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ORIGINAL ARTICLE

Mangromicins, six new anti-oxidative agents isolated from a culture broth of the actinomycete, *Lechevalieria aerocolonigenes* K10-0216

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We have been continually searching for novel chemical compounds from culture broths of various actinomycetes using a physicochemical screening system. During the course of this program, we have previously reported the discovery of two new natural products, designated mangromicins A and B, discovered in a broth of a rare actinomycete strain, *Lechevalieria aerocolonigenes* K10-0216. Mangromicins have a unique and rare structure, a cyclopentadecane skeleton with a tetrahydrofuran unit and a 5,6-dihydro-4-hydroxy-2-pyrone moiety. New mangromicin analogs were isolated by using an improved production medium. As a consequence, six analogs, together with mangromicins A and B, were isolated from a cultured broth of *L. aerocolonigenes* K10-0216. We named them mangromicins D, E, F, G, H and I. All mangromicins showed radical scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals and nitric oxide generated from LPS-stimulated RAW264.7 cells, a murine macrophage cell line. Among the analogs, mangromicins A and I showed the most potent DPPH radical scavenging activity and nitric oxide scavenging activity, respectively.

INTRODUCTION

Of the 1073 small molecule organic compounds introduced worldwide as drugs during the period 1981–2010, 66% were natural products, derivatives or mimics of natural products.[1] Natural products from microbial cultured broths have been screened by bioassay programs to identify useful bioactivity properties.[2] Many bioactive compounds have been discovered by this approach, such as kanamycin,[3] leucomycin[4] and avermectin,[5] all well-known useful compounds of microbial metabolite origin. However, the search for new compounds from microorganisms is often criticized for resulting in the re-discovery, re-isolation and re-characterization of previously known compounds. Therefore, it is important to avoid this duplication as early as possible in the process.

We have discovered new natural products, trehangelins[6] and mangromicins,[7] from culture broths of microorganisms using physicochemical (PC) screening. As PC screening is a method utilizing the PC properties of compounds, such as polarity, molecular formula and UV spectra, this screening method does not depend on biological activity. The search for new compounds via this screening system is guided by liquid chromatography-UV detection (LC/UV), liquid chromatography-MS (LC/MS), and color reaction (for example, detection of alkaloids by Dragendorff's reagent).

We constructed an in-house database based on the PC data of various known compounds in microbial culture broths. Using this database and external databases, such as the Dictionary of Natural Products (http://dnp.chemnetbase.com/), a cultured broth that was predicted to contain new compounds was selected. The structure elucidation of new compounds that were isolated from the cultured broth was carried out. Subsequently, the purified new compound was investigated for biological activities (antimicrobial, antitumor, antiparasitic activities, etc.) by specific customized bioassays.

PC screening system has already identified several new natural compounds, for example, staurosporine,[8] dityromycin[9] and trehangelins.[6] Mangromicins A (**1**) and B (**2**) were also discovered from a cultured broth of the actinomycete species, *Lechevalieria aerocolonigenes* K10-0216, isolated from mangroves in Iriomote Island, Okinawa prefecture, Japan (Figure 1).[7,10,11] Mangromicins have a unique cyclopentadecane skeleton with a tetrahydrofurane unit and a 5,6-dihydro-4-hydroxy-2-pyrone moiety. Moreover, we discovered six new analogs (mangromicins D–I (**3–8**)) from a broth of same strain K10-0216 using an improved production medium.[12] In this study, we report the fermentation, isolation, structure elucidation and anti-oxidative activities of these mangromicin analogs.

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RESULTS AND DISCUSSION

Improvement of fermentation

We previously reported that mangromicins A (1) and B (2) were isolated from the cultured broth of *L. aerocolonigenes* K10-0216 using a production medium consisting of 2% soluble starch, 0.5% glycerol, 1.0% de-fatted wheat germ, 0.3% meat extract, 0.3% dry yeast and 0.3% CaCO$_3$ (basal medium). However, the yields of both 1 and 2 were 0.24 µg ml^{-1} in the basal medium (Supplementary Figure S1). Therefore, we improved the production medium to increase the productivity of both 1 and 2. The productivity of 1 and 2 were examined by altering the amount of all components in the basal medium. The composition of the modified medium was fixed at 5% soluble starch, 0.5% glycerol, 1.0% de-fatted wheat germ, 1.0% dry yeast and 0.3% CaCO$_3$. The productivity of 1 and 2 in the modified medium was increased 100-fold compared with the original medium (Supplementary Figure S1). In addition, many new analogs were detected by LC/UV and LC/MS which were subsequently isolated and their chemical structures were elucidated.

Isolation of six new mangromicin analogs

The procedure for isolation of 1–8 is summarized in Scheme 1. Isolation of all mangromicin analogs was guided by PC properties using LC/UV and LC/MS equipment. The above-described modified medium was used for production of mangromicin analogs. The 8-day-old culture broth (10 l) was centrifuged to separate mycelium
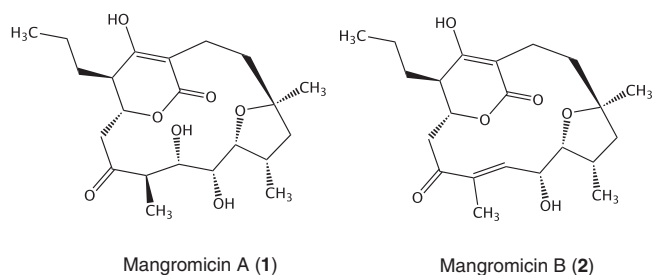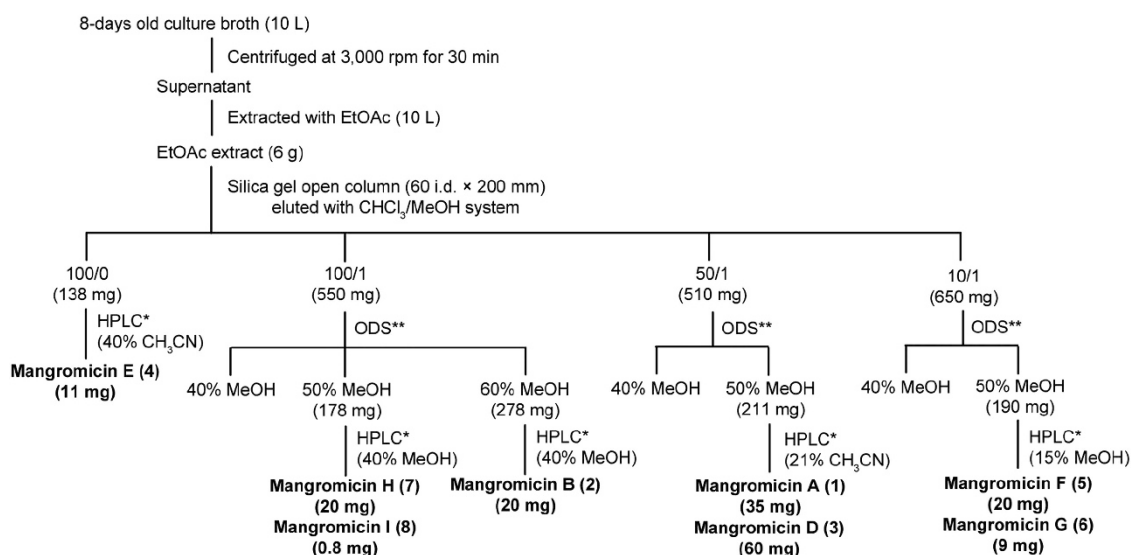
and supernatant. The supernatant was extracted three times with ethyl acetate (10 l). The organic layer was concentrated to dryness *in vacuo* to afford a crude material (6 g). The ethyl acetate extract was applied to silica gel column chromatography FL100D (60 i.d. × 200 mm, Fuji Silysia Co., Tokyo, Japan), which was eluted with each 1 l of a mixture of CHCl$_3$-MeOH (100:0, 100:1, 50:1 and 10:1) in that order. The CHCl$_3$-MeOH (100:0) fraction (138 mg) was dissolved in a small amount of MeOH and purified by HPLC on an Inertsil ODS-4 column (10 i.d. × 250 mm) with 40% MeCN at 4.7 ml min^{-1} (monitoring at UV 254 nm). The yield of mangromicin E (4) was 11 mg. The CHCl$_3$-MeOH (100:1) fraction (550 mg) was applied on an ODS column (40 i.d. × 150 mm, Senshu Scientific Co., Tokyo, Japan). After washing with 40% MeOH, the fractions containing mangromicins H (7) and I (8) were eluted with 50% MeOH, followed by concentration *in vacuo*. This fraction (178 mg) was dissolved in a small amount of MeOH and purified by HPLC on an Inertsil ODS-4 column (10 i.d. × 250 mm) with 40% MeOH at 4.7 ml min^{-1} (monitoring at UV 254 nm). The yields of mangromicins H (7) and I (8) were 20 and 0.8 mg, respectively. The same ODS-treated fraction containing mangromicin B (2) was eluted with 60% MeOH, followed by concentration *in vacuo*. Next, the same ODS-treated fractions containing mangromicin B (2) was eluted with 60% MeOH, followed by concentration *in vacuo*. This fraction (278 mg) was dissolved in a small amount of MeOH and purified by HPLC on an Inertsil ODS-4 column (10 i.d. × 250 mm) with 40% MeOH at 4.7 ml min^{-1} (monitoring at UV 254 nm). The yield of mangromicin B (2) was 20 mg. The CHCl$_3$-MeOH (50:1) fraction (510 mg) was applied on an ODS column (40 i.d. × 150 mm, Senshu Scientific Co.). After washing with 40% MeOH, the fractions containing mangromicins A (1) and D (3) were eluted with 50% MeOH, and then concentrated *in vacuo*. This fraction (211 mg) was dissolved in a small amount of MeOH and purified by HPLC on an Inertsil ODS-4 column (10 i.d. × 250 mm) with 15% MeOH at 4.7 ml min^{-1} (monitoring at UV 254 nm). The yields of mangromicins A (1) and D (3) were 35 and 60 mg, respectively. The CHCl$_3$-MeOH (10:1) fraction (650 mg) was applied on an ODS column (40 i.d. × 150 mm). After washing with 40% MeOH, the fractions containing

Figure 1 Structures of mangromicins A (1) and B (2).

Scheme 1 Purification of mangromicin analogs.

mangromicins F (**5**) and G (**6**) were eluted with 50% MeOH, followed concentration *in vacuo*. This fraction (190 mg) was dissolved in a small amount of MeOH and purified by HPLC on an Inertsil ODS-4 column (10 i.d. × 250 mm) with 15% MeOH at 4.7 ml min^{-1} (monitoring at UV 254 nm). The yields of mangromicins F (**5**) and G (**6**) were 20 and 9 mg, respectively.

PC properties of six new mangromicin analogs

The PC properties of compounds **3–8** are summarized in Table 1. All analogs are readily soluble in MeOH, EtOH and MeCN. Compounds **3–8** showed absorption maxima at 231–251 nm in UV spectra. The IR absorption at 3421–3451 and 1651–1655 cm^{-1} in **3–8** suggested the presence of hydroxyl and carbonyl groups. The PC properties are similar for not only these six compounds but also for mangromicins A (**1**) and B (**2**), confirming that these compounds are structurally related.

Structure elucidation of mangromicin H (7)

Structure analysis was initially performed using mangromicin H (**7**) as it had the lowest MW among the analogs. Mangromicin H (**7**) was obtained as a pale yellow powder determined to have the molecular formula of $C_{21}H_{32}O_6$ by HRESIMS [M + H]$^+$ *m/z* 381.2277 (calcd for $C_{21}H_{33}O_6$, 381.2272). The 1D and 2D NMR spectra of **7** were measured in CD$_3$OD.

The ^{1}H and ^{13}C NMR spectral data of **7** are listed in Tables 2 and 3, respectively. The ^{1}H NMR data indicated the presence of three oxygenated sp^3 methines, three sp^3 methines containing two methines coupled to methyl groups, six methylenes, one primary methyl, two secondary methyls and one tertiary methyl. The ^{13}C NMR spectrum showed the resonances of 21 carbons, which were classified into two olefinic carbons (δ_c 102.8 and 169.3), two carbonyl carbons (δ_c 169.6 and 212.8), one oxygenated sp^3 quaternary carbon (δ_c 83.8), three oxygenated sp^3 methine carbons (δ_c 73.7, 69.2 and 82.0), three sp^3 methine carbons (δ_c 46.1, 49.3 and 37.0), six sp^3 methylene carbons (δ_c 44.3, 37.7, 49.4, 36.5, 19.5 and 25.8) and four methyl carbons (δ_c 11.9, 15.5, 15.5 and 25.3) by HSQC spectra. The ^{1}H–^{1}H COSY and HOHAHA spectra revealed four partial structures: (a) C-5/C-6, (b) C-8/C-13, C-8/C-8-Me and C-12/C-12-Me, (c) C-15/C-16, (d) C-4/C-2′-Me, as shown in Figure 2. The presence of a 5,6-dihydro-4-hydroxy-2-pyrone moiety was confirmed by the HMBC correlations from H$_3$-2′-Me to C-4; from H$_2$-1′ to C-3, C-4 and C-5;

from H-4 to C-1′ and C-2′-Me; from H-5 to C-3, C-4, C-6 and C-1′; from H-16 to C-1, C-2 and C-3. A tetrahydrofurane unit with a secondary methyl and a tertiary methyl linked to C-12 and C-14 positions was confirmed by the HMBC correlations from H-12 to C-14; from H$_2$-13 to C-11; from H$_3$-12-Me to C-11, C-12 and C-13; from H$_3$-14-Me to C-13 and C-14. The HMBC correlations from H$_3$-14-Me to C-15; from H$_2$-15 to C-14, C-14-Me and C-16; from H$_2$-16 to C-14, and C-15 showed that a 5,6-dihydro-4-hydroxy-2-pyrone moiety and tetrahydrofurane unit were connected by an ethylene bond. Finally, the HMBC correlations from H-6 to C-5 and C-7; from H$_3$-8-Me to C-7 revealed the connectivity between C-6 and C-8 via ketone carbon at C-7, which connected two partial structures a and b. Therefore, the planar structure of **7** was elucidated as shown in Figure 2a, and it was designated as mangromicin H, 4-de-*n*-propyl-4-ethyl-9-dehydroxy analog of mangromicin A (**1**).

The relative configuration of **7** was estimated by ^{1}H–^{1}H coupling constant analysis and ROESY experiments (Figure 2b). The ROESY correlations were observed between H$_2$-13b/H$_3$-12-Me and H$_2$-13b/H$_3$-14-Me. These results indicated that H$_3$-14-Me and H$_3$-12-Me are located on the identical surface of the tetrahydrofurane unit. Moreover, the ROESY correlations were observed between H$_3$-8-Me/H-10 and H-10/H$_3$-12-Me. In addition, *J*-coupling between H$_{\alpha1}$ and H$_{\beta1}$ was almost zero, and between H$_{\alpha2}$ and H$_{\beta2}$ was a small coupling constant (0.8 Hz). Therefore, the relative configuration from C-8 to C-14 in **7** was determined as shown in Figure 2b. ROESY and NOE correlations were observed between H-4 and H-6a and between H-4 and H-5. No coupling constants were observed between H-4/H-5 and H-5/H-6a. These results reveal that the *n*-ethyl group at C-4 and methylene at C-6 bound to 5,6-dihydro-4-hydroxy-2-pyrone moiety are located on the opposite surface. Therefore, it was suggested that **7** has the relative configuration of 4R*, 5R*, 8R*, 9S*, 10R*, 11R*, 12S* and 14S* (Figure 2b).

Structure elucidation of mangromicin D (3)

Mangromicin D (**3**) was obtained as a pale yellow powder determined to have the molecular formula of $C_{22}H_{34}O_7$ by HRESIMS [M + H]$^+$ *m/z* 411.2383 (calcd for $C_{22}H_{35}O_7$, 411.2392). As some broadened signals were observed in CD$_3$OD, the 1D and 2D spectra of **3** in DMSO(dimethylsulphoxide)-d_6 were also measured at 80 °C to yield sharper signals.

Table 1 PC properties of mangromicin analogs

| | Mangromicin | | | | | |
	D (3)	E (4)	F (5)	G (6)	H (7)	I (8)
Appearance	Pale yellow	Pale yellow	Pale yellow	Pale yellow	Pale yellow	Pale yellow
Molecular formula	$C_{22}H_{34}O_7$	$C_{22}H_{32}O_6$	$C_{21}H_{32}O_7$	$C_{22}H_{34}O_7$	$C_{21}H_{32}O_6$	$C_{22}H_{32}O_7$
MW	410.5012	392.4859	396.4746	410.5012	380.4752	408.4853
ESI-MS(m/z)						
Calcd	411.2392	393.2277	397.2218	411.2375	381.2272	409.2222
Found	411.2383	393.2277	397.2226	411.2385	381.2277	409.2226
[α]$_D^{25.3}$ (*c* 0.1, MeOH)	+ 27.96	+ 30.48	+ 51.56	+ 29.16	+ 33.80	NT
UV λ$_{max}^{MeOH}$ nm (ε)	251 (1806)	231 (314)	250 (1387)	251 (1231)	251 (1521)	246 (490)
IR ν$_{max}^{KBr}$ cm^{-1}	3421, 1655	3451, 1651	3433, 1651	3425, 1651	3430, 1651	3431, 1651
Soluble in	MeOH	MeOH	MeOH	MeOH	MeOH	MeOH
	EtOH MeCN	EtOH MeCN	EtOH MeCN	EtOH MeCN	EtOH MeCN	EtOH MeCN

Abbreviation: NT, not tested.

Table 2 ¹H NMR data for mangromicin analogs

Position	D (3)[a] δ_H, mult (J in Hz)	E (4)[b] δ_H, mult (J in Hz)	F (5)[b] δ_H, mult (J in Hz)	G (6)[b] δ_H, mult (J in Hz)	H (7)[b] δ_H, mult (J in Hz)	I (8)[b] δ_H, mult (J in Hz)
2		4.63 dd (2.0, 9.6)				
4	2.12 t (7.2)	2.83 ddd (6.0, 6.0, 8.4)	2.05 m	2.13 m	2.01 dd (2.4, 4.0)	2.05 dt (4.0, 14.8)
5	4.56 d (8.4)	4.94 ddd (6.0, 12.0, 12.6)	4.83 m	4.73 dd (1.6, 11.8)	4.79 d (12.0)	4.93 dd (0.8, 11.2)
6a	2.33 m	2.51 m	2.38 m	2.15 dd (17.4, 1.6)	2.36 d (18.4)	2.40 m
6b	4.16 dd (8.4, 16.4)	3.71 t (12.6)	4.34 dd (11.6, 18.4)	4.46 dd (17.4, 11.8)	4.48 dd (12.0, 18.4)	4.78 dd (11.2, 18.4)
8	2.96 m		3.33 m	2.72 m	2.72 m	
9a	1.68 m	6.67 d (8.8)	1.61 m	1.90 m	1.76 m	4.20 d (8.4)
9b	1.84 m		1.80 m	2.32 m	2.42 m	
10	3.50 m	4.70 d (8.8)	3.65 m	3.58 ddd (10.4, 3.6, 0.4)	3.57 ddd (0.8, 3.2, 5.6)	3.20 d (8.4)
11	3.38 dd (2.0, 7.2)	3.60 m	3.55 m	3.33 m	3.40 m	3.42 d (8.8)
12	2.30 m	2.42 m	2.44 m	2.60 m	2.44 dt (3.2, 11.4)	2.55 m
13a	1.54 m	1.45 dd (8.4, 12.6)	1.59 dd (2.0, 5.2)	1.44 m	1.64 m	1.63 m
13b	1.64 dd (8.7, 8.7)	2.10 dd (8.4, 12.6)	1.72 m	2.15 m	1.70 m	1.85 dd (11.6, 13.2)
15a	1.18 dt (2.0, 9.8)	1.28 m	1.26 dt (3.6, 14.2)	1.37 m	1.25 dt (4.0, 14.4)	1.27 dt (4.0, 14.6)
15b	2.38 m	2.56 m	2.49 ddd (4.0, 13.6, 14.2)	2.56 m	2.48 m	2.38 m
16a	1.93 dd (4.8, 9.8)	1.67 m	2.06 m	2.05 dt (4.0, 7.2)	2.04 m	2.11 m
16b	2.71 m	2.45 m	2.85 ddd (4.4, 13.6, 15.4)	2.73 m	2.88 ddd (4.0, 10.4, 12.0)	2.90 ddd (3.8, 13.2, 14.6)
1′	1.48 s	1.32 m, 1.97 m	1.65 m	1.61 m, 1.57 m	1.64 m, 1.70 m	1.68 m, 1.72 m
2′	1.30 m, 1.44 m	1.38 m, 1.48 m		1.38 m, 1.53 m		
2′-(Me)			1.03 t (7.4)		1.03 t (8.0)	1.05 t (7.6)
3′-(Me)	0.88 t (6.4)	0.98 t (7.2)		0.96 t (12.8)		
8-CH₂	3.30 m, 3.50 m		3.52 m, 3.67 m			5.68 d (2.0), 6.48 d (2.0)
8-Me		1.79 d (1.2)		1.03 (3H, d, 13.7)	1.06 d (5.6)	
9-OMe						3.24 s
12-Me	1.0 d (7.0)	1.20 d (7.6)	1.07 d (6.8)	1.09 d (13.7)	1.05 d (3.6)	1.06 d (6.8)
14-CH₂				3.47 d (10.8), 3.96 d (10.8)		
14-Me	1.26 s	1.42 s	1.35 s		1.34 s	1.38 s

[a]In DMSO-d_6 (80 °C).
[b]In CH₃OD.

From a comparison of the ¹H and ¹³C NMR of 3 and 7, the signals of a *n*-propyl (1′; δ_H 1.48/δ_C 32.9, 2′; δ_H 1.30 and 1.44/δ_C 19.1, 3′; δ_H 0.88/δ_C 13.3) and hydroxymethyl (8-CH₂; δ_H 3.30 and 3.50/δ_C 62.3) groups were observed in 3 instead of those of an ethyl (1′; δ_H 1.64 and 1.70/δ_C 25.8, 2′; δ_H 1.03/δ_C 11.9) together with a secondary methyl (8-Me; δ_H 1.06/δ_C 15.5) in 7. In the COSY correlations and HMBC correlations, the conectivity of the *n*-propyl group at C-4 (from H₂-1′ to C-4, from H₂-2′ to C-4 and from H-4 to C-1′ and C-2′) and a hydroxymethyl group at C-8 (from H₂-8 to C-7 and C-9, and from H-9 to CH₂-8) in 3 was confirmed (Supplementary Figure S2). In addition, the presence of an enol-type hydroxy group at C-3 was detected by the HMBC correlations from 3-OH (δ_H br. 9.55–9.83 in DMSO-d_6, Supplementary Figure S7) to C-2 and C-4. Therefore, the planar structure of 3 was elucidated as shown in Figure 3, and it was designated as mangromicin D, a 8-oxymethylene-9-dehydroxy analog of mangromicin A (1).

Structure elucidation of mangromicin E (4)
Mangromicin E (4) was obtained as a pale yellow powder determined to have the molecular formula of $C_{22}H_{32}O_6$ by HRESIMS [M + H]⁺ *m/z* 393.2277 (calcd for $C_{22}H_{33}O_6$, 393.2277). The 1D and 2D NMR spectra of 4 were obtained in CD₃OD.

From a comparison of the ¹H and ¹³C NMR of 4 and 7, the signals of one sp^3 methine (2-CH; δ_H 4.63/δ_C 55.2), one carbonyl carbon (C-2; δ_c 203.6), two olefinic carbons (C-8; δ_c 136.7 and C-9; 148.0), one sp^2 methine (9-CH; δ_H 6.71) and a *n*-propyl (1′; δ_H 1.32 and 1.97/δ_C 28.5, 2′; δ_H 1.38 and 1.48/δ_C 21.1, 3′; δ_H 0.98/δ_C 14.3) group were observed in 4 instead of those of two olefinic carbons (C-2 and C-3; δ_c 102.8 and 169.3), a methine (8-CH; δ_H 2.72/δ_C 49.3), a methylene (H₂-9; δ_H 1.76 and 37.7) and an ethyl (1′; δ_H 1.64 and 1.70/δ_C 25.8, 2′; δ_H 1.03/δ_C 11.9) group in 7. In the COSY correlations and HMBC correlations, the conectivity of the *n*-propyl group at C-4 (from H₂-1′ to C-3, C-4 and C-5, from H₂-2′ to C-4 and from H-4 to C-1′ and C-2′) in 4 was confirmed (Supplementary Figure S2). Therefore, the planar structure of 4 was elucidated as shown in Figure 3, and it was designated as mangromicin E, a 3-keto analog of mangromicin B (2). It is likely that akaerolide and mangromicin E are in relationship of stereoisomers.[13]

Structure elucidation of mangromicin F (5)
Mangromicin F (5) was obtained as a pale yellow powder determined to have the molecular formula of $C_{21}H_{32}O_7$ by HRESIMS [M + H]⁺ *m/z* 397.2226 (calcd for $C_{21}H_{33}O_7$, 397.2218). The 1D and 2D NMR spectra of 5 were obtained in CD₃OD.

Table 3 ^{13}C NMR data for mangromicin analogs

Position	D (3)[a]	E (4)[b]	F (5)[b]	G (6)[b]	H (7)[b]	I (8)[b]
	δ_C, mult	δ_C, mult	δ_C, mult	δ_C, mult	δ_C, mult	δ_C, mult
1	164.4 C	172.2 C	169.2 C	169.0 C	169.6 C	168.3 C
2	101.8 C	55.2 CH	103.3 C	103.0 C	102.8 C	103.4 C
3	163.6 C	204.3 C	167.5 C	167.9 C	169.3 C	169.3 C
4	41.4 CH	50.9 CH	45.4 CH	44.2 CH	46.1 CH	45.6 CH
5	71.2 CH	77.8 CH	73.6 CH	73.9 CH	73.7 CH	73.8 CH
6	45.5 CH$_2$	38.4 CH$_2$	46.6 CH$_2$	44.0 CH$_2$	44.3 CH$_2$	44.0 CH$_2$
7	210.1 C	203.6 C	213.7 C	212.4 C	212.8 C	197.7 C
8	49.2 CH	136.7 C	49.9 CH	43.6 CH	49.3 CH	147.5 C
9	33 CH$_2$	148.0 CH	35.1 CH$_2$	38.6 CH$_2$	37.7 CH$_2$	82.3 CH
10	66.3 CH	69.1 CH	68.9 CH	69.2 CH	69.2 CH	79.5 CH
11	81.2 CH	83.3 CH	84.6 CH	82.1 CH	82.0 CH	80.1 CH
12	35.0 CH	37.6 CH	37.2 CH	36.9 CH	37.0 CH	37.5 CH
13	47.9 CH$_2$	50.5 CH$_2$	49.7 CH$_2$	41.8 CH$_2$	49.4 CH$_2$	48.0 CH$_2$
14	81.2 C	83.5 C	83.2 C	87.0 C	83.8 C	85.0 C
15	34.4 CH$_2$	33.2 CH$_2$	36.0 CH$_2$	34.2 CH$_2$	36.5 CH$_2$	37.3 CH$_2$
16	18.1 CH$_2$	21.4 CH$_2$	19.5 CH$_2$	18.9 CH$_2$	19.5 CH$_2$	19.4 CH$_2$
1′	32.9 CH$_2$	28.5 CH$_2$	24.8 CH$_2$	34.2 CH$_2$	25.8 CH$_2$	25.7 CH$_2$
2′	19.1 CH$_2$	21.1 CH$_2$	21.1 CH$_2$	21.3 CH$_2$		
2′-Me					11.9 CH$_3$	11.8 CH$_3$
3′-Me	13.3 CH$_3$	14.3 CH$_3$	14.3 CH$_3$	14.2 CH$_3$		
8-CH$_2$	62.3 CH$_2$					126.6 CH$_2$
8-Me		11.8 CH$_3$	11.8 CH$_3$	15.3 CH$_3$	15.5 CH$_3$	
9-OMe						57.0 CH$_3$
12-Me	14.6 CH$_3$	18.8 CH$_3$	18.7 CH$_3$	13.7 CH$_3$	15.5 CH$_3$	13.7 CH$_3$
14-CH$_2$				65.9 CH$_2$		
14-Me	24.0 CH$_3$	25.5 CH$_3$	25.5 CH$_3$		25.3 CH$_3$	24.5 CH$_3$

[a]In DMSO-d_6 (80 °C).
[b]In CH$_3$OD.

Figure 2 Structure of mangromicin H (**a**) ^{1}H–^{1}H COSY and HOHAHA correlations (bold) and selected HMBC correlations (arrow) of mangromicin H (**7**). (**b**) Key ROESY and NOE correlations (line) and coupling constants (dot) of mangromicin H (**7**). (**c**) Probable configurations around H$_{\beta1}$-C4-C5-H$_{\alpha1}$ and H$_{\alpha2}$-C10-C11-H$_{\beta2}$ are indicated in Newman projections.

From a comparison of the ^{1}H and ^{13}C NMR of **5** and **7**, the signals of a hydroxymethyl (8-CH$_2$; δ_H 3.52 and 3.67/δ_C 64.8) group were observed in **5** instead of those of a secondary methyl (8-Me; δ_H 1.06/δ_C 15.5) in **7**. In the COSY correlations and HMBC correlations, the conectivity of a hydroxymethyl group at C-8 (from H$_2$-8 to C-7) in **5** was confirmed (Supplementary Figure S2). Therefore, the planar structure of **5** was elucidated as shown in Figure 3, and it was designated as mangromicin F, an 8-oxymethylene analog of mangromicin H (**7**).

Structure elucidation of mangromicin G (6)

Mangromicin G (**6**) was obtained as a pale yellow powder determined to have the molecular formula of C$_{22}$H$_{34}$O$_7$ by HRESIMS [M + H]$^+$ m/z 411.2385 (calcd for C$_{21}$H$_{35}$O$_7$, 411.2375). The 1D and 2D NMR spectra of **6** were obtained in CD$_3$OD.

From a comparison of the ^{1}H and ^{13}C NMR of **6** and **7**, the signals of a n-propyl (1′; δ_H 1.61 and 1.57/δ_C 34.2, 2′; δ_H 1.38 and 1.53/δ_C 21.3, 3′; δ_H 0.96/δ_C 14.2) and a hydroxymethyl (14-CH$_2$; δ_H 3.47 and 3.96/δ_C 65.9) group were observed in **6** instead of those of an ethyl (1′; δ_H 1.64 and 1.70/δ_C 25.8, 2′; δ_H 1.03/δ_C 11.9) and a tertiary methyl (14-Me; δ_H 1.34/δ_C 25.3) observed in **7**. In the COSY correlations and HMBC correlations, the connectivity of an n-propyl group at C-4 (from H$_2$-1′ to C-3, C-4, C-5 and C-2′, from H-4 to C-3 and C-5 and from H-5 to C-4 and C-1′) and a hydroxymethyl group at C-14 (from H$_2$-8 to C-13, C-14 and C-15, and from H-13 and H-15 to CH$_2$-8) in **5** was confirmed (Supplementary Figure S2). Therefore, the planar structure of **6** was elucidated as shown in Figure 3, and it was designated as mangromicin G, a 4-de-ethyl-4-n-propyl-14-oxymethylene of mangromicin H (**7**).

Structure elucidation of mangromicin I (8)

Mangromicin I (**8**) was obtained as a pale yellow powder determined to have the molecular formula of C$_{22}$H$_{32}$O$_7$ by HRESIMS [M + H]$^+$ m/z 409.2226 (calcd for C$_{22}$H$_{33}$O$_7$, 409.2222). The 1D and 2D NMR spectra of **7** were obtained in CD$_3$OD.

From a comparison of the ^{1}H and ^{13}C NMR of **8** and **7**, the signals of a methylene (8-CH$_2$; δ_H 5.68 and 6.48/δ_C 126.6), one methine (9-CH; δ_H 4.20/δ_C 82.3) and methoxy (9-OCH$_3$; δ_H 3.24/δ_C 57.0) group were observed in **8**, instead of those of a secondary methyl (8-Me; δ_H 1.06/δ_C 15.5) and a methylene (H$_2$-9; δ_H 1.76 and 37.7) observed in **7**. In the COSY correlations and HMBC correlations, the conectivity of an exomethylene group at C-8 (from H$_2$-8 to C-7, C-8 and C-9 and from H-9 to H$_2$-8) in **8** was confirmed (Supplementary Figure S2). Therefore, the planar structure of **8** was elucidated as shown in Figure 3, and it was designated as mangromicin I, an 8-de-methyl-8-exomethylene-9-de-methylene-9-methoxy analog of mangromicin H (**7**).

Biological activities of mangromicins

Eight mangromicin analogs were evaluated in a panel of assays, such as antimicrobial activities for pathogenic bacteria and fungi, antivirus activity, cytotoxicity for cancer cells and antiparasitic activity for parasitic protozoa. In consequence, all mangromicin analogs except for mangromicin E (**4**) and I (**8**) had more potent scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals than that of α-tocopherol. Among the eight compounds, mangromicin A (**1**) showed the most potent anti-oxidant activity s (IC$_{50}$ = 2.4 μM, Table 4, Supplementary Figure 3S).
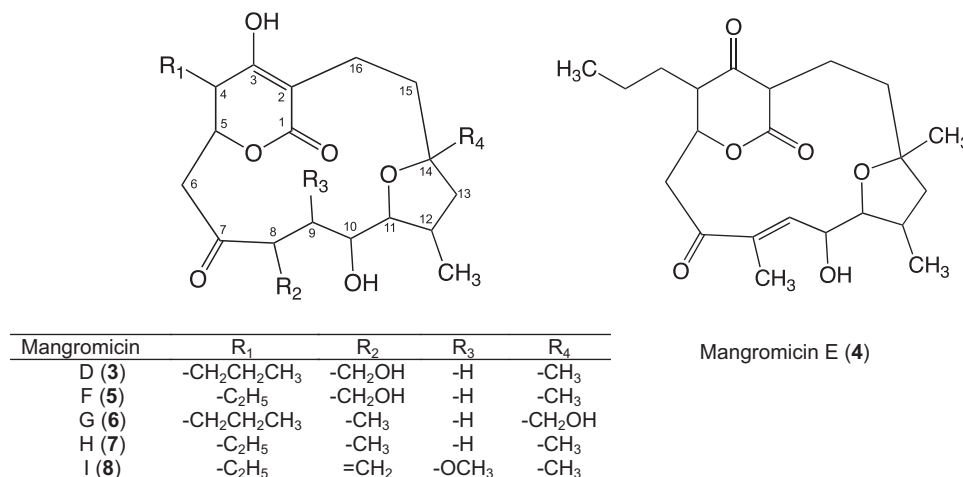
Mangromicin	R_1	R_2	R_3	R_4
D (**3**)	-CH$_2$CH$_2$CH$_3$	-CH$_2$OH	-H	-CH$_3$
F (**5**)	-C$_2$H$_5$	-CH$_2$OH	-H	-CH$_3$
G (**6**)	-CH$_2$CH$_2$CH$_3$	-CH$_3$	-H	-CH$_2$OH
H (**7**)	-C$_2$H$_5$	-CH$_3$	-H	-CH$_3$
I (**8**)	-C$_2$H$_5$	=CH$_2$	-OCH$_3$	-CH$_3$

Figure 3 Structures of mangromicins D (**3**), E (**4**), F (**5**), G (**6**), H (**7**) and I (**8**).

Table 4 Scavenging effects of mangromicin analogs on DPPH free radicals

Compound	DPPH free radical scavenging activity (IC$_{50}$, µM)
Mangromicin A	2.4 ± 0.10
Mangromicin B	3.8 ± 0.03
Mangromicin D	3.1 ± 0.01
Mangromicin E	31.1 ± 0.33
Mangromicin F	3.9 ± 0.05
Mangromicin G	6.5 ± 0.05
Mangromicin H	4.1 ± 0.04
Mangromicin I	16.3 ± 0.15
α-tocopherol	11.4 ± 0.77

Figure 4 Scavenging effects of mangromicin analogs on NO generated from LPS-stimulated a murine macrophage RAW264.7 cell line.

In addition, mangromicin analogs were investigated for scavenging activity with respect to nitric oxygen (NO) generated from LPS-stimulated RAW264.7 cells, a murine macrophage cell line. All mangromicin analogs (at final concentration of 100 µM) showed no cytotoxic activity against RAW264.7 cells (data not shown). As shown in Figure 4, mangromicin I (**7**) had the most potent NO scavenging activity. To further understand the effect of mangromicin B (**2**), which was the second-most potent analog, on the mRNA expression profiles of iNOS in murine macrophage RAW264.7 cells, RT-PCR (reverse transcription-polymerase chain reaction) tests were conducted. As mangromicin I (**8**) was in short supply, mangromicin B (**2**) was used for the RT-PCR experiment. Mangromicin B (**2**) did not attenuate mRNA expression levels of iNOS when compared with LPS-stimulated cells (Supplementary Figure S4). It was therefore suggested that mangromicin B (**2**) has an ability to scavenge NO generated from LPS-stimulated RAW264.7 cells.

Interestingly, the anti-oxidant activity of mangromicin E (**4**) was reduced about 10 times than that of mangromicin B (**2**). As mangromicin E (**4**) had a carbonyl group at the C-3 position instead of hydroxy group of mangromicin B (**2**), it was suggested that the hydroxy group at the C-3 position is important in the scavenging activity for DPPH free radicals. Conversely, mangromicins B (**2**), E (**4**) and I (**8**) showed more potent NO scavenging activity among the analogs tested. As these three analogs had an α,β-unsaturated ketone carbonyl group at the C-7 position, it was suggested that this common partial structure is important in the NO scavenging activity.
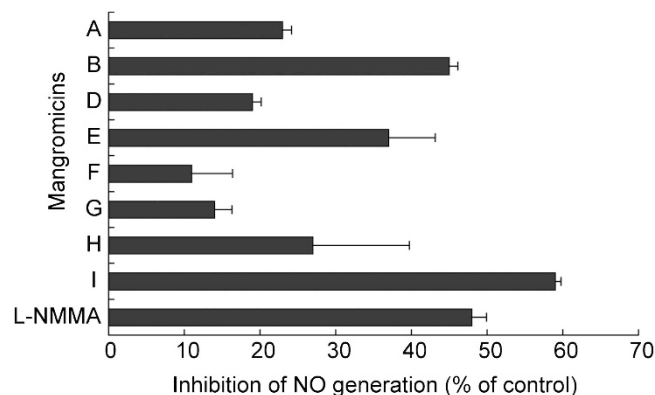
Thus, there was poor correlation between DPPH free radical scavenging activity and NO scavenging activity in mangromicin analogs. Therefore, the functional site for radical scavenging activity in mangromicin analogs may be different according to radical species.

METHODS
General experimental procedures
NMR spectra were measured using an Agilent Technologies NMR System 400 and INOVA 600 (Varian Co., Palo Alto, CA, USA) or JEOL JNM-ECA 500 spectrometer (JEOL Ltd., Tokyo, Japan), with ^{1}H-NMR at 400, 500 or 600 MHz and ^{13}C-NMR at 100, 125 or 150 MHz in CD$_3$OD or DMSO-d_6. The chemical shifts are expressed in p.p.m. and are referenced to residual CD$_3$OD (3.31 p.p.m.) or DMSO-d_6 (2.48 p.p.m.) in the ^{1}H-NMR spectra and CD$_3$OD (49 p.p.m.) or DMSO-d_6 (39.5 p.p.m.) in the ^{13}C-NMR spectra. LC-ESI-MS spectra were measured using an AB Sciex QSTAR Hybrid LC/MS/MS Systems (AB Sciex, Framingham, MA, USA). IR spectra (KBr) were taken on a Horiba FT-710 Fourier transform IR spectrometer (Horiba Ltd, Kyoto, Japan). UV spectra were measured with a Hitachi U-2810 spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan). Optical rotation was measured on a JASCO model DIP-1000 polarimeter (Jasco Co., Tokyo, Japan). Fluorescence was monitored using a Corona Grating Microplate Reader SH-9000 Lab (Corona Electric Co., Ltd., Ibaraki, Japan).

Reagents
Reagents were purchased from the following sources: LPS was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). DPPH, IFN-γ and NG-monomethyl-

L-arginine were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Diaminofluorescein-2 (DAF-2) was purchased from Sekisui Medical Co., Ltd. (Tokyo, Japan). All other reagents used were of analytical grade.

Fermentation

The strain K10-0216 was grown and maintained on agar slants consisting of 1.0% starch, 0.3% NZ amine, 0.1% yeast extract, 0.1% meat extract, 1.2% agar and 0.3% $CaCO_3$. A loop of spores of strain K10-0216 was inoculated into 100 ml of seed medium, consisting of 2.4% starch, 0.1% glucose, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract and 0.4% $CaCO_3$ (adjusted to pH 7.0 before sterilization) in a 500-ml Erlenmeyer flask. The flask was incubated on a rotary shaker (210 r.p.m.) at 27 °C for 3 days. A 1-ml portion of the seed culture was transferred to 500-ml Erlenmeyer flasks (total 150) containing 100 ml of starch medium, consisting of 5.0% soluble starch, 0.5% glycerol, 1.0% de-fatted wheat germ, 1.0% dry yeast and 0.3% $CaCO_3$ (adjusted to pH 7.0 before sterilization) and fermentation was carried out on a rotary shaker (210 r.p.m.) at 27 °C for 7 days.

Biological activities of mangromicins

Effect of mangromicins on DPPH free radicals: antioxidative activity was estimated by the DPPH free radical scavenging method, as described previously with a minor modification.[14] DPPH was dissolved in dimethylformamide ($10\,mg\,ml^{-1}$) and then diluted to $120\,\mu mol\,l^{-1}$ with $0.1\,mol\,l^{-1}$ Tris-HCl buffer (pH 7.6) containing 5% Triton X-100. Ten microliters of the sample solution was then added to $90\,\mu l$ of a DPPH solution. After incubation for 30 min in the dark at room temperature, the absorbance at 517 nm was measured with a microplate reader (Corona Electric).

Effect of mangromicins on NO production generated from LPS-stimulated RAW 264.7 cells: RAW 264.7 cells (a mouse macrophage cell line) were obtained from the American Type Culture Collection (Rockville, MD, USA). Raw 264.7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, streptomycin ($100\,U\,ml^{-1}$) and penicillin G ($100\,U\,ml^{-1}$) at 37 °C in an atmosphere of 5% CO_2 and 95% air, as described previously.[15]

RAW 264.7 cells were plated in wells of a 96-well microplate (1×10^5 cells/well), and were cultured for 6 h to make macrophages adherent. Cells were subsequently cultured for 16 h with $200\,\mu l$ of fresh medium containing LPS ($100\,ng\,ml^{-1}$) and IFN-γ ($10\,U\,ml^{-1}$). The activated cells adherent in wells were washed twice with KRP (Krebs–Ringer–Phosphate) buffer, $200\,\mu l$ of medium containing each mangromicin analog (a final concentration at $100\,\mu mol\,l^{-1}$) diluted in DMSO with $1\,mM$ L-arginine and $10\,\mu M$ DAF-2 was added to each well. DAF-2, a sensitive fluorescent indicator, was used for the detection of NO.[16,17] The final DMSO concentration was <0.1%. At this concentration, DMSO did not show any NO-induction without stimulation with LPS. After incubation for 2 h, the supernatants were transferred to black microplates and the florescence was measured with a microplate reader (Corona Electric) calibrated for excitation at 495 nm and emission at 515 nm.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)