5 mg) with a catalytic amount of 4-(dimethylamino)pyridine and 5 μL of freshly distilled triethylamine, in 1 mL of freshly distilled CH_2Cl_2, and the solution was allowed to stand at room temperature for 12 h under an argon atmosphere. The residue, obtained after evaporation of the solvent, was subjected to reverse-phase HPLC with use of a linear gradient from water (100%) to CH_3CN (100%), UV detector λ = 260 nm, to obtain 0.1 mg of the (*R*)-(+)-MTPA ester of **2**. The same procedure was used to obtain 0.4 mg of the (*S*)-(−)-MTPA ester of **2**. 3-(+)-(*R*)-MTPA ester of **2**: selected ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.68 (1H, dd, H-3), 3.14 (1H, quintet, H-2), 1.70 (1H, overlapped, H-4), 1.36 (3H, d, J = 6.8 Hz, H-9), 0.92 (3H, d, J = 7.5 Hz, H-10), 0.89 (3H, d, J = 6.8 Hz, H-11), 0.87 (3H, t, J = 7.1 Hz, H-8). 3-(−)-(*S*)-MTPA ester of **2**: selected ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.68 (1H, dd, H-3), 3.12 (1H, quintet, H-2), 1.77 (1H, overlapped, H-4), 1.32 (3H, d, J = 6.7 Hz, H-9), 0.96 (3H, d, J = 7.1 Hz, H-10), 0.91 (3H, d, J = 6.5 Hz, H-11), 0.87 (3H, t, J = 7.1 Hz, H-8).

Methanolysis of Homophymine A (1). A solution of 2 mg of homophymine A (**1**) was treated with 2.1 mg of NaOMe in dry methanol (200 μL) at room temperature for 24 h. The reaction mixture was neutralized with 0.1 N HCl, poured into ice−water, and then extracted with *n*-BuOH. The *n*-BuOH phase was evaporated under reduced pressure and the crude product (0.8 mg) was subjected to ESI Q/TOF analysis. Opened methyl ester *m/z* 1630.74 [M + H]$^+$.

Preparation of L-ThrOMe and L-alloThrOMe. A solution of 50 mg of *N*-Boc-L-Thr was treated with a large excess of diazomethane in dry dichloromethane. The yellow solution was stirred at room temperature for 30 min and then it was concentrated under reduced pressure to give a residue (50 mg) that was subject to the next reaction without purification. 2,6-Di-*tert*-butylpyridine (1.4 mL) and methyl trifluoromethanesulfonate (0.7 mL) were added sequentially to a solution of *N*-Boc-L-Thr methyl ester (0.2 mmol) in CH_2Cl_2 at 0 °C under argon atmosphere. The mixture was allowed to warm to room temperature where stirring was continued for 14 h. Saturated $NaHCO_3$ solution was added and the organic phase was washed with water, dried ($MgSO_4$), and then concentrated in vacuo. Purification by column chromatography on silica gel with *n*-hexane:EtOAc (99:1) as eluent gave the methyl ether in quantitative yield as a colorless oil. A 1 mg portion of *N*-Boc-L-ThrOMe methyl ester was dissolved in degassed 6 N HCl (0.5 mL) in an evacuated glass tube and heated at 120 °C for 16 h. The solvent

was removed in vacuo and the resulting material was subjected to Marfey's derivatization with L- and D-FDAA. L-*allo*ThrOMe was obtained following the same procedure as described for L-ThrOMe.

Evaluation of Antiviral Activity. The effects of homophymine A (**1**) were evaluated in PBMC cell lines infected with HIV-1 (III B strain) as previously described.[34] The compounds were solubilized in DMSO at an initial concentration of 10^{-2} M. The 1% dilution in culture medium as well as serial dilutions were used in the antiviral assays. The production of virus was measured by quantification of the reverse transcriptase activity associated with the virus particles released in the supernatant after 5 days of cell culture in the presence of different concentrations of the compound. The 50% inhibitory concentration of virus multiplication (IC_{50}) was derived from the computer-generated median effect plot of the dose−effect data.[35] In parallel experiments, cytotoxicity of homophymine A (**1**) for uninfected cells was measured after an incubation of 7 d in its presence, using a colorimetric assay (MTT test).[36]

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Supporting Information Available: General experimental methods and spectroscopic data for homophymine A (**1**), HTMOA (**2**), ADHA (**3**) and AHDMHA (**4**). This material is available free of charge via the Internet at http://pubs.acs.org.

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