GTRI-BB, a New Cytotoxic Isochromanquinone Produced by *Micromonospora* sp. SA-246

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Crisamicin A, an isochromanquinone antibiotic, which is produced by *Micromonospora* sp. has antimicrobial properties[1~3]. In our previous studies, its derivatives, 9- and 1-hydroxycrisamicin A, also showed antimicrobial activity chiefly against Gram-positive bacteria[4,5], but not against Gram-negative bacteria and fungi, and was inhibitory to the growth of some human cancer cell lines[4]. Currently we also found a new antibiotic, referred as GTRI-BB (Fig. 1), from the microbial culture that differed from the above antibiotics and crisamicin C[6], but was structurally similar to crisamicin A. We report in this paper its structure elucidation and biological properties including cytotoxic and antimicrobial activities.

The GTRI-BB-producing strain, *Micromonospora* sp. SA246, which was already described in the previous study[4], was used in this study. Potato-dextrose broth was used for the culture of the microorganism. SA 246 was cultured in 500 ml flasks containing each 100 ml of the medium for 14 days on a rotary shaker (250 rpm) in a growth chamber at 27°C.

The culture filtrate and 70% aq. acetone extract of the mycelial cake were combined and extracted with ethyl acetate. The dark reddish extract was concentrated *in vacuo* and applied to silica gel column chromatography, eluting with a linear gradient of ethyl acetate - methanol (100 : 1 ~ 100 : 50). Active fractions showing cytotoxicity against cancer cell lines and antimicrobial activity against *Bacillus subtilis* IAM 1069 were collected and subjected to the 2nd chromatography using a silica gel column with chloroform - methanol gradient elution (100 : 1 ~ 100 : 20). The active fractions were concentrated and chromatographed on TLC (Merck silica gel 60 F_{254} plate, 250 mm) using the solvent system of chloroform : methanol (25 : 1). An active band with the *Rf* value of 0.49, which was different from those of other isochromanquinone antibiotics, was scraped off and extracted with the mixture of chloroform - methanol (20 : 1). The extract was further purified by HPLC on a 20 mm i.d.×250 mm Nucleosil C18, 7 μm column in a 62% aq. acetonitrile to afford GTRI-BB with a retention time of 27.7 minutes. The production of GTRI-BB from the microbial culture was very low relative to other crisamicin A derivatives, amounting less than about one tenth of 9- or 1-hydroxycrisamicin A.

The purified dried material was yellow powder. It was readily soluble in DMSO, CH_3CN, and $CHCl_3$ (containing a small portion of MeOH), but insoluble in water and *n*-hexane. UV-visible spectrum measured with a Shimazu UV-260 spectrophotometer showed the absorption peaks at 233, 270 and 460 nm. ESI-mass spectrum measured on a JEOL HX 100 mass spectrometer had the $[M-H]^-$ peak at 629.2, assuming the molecular weight to be 630, which is 16 mass units higher than 1- or 9-hydroxycrisamicin A.

The various NMR spectra were obtained on a Bruker NMR spectrometer at 400 MHz in $CDCl_3$ with TMS as an internal standard. ^1H and ^{13}C NMR spectral data of GTRI-BB summarized in Table 1 were almost identical to those of crisamicin A and its hydroxyl derivatives[4,5]. This suggests that GTRI-BB may be one of crisamicin A derivatives. ^1H NMR of GTRI-BB showed two hydrogen-bonded hydroxyl groups at 11.88 and 11.93 ppm, four aromatic methine, seven oxygenated methine, two methylene, and three

Fig. 1. Structure of GTRI-BB.

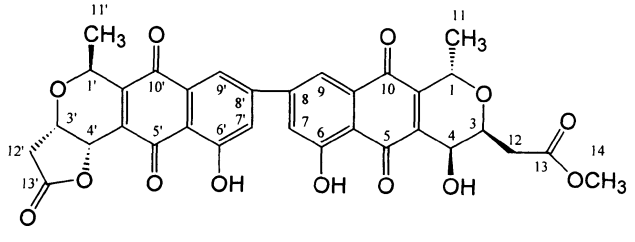

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Table 1. ^{1}H and ^{13}C NMR spectral data for GTRI-BB in $CDCl_3$.

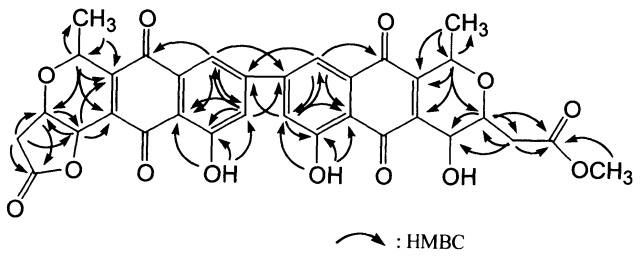
No.	d_C	d_H
1	67.5	5.06 (q, J = 6.8 Hz)
3	67.7	4.38 (m)
4	59.9	4.74
4a	140.5	
5	186.9[a]	
5a	114.4	
6	162.0	
6-OH		11.88 (s)
7	122.8	7.60 (d, J = 1.6 Hz)
8	146.4	
9	118.2	7.94 (d, J = 1.6 Hz)
9a	132.2[b]	
10	181.8	
10a	148.2	
11	17.7	1.58 (d, J = 6.8 Hz)
12	35.5	2.88 (m)
13	171.4	
14	51.9	3.78 (s)
1'	66.7	5.12 (q, J = 6.8 Hz)
3'	66.5	4.74
4'	68.3	5.29 (d, J = 2.8 Hz)
4a'	134.6	
5'	188.4[a]	
5a'	114.6	
6'	161.9	
6'-OH		11.93 (s)
7'	122.4	7.57 (d, J = 1.6 Hz)
8'	146.3	
9'	117.9	7.92 (d, J = 1.6 Hz)
9a'	132.5[b]	
10'	182.5	
10a'	150.9	
11'	18.5	1.58 (d, J = 6.8 Hz)
12'	36.8	3.00 (dd, J = 17.8, 5.2 Hz)
		2.74 (d, J = 17.8 Hz)
13'	173.8	

Protons of H-4 and H-3' were overlapped.

[a,b] Chemical shifts interchangeable.

methyl signals. ^{14}C NMR and DEPT spectra revealed signals assigned to followings: four quinone carbonyl carbons (188.4, 186.9, 182.5, and 181.8 ppm), two carbonyl carbons (173.8 and 171.4 ppm), four aromatic methine carbons (122.8, 122.4, 118.2, and 117.9 ppm), two oxygenated sp^2 quaternary carbons (162.0 and 161.9 ppm), 10 sp^2 quaternary carbons (114.4~150.9 ppm), and 11 sp^3 carbons (17.7~68.3 ppm). Direct comparison of NMR spectral data showed that GTRI-BB contained one more oxygenated methyl proton signal at 3.78 ppm and one more methyl carbon signal at 51.9 ppm than other crisamicin A derivatives. Except these exceptions, the NMR spectral data matched well with crisamicin A. A significant chemical shift was noted in C-4 at 59.9 ppm, compared to other crisamicin A derivatives with C-4 around 69.0 ppm, suggesting cleavage site of γ-lactone ring. This means the oxygenated methine of C-4 in crisamicin A may have a hydroxyl group, leading to a cleavage of γ-lactone ring. Considering the chemical shift values and the molecular weight, the increased molecule would be methylated and hydroxylated into crisamicin A. The structure of GTRI-BB was confirmed by ^{1}H-^{13}C long-range correlations by the HMBC experiments, which showed a critical long-range coupling between 14-H (3.78 ppm) and 13-C (171.4 ppm) as shown in Fig. 2. Other long-range correlations were consistent with the structure of GTRI-BB. Therefore, GTRI-BB was assigned as a crysamicin analog that one of two γ-lactone rings in crysamicin A is opened. The above NMR data matched well with the molecular weight of the compound, proving that it should have a molecular formula of $C_{33}H_{26}O_{13}$. Also the stereochemistry of compound GTRI-BB was suggested to be the same stereostructure as that of crysamicin A, as shown in Fig. 1. This proposition was supported by the NMR data consistent with those of crisamicin A and isolation of crisamicin A from the culture broth of the GTRI-BB-producing strain.

Fig. 2. HMBC correlations of GTRI-BB.



Cytotoxicity of the purified compound against a panel of human cancer cell lines was tested. Each of cell lines was suspended in the 10% calf serum-containing RPM 1640 medium and diluted to give appropriate cell densities for inoculation onto 96-well microtiter plates, preincubating for 24 hours at 37°C in 5% CO_2 to allow stabilization. The compound was added into the microtiter plates, and incubated for 48 hours. The cell growth was assayed by

Table 2. Cytotoxicity of GTRI-BB on human tumor cell lines.

Cell line	GI_{50} (μg/ml)[a]	
	GTRI-BB	Adriamycin
PC-3 (Prostate)	0.21	0.94
ACHN (Renal)	0.08	0.60
A549 (Lung)	0.25	0.95
SW 620 (Colon)	0.11	0.58
K562 (Leukemia)	0.31	0.91
Du 145 (Prostate)	0.20	0.81
UACC 62 (Melanoma)	0.08	0.28

Each of cell lines were incubated for 48 hours in the presence of GTRI-BB at 37°C in 5% CO_2 after 24-hour preincubation.

[a]Concentration of 50% growth inhibition.

SRB method[7]. A dose-response curve was plotted and a concentration giving 50% inhibition of cell growth (IC_{50}) was calculated. GTRI-BB exhibited stronger inhibitory effect on the growth of the tumor cell lines than adriamycin which is a commercial anticancer compound (Table 2). GTRI-BB was more cytotoxic than 9-hydroxycrisamicin A, showing more than twice toxicity to the same kinds of cell lines such as melanoma, lung and colon cancers. This indicates the structural modification may enhance the cytotoxic efficacy.

Specific pathogen-free (SPF) BDF1 mice (females, 18~21 g) were used as test animals for the anticancer activity of GTRI-BB. Mice were intraperitoneally implanted with B16 melanoma (10^5 cells/animal), and from 4 hours later, the chemical solutions (0.1 mg, 0.3 mg and 1.0 mg/kg) were intraperitoneally administered daily at 0.2 ml per 20 g body weight 13 times up to on the 12th day after the cancer cell implementation. For negative and positive controls, 0.8% tween 80 (vehicle alone) and 1 mg/kg adriamycin were administered as above. Mortality and body weight changes of treated mice were examined daily and at the intervals of 2 days, respectively, until 30 days after the first treatment. Contrary to the cytotoxic efficacy of GTRI-BB, no significant effect on the survival of mice implanted with B16 melanoma at 3 concentration levels of GTRI-BB (0.1, 0.3 and 1.0 mg/kg), while 1 mg/kg of adriamycin was very effective for sustaining the survival of mice (survival extension efficacy of 90.2% than the vehicle control). GTRI-BB rather shortened the mouse life span at 1 mg/kg concentration, indicating nonspecific toxicity. The mice treated with GTRI-BB or adriamycin showed no significant changes of body weight during the first 12 days except for 1 mg/kg of GTRI-BB that showed weight loss for 5 days after treatment compared with the vehicle alone. This suggests that BTRI-BB should have no value as the internal use for antitumorous therapy. However, its high toxicity especially to melanoma may implicate that the compound can be applied topically or locally with a proper delivery

Table 3. Antimicrobial activity of GTRI-BB.

Test organism	Diameter of inhibition zone[a] (mm)
Gram−negative bacteria	
Escherichia coli	0
Pseudomonas aeruginosa	0
P. fluorescens	0
Salmonella typhimurium	0
Gram−positive bacteria	
Staphylococcus aureus R−209	23
Bacillus subtilis	14
Sarcina lutea	11
Streptococcus sp.	10
Yeasts	
Candida albicans	0
Saccharomyces cerevisiae	0
Fungi	
Magnaporthe grisea	0
Alternaria mali	0
Fusarium solani	0
Colletotrichum sp.	0

[a] Paper discs (8 mm in diameter) with 40 μg of GTRI−BB were placed on agar media, and inhibition diameter was examined.

system.

Antimicrobial activity was determined by paper disc method using Mueller-Hinton agar and potato-dextrose agar (PDA) for bacteria and fungi, respectively. GTRI-BB showed antibiotic activity only against the Gram-positive bacteria tested, but no effect against the Gram-negative bacteria and fungi (Table 3) as in other crisamicin derivatives. This suggests that the secondary structural modification of crisamicin A may be not related to the changes of antimicrobial activity.

References

1) NELSON, R. A.; J. A. POPE, G. M. LUEDEMANN, L. E. MCDANIEL & C. P. SCHAFFNER: Crisamicin A, a new antibiotic from *Micromonospora*. I. Taxonomy of the producing strain, fermentation, isolation, physicochemical characterization and antimicrobial properties. J. Antibiotics 39: 335~344, 1986
2) LING, D. L.; S. SHIELD & K. L. RINEHART: Isolation and structure determination of crisamicin A, a new antibiotic from *Micromonospora purpureochromogenes* subsp. *halotolerans*. J. Antibiotics 39: 345~353, 1986
3) NELSON, R. A.; J. A. POPE, R. C. PANDEY, L. E. MCDANIEL, C. P. SCHAFFNER, R. L. BEVERIDGE, P. H. HOOPS & F. JORDAN: Studies on the biosynthesis of the antibiotic crisamicin A and carbon-13 magnetic resonance assignments. J. Antibiotics 41: 1659~1667, 1988
4) YEO, W. H.; B. S. YUN, N. I. BACK, Y. H. KIM, S. S. KIM, E. K. PARK, K. S. WHANG & S. H. YU: 9-Hydroxycrisamicin A, a new cytotoxic isochromanquinone antibiotic produced by *Micromonospora* sp. SA246. J. Antibiotics 39: 546~550, 1997
5) YEO, W. H.; O. K. LEE, B. S. YUN, J. S. YOO, Y. K. KIM, E. K. PARK, S. S. KIM, Y. H. KIM, S. K. KIM, I. D. YOO, K. S. WHANG & S. H. YU: 1-Hydroxycrisamicin A, a new antibacterial isochromanquinone antibiotic produced by *Micromonospora* sp. SA246. J. Antibiotics 51: 82~84, 1998
6) RUSSELL, W. L.; R. C. PANDEY & C. P. SCHAFFNER: Crisamicin C, a new isochromanquinone antibiotic. Isolation, structure determination, and biosynthesis. J. Antibiotics 41: 149~156, 1988
7) SKEHAN, P.; R. STORENG, D. SCUDIERO, A. MONKS, J. MCMAHON, D. VISTICA, J. T. WARREN, H. BOKESCH, S. KENNEY & M. R. BOYD: New colorimetric cytotoxicity assay for anticancer-drug screening. J. Natl. Cancer Inst. 82: 1107~1112, 1990