DEMALONYL DERIVATIVES OF GUANIDYLFUNGIN A AND COPIAMYCIN: THEIR SYNTHESIS AND ANTIFUNGAL ACTIVITY

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Guanidylfungin A was chemically modified by alkylation, reduction and/or demalonylation. Demalonylmethylguanidylfungin A became soluble in water and showed approximately eight-fold higher activity against fungi and Gram-positive bacteria than guanidylfungin A along with strongly fungicidal effect. Similarly, copiamycin was converted to demalonylmethylcopiamycin, which also showed higher antifungal activity than copiamycin itself.

Recently, the structures of macrocyclic lactone antibiotics, such as azalomycins F_{3a}, F_{4a}, and F_{5a}[1,2], copiamycin[3,8], neocopiamycin A[4], scopafungin[5], niphimycin I[6~8], and guanidylfungins A and B[9,10] were successively reported. These antibiotics have a macrocyclic polyhydroxyl lactone ring with a malonyl monoester and an intramolecular hemiketal, and a side chain with a terminal guanidine. They are active against Gram-positive bacteria and fungi with fungistatic effect even at high concentrations and some are effective for fungal infections[11,12].

In the course of our studies of the guanidylfungins[9,10], we found that demalonylmethylguanidylfungin A (**6**) showed higher activity than guanidylfungin A against fungi and Gram-positive bacteria.

In this paper we report synthesis and antimicrobial activity of demalonyl derivatives of guanidylfungin A and copiamycin.

Preparation of Demalonyl Derivatives (Scheme 1)

Guanidylfungin A (**1**) was methylated with methanolic hydrochloride to give methylguanidylfungin A (**2**)[10]. This procedure was applied to prepare other alkyl derivatives, *i.e.*, ethyl- (**3**), butyl- (**4**), and allylguanidylfungin A (**5**) (see Experimental). Methylguanidylfungin A (**2**) was selectively demalonylated with base to produce demalonylmethylguanidylfungin A (**6**) without opening the lactone ring. Reduction of **1** with sodium borohydride yielded **7**, which was also demalonylated with base to give **8**. The lactone ring was stable to base and was not opened during either demalonylation reaction. The stability may be attributed to the conformation of the macrocyclic molecule. However, direct hydrolysis of **1** with base gave a complex mixture of products. This is possibly due to the degradation reactions such as retro aldol cleavage of β-ketol during alkaline treatment[13].

The structures of **3** to **8** were determined by secondary ion mass spectrometry (SIMS) and ^{13}C

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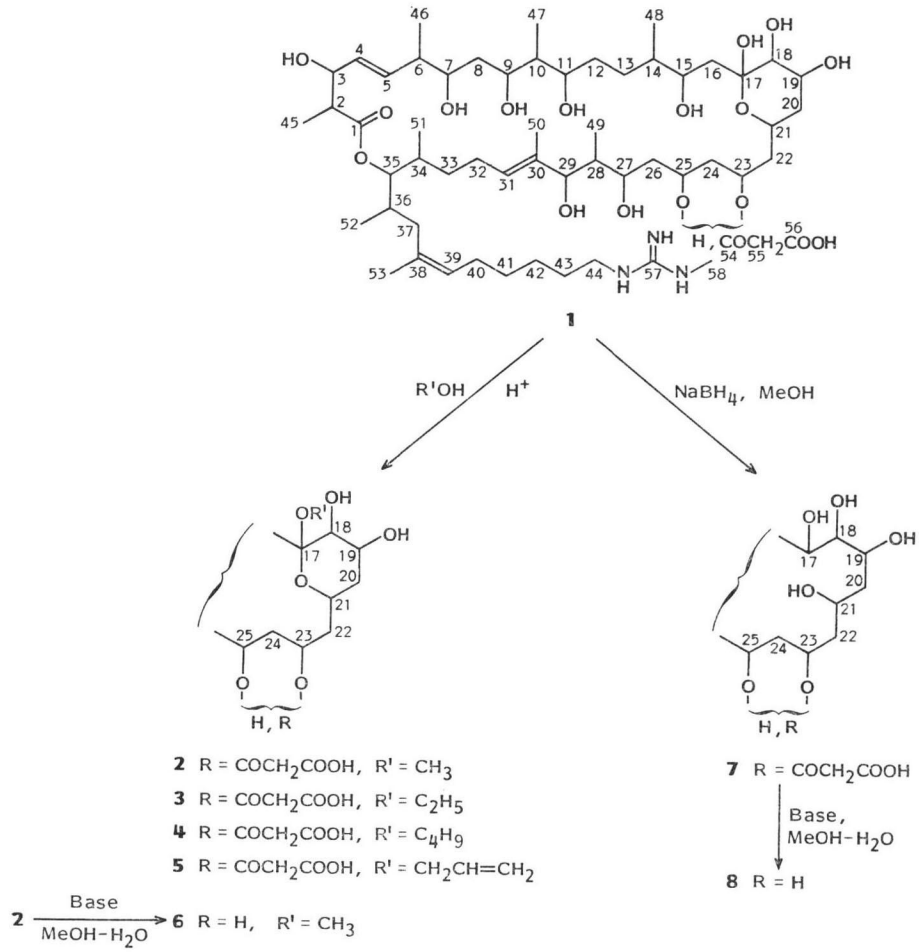
Scheme 1. Preparation procedures of guanidylfungin A derivatives.



NMR spectra (see Experimental). The absence of the malonyl group in **6** and **8** was confirmed by their ^{13}C NMR spectra (Fig. 1), in which the signals owing to carbonyl carbons (δ_c 173.8 and 171.3) of the malonyl group disappeared.

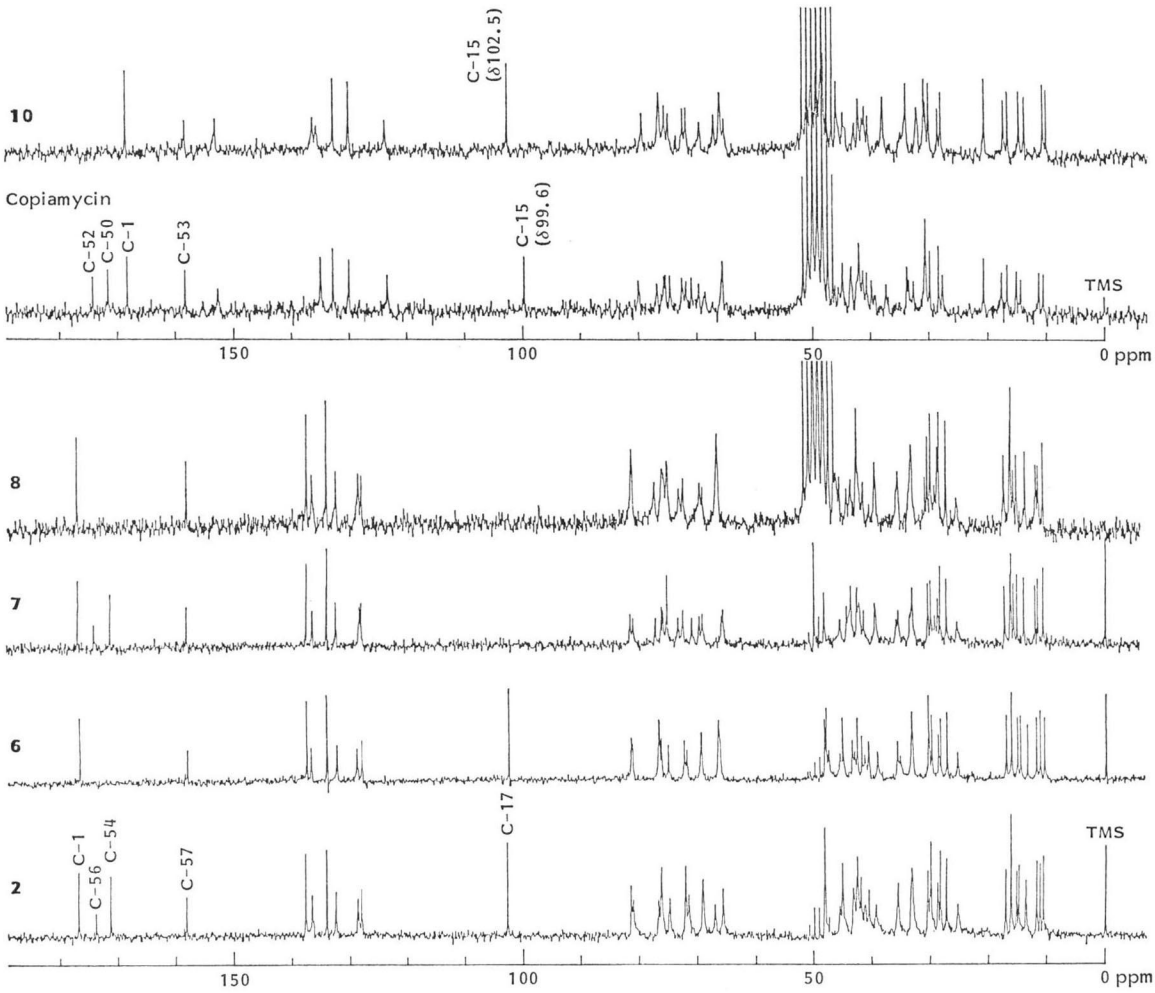
Copiamycin was also converted to methylcopiamycin (**9**) and then to demalonylmethylcopiamycin hydrochloride (**10**) (Fig. 2). The structures of **9** and **10** were determined by the ^{13}C NMR spectra (Fig. 1) and SIMS (see Experimental).

Antimicrobial Activity of Derivatives

The antimicrobial spectra of **2** to **10** were measured by serial two-fold agar dilution method (Table 1).

Compounds **2**, **3**, **4**, and **5** were as active as **1** or were slightly less active than **1**, against fungi and Gram-positive bacteria. Demalonylmethylguanidylfungin A hydrochloride (**6**) had four- to eight-fold the activity against these organisms compared to **1** and **2**. Compound **7** almost lost antimicrobial activity except against *Aspergillus fumigatus*. Compound **8** showed higher antimicrobial activity than **7** and was selectively active against *A. fumigatus*.

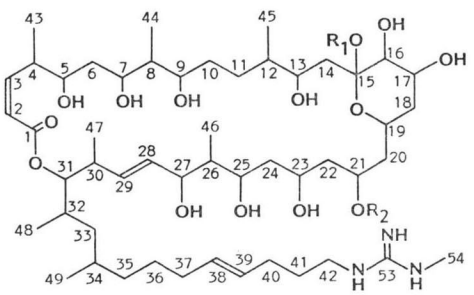
Fig. 1. ^{13}C NMR of methylguanidylfungin A (**2**), copiamycin and their derivatives (25 MHz in CD_3OD).



These results indicate that a) the six-membered hemiketal ring plays an important role in the antimicrobial activity and b) the malonyl group is less important for the antimicrobial activity. Guanidylfungin A is an amphoteric compound. The demalonylation yielded a basic compound and the solubility of its hydrochloride was much increased (**6** was soluble in water up to about 15 mg/ml, whereas the solubility of **1** was below 1 mg/ml). The enhanced activity may partly be explained by the increased solubility in water.

These findings on the activity of guanidylfungin A resemble those[14] of polyene macrolide

Fig. 2. Structures of copiamycin and its derivatives.



Copiamycin	$R_1=H$	$R_2=\overset{50}{C}O\overset{51}{C}H_2\overset{52}{C}OOH$
Methyl-copiamycin (**9**)	$R_1=CH_3$	$R_2=COCH_2COOH$
Demalonylmethyl-copiamycin (**10**)	$R_1=CH_3$	$R_2=H$

Table 1. Antimicrobial spectra of guanidylfungin A (**1**), copiamycin (CPM), and their derivatives.

Organisms tested	Medium*	MIC (μg/ml)										
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	CPM	**9**	**10**
Staphylococcus aureus FDA 209P	1	6.25	12.5	6.25	12.5	6.25	0.78	>100	100	12.5	25	3.12
Bacillus subtilis PCI 219	1	12.5	25	12.5	25	12.5	1.56	>100	100			
Escherichia coli K-12	1	>100	>100	>100	>100	>100	>100	>100	>100			
Candida albicans IAM 4888	2	12.5	25	25	25	25	3.12	>100	100			
C. albicans Yu 1200	3	50	50				6.25		100	25	25	6.25
Saccharomyces cerevisiae IAM 4020	2	50	50	50	50	50	6.25	>100	100			
Aspergillus fumigatus IAM 2153	2	25	12.5	25	50	25	3.12	50	12.5			
Mucor racemosus	2	12.5	6.25	6.25	25	12.5	3.12	>100	>100			
Paecilomyces variotii IAM 5001	2	12.5	12.5	12.5	25	12.5	1.56	>100	50			
Sporotrichum schenckii	2	12.5	25	25	25	25	3.12	>100	50			

* Medium 1: Heart infusion agar (37°C, 20 hours), 2: potato dextrose agar (30°C, 2 days), 3: Sabouraud dextrose agar (37°C, 2 days).

antibiotics such as amphotericin B and nystatin. These polyene antibiotics are amphoteric; their methyl esters are soluble in water and are as active as the parent compounds.

The minimum fungicidal concentrations (MCC) against *Candida albicans* Yu 1200 of **2**, **6**, and **8** were >200, 25, and 200 μg/ml, respectively; this shows that the activity of the demalonyl derivatives is not fungistatic but fungicidal. Thus, the mode of their antifungal action seems to be somewhat different from that of the native compounds.

The MICs against *C. albicans* Yu 1200 of **9** (methylcopiamycin) and **10** (demalonylmethylcopiamycin hydrochloride) were 50 and 6.25 μg/ml, respectively (Table 1), and the MCCs of **9** and **10** were >200 and 25 μg/ml, respectively. As expected, the antifungal activity was enhanced and the activity became fungicidal, by demalonylation.

The demalonyl derivatives **6**, **8**, and **10** may also exhibit synergism with imidazole antifungal agents such as clotrimazole and miconazole, as do copiamycin, neocopiamycin A[4,12], and guanidylfungin A (K. TAKESAKO, unpublished data).

Experimental

General

Melting points were taken using a Yazawa BY-1 and are uncorrected. UV spectra were measured on a Shimadzu UV-250 spectrometer. IR spectra were recorded using a Hitachi apparatus (model 270-30). Mass spectra were measured on a Hitachi M-80A. NMR spectra were recorded with a Jeol JNM FX-100 spectrometer and chemical shifts are given in ppm (in δ) relative to TMS as an internal or external standard.

Alkylation of Guanidylfungin A (1)

To a suspension of **1** (0.6 g) in MeOH (2 ml) was added 1 N methanolic HCl (2 ml) with stirring. After 20 minutes of stirring the solution was subjected to reversed phase HPLC using MeOH - 0.01 M aq NH_4OAc (80: 20). The fractions containing **2** were collected, concd and precipitated from hot aq acetone to give a white powder (0.3 g): mp, SIMS, ^1H and ^{13}C NMR (see ref 10).

Similarly, a suspension of **1** (50~100 mg) in ethanol, butanol or allyl alcohol was treated with ethanolic, butanolic or allyl alcoholic HCl to give **3** (15 mg), **4** (15 mg) or **5** (29 mg), respectively.

3: MP 147~152°C; SIMS *m/z* 1,158 (M+H); ^{13}C NMR (25 MHz in CD_3OD) δ 176.8 (s), 173.9 (s), 171.3 (s), 158.2 (s), 137.7 (s), 136.6 (d), 134.0 (s), 132.4 (d), 128.7 (d), 128.0 (d), 102.7 (s), 81.4 (d), 81.0 (d), 76.7 (d), 76.2 (dd), 74.8 (d), 72.1 (dd), 71.5 (d), 69.1 (dd), 67.0 (d), 65.6 (d), 56.3 (t, OCH_2CH_3), 48.1 (d), 45.4 (t), 45.1 (dt), 43.2 (dt), 42.5 (tt), 41.9 (d), 41.3 (t), 40.5 (d), 39.3 (t), 36.5 (t), 35.5 (d), 33.2 (tdt), 30.4 (t), 29.9 (tt), 28.7 (t), 28.3 (q), 27.3 (t), 25.3 (t), 17.0 (q), 16.1 (qq), 15.9 (q, OCH_2CH_3), 15.2 (q), 14.8 (q), 13.7 (q), 11.7 (q), 11.2 (q), 10.6 (q).

4: MP 134~136°C; SIMS *m/z* 1,186 (M+H); ^{13}C NMR (25 MHz in CD_3OD) δ 176.9, 174.5, 171.3, 158.2, 137.6, 136.6, 134.1, 132.5, 128.9, 128.0, 102.6, 81.5, 81.0, 76.8, 76.2, 74.9, 72.2, 71.7, 69.7, 69.0, 67.1, 65.5, 60.7 (t, $OCH_2(CH_2)_2CH_3$), 45.5, 45.1, 43.3, 42.6, 41.8, 41.3, 40.5, 39.3, 36.5, 35.5, 33.4, 33.2, 30.5, 30.1, 29.9, 28.7, 28.4, 27.3, 25.3, 20.7, 17.0, 16.1, 15.2, 14.7, 14.7 (q, $OCH_2(CH_2)_2CH_3$), 13.6, 12.0, 11.1, 10.6.

5: MP 149~156°C; SIMS *m/z* 1,170 (M+H); ^{13}C NMR (25 MHz in CD_3OD) δ 176.7, 173.9, 171.3, 158.1, 137.6, 136.8 (d, $OCH_2CH=CH_2$), 136.5, 134.0, 132.4, 128.7, 127.9, 115.7 (t, $OCH_2CH=CH_2$), 103.0, 81.3, 81.0, 76.7, 76.2, 74.8, 72.0, 71.4, 69.1, 67.0, 65.6, 61.8 (t, $OCH_2CH=CH_2$), 45.4, 45.0, 43.1, 42.5, 42.0, 41.2, 40.5, 39.3, 36.6, 35.4, 33.1, 30.4, 29.9, 28.7, 28.3, 27.2, 25.3, 16.9, 16.1, 15.1, 14.7, 13.6, 11.7, 11.1, 10.5.

Reduction of 1

To a suspension of **1** (0.5 g) in MeOH (50 ml) was added a solution of $NaBH_4$ (1 g) in MeOH (10 ml) with stirring, and the solution was stirred at room temp overnight. The reaction mixture

was neutralized with dil aq HCl and concd to dryness under reduced pressure. The residue was washed with H_2O, extracted with MeOH and purified by preparative reversed phase HPLC with MeOH - 0.01 M NH_4OAc (80: 20) to give **7** (410 mg): mp 118~122°C; SIMS *m/z* 1,132 (M+H); ^{13}C NMR (25 MHz in CD_3OD) δ 176.7, 173.9, 171.3, 158.2, 137.5, 136.6, 134.0, 132.5, 128.3, 128.0, 81.4 (d), 81.0 (d), 77.1 (d), 76.1 (d), 75.8 (d), 75.2 (dd), 73.2 (d), 72.4 (d), 70.8 (d), 69.6 (d), 69.1 (d), 65.8 (d), 65.6 (d), 48.2, 45.5, 44.3, 44.0, 43.5, 42.3, 41.4, 39.4, 35.8, 35.4, 33.5, 33.1, 30.4, 29.9, 29.2, 28.6, 28.3, 27.2, 25.4, 17.1, 16.3, 16.1, 15.7, 15.1, 13.9, 12.0, 11.5, 10.6.

Demalonylation of **2** and **7**

To a solution of **2** (0.5 g) in MeOH (60 ml) was added 2 N KOH in MeOH - H_2O (2: 1, 20 ml) and the solution was allowed to stand at room temp overnight. The solution was neutralized with dil aq HCl and concd to dryness. The residue was washed with H_2O and extracted with MeOH to give **6** (380 mg): mp 122~125°C; SIMS *m/z* 1,058 (M+H); ^{13}C NMR (25 MHz in CD_3OD) δ 176.7 (s), 158.1 (s), 137.4 (s), 136.7 (d), 134.0 (s), 132.3 (d), 128.8 (d), 127.9 (d), 102.4 (s), 81.3 (d), 81.1 (d), 76.6 (dd), 76.0 (d), 75.0 (d), 72.3 (d), 71.8 (d), 69.3 (dd), 66.4 (dd), 66.1 (d), 48.1 (q, OCH_3), 47.8, 45.5, 45.0, 43.4, 43.0, 42.5, 41.8, 41.3, 40.6, 39.0, 35.7, 35.1, 33.2, 30.4, 29.8, 28.7, 28.3, 27.3, 25.3, 17.0, 16.0, 15.1, 14.5, 13.4, 11.8, 11.2, 10.5.

Anal Calcd for $C_{56}H_{103}N_3O_{15}\cdot HCl$: C 61.51, H 9.52, N 3.84.
Found: C 60.79, H 9.52, N 3.62.

To a solution of **7** (130 mg) in MeOH (20 ml) was added 2 N KOH in H_2O (6 ml) and the solution was allowed to stand at room temp overnight. Then the reaction mixture was neutralized with dil aq HCl and subjected to a column of Diaion HP-20 (50 ml) after evaporation of MeOH. The column was washed with H_2O and eluted with MeOH. The eluate was concd to dryness to give **8** (30 mg): mp 110~116°C; SIMS *m/z* 1,046 (M+H); ^{13}C NMR (25 MHz in CD_3OD) δ 177.0 (s), 158.4 (s), 137.5 (s), 136.5 (d), 134.2 (s), 132.5 (d), 128.4 (d), 128.0 (d), 81.5 (dd), 77.5 (d), 76.0 (dd), 75.5 (dd), 72.7 (d), 70.8 (d), 70.0 (d), 69.3 (d), 67.0 (ddd), 46.0, 45.5, 43.6, 42.6, 42.2, 41.4, 39.8, 39.4, 35.9, 35.7, 33.4, 33.0, 30.4, 29.9, 29.5, 28.7, 28.3, 27.3, 25.5, 17.0, 16.3, 16.1, 15.7, 15.1, 13.9, 12.0, 11.6, 10.7.

Production and Isolation of Copiamycin

Streptomyces hygroscopicus var. *crystallogenes* (IFM 1236) was used for production of copiamycin. Spores of the strain grown on oatmeal agar were inoculated into 500-ml Erlenmeyer flasks containing 100 ml of a medium composed of glucose 1.0%, Polypeptone 0.2%, beef extract 0.1% and yeast extract 0.1%, and the flasks were incubated with shaking for 40 hours at 27°C. The culture broth (200 ml) was transferred into a 30-liter fermentor containing 20 liters of the medium described above. The fermentation was carried out at 27°C for 3 days under aeration (15 liters/minute) and agitation (250 rpm).

The mycelial cake collected by centrifugation from the cultured broth (40 liters) was extracted with acetone - H_2O (7: 3). The extract was mixed with Celite (50 g) and the solvent was evaporated off to give a brown powder. The powder was applied to a silica gel column prepared in $CHCl_3$ and developed with $CHCl_3$, $CHCl_3$ - MeOH (4: 1) and $CHCl_3$ - MeOH - H_2O (65: 25: 4) and (5: 4: 1). The $CHCl_3$ - MeOH - H_2O (5: 4: 1) eluate was collected and concd to give a yellowish powder (6 g), which was a mixture of copiamycin and neocopiamycin A. The powder (2 g) was then chromatographed on a silica gel column with 2-BuOH and 2-BuOH - H_2O (4: 1). The fractions containing copiamycin were collected and concd to give a white powder (480 mg): mp 142~145°C; UV λ_{max}^{MeOH} nm (ε) 215 (sh, 19,000); IR (KBr) cm^{-1} 3400, 2970, 2940, 1720, 1670, 1650, 1600, 1460, 1380, 1300, 1250, 1150, 1070, 980; SIMS *m/z* 1,058 (M+H), 970 (M−$COCH_2COOH$); ^{13}C NMR (see Fig. 1).

Preparation of Methylcopiamycin (**9**) and Demalonylmethylcopiamycin (**10**)

Copiamycin (200 mg) was treated with methanolic HCl for 10 minutes and the solution was neutralized with Amberlite IRA-45 (OH^- type). The resin was filtered off and the filtrate was concd to dryness. The residue was applied to a silica gel column and developed with $CHCl_3$ and $CHCl_3$ - MeOH - H_2O (65: 25: 4). The fractions containing methylcopiamycin (**9**) were collected and concd to give a white powder (165 mg): SIMS *m/z* 1,072 (M+H), 984 (M−$COCH_2COOH$).

To a solution of **9** (130 mg) in MeOH (10 ml) was added 1 N KOH in H_2O (10 ml). The solution was allowed to stand at room temp for 4 hours and then concd to dryness after neutralization with dil aq HCl. The residue was extracted with MeOH and the extract was subjected to a silica gel column. The column was developed with $CHCl_3$ and $CHCl_3$ - MeOH - H_2O (65: 25: 4). The fractions containing demalonylmethylcopiamycin hydrochloride (**10**) were collected and concd to give a white powder (50 mg): mp 131~136°C; UV λ_{max}^{MeOH} nm (ε) 215 (sh, 15,500); IR (KBr) cm^{-1} 3400, 2970, 2940, 1700, 1680, 1650, 1465, 1380, 1250, 1150, 1100, 980; SIMS *m/z* 986 (M+H); ^{13}C NMR (25 MHz in CD_3OD) δ 168.3 (s), 158.1 (s), 152.9 (d), 136.3 (d), 135.6 (d), 132.7 (d), 130.0 (d), 123.6 (d), 102.5 (s), 79.3 (d), 76.6 (dd), 75.4 (d), 74.8 (d), 72.3 (d), 71.7 (d), 69.4 (d), 66.9 (d), 65.9 (dd), 65.2 (d), 45.8, 44.6, 42.6, 41.9, 41.3, 40.9, 40.3, 37.7, 33.7, 31.9, 30.6, 30.4, 29.8, 28.3 (q), 27.8, 20.4, 17.1, 16.3, 14.4, 13.5, 10.4, 9.8.

Determination of Minimum Fungicidal Concentrations

Concentrations of guanidylfungin A derivatives were adjusted by serial two-fold dilution with Sabouraud dextrose (2%) broth using a microtiter plate. To each microtiter well of various concentrations of drugs, *C. albicans* was inoculated to a final concentration of 1×10^5 cells/ml by counting with a hemacytometer in a total volume of 150 μl. The microtiter plate was incubated at 30°C for 20 hours. Then a portion (100 μl) was removed from each well, spread on Sabouraud dextrose agar and incubated at 30°C. After 2 days of incubation the number of colonies was counted. The minimum fungicidal concentration was defined as the lowest concentration of drug that gave no more than 10 colonies.

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