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Aeruginoguanidines 98-A–98-C: cytotoxic unusual peptides from the cyanobacterium *Microcystis aeruginosa*

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Abstract—Aeruginoguanidines 98-A–98-C (**1**–**3**) were isolated from the cyanobacterium *Microcystis aeruginosa* (NIES-98). The structures of aeruginoguanidines 98-A–98-C (**1**–**3**) were determined by two-dimensional $^{1}H-^{1}H$ and $^{1}H-^{13}C$ NMR correlation experiments and confirmed by mass spectral analysis. The absolute stereochemistry of **1**, consisting of Hphpa trisulfate (1-(4-hydroxy-3-hydroxymethyl)phenyl-1-hydroxy-2-propylamine 4′,3′,1-tri-*O*-sulfate), MpArg (N^{α}-methyl-N^{ω}-prenylarginine) and MgArg ((*Z*)-N^{α}-methyl-N^{ω}-geranylarginine), was determined by the NMR analyses of phenylglycine methyl ester (PGME) or Boc phenylglycine (BPG) derivatives of acid hydrolysates. These compounds showed moderate cytotoxicity against the P388 murine leukemia cells. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

The freshwater cyanobacterium *Microcystis aeruginosa* has been extensively studied from the environmental, toxicological, biological, and chemical points of view because they are responsible for water blooms that frequently produce potent hepatotoxins.[1] These hepatotoxic cyclic peptides, microcystins, have been isolated from in a number of strains of *Microcystis* and some other cyanobacteria.[2] Moreover, these peptides have been found to be a highly effective inhibitors of protein phosphatase 1 and 2A.[3] On the other hand, in the course of our screening program of new protease inhibitors from microalgae, we have reported that *M. aeruginosa* is very rich source of the protease inhibitory peptides such as aeruginosins,[4] microginins,[5] micropeptins,[6] and microviridins.[7]

In the course of isolation of protease inhibitors, aeruginosins 98-A–98-C[4b] from *M. aeruginosa* (NIES-98), we found that this strain produces unusual peptides in large quantities. We now report the isolation and structural elucidation of aeruginoguanidines 98-A (**1**), 98-B (**2**), and 98-C (**3**) from the freshwater cyanobacterium *M. aeruginosa* (NIES-98).

2. Results and discussion

M. aeruginosa (NIES-98) was obtained from the NIES-collection[8] and cultured in our laboratory to yield 115 g (dry weight) from 420 L of culture. The 80% methanol extract of freeze-dried alga was partitioned between water and diethyl ether. The aqueous layer was further extracted with *n*-butanol and *n*-butanol layer was subjected to ODS flash column chromatography followed by reversed-phase HPLC with aqueous MeCN containing 0.05% TFA to yield aeruginoguanidines 98-A–98-C (**1**–**3**).

The molecular formula of **1** was established to be $C_{39}H_{67}N_9O_{14}S_3$ by the HRFABMS and NMR spectral data (Table 1). The IR spectrum showed the presence of sulfates (1260 cm^{-1}), guanidinyl groups (1620 cm^{-1}), and amide carbonyl groups (1660 cm^{-1}).

Aeruginoguanidine 98-A (**1**) was composed of three curious units, Hphpa trisulfate (1-(4-hydroxy-3-hydroxymethyl)-phenyl-1-hydroxy-2-propylamine 4′,3′,1-tri-*O*-sulfate), MpArg (N^{α}-methyl-N^{ω}-prenylarginine), and MgArg ((*Z*)-N^{α}-methyl-N^{ω}-geranylarginine), from the detailed analyses of 2D NMR and FABMS spectra.

2.1. Hphpa trisulfate

The positive and negative FABMS of **1** using glycerol as a matrix revealed desulfated ions at *m*/*z* 822 $(M-2SO_3+H)^+$, 724 $(M-3SO_3-H_2O+H)^+$ and 900 $(M-SO_3-H)^-$, which supported the presence of three sulfate groups. ^{1}H and ^{13}C NMR spectra of **1** suggested the presence of trisubstituted benzene, and the positions of substituents (C-4, 6, 7; δ

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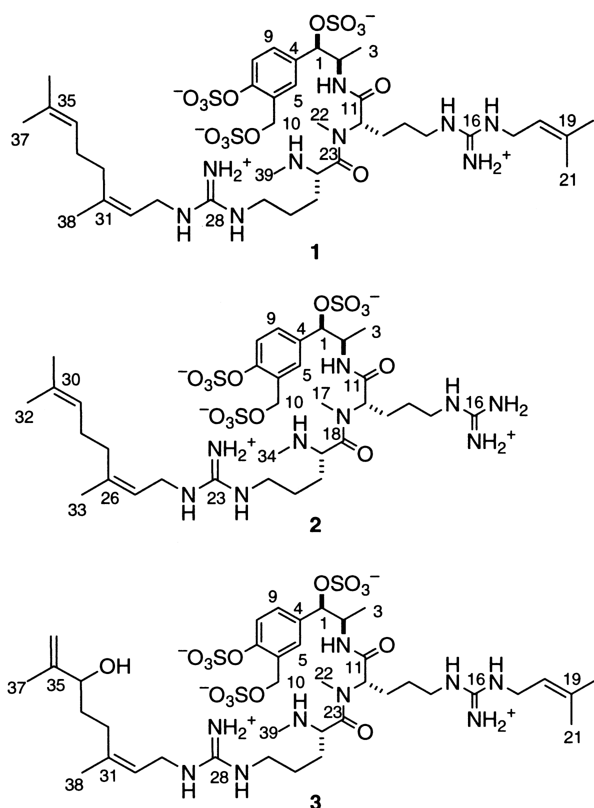

133.0, 128.7, 149.7) were determined by $^{1}H-^{1}H$ COSY, HMQC, and HMBC spectra (Table 1 and Fig. 1). In the $^{1}H-^{1}H$ COSY spectrum, the connectivities from H-1 (δ 5.14) to H-3 (δ 0.92) and 2-NH (δ 7.57), were determined. The correlations in the HMBC spectrum from H-1 to C-4, C-5 (δ 127.0) and C-9 (δ 126.6) revealed the connectivity between C-1 and C-4 (Fig. 1). The HMBC correlations from primary hydroxy methylene protons (H-10; δ 4.92 and 4.97) to C-5, C-6 and C-7 allowed a hydroxy methyl group to connect with C-6 (Fig. 1). The aromatic proton (H-8; δ 7.30, d J=8.4 Hz), which correlated with H-9 (δ 7.06, dd J=8.4, 2.0 Hz) in the $^{1}H-^{1}H$ COSY, was confirmed the correlations with C-4 and C-6 by HMBC spectrum (Fig. 1). These results suggested that this unit has a feature of Hphpa (1-(4-hydroxy-3-hydroxymethyl)phenyl-1-hydroxy-2-propylamine). Judging from the chemical shifts data, the structure of this unit was determined to be Hphpa trisulfate whose sulfate groups were adjacent to C-1 (δ 77.6), 7 and 10 (δ 62.7).

2.2. MpArg and MgArg

The ^{13}C NMR signals at δ 155.6 and 155.7 showed the existence of two guanidino groups. The connectivity from the former guanidinyl carbon (δ 155.6) to amide carbonyl (MpArg C-11) and *N*-methyl (MpArg C-22) groups was determined by $^{1}H-^{1}H$ COSY and HMBC experiments. Furthermore, the HMBC correlation from the broad methylene protons (H-17; δ 3.87) of the prenyl group, which was easily determined by $^{1}H-^{1}H$ COSY and HMBC spectra, to this guanidinyl carbon confirmed that this unit was MpArg (N^{α}-methyl-N^{ω}-prenylarginine). These experiments also showed the connectivity from the latter

Table 1. NMR data for aeruginoguanidine 98-A (**1**) in DMSO-d_6

	^{13}C mult	^{1}H J (mult, Hz)	HMBC (C, no)
Hphpa trisulfate			
1	77.6 d	5.14 d 6.1	2, 3, 4, 5, 9
2	48.9 d	4.05 qdd 6.7, 6.9, 6.1	1, 3, 4
3	14.7 q	0.92 d 6.7	1, 2
4	133.0 s		
5	127.0 d	7.17 d 2.0	1, 7, 9, 10
6	128.7 s		
7	149.7 s		
8	120.5 d	7.30 d 8.4	4, 6, 7
9	126.6 d	7.06 dd 8.4, 2.0	1, 5, 7
10a	62.7 t	4.92 d 12.8	5, 6, 7
10b		4.97 d 12.8	5, 6, 7
NH		7.57 d 6.9	11
MpArg			
11	169.5 s		
12	56.7 d	4.83 dd 9.6, 6.0	11, 13, 22
13a	24.9 t	1.60 m	
13b		1.79 m	
14	23.8 t	1.40 m	
15a	47.0 t	3.18 m	16
15b		3.23 m	16
16	155.6 s		
17	45.7 t	3.87 br	16, 17, 18
18	118.3 d	5.08 m	20, 21
19	136.8 s		
20	25.4 q	1.69 s	18, 19, 21
21	17.6 q	1.65 s	18, 19, 20
22	30.6 q	3.01 s	12, 23
17═NH		7.12 br	
MgArg			
23	168.4 s		
24	58.0 d	4.48 br	
25a	25.2 t	1.81 m	
25b		1.97 m	
26a	21.5 t	1.60 m	
26b		1.77 m	
27a	46.4 t	3.18 br	28
27b		3.35 br	
28	155.7 s		
29	45.3 d	3.92 br	28, 30, 31
30	119.0 d	5.12 t 6.6	32, 38
31	140.3 s		
32a	31.42 t	2.05	
32b		2.08 m	
33	25.7 t	2.04 m	
34	123.8 d	5.10 m	
35	131.3 s		
36	25.5 q	1.64 s	34, 35, 37
37	17.6 q	1.58 s	34, 35, 36
38	23.0 q	1.72 s	30, 31
39	31.37 q	2.52 s	24
24–NH		8.75 br	
27–NH		7.18 br	
29–NH		7.18 br	
28═NH		7.12 br	

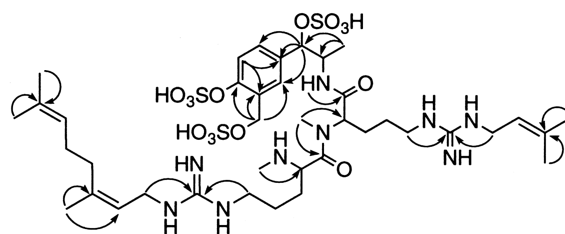

Figure 1. Key HMBC correlations (arrows) of **1**.

guanidinyl carbon (δ 155.7) to another *N*-methyl group (MgArg C-39) and presence of the geranyl group. The *Z*-geometry of the trisubstituted double bond was deduced from the NOESY cross-peak; H-30 (δ 5.12)/H-38 (δ 1.72) and the chemical shifts of H-38 (δ_H 1.72, δ_C 23.0). Moreover, a correlation between the broad methylene protons (MgArg H-29; δ 3.92) of the geranyl group and the latter guanidinyl carbon was observed in HMBC spectrum. The connectivity between MgArg C-23 (δ 168.4) and C-24 (δ 58.0) was deduced by NOESY (MpArg H-22 (δ 3.01)/MgArg H-24) and the HMBC correlations from H-22 to C-23 (δ 168.4) of MgArg, though correlations between C-23 and protons in MgArg were not detected by HMBC spectrum.

The sequence of **1** was deduced by HMBC (Hphpa trisulfate H-2/MpArg C-11) and NOESY correlation (MpArg H-22/MgArg H-24).

The absolute stereochemistry of the MpArg unit was determined as follows. The (*R*)- and (*S*)-PGME (phenylglycine methyl ester) amides of MiArg (**4**; N^{α}-methyl-N^{ω}-isoamylarginine), obtained by the acid hydrolysis of a hydrogenated product of **1**, were prepared by treatment with (*R*)- and (*S*)-PGME in DMF using the coupling and additive reagents (PyBOP, HOBt), and $\Delta\delta$ values ($\delta_S-\delta_R$) were determined at 600 MHz.[9] Negative $\Delta\delta$ were found for the protons on *N*-Me side of the PGME plane, whereas positive values were found for protons on C-3 to C-10 and C-10′ side (Fig. 2). Therefore, the absolute stereochemistry at C-2 of the MpArg unit was *S* configuration. The absolute stereochemistry of MgArg was also determined to be *S* configuration by the NMR analysis of (*R*)- and (*S*)-PGME amides of MdoArg (**5**; N^{α}-methyl-N^{ω}-2,6-dimethyloctyl-arginine), obtained by the acid hydrolysis of hydrogenated product of **1** (Fig. 3).

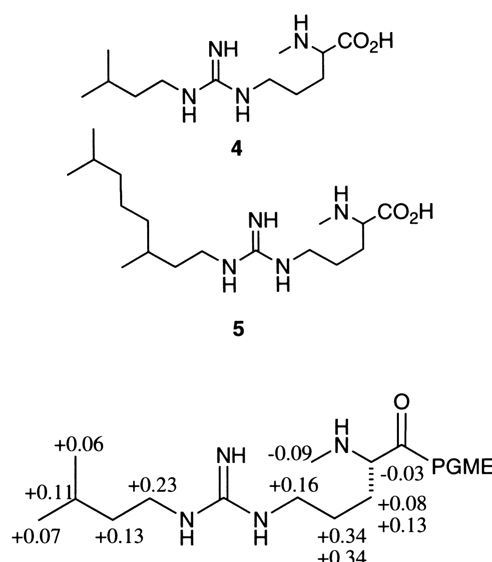

Figure 2. $\Delta\delta$ ($\delta_S-\delta_R$) values in ppm obtained at 600 MHz for (*R*)- and (*S*)-PGME amides of MiArg.

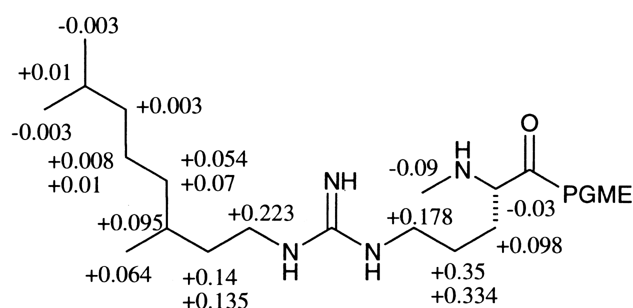

Figure 3. $\Delta\delta$ ($\delta_S-\delta_R$) values in ppm obtained at 600 MHz for (*R*)- and (*S*)-PGME amides of MdoArg.

The assignment of the absolute configuration of the Hphpa trisulfate unit was quite difficult for the insolubility of aeruginoguanidines in various solvents except DMF and DMSO.[10] Sufficient sample of hydroxyamine could not be obtained by acid hydrolysis under various conditions. Hydrazinolysis of **1** was also failure. Finally, small sample of Hphpa (**6**; 0.8 mg) obtained by acid hydrolysis of **1** (50 mg) using 3 M HCl (100°C for 3 h) was used for the microanalysis of the absolute structure determination. The absolute configuration at C-2 of Hphpa was achieved by the Boc-phenylglycine (BPG) method.[11] The (*R*)- and (*S*)-BPG amides of Hphpa were prepared by treatment with (*R*)- and (*S*)-BPG in DMF using the coupling and additive reagents (PyBOP, HOBt), and $\Delta\delta$ values ($\delta_S-\delta_R$), which were analyzed with 600 MHz NMR, indicated that the absolute configuration at C-2 of Hphpa was *R*-configuration (Fig. 4). The absolute configuration at C-1 of Hphpa was determined by the comparison with four isomers ((1*R*,2*R*) and (1*S*,2*S*) norephedrine were prepared from (1*S*,2*R*) and (1*R*,2*S*) norephedrine, respectively) of norephedrine (Table 2). The similarity of the coupling constants and optical rotations between (1*R*,2*R*) norephedrine and Hphpa indicated that the configurations at C-1 and C-2 of Hphpa were *R*.

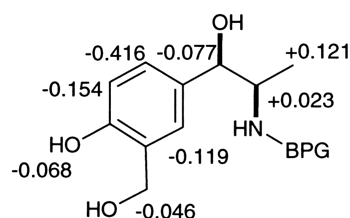

Figure 4. $\Delta\delta$ ($\delta_S-\delta_R$) values in ppm obtained at 600 MHz for (*R*)- and (*S*)-BPG amides of Hphpa.

The fragment patterns of the positive and negative FABMS of aeruginoguanidine 98-B (**2**) using glycerol as a matrix revealed the presence of three sulfate groups. The molecular formula of **2** was established to be $C_{34}H_{59}N_9O_{14}S_3$ by the HRFABMS and NMR spectral data (Table 3). The ^{1}H and ^{13}C NMR spectra of **2** were similar to those of **1**, but two methyl protons of the prenyl group were not detected in ^{1}H NMR spectrum. The ^{1}H–^{1}H COSY, HMQC and HMBC data showed the presence of Hphpa trisulfate, MgArg and *N*-Me Arg, which lacked a prenyl group. The sequence of **2** was deduced by HMBC (Hphpa trisulfate H-2/*N*-MeArg C-11) and NOESY correlations (*N*-MeArg H-17/MgArg H-19). The correspondence of ^{1}H and ^{13}C NMR spectra at C-1, C-2, C-12, C-18 (C-24 in **1**) between **1** and **2** indicated that the absolute stereochemistry of **2** was 1*R*,2*R*,12*S*,18*S*.

Table 2. Physical and chemical data for Hphpa and norephedrine

	$[\alpha]_D^{21}$ (EtOH)	^{1}H NMR (DMSO-d_6, 300 K)	
		H-1	H-2
Hphpa from **1**	−31.3° (*c* 0.0667)	4.27 (dd, *J*=9.9, 3.4 Hz)	3.16 (m)
(1*S*,2*R*) Norephedrine	+35.0° (*c* 1.0)	4.90 (br)	3.38 (br)
(1*R*,2*S*) Norephedrine	−35.3° (*c* 1.0)	4.90 (br)	3.38 (br)
(1*R*,2*R*) Norephedrine	−29.8° (*c* 0.5)	4.39 (dd, *J*=9.0, 3.4 Hz)	3.24 (m)
(1*S*,2*S*) Norephedrine	+31.2° (*c* 1.0)	4.39 (dd, *J*=9.0, 3.4 Hz)	3.24 (m)

Table 3. NMR data for aeruginoguanidines 98-B (**2**) and 98-C (**3**) in DMSO-d_6

Aeruginoguanidine 98-B (**2**)		
	^{13}C	^{1}H *J* (mult, Hz)
Hphpa trisulfate		
1	77.5 d	5.16 d 5.8
2	48.7 d	4.07 qdd 7.1, 6.9, 5.8
3	14.6 q	0.92 d 6.9
4	132.9 s	
5	127.0 d	7.15 d 2.3
6	128.9 s	
7	149.7 s	
8	120.5 d	7.30 d 8.6
9	126.8 d	7.06 dd 8.6, 2.3
10a	62.9 t	4.92 d 12.8
10b		4.97 d 12.8
2–NH		7.54 d 7.1
N-Me Arg		
11	169.5 s	
12	56.5 d	4.86 dd 10.2, 5.4
13a	25.0 t	1.66 m
13b		1.80 m
14a	25.4 t	1.32 m
14b		1.40 m
15	40.2 t	3.10 m
16	158.6 s	
17	30.6 q	2.99 s
15–NH		7.43 t 5.9
MgArg		
18	169.4 s	
19	58.0 d	4.48 br
20a	25.1 t	1.81 m
20b		1.97 m
21b		1.79 m
22a	46.4 t	3.20 br
22b		3.35 br
23	155.7 s	
24	45.3 d	3.92 brd 6.5
25	119.0 d	5.12 td 6.5,1.8
26	140.3 s	
27a	31.42 t	2.05 m
27b		2.07 m
28	25.9 t	2.04 m
29	123.8 d	5.09 m
30	131.3 s	
31	25.5 q	1.64 d 0.7
32	17.6 q	1.57 d 1.2
33	23.0 q	1.72 d 1.1
34	31.38 q	2.52 br
19–NH		8.76 br
23═NH		7.17 br

Aeruginoguanidine 98-C (**3**)		
	^{13}C	^{1}H *J* (mult, Hz)
Hphpa trisulfate		
1	77.5 d	5.16 d 6.4
2	48.9 d	4.06 qdd 6.8, 6.7, 6.4
3	14.5 q	0.92 d 6.8
4	132.8 s	
5	127.1 d	7.17 s
6	128.9 s	
7	149.8 s	
8	120.5 d	7.30 d 8.4
9	126.6 d	7.06 d 8.4
10a	62.9 t	4.92 d 12.8
10b		4.97 d 12.8
2–NH		7.57 d 6.7
MpArg		
11	169.6 s	
12	56.7 d	4.84 dd 9.5, 5.9
13a	21.7 t	1.62 br
13b		1.77 br
14	23.8 t	1.40 m
15a	46.9 t	3.18 m
15b		3.22 m
16	155.6 s	
17	45.7 t	3.87 br
18	118.3 d	5.08 d 6.4
20	25.4 q	1.69 s
21	17.8 q	1.65 s
22	30.6 q	3.00 s
16═NH		7.16 br
MdoArg		
23	168.4 s	
24	58.0 d	4.47 br
25a	25.1 t	1.82 br
25b		1.97 br
26a	24.9 t	1.63 br
26b		1.78 br
27a	46.7 t	3.23 m
27b		3.37 br
28	155.7 s	
29	45.6 t	3.92 br
30	118.9 d	5.12 t 6.1
31	140.5 s	
32	27.8 t	2.06 m
33a	33.1 t	1.45 m
33b		1.52 m
34	73.3 d	3.85 m
35	148.2 s	
36a	109.9 t	4.74 br
36b		4.88 br
37	17.8 q	1.64 br
38	23.0 q	1.70 s
39	31.4 q	2.52 s
24–NH		8.76 br
28═NH		7.18 br

The fragment pattern of the positive FABMS of aeruginoguanidine 98-C (**3**) using glycerol as a matrix revealed the presence of three sulfate groups. The molecular formula of **3** was established to be $C_{39}H_{67}N_9O_{15}S_3$ by the HRFABMS and NMR spectral data (Table 3). The ^1H and ^{13}C NMR spectra of **3** resembled those of **1**. The $^1H-^1H$ COSY, HMQC and HMBC data showed the presence of Hphpa trisulfate and MpArg. The ^1H and ^{13}C NMR spectrum of **3** lacked methine and methyl signals (δ_H 5.10, δ_C 123.8 and δ_H 1.64, δ_C 25.5) of geranyl group in **1**, but revealed the presence of an oxymethine and a terminal methylene signals (δ_H 3.85, δ_C 73.3; δ_H 4.74, 4.88, δ_C 109.9). Therefore, **3** was suggested to be substituted MhdoArg ((*Z*)-N^{α}-methyl-N^{ω}-6-hydroxy-3,7-dimethyl-2,7-octadienylarginine), which was deduced by $^1H-^1H$ COSY, HMQC and HMBC spectra, in place of MgArg of **1**. The sequence of **3** was deduced by HMBC (Hphpa trisulfate H-2/*N*-MeArg C-1) and NOESY correlations (MpArg H-22/MhdoArg H-24).

The correspondence of ^1H and ^{13}C NMR spectra at C-1, C-2, C-12, C-24 between **1** and **3** indicated that the absolute stereochemistry of **3** was 1*R*,2*R*,12*S*,24*S*. Studies on the absolute configuration at C-34 is now in progress.[12]

2.3. Biological activities

Aeruginoguanidines 98-A–98-C showed moderate cytotoxicity against P388 leukemia cells with IC_{50} values of 26, 27 and 50 μg/mL, respectively.

3. Discussion

Aeruginoguanidines 98-A–98-C (**1**–**3**) are novel unusual peptides consisting of Hphpa trisulfate and two *N*-Me Arg derivatives. Since all these units are very unique structures, their biosynthesis pathway is interesting.

Aeruginoguanidine 98-A is also produced by the other strains (NIES-91, 99, 101, 299) besides *M. aeruginosa* (NIES-98).[13] Among these strains, *M. aeruginosa* (NIES-299) produced aeruginoguanidine 98-A up to about 1 g per 100 g of dried algal cells. To our knowledge, a secondary metabolite which is produced over 1% of dried algal cells in cyanobacteria has never been known. Aeruginoguanidines are composed of very hydrophylic part and hydrophobic part. Moreover, aeruginoguanidines are insoluble in H_2O and many organic solvents at a concentration of 1 mg/mL. Therefore, we are interested in how these peptides are kept in the cyanobacterial cells.

4. Experimental

4.1. General experimental procedures

UV spectrum was recorded on a Hitachi 330 spectrophotometer. Optical rotation was measured on a JASCO DIP-1000 polarimeter. IR spectrum was measured on a JASCO FT/IR-5300 spectrometer. ^1H and ^{13}C NMR spectra were obtained with either Bruker AM600 or JEOL A600 in DMSO-d_6 at 27.0°C. The resonances of residual DMSO-d_6 at δ_H 2.49 and δ_C 39.5 were used as internal references for ^1H and ^{13}C NMR spectra, respectively. FAB mass spectra, containing high resolution FABMS, were recorded by employing a JEOL JMS SX-102 mass spectrometer.

4.2. Culture conditions

Culture conditions were the same as previously described.[4b]

4.3. Extraction and isolation

Freeze-dried alga (*M. aeruginosa* (NIES-98); 115.4 g from 420 L of culture) was extracted with 80% MeOH (2 L×3) and MeOH (1.8 L×1). Combined 80% MeOH and MeOH extracts were concentrated to an aqueous suspension which was then extracted with ether. The aqueous layer was extracted with *n*-BuOH. The *n*-BuOH layer was evaporated under reduced pressure to green dry solid (11.4 g), which was subjected to flash chromatography on ODS (YMC-ODS AM120Å, 10×7 cm) with aqueous MeOH (20, 30, 40, 50, 60, 100%) followed by CH_2Cl_2.

The 40% (377 mg) and 50% MeOH (535 mg) fractions were combined, concentrated and subjected to flash chromatography on ODS (YMC-ODS AM120Å, 5×8 cm) with aqueous MeOH (20, 30, 40, 50, 60, 100%) followed by CH_2Cl_2. The 40% MeOH (113 mg) fraction was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10×250 mm; 20–50% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield **2** (9.6 mg). The 50% MeOH fraction (249 mg) was subjected to reversed-phase HPLC (YMC-Pack AM-324 120Å, 10×300 mm; 20–44% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield **1** (38.6 mg) and **2** (41.3 mg). The fraction (19.1 mg) containing **3** was subjected to reversed-phase HPLC (YMC-Pack AM-324 120Å, 10×300 mm; 23–28% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield **3** (12.9 mg). The MeOH fraction (230 mg) was subjected to reversed-phase HPLC (YMC-Pack AM-324 120Å, 10×300 mm; 32% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield **1** (109.3 mg).

4.3.1. Aeruginoguanidine 98-A (1). $[\alpha]_D^{28}=+4.9°$ (*c* 1.0, DMF); UV (60% EtOH) λ_{max} 268 nm (ε 1010); IR (KBr) 1660, 1620, 1260, 1050, 1010 cm^{-1}; HRFABMS *m/z* 980.3946 $[M-H]^-$ ($C_{39}H_{66}N_9O_{14}S_3$, Δ −5.5 mmu).

4.3.2. Aeruginoguanidine 98-B (2). $[\alpha]_D^{28}=+3.7°$ (*c* 1.0, DMF); UV (60% EtOH) λ_{max} 269 nm (ε 1420); HRFABMS *m/z* 912.3276 $[M-H]^-$ ($C_{34}H_{58}N_9O_{14}S_3$, Δ +1.1 mmu), HMBC correlations: H-1/C-3, C-4, C-5 and C-9, H-2/C-1 and C-11, H-3/C-1 and C-2, H-5/C-1, C-7, C-9 and C-10, H-8/C-4, C-6 and C-7, H-9/C-1, C-4 and C-7, H-10a/C-5, C-6 and C-7, H-10b/C-5, C-6 and C-7, 2-NH/C-11, H-12/C-11, C-13, C-14 and C-18, H-15/C-13, C-14 and C-16, H-17/C-11 and C-18, H-22a/C-23, H-24/C-23, C-25 and C-26, H-25/C-27 and C-33, H-27a/C-25, C-26, C-28, C-29 and C-33, H-27b/C-25, C-26, C-28, C-29 and C-33, H-28/C-27, C-29 and C-30, H-29/C-28, C-31 and C-32, H-31/C-29, C-30, C-32, H-32/C-29, C-30 and C-31, H-33/C-25 and C-27, H-34/C-19.

4.3.3. Aeruginoguanidine 98-C (3). $[\alpha]_D^{28}=-1.4°$ (*c* 0.5, DMF); UV (50% MeOH) λ_{max} 265 nm (ε 1030); HRFABMS *m/z* 996.3851 $[M-H]^-$ ($C_{39}H_{66}N_9O_{15}S_3$, Δ +1.1 mmu), HMBC correlations: H-1/C-2, C-3, C-4 and C-5, H-2/C-1, C-3 and C-4, H-3/C-1 and C-2, H-5/C-1, C-7, C-9 and C-10, H-8/C-4, C-6 and C-7, H-9/C-1, C-5 and C-7, H-10a/C-5, C-6 and C-7, H-10b/C-5, C-6 and C-7, 2-NH/C-11, H-12/C-11, C-14 and C-22, H-15a/C-14, C-16 and C-17, H-17/C-15, C-16, C-18 and C-19, H-18/C-20 and C-21, H-20/C-18, C-19 and C-21, H-21/C-18, C-19 and C-20, H-22/C-12 and C-23, H-29/C-27, C-30 and C-31, H-30/C-32 and C-38, H-32/C-31, H-34/C-32, C-33, C-35, C-36 and C-37, H-36a/C-34 and C-37, H-36b/C-34 and C-37, H-37/C-34, C-35 and C-36, H-38/C-30, C-31 and C-32, H-39/C-24.

4.3.4. MiArg (N^{α}-methyl-N^{ω}-isoamylarginine) and MdoArg (N^{α}-methyl-N^{ω}-2,6-dimethyloctylarginine). 1 (100 mg) was dissolved in DMF (3.0 mL) and EtOH (5.0 mL) and then 30 mg of palladium black was added to a solution, and the mixture was stirred at room temperature for 4 days under hydrogen. The reaction mixture was filtered and concentrated. This crude residue was dissolved in DMF (3.0 mL) and 6 M HCl (5.0 mL), and heated at 110°C for 2.5 days. After the solvent was removed by evaporation, the reaction mixture was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10×250 mm; 0–80% MeCN containing 0.05% TFA; UV detection at 210 nm; flow rate 2.0 mL/min) to yield MdoArg (33.4 mg) and a fraction containing MiArg (32.8 mg). The fraction containing MiArg was further subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10×250 mm; 13% MeCN containing 0.05% TFA; UV detection at 210 nm; flow rate 2.0 mL/min) to yield MiArg (16.9 mg). N^{α}-methyl-N^{ω}-isoamylarginine: $[\alpha]_D^{23}=+18.6°$ (*c* 0.1, MeOH); HRFABMS *m/z* 259.2159 $[M+H]^+$ $C_{12}H_{27}N_4O_2$, Δ +2.5 mmu); ^1H NMR spectrum (DMSO-d_6; 300 K), δ 0.87 (m, 6H, H-10 and 10′), 1.37 (m, 2H, H-8), 1.55 (m, 2H, H-4 and H-9), 1.58 (m, 1H, H-3), 1.70 (m, 1H, H-4), 1.77 (m, 1H, H-3), 2.45 (s, 3H, *N*-Me), 3.12 (m, 1H, H-5), 3.23 (m, 2H, H-7), 3.35 (m, 1H, H-2), 3.40 (m, 1H, H-5), ^{13}C NMR spectrum (DMSO-d_6; 300 K), 22.2 (C-10 and C-10′), 23.0 (C-4), 25.4 (C-9), 25.6 (C-3), 32.0 (*N*-Me), 35.5 (C-8), 46.8 (C-7), 47.6 (C-5), 59.5 (C-2), N^{α}-methyl-N^{ω}-2,6-dimethyloctylarginine: $[\alpha]_D^{23}=+18.6°$ (*c* 0.1, MeOH); HRFABMS *m/z* 329.2874 $[M+H]^+$ ($C_{17}H_{37}N_4O_2$, Δ −4.3 mmu); ^1H NMR spectrum (DMSO-d_6; 300 K), δ 0.84 (m, 6H, H-14 and 14′), 0.87 (m, 3H, H-9′), 1.07 (m, 1H, H-10), 1.12 (m, 2H, H-12), 1.20 (m, 1H, H-11), 1.26 (m, 1H, H-10), 1.28 (m, 1H, H-11), 1.30 (m, 1H, H-7), 1.41 (m, 1H, H-9), 1.51 (m, 2H, H-8 and H-13), 1.56 (m, 1H, H-4), 1.65 (m, 1H, H-3), 1.69 (m, 1H, H-4), 1.79 (m, 1H, H-3), 2.52 (s, 3H, *N*-Me), 3.20 (m, 1H, H-5), 3.25 (m, 2H, H-7), 3.36 (m, 1H, H-5), 3.60 (m, 1H, H-2), ^{13}C NMR spectrum (DMSO-d_6; 300 K), 19.1 (C-9′), 22.5 (C-4), 22.6 (C-14 and C-14′), 24.0 (C-11), 25.5 (C-3), 27.5 (C-13), 30.0 (C-9), 31.2 (*N*-Me), 34.0 (C-8), 36.5 (C-10), 38.8 (C-12), 46.5 (C-7), 47.5 (C-5), 61.0 (C-2).

4.3.5. N^{α}-Methyl-N^{ω}-isoamylarginine (*S*,*R*)-PGME amides. A solution of N^{α}-methyl-N^{ω}-isoamylarginine (3.0 mg) in DMF (0.5 mL) was added with (*S*)-PGME (2.2 mg), PyBOP (7.0 mg), HOBt (1.8 mg) and *N*-methylmorphorine (3.0 μL) at 0°C, and the mixture was stirred at room temperature for 1.5 h. After the solvent was removed by lyophilization, the reaction mixture was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10×250 mm; 10–70% MeCN containing 0.05% TFA; UV-detection 210 nm; flow rate 2.0 mL/min) to yield N^{α}-methyl-N^{ω}-isoamylarginine (*S*)-PGME amide (2.3 mg): HRFABMS *m/z* 406.2780 $[M+H]^+$ ($C_{21}H_{36}N_5O_3$, Δ −3.8 mmu); ^1H NMR specrum (CD_3OD; 300 K), N^{α}-methyl-N^{ω}-isoamylarginine δ 0.980 (d, *J*=6.8 Hz, 6H, H-10 and 10′), 1.552 (m, 2H, H-8), 1.670 (m, 1H, H-9), 1.788 (m, 1H, H-4), 1.920 (m, 2H, H-3 and H-4), 1.970 (m, 1H, H-3), 2.630 (s, 3H, *N*-Me), 3.381 (m, 2H, H-5), 3.420 (m, 2H, H-7), 3.862 (dd, *J*=7.3, 4.3 Hz, 1H, H-2), (*S*)-PGME δ 3.710 (s, 3H, CO_2Me), 5.520 (s, 1H, H-2), 7.370–7.426 (m, 5H, phenyl). N^{α}-methyl-N^{ω}-isoamylarginine (*R*)-PGME amide was also derivatized as described above. N^{α}-methyl-N^{ω}-isoamylarginine (*R*)-PGME amide (0.9 mg): HRFABMS *m/z* 406.2818 $[M+H]^+$ ($C_{21}H_{36}N_5O_3$, Δ 0.0 mmu); ^1H NMR spectrum (CD_3OD; 300 K), N^{α}-methyl-N^{ω}-isoamylarginine δ 0.915 (d, *J*=6.8 Hz, 3H, H-10), 0.920 (d, *J*=6.4 Hz, 3H, H-10′), 1.420 (m, 2H, H-7), 1.445 (m, 1H, H-4), 1.556 (m, 1H, H-9), 1.576 (m, 1H, H-4), 1.839 (m, 2H, H-3), 2.720 (s, 3H, *N*-Me), 3.190 (m, 2H, H-7), 3.220 (ddd, *J*=9.8, 6.0, 3.8 Hz, 2H, H-5), 3.890 (t *J*=6.8 Hz, 1H, H-2), (*R*)-PGME δ 3.720 (s, 3h, CO_2Me), 5.560 (s, 1H, H-2), 7.360–7.420 (m, 5H, phenyl).

4.3.6. N^{α}-Methyl-N^{ω}-2,6-dimethyloctylarginine (*S*,*R*)-PGME amides. A solution of N^{α}-methyl-N^{ω}-dimethyloctylarginine (3.0 mg) in DMF (0.5 mL) was added with (*S*)-PGME (2.2 mg), PyBOP (7.0 mg), HOBt (1.8 mg) and *N*-methylmorphorine (3.0 μL) at 0°C, and the mixture was stirred at room temperature for 1.5 h. After the solvent was removed by lyophilization, the reaction mixture was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10×250 mm; 20–100% MeCN containing 0.05% TFA; UV-detection 210 nm; flow rate 2.0 mL/min) to yield N^{α}-methyl-N^{ω}-dimethyloctylarginine (*S*)-PGME amide (2.0 mg): HRFABMS *m/z* 476.3591 $[M+H]^+$ ($C_{26}H_{46}N_5O_3$, Δ −1.0 mmu); ^1H NMR spectrum (CD_3OD; 300 K), N^{α}-methyl-N^{ω}-dimethyloctylarginine δ 0.877 (m, 6H, H-14 and 14′), 0.965 (d, *J*=6.4 Hz, 3H, H-9′), 1.174 (m, 1H, H-10), 1.175 (m, 1H, H-12), 1.280 (m, 1H, H-11), 1.380 (m, 1H, H-10), 1.480 (m, 1H, H-8), 1.520 (m, 1H, H-9), 1.540 (m, 1H, H-13), 1.680 (m, 1H, H-8), 1.800 (m, 1H, H-4), 1.924 (m, 1H, H-4), 1.948 (m, 2H, H-3), 2.630 (s, 3H, *N*-Me), 3.398 (m, 2H, H-5), 3.428 (m, 2H, H-7), 3.870 (dd, *J*=7.7 Hz, 4.3, 1H, H-2), (*S*)-PGME δ 3.720 (s, 3H, CO_2Me), 5.515 (s, 1H, H-2), 7.380–7.440 (m, 5H, phenyl). N^{α}-methyl-N^{ω}-dimethyloctylarginine (*R*)-PGME amide was also derivatized as described above. N^{α}-methyl-N^{ω}-dimethyloctylarginine (*R*)-PGME amide (4.7 mg): HRFABMS *m/z* 476.3591 $[M+H]^+$ ($C_{26}H_{46}N_5O_3$, Δ −1.0 mmu); ^1H NMR spectrum (CD_3OD; 300 K), N^{α}-methyl-N^{ω}-dimethyloctylarginine δ 0.879 (d, *J*=6.4 Hz, 3H, H-14), 0.881 (d, *J*=6.8 Hz, 3H, H-14′), 0.901 (d, *J*=6.4 Hz, 3H, H-9′), 1.120 (m, 1H, H-10), 1.172 (m, 2H, H-12), 1.272 (m, 1H, H-11), 1.310 (m, 1H, H-10), 1.340 (m, 1H, H-8), 1.368 (m, 1H, H-11), 1.425 (m, 1H, H-9), 1.450 (m, 1H, H-4), 1.530 (m, 1H, H-13), 1.545 (m, 1H, H-8), 1.590 (m, 1H, H-4), 1.850 (m, 2H, H-3), 2.720 (s, 3H, *N*-Me), 3.205 (m, 2H, H-7), 3.220 (m, 2H, H-5), 3.900 (dd,

J=7.7, 5.6 Hz, 1H, H-2), (*R*)-PGME δ 3.720 (s, 3H, CO_2Me), 5.560 (s, 1H, H-2), 7.370–7.450 (m, 5H, phenyl).

4.3.7. Hphpa (1-(4-hydroxy-3-hydroxymethyl)phenyl-1-hydroxy-2-propylamine). 1 (50 mg) was dissolved in 3 M HCl (5.0 mL), and heated at 100°C for 3 h. After the solvent was removed by lyophilization, the reaction mixture was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10×250 mm; 5% MeCN containing 0.05% TFA; UV detection at 210 nm; flow rate 2.0 mL/min) to yield Hphpa (0.8 mg); $[\alpha]_D^{21}=-31.3°$ (*c* 0.0667, EtOH); HRFABMS *m/z* 198.1147 $[M+H]^+$ ($C_{10}H_{16}NO_3$, Δ +1.6 mmu); ^1H NMR spectrum (DMSO-d_6, 300 K), δ 0.90 (d, *J*=6.8 Hz, 3H, H-3), 3.16 (m, 1H, H-2), 4.27 (dd, *J*=9.9, 3.4 Hz, 1H, H-1), 4.47 (br, 2H, H-7′), 4.99 (t *J*=4.7 Hz, 1H, 7′-OH), 5.99 (d, *J*=3.4 Hz, 1H, 1-OH), 6.74 (d, *J*=8.1 Hz, 1H, H-5′), 7.01 (brd, 1H, H-6′), 7.27 (br, 1H, H-2′), 7.78-7.88 (br, 2H, NH_2), 9.41 (s, 1H, 4′-OH); ^{13}C NMR spectrum (DMSO-d_6, 300 K), δ 15.1 (C-3), 52.4 (C-2), 58.0 (C-7′), 74.6 (C-1), 114.1 (C-5′), 125.8 (C-2′ and C-6′).

4.3.8. (*R*,*S*)-BPG-Hphpa Amides. A solution of Hphpa (0.2 mg) in DMF (0.4 mL) was added with (*S*)-BPG (2.0 mg), PyBOP (4.0 mg), HOBt (2.0 mg) and *N*-methylmorphorine (10 μL) at 0°C, and the mixture was stirred at room temperature for 12 h. After the solvent was removed by lyophilization, the reaction mixture was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10×250 mm; 10–90% MeCN containing 0.05% TFA; UV-detection 210 nm; flow rate 2.0 mL/min) followed by reversed-phase HPLC (Cosmosil 5C18-MS, 10×250 mm; 35% MeCN containing 0.05% TFA; UV-detection 210 nm; flow rate 2.0 mL/min) to yield (*S*)-BPG-Hphpa amide (0.1 mg): HRFABMS *m/z* 431.2207 $[M+H]^+$ ($C_{23}H_{31}N_2O_6$, Δ +2.5 mmu); ^1H NMR spectrum (DMSO-d_6, 300 K), (*S*)-BPG δ 1.382 (s, 9H, *tert*-butyl), 5.130 (d, *J*=7.3 Hz, 1H, H-2), 7.224 (m, 1H, phenyl), 7.241 (d, *J*=7.3 Hz, 1H, NH), 7.275 (t *J*=6.8 Hz, 2H, phenyl), 7.315 (m, 2H, phenyl), Hphpa δ 0.800 (d, *J*=6.4 Hz, 3H, H-3), 3.847 (m, 1H, H-2), 4.415 (d, *J*=5.1 Hz, 1H, H-1), 4.448 (s, 2H, H-7′), 6.660 (d, *J*=8.1 Hz, 1H, H-5′), 6.922 (brd, *J*=8.1 Hz, 1H, H-6′), 7.234 (s, 1H, H-2′), 7.708 (d, *J*=8.6 Hz, 1H, NH), 9.160 (s, 1H, 4′-OH). (*R*)-BPG-Hphpa amide was also derivatized as described above. (*R*)-BPG-Hphpa amide (0.1 mg): HRFABMS *m/z* 431.2207 $[M+H]^+$ ($C_{23}H_{31}N_2O_6$, Δ +2.5 mmu); ^1H NMR spectrum (DMSO-d_6, 300 K), (*R*)-BPG δ 1.376 (s, 9H, *tert*-butyl), 5.141 (d, *J*=9.0 Hz, 1H, H-2), 7.224 (m, 1H, phenyl), 7.241 (d, *J*=7.3 Hz, 1H, NH), 7.275 (t *J*=6.8 Hz, 2H, phenyl), 7.315 (m, 2H, phenyl), Hphpa δ 0.921 (d, *J*=6.8 Hz, 3H, H-3), 3.870 (m, 1H, H-2), 4.338 (m, 1H, H-1), 4.402 (brd, *J*=5.6 Hz, 2H, H-7′), 4.846 (t *J*=5.6 Hz, 1H, 7′-OH), 5.188 (br, 1H, 1-OH), 6.506 (m, 2H, H-5′ and H-6′), 7.115 (s, 1H, H-2′), 7.672 (d, *J*=8.1 Hz, 1H, NH), 9.092 (s, 1H, 4′-OH).

4.3.9. Norephedrine. (1*S*,2*R*) and (1*R*,2*S*) norephedrines were purchased from Wako Pure Chemical Industries, and subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10×250 mm; 8% EtOH containing 0.05% TFA; UV-detection 210 nm; flow rate 2.0 mL/min) to yield (1*S*,2*R*) norephedrine: $[\alpha]_D^{21}=+35.0°$ (*c* 1.0, EtOH); ^1H NMR (DMSO-d_6, 600 MHz), δ 0.92 (d, *J*=6.8 Hz, 3H), 3.38 (br, 1H), 4.90 (br, 1H), 6.01 (d, *J*=1.7 Hz, 1H), 7.29 (t, *J*=6.8 Hz, 1H), 7.33–7.40 (m, 4H), 7.92–8.14 (br, 2H), (1*R*,2*S*) norephedrine: $[\alpha]_D^{21}=-35.3°$ (*c* 1.0, EtOH); ^1H NMR spectrum was in correspondence with (1*S*,2*R*) norephedrine.

(1*S*,2*R*) Norephedrine (100 mg) was dissolved in pyridine (0.5 mL) and acetic anhydride (0.5 mL), and the mixture was stirred at room temperature for 16 h. After the solvent was removed by evaporation and lyophilization, the reaction mixture was dissolved in EtOAc, washed with H_2O, saturated aqueous NaCl, and dried over $MgSO_4$ to yield the crude diacetate. This crude material was dissolved in MeOH (0.75 mL) and 4N NaOH (0.25 mL), and the mixture was stirred at room temperature for 30 min. The reaction mixture was concentrated, neutralized with 1 M HCl, dissolved in EtOAc, washed with H_2O, saturated aqueous NaCl, and dried over $MgSO_4$ to yield the crude acetylamide.

To this crude acetylamide, 300 μL of thionyl chloride was added at 0°C. The mixture was stirred at 0°C for 15 min under argon, and the solvent was removed by lyophilization. This reaction mixture was dissolved in 6 M HCl (2.0 mL), and heated at 100°C for 3 h under argon. After the solvent was removed by lyophilization, the reaction mixture was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10×250 mm; 6% EtOH containing 0.05% TFA; UV-detection 210 nm; flow rate 2.0 mL/min) to yield (1*R*,2*R*) norephedrine (86 mg; 86%): $[\alpha]_D^{21}=-29.8°$ (*c* 0.5, EtOH); ^1H NMR (DMSO-d_6, 600 MHz), δ 0.93 (d, *J*=6.4 Hz, 3H), 3.24 (m, 1H), 4.39 (dd, *J*=9.0, 3.4 Hz, 1H), 6.20 (d, *J*=3.4 Hz, 1H), 7.31–7.40 (m, 5H), 7.78–7.90 (br, 2H). (1*S*,2*S*) Norephedrine ($[\alpha]_D^{21}=+31.2°$ (*c* 1.0, EtOH); ^1H NMR spectrum was correspondence with (1*S*,2*S*) norephedrine) was obtained from (1*R*,2*S*) norephedrine (100 mg) in 82% yield.

4.4. Assays for cytotoxic activity

P388 murine leukemia cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in 95% air, with RPMI-1640 media containing 10% fetal bovine serum, 500 units/mL penicillin, and 50 μg/mL streptomycin. The cells (2.5×104 cells/mL) were incubated with samples (ranging in concentration from 25 to 0.01 μg/mL) in 0.2 mL of cultured medium at 37°C for 3 days. After incubation, the cells were treated with tetrazolium salt (MTT) for 4 h, the plate was centrifuged at 3000*g* for 5 min, and the resulting supernatant was removed by suction. The precipitates were dissolved in dimethyl sulfoxide, and absorbance was measured by a microtiter plate reader at a wavelength of 570 nm (reference wavelength at 630 nm). Cytotoxic activity was evaluated based on a comparison of the survival ratio of the cells with the control value.

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12. Although we tried the esterification of **3** with MTPA by the improved Mosher method, this experiment resulted only in recovered the starting material.
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