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Bottromycin derivatives: Efficient chemical modifications of the ester moiety and evaluation of anti-MRSA and anti-VRE activities

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ABSTRACT

Novel bottromycin derivatives were synthesized from bottromycin A₂ via a hydrazide derivative as a common intermediate. Seventeen derivatives were subjected to in vitro evaluation against drug-resistant gram-positive bacteria. Some compounds showed potent anti-MRSA and anti-VRE activity, as did bottromycin A₂. Notably, a propyl ketone derivative exhibited good antibacterial activity with excellent metabolite stability.

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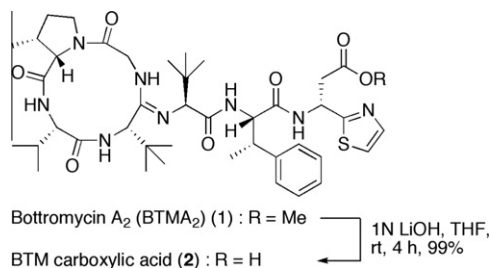
The increasing prevalence of multidrug-resistant gram-positive bacteria, including methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) strains, has created increasingly serious problems.^{1,2} In particular, MRSA has been recognized as one of the major pathogens causing nosocomial infections. Clinically-used linezolid and teicoplanin produce undesirable side effects, such as anaphylactoid symptoms, acute renal failure and disruption of liver function. More recently, vancomycin-resistant *Staphylococcus aureus* (VISA) has been increasingly identified even though vancomycin is the last resort anti-MRSA antibiotic.³ Therefore, there is an urgent and growing need for development of new antibiotics with novel modes of action to help overcome these problems.

During the screening for anti-MRSA and anti-VRE agents from the natural product library in our institute, bottromycin A₂ (BTMA₂) (**1**) (Scheme 1) was found to exhibit potent antibiotic activity against clinically-isolated MRSA and VRE strains. Bottromycin A₂ was first isolated from the fermentation broth of *Streptomyces bottropensis* by Waisvisz et al. in 1957, and found to be an antibacterial peptide effective against gram-positive bacteria and mycoplasma.⁴ In 1991, Yokoyama et al. first reported the potent anti-MRSA properties of **1**.⁵ We have independently confirmed that **1** has potent anti-MRSA and anti-VRE activities with MIC value of 1.0 µg/mL and 0.5 µg/mL, respectively.⁶ As for the mechanism of action, **1** inhibits bacterial protein synthesis by blocking aminoacyl-tRNA from connecting to the A site on the 50S ribosome.⁷

The mode of action of **1** is different from that of other antibiotics.^{8,9} Therefore, **1** represents a promising novel antibiotic for treatment of bacterial infections. We have accomplished the first asymmetric total synthesis and determination of the absolute stereochemistry of bottromycin A₂.⁶ We herein report the synthesis, anti-MRSA and anti-VRE activities and metabolite stability of several bottromycin derivatives.

At the outset, we needed to prepare sufficient quantities of **1** by fermentation. We earlier discovered that **1** is produced in the culture broth of *Streptomyces* sp. KM-9459. Therefore, fermentation of KM-9459 strain was carried out in a liquid culture medium.¹⁰

It should be stressed that **1** does not show good in vivo efficacy because of its instability in oral and parenteral administrations.¹¹ In addition, Miller et al. reported that some amide derivatives of the methyl ester moiety of **1** showed in vivo efficacy against *S. aureus* infection in mice.¹² From these results, we predicted that the



Scheme 1. Synthesis of BTM carboxylic acid (**2**).

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deactivation of **1** (in vivo tests) is caused by hydrolysis of the terminal methyl ester moiety. To confirm the instability, we performed hydrolysis of **1** with 1 N LiOH to produce the corresponding terminal acid (**2**) to compare with the metabolites of **1** (Scheme 1).¹³ Subsequently, bottromycin degradation product was compared to **2** (Fig. 1). Bottromycin A₂ was completely decomposed in mouse plasma after 10 min, as the LC–MS chromatogram signal at 10.83 min of **1** disappeared. Additionally, the retention time of the degradation product was identical to that of **2**, which showed very weak anti-MRSA activities (MIC ≥ 64 μ g/mL). These results suggested that the deactivation of **1** in vivo was due mainly to the instability of the terminal ester moiety. Therefore, we designed amide, urea and ketone derivatives, which did not undergo hydrolysis with plasma.

At first, we attempted the synthesis of derivatives via **2**. But **2** showed amphipathic property, making it difficult to handle on large-scale preparation. In order to improve handling and make it simpler to synthesize various derivatives, a synthetic strategy of bottromycin derivatives via a key intermediate hydrazide (**3**) was drawn up (Scheme 2).¹⁴ The hydrazide (**3**) was prepared from **1** to act as a precursor an active azide ester.

The reaction sequence for preparation of **3** and BTM amide derivatives are outlined in Scheme 3. BTM hydrazide (**3**) was

prepared from bottromycin A₂ in 97% yield. Treatment of **3** in *N*-methyl-2-pyrrolidinone (NMP), with *n*-butyl nitrite under acidic condition, afforded the azide ester, which was used to the condensation process without purification. Six amide derivatives, representing a diverse range of acyclic and cyclic groups, were subjected to the condensation process. Each of these amines was reacted to azide ester to give amides (**4–9**) in moderate to good isolated yields (56–78%). Furthermore, the Boc group of **9** was removed to afford the free piperazino amide (**10**) in 78% yield. Then 3-bromo propyne or benzyl bromide was added to a solution of **10** in DMF, to give 4-propargyl piperazino amide (**11**) and 4-benzyl piperazino amide (**12**), respectively, in 72% and 71% yields. Several urea derivatives were synthesized as follows (Scheme 4): firstly, acyl azide was obtained in a manner similar to previously described. Subsequently, BTM isocyanate was synthesized by Curtius rearrangement of the BTM acyl azide in toluene. The BTM isocyanate was then converted to *N*'-thiazolidylurea (**13**) with thiazolidine in 29% yield from **3**. In the same way, *N*'-benzyl-urea (**14**) was synthesized with benzyl amine in 38% yield. Next, thioester and ketone derivatives were prepared (Scheme 5). Ethanethiol, 1-propanethiol or 2-propanethiol was added to a solution of BTM acyl azide in NMP to produce the desired thioester derivatives (**15–17**) in low to good yields (14–81%). The resulting ethyl thioes-

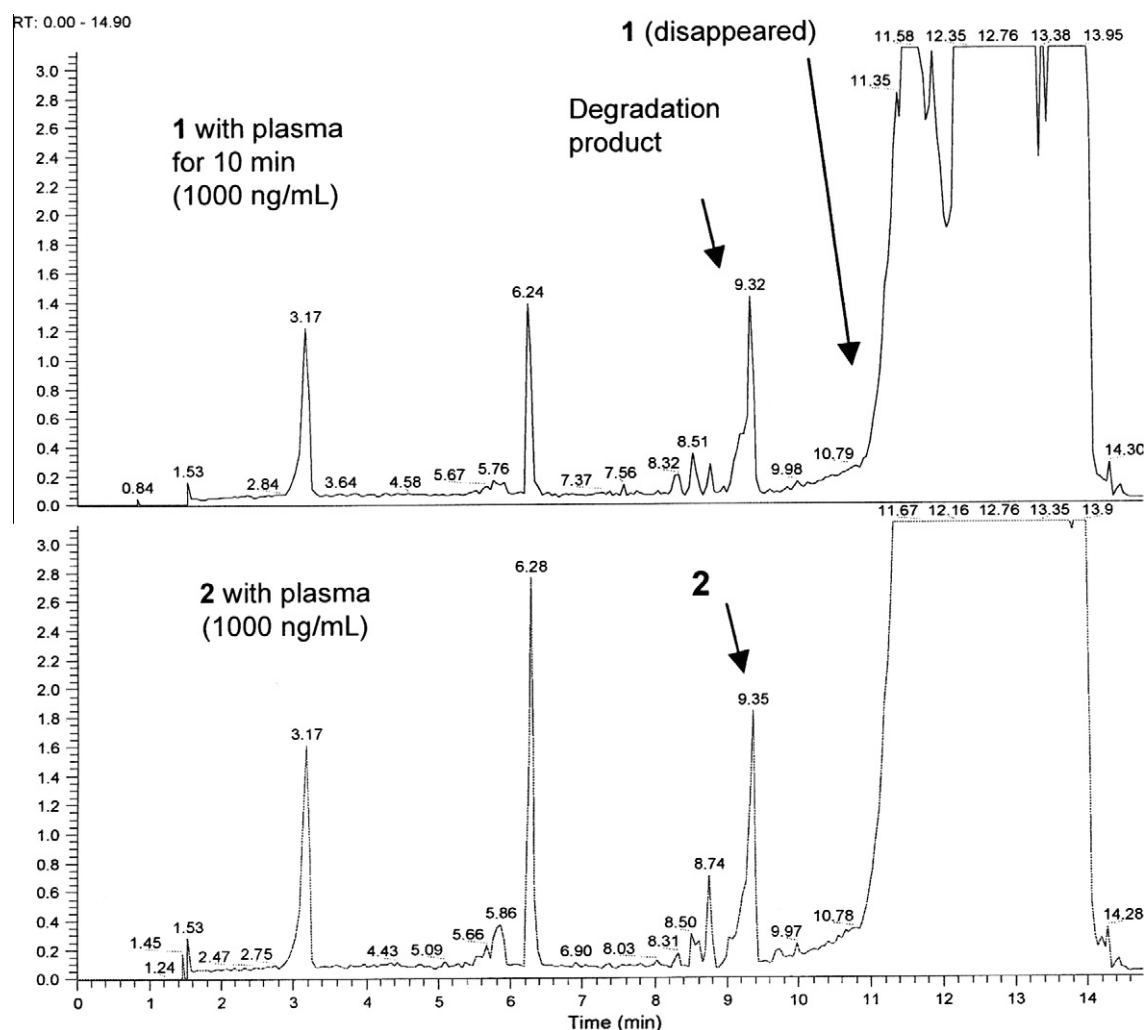
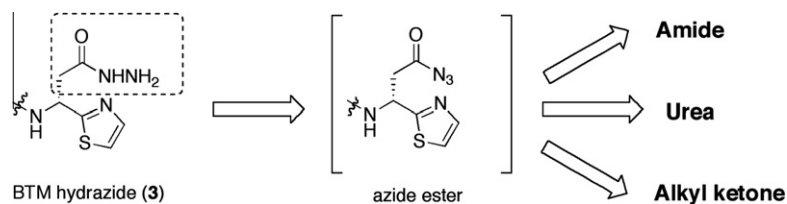
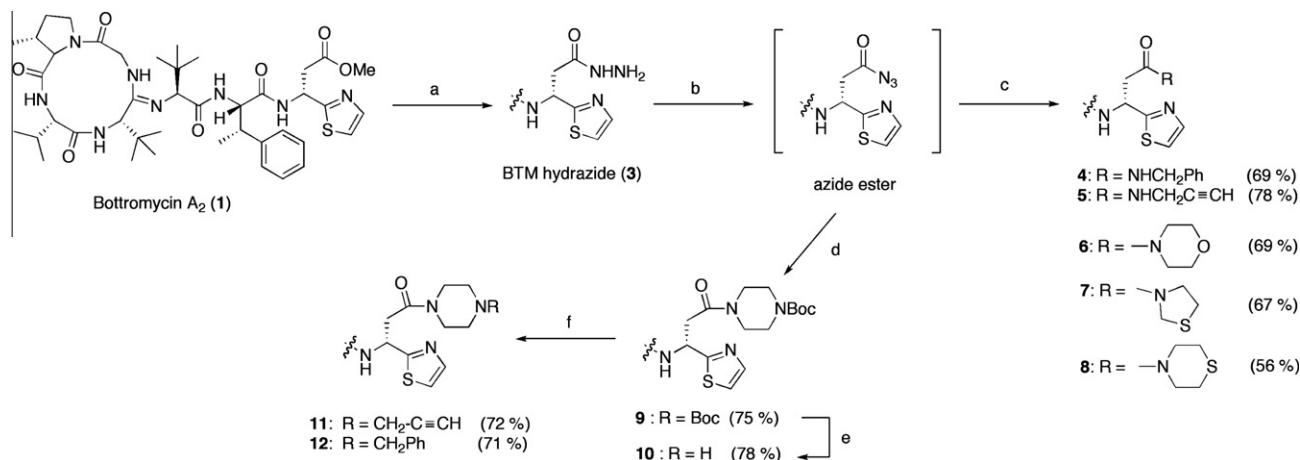


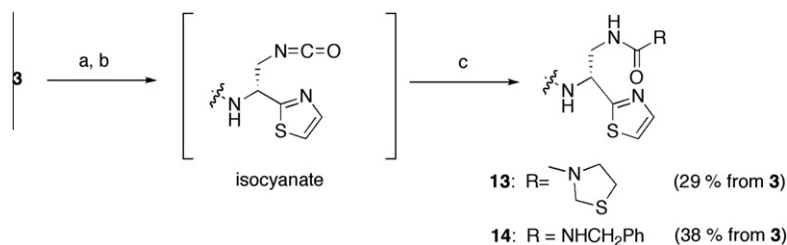
Figure 1. LC/MS chromatograms of bottromycin degradation product and bottromycin carboxylic acid (**2**) in mouse plasma. (A) Degradation product of bottromycin A₂ in mouse plasma; (B) authentic bottromycin carboxylic acid (**2**) in mouse plasma. Ion-trap mass spectrometry (ionization mode): positive ESI, sample volume injected: 20 μ L, analytical column: Zorabox SB-C18 (3.5 μ m, 50 mm \times 4.6 mm ID), solvent A: 0.1% AcOH, solvent B: 0.1% AcOH-containing MeCN, total flow rate: 1.0 mL/min, split ratio: 1:4 (mass spectrometer:waste).



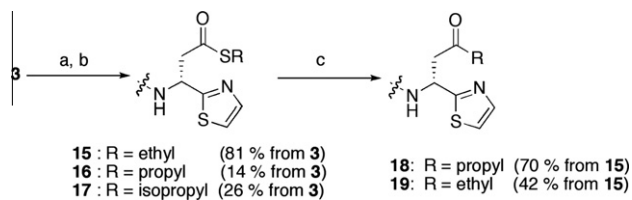
Scheme 2. Synthetic strategy of bottromycin derivatives from BTM hydrazide (**3**) via azide ester.



Scheme 3. Synthesis of amide derivatives (**4–12**). Reagents and conditions: (a) hydrazine, EtOH, 90 °C, 4 h, 97%; (b) 4 N HCl/dioxane, *n*-butyl nitrite, NMP, rt, 30 min; (c) primary or secondary amines, TEA, 60 °C; (d) 1-(*tert*-butoxycarbonyl)piperazine, TEA, 60 °C, 2 h; (e) 4 N HCl/dioxane, CH₂Cl₂, rt, 2 h; (f) 3-bromo propyne or benzyl bromide, K₂CO₃, DMF, rt.



Scheme 4. Synthesis of urea derivatives (**13, 14**). Reagents and conditions: (a) same as step b in [Scheme 3](#); (b) TEA, toluene, 60 °C; (c) thiazolidine or benzylamine, toluene, 60 °C.



Scheme 5. Synthesis of ketone derivatives (**18, 19**) via the thioester (**15**). Reagents and conditions: (a) same as step b in [Scheme 3](#); (b) R-mercaptane, TEA, NMP, rt; (c) R-ZnI, Pd(PPh₃)₂Cl₂, CH₂Cl₂/THF, rt.

ter (**15**) was then converted to ketone derivatives (**18, 19**) in 70% and 42% yields, respectively, using the procedure of Fukuyama et al.^{15–17}

The efficiency of the chemical modifications via the hydrazide intermediate of **1** allowed the preparation of each derivatives at the terminal methyl ester moiety of thiazolyl β-alanine in significant purity for in vitro testing against six gram-positive strains [*S. aureus* FDA209P, *S. aureus* Smith; MRSA HH-1, MRSA 92-1191; VRE (*Enterococcus faecalis* NCTC12201 and *E. faecalis* NCTC12203)],

using standard serial-dilution techniques ([Table 1](#)). The BTM hydrazide (**3**) actually showed less anti-MRSA activity than **1**. BTM piperazino amide and urea derivatives (**4–14**) exhibited 4- to 32-fold less activity against *S. aureus*, MRSA and VRE than **1**. Although these derivatives showed weaker activity, they exhibited improved stability, as expected. Particularly, amide derivatives (**5–8**) and urea derivatives (**13, 14**) showed good stability. Additionally, comparison of **6** and **8**, indicated that introduction of sulfur atom has a tendency to improve antibacterial properties. As envisaged, the BTM thioester derivatives (**15–17**) were unstable in mouse plasma. However, it is noteworthy that **15–17** showed 2- to four-fold more potent antibacterial activity. The thioesters were the first compounds exhibiting better impact than **1**. These results suggest that **1** has significant promise as a lead compound for new drugs. Moreover, the antibacterial activities of the ketone derivatives (**18, 19**) were almost the same as that of **1**. In particular, the propyl ketone (**18**) produced results very similar to vancomycin. The stability in mouse plasma of **18** and **19** was notably better, compared with thioester derivatives. Subsequent biological evaluation using MRSA-infected mice revealed that **18** has superior efficacy. Notably, 100 mg/kg of **18** given orally caused mice to sur-

Table 1

Minimum inhibitory concentrations (MICs) value of BTM derivatives

Compound/ strains	<i>S. aureus</i> FDA209P ^a (μg/mL)	<i>S. aureus</i> Smith ^a (μg/mL)	MRSA HH-1 ^b (μg/mL)	MRSA 92-1191 ^b (μg/mL)	VRE NCTC12201 ^c (μg/mL)	VRE NCTC12203 ^d (μg/mL)	Rates of residual anti-MRSA activity ^e (%)
BTMA ₂ (1)	1	1	1	2	1	0.5	0
2	64	64	64	128	128	32	—
3	16	16	16	32	8	4	86
4	8	8	8	8	8	2	71
5	8	8	8	16	4	2	100
6	16	8	16	32	16	4	100
7	4	4	8	8	8	2	100
8	8	4	8	8	4	4	100
9	8	4	8	8	8	4	42
10	64	32	64	128	32	32	—
11	16	16	16	32	16	16	67
12	4	4	4	4	4	4	84
13	4	4	4	4	4	2	100
14	8	16	16	16	8	4	100
15	≤0.25	0.5	≤0.25	0.5	≤0.25	≤0.25	0
16	0.5	0.5	≤0.25	1	1	≤0.25	0
17	0.5	0.5	0.5	1	1	≤0.25	0
18	1	1	1	2	2	0.5	100
19	1	1	2	2	2	1	100
VCM ^f	1	1	0.5	1	>128	>128	—
LZD ^g	2	2	2	2	2	2	—

^a *Staphylococcus aureus* FDA209P and Smith: susceptible strains.^b MRSA HH-1 and 92-1191: MRSA strains isolated from clinical patients.^c *Enterococcus faecalis* NCTC12201: encoded by *van A* gene.^d *E. faecium* NCTC12203: encoded by *van A* gene.^e Residual ratio of activity is that residual ratio is shown by percentage after calculating concentration by using working curve. The working curve is encoded from inhibition zone after blending sample solution and normal rat serum.^f Vancomycin.^g Linezolid.

vive at least 5 days after administration, whereas all non-treated animals died within the same timeframe. Thus, the propyl ketone (18) we synthesized appears to be a promising lead compound.

In conclusion, we have synthesized and evaluated the antibacterial activities of BTM derivatives against gram-positive bacteria, including drug-resistant strains such as MRSA and VRE. The thioester derivatives (15–17) and ketone derivatives (18, 19) showed good antibacterial properties. In particular, the propyl ketone (18) exhibited potent activity against *S. aureus*, MRSA and VRE, directly comparable to that of vancomycin. Additionally, 18 was very stable in mouse plasma. Based on these findings, further structural optimization and structure–activity relationship studies of this class of compounds are currently in progress.

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References and notes

- Leclercq, R.; Derlot, E.; Duval, J.; Courvalin, P. *N. Engl. J. Med.* **1988**, 319, 157.
- Uttley, A. H. C.; Collins, C. H.; Naidoo, J.; George, R. C. *Lancet* **1988**, 331, 57.
- Sievert, D. M.; Boulton, M. L.; Stoltman, G.; Johnson, D.; Stobierski, M. G.; Downes, F. P.; Somsel, P. A.; Rudrik, J. T.; Brown, W.; Hafeez, W.; Lundstorm, T.; Flanagan, E.; Johnson, R.; Chang, S. *MMWR. Morb. Mortal. Wkly. Rep.* **2002**, 51, 565.
- (a) Waisvisz, J. M.; van der Hoeven, M. G.; van Peppen, J.; Zwennis, W. C. M. *J. Am. Chem. Soc.* **1957**, 79, 4520; (b) Waisvisz, J. M.; van der Hoeven, M. G.; Hölscher, J. F.; te Nijenhuis, B. J. *Am. Chem. Soc.* **1957**, 79, 4522; (c) Waisvisz, J. M.; van der Hoeven, M. G.; te Nijenhuis, B. J. *Am. Chem. Soc.* **1957**, 79, 4524; (d) Waisvisz, J. M.; van der Hoeven, M. G. *J. Am. Chem. Soc.* **1958**, 80, 383.
- Sowa, S.; Masumi, N.; Inoue, Y.; Nakamura, S.; Takesue, Y.; Yokoyama, T. *Hiroshima J. Med. Sci.* **1991**, 40, 137.
- Shimamura, H.; Gouda, H.; Nagai, K.; Hirose, T.; Ichioka, M.; Furuya, Y.; Kobayashi, Y.; Hirano, S.; Sunazuka, T.; Ōmura, S. *Angew. Chem., Int. Ed.* **2009**, 48, 914.
- (a) Otake, T.; Kaji, A. *J. Biol. Chem.* **1976**, 251, 2299; (b) Otake, T.; Kaji, A. *FEBS Lett.* **1981**, 123, 173; (c) Otake, T.; Kaji, A. *FEBS Lett.* **1983**, 153, 53.
- Dudley, H. W.; Ben, B. *Angew. Chem., Int. Ed.* **1999**, 38, 1172.
- Shinabarger, D. *Expert Opin. Investig. Drugs* **1999**, 8, 1195.
- Fermentation and isolation of BTMA₂: A loopful of spores of *Streptomyces* sp. KM-9459 was inoculated into 100 mL of first seed medium consisting of 2.4% starch, 0.5% yeast extract, 0.4% CaCO₃, 0.3% peptone, 0.3% meat extract, 0.1% glucose (adjusted to pH 7.0 before sterilization) in Erlenmeyer flasks. The inoculated medium was incubated in a rotary shaker (210 rpm) at 27 °C for 2 days. The first seed culture (1 mL) was transferred to 500 mL Erlenmeyer flasks (total nine flasks) containing 100 mL of second seed medium of 4.0% glucose, 1.0% lactose, 1.5% powdered soybeans (defatted), 0.6% yeast extract, 0.3% CaCO₃, 0.001% CoCl₂·6H₂O (pH 7.0–7.1, unadjusted), and the inoculated medium was incubated in a rotary shaker (210 rpm) at 27 °C for 2 days. Then, the second seed culture (900 mL) was divided into half (450 mL × 2), each of which were transferred into two JAR fermentors of 30 L contained 14.55 L each of main medium consisting the same composite as second medium, and stirred (300 rpm) with 5 L/min venting at 28 °C for 7 days, respectively. The resulted culture broths (15 L × 2) were combined and extracted with EtOAc (30 L) and the organic layer was concentrated. The residue was dissolved into two layer in a solution of hexane: 90% MeOH aq solution (1:1) and shaken well. Then, the two layers were separated, and the 90% MeOH aq layer was concentrated. The resulting residue was applied to silica gel flash chromatography [100% CHCl₃ → hexane/EtOAc (1:1) → hexane/acetone (1:1) → CHCl₃/MeOH (9:1)] to fractionate. The active materials were eluted by CHCl₃/MeOH (9:1), which was concentrated and purified by silica gel flash chromatography [CHCl₃/MeOH (60:1 → 30:1)] to furnish BTMA₂ (909.9 mg) as a colorless solid.
- Tanaka, N.; Nishimura, T.; Nakamura, S.; Umezawa, H. *J. Antibiot.* **1966**, 19, 149.
- Miller, W. J.; Chaiet, L.; Rasmussen, G.; Christensen, B.; Hannah, J.; Miller, A. K.; Wolf, F. J. *J. Med. Chem.* **1968**, 11, 746.
- Instrument data of bottromycin carboxylic acid (2) IR (KBr), ν (cm⁻¹): 3317, 2972, 1672, 1522, 1419, 1196, 1144; ¹H NMR (300 MHz, CD₃OD), δ (ppm): 7.73 (1H, d, *J* = 3.0 Hz), 7.52 (1H, d, *J* = 3.6 Hz), 7.40–7.09 (5H, complex m), 5.80–5.70 (1H, m), 4.76 (1H, m), 4.59 (1H, d, *J* = 17.9 Hz), 4.57 (1H, d, *J* = 10.8 Hz), 4.34–3.88 (6H, complex m), 3.81 (1H, s), 3.25–3.05 (4H, complex m), 2.24–2.08 (3H, m), 1.96–1.80 (2H, m), 1.26 (3H, d, *J* = 7.2 Hz), 1.04–0.91 (18H, br), 0.81 (3H, d, *J* = 6.8 Hz); ¹³C NMR (67.5 MHz, CD₃OD), δ (ppm): 176.9, 176.5, 174.8, 173.2, 168.5, 168.2, 166.9, 144.7, 143.6, 129.3 (C × 2), 128.9 (C × 2), 128.2, 120.0, 69.2, 65.6, 62.4, 50.4, 48.0, 46.8, 42.2, 37.2, 36.6, 34.9, 34.7, 30.0, 28.1 (C × 4), 27.3 (C × 3), 20.3, 19.7, 19.3, 15.8; Three carbon signals of 2 for ¹³C NMR could not be detected, it may cause by the equilibrium of rotational isomers due to

- amides and/or amidine bonds; HRMS (FAB) (matrix:thioglycerol + glycerol), m/z : 809.4390 $[M+H]^+$, calcd for $C_{41}H_{60}N_8O_7S$: 809.4384 $[M+H]$.
14. Instrument data of BTM hydrazide (**3**) IR (KBr), ν (cm^{-1}): 3325, 2966, 2881, 1653, 1533, 1506, 1257; ^1H NMR (300 MHz, CDCl_3), δ (ppm): 7.76 (1H, d, $J = 5.5$ Hz), 7.55 (1H, d, $J = 3.5$ Hz), 7.34–7.28 (5H, m), 7.14 (1H, d, $J = 3.2$ Hz), 6.79 (1H, d, $J = 9.1$ Hz), 5.44 (1H, br), 4.96 (1H, dd, $J = 4.0, 9.4$ Hz), 4.57 (1H, d, $J = 10.6$ Hz), 3.89 (1H, s), 3.83 (2H, br, s), 3.74–3.24 (7H, m), 2.94 (1H, dd, $J = 4.0, 14.6$ Hz), 2.88–2.72 (1H, m), 2.45 (1H, m), 2.36–2.25 (1H, m), 1.99–1.91 (2H, m), 1.66–1.47 (1H, m), 1.41 (3H, d, $J = 6.9$ Hz), 1.14–0.85 (21H, m), 0.80 (3H, d, $J = 6.0$ Hz), 0.77 (3H, d, $J = 6.2$ Hz); ^{13}C NMR (67.5 MHz, CDCl_3), δ (ppm): 174.0, 173.6, 173.0, 172.3, 171.1, 169.0, 168.7, 156.6, 142.1, 140.7, 128.7 ($\text{C} \times 2$), 128.1 ($\text{C} \times 2$), 126.8, 119.2, 70.4, 68.5, 65.4, 56.7, 53.1, 50.6, 48.0, 46.9, 42.2, 38.5, 38.3, 35.2, 33.1, 30.1, 27.9 ($\text{C} \times 3$), 27.7 ($\text{C} \times 3$), 27.0, 20.1, 19.7, 18.7, 15.2; HRMS (FAB) (matrix:thioglycerol + glycerol) m/z : 823.4686 $[M+H]^+$, calcd for $C_{41}H_{63}N_{10}O_6S$: 823.4653 $[M+H]$.
15. Tokuyama, H.; Yokoshima, S.; Yamashita, T.; Fukuyama, T. *Tetrahedron Lett.* **1998**, 39, 3189.
16. Knochel, P.; Yeh, M. C. P.; Berk, S. C.; Talbert, J. J. *Org. Chem.* **1988**, 53, 2390.
17. Instrument data of BTM propyl ketone (**18**) IR (KBr), ν (cm^{-1}): 3282, 2968, 2881, 1649, 1545, 1498, 1255; ^1H NMR (270 MHz, CDCl_3), δ (ppm): 7.65 (1H, d, $J = 3.2$ Hz), 7.61–7.48 (1H, m), 7.39–7.12 (5H, m), 7.17 (1H, d, $J = 3.2$ Hz), 6.97 (1H, d, $J = 10.3$ Hz), 6.91 (1H, d, $J = 8.9$ Hz), 5.64 (1H, dd, $J = 7.3, 13.5$ Hz), 5.01 (1H, dd, $J = 4.1, 8.9$ Hz), 4.59 (1H, d, $J = 10.3$ Hz), 3.96 (1H, d, $J = 8.4$ Hz), 3.92 (2H, s, br), 3.82–3.65 (2H, m), 3.62–3.45 (2H, m), 3.45–3.34 (1H, m), 3.27 (1H, dd, $J = 7.3, 18.2$ Hz), 3.16 (1H, dd, $J = 5.5, 16.1$), 2.97–2.84 (2H, m), 2.89–2.70 (1H, m), 2.59–2.35 (1H, m), 2.27 (1H, dd, $J = 5.7, 11.9$ Hz), 2.12–1.94 (1H, m), 2.06 (2H, s, br), 1.75–1.49 (1H, m), 1.37 (3H, d, $J = 7.3$ Hz), 1.24 (3H, t, $J = 7.4$ Hz), 1.12 (3H, d, $J = 7.0$ Hz), 1.03–0.87 (18H, br), 0.80 (3H, d, $J = 6.5$ Hz), 0.72 (3H, d, $J = 6.5$ Hz); ^{13}C NMR (67.5 MHz, CDCl_3), δ (ppm): 207.6, 174.4, 172.8, 172.0, 171.0, 170.5, 169.1, 157.0, 142.8, 141.2, 128.5 ($\text{C} \times 2$), 128.2 ($\text{C} \times 2$), 126.9, 119.6, 70.4, 68.8, 65.5, 57.3, 53.8, 47.9, 47.8, 47.1, 47.0, 45.1, 41.6, 38.5, 35.4, 32.9, 30.2, 27.7 ($\text{C} \times 3$), 27.6 ($\text{C} \times 3$), 26.9, 20.2, 19.5, 16.9, 16.1, 15.5, 13.6; HRMS (FAB) (matrix:thioglycerol + glycerol): m/z : 835.4924 $[M+H]^+$, calcd for $C_{44}H_{66}N_8O_6S$: 835.4904 $[M+H]$.