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NOTE

# Two novel benzastatin derivatives, JBIR-67 and JBIR-73, isolated from *Streptomyces* sp. RI18

Keiichiro Motohashi[1], Aya Nagai[1], Motoki Takagi[1] and Kazuo Shin-ya[2]

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Members of the class *Actinobacteria* are known to produce pharmaceutically important compounds and have been extensively studied. Thus far, soil has been the primary source of *Actinobacteria*. However, the rate of discovery of novel compounds from soil *Actinobacteria* strains has decreased significantly. Therefore, we explored the possibility of isolating unusual *Actinobacteria* capable of producing new metabolites by employing specially devised methods instead of the conventional selection methods using antibiotics, and attempted to isolate rare *Actinobacteria* from soil samples using the membrane filter (MF) method.[1] In fact, we have already isolated a new *Streptomyces* sp. using this method,[2] and have described new promothiocin derivatives from the isolated *Streptomyces*.[3] In the course of searching for metabolites from cultures of *Actinobacteria* isolated using the MF method, we isolated novel compounds designated JBIR-73 (**1**) and JBIR-67 (**2**), which possess unique structures, as well as the known benzastatin derivatives, virantmycin[4] and 7-hydroxyl benzastatin D,[5] from *Streptomyces* sp. RI18 (Figure 1a).

*Streptomyces* sp. RI18 was isolated from a soil sample collected in Shuri, Okinawa Prefecture, Japan, using the MF method.[1,2] To identify the genus of the strain RI18, we compared the 16S rRNA gene sequence of RI18 with those available in the DNA Data Bank of Japan using the basic local alignment search tool. The strain was identified as the genus *Streptomyces*.

The strain was cultivated in 50-ml test tubes containing 15 ml of a seed medium consisting of starch (Kosokagaku, Tokyo, Japan) 1.0%, polypepton (Nihon Pharmaceutical, Tokyo, Japan) 1.0%, molasses (Dai-Nippon Meiji Sugar, Tokyo, Japan) 1.0% and meat extract (Extract Ehlrich, Wako Pure Chemical Industry, Osaka, Japan) 1.0%, pH 7.2 (before sterilization). The test tubes were shaken on a reciprocal shaker (355 r.p.m.) at 27 °C for 2 days. Aliquots (2.5 ml) of the broth were transferred to 500-ml baffled Erlenmeyer flasks (20 flasks) containing 100 ml of a production medium consisting of starch 1.0%, glucose 1.0%, glycerol 1.0%, polypepton 0.5%, yeast extract (BD Biosciences, San Jose, CA, USA) 0.2%, corn steep liquor (Oriental Yeast, Tokyo, Japan) 1 ml, NaCl 0.1% and $CaCO_3$ 0.32%, pH 7.4 (before sterilization), and cultured on a rotary shaker (180 r.p.m.) at 27 °C for 5 days.

The fermentation broth (2 l) was centrifuged, and the mycelial cake was extracted in $Me_2CO$ (500 ml). After *in vacuo* concentration, the aqueous concentrate was extracted with EtOAc (100 ml×3). The extract was dried over $Na_2SO_4$ and evaporated to dryness. The residue (0.79 *g*) was subjected to normal-phase medium-pressure liquid chromatography (Purif-Pack SI-60; Shoko Scientific, Yokohama, Japan), and successively eluted with a gradient system of *n*-hexane-EtOAc (0–30% EtOAc) and $CHCl_3$-MeOH (0–50% MeOH). Virantmycin was isolated from the fraction eluted with 2% MeOH. The fraction eluted with 3% MeOH (57.2 mg) was purified by preparative reverse-phase high-performance liquid chromatography using an L-column2 ODS column (20 i.d.×150 mm; Chemical Evaluation and Research Institute, Tokyo, Japan), with 65% MeOH-$H_2O$ containing 0.1% formic acid (flow rate 10 ml $min^{-1}$) to yield **2** (0.88 mg; retention time 11.3 min) and 7-hydroxyl benzastatin D. The supernatant of the fermentation broth was applied to a Diaion HP-20 column (Mitsubishi Chemical., Tokyo, Japan). The column was washed with 30% aqueous MeOH and eluted with 100% MeOH. The 100% MeOH eluate was evaporated *in vacuo*, and the residue (0.99 g) was subjected to reverse-phase medium pressure-liquid chromatography (Purif-Pack ODS-100; Shoko Scientific.) with an aqueous MeOH linear gradient system (20–100% MeOH). The 70% MeOH eluate (85.1 mg) was subjected to preparative reverse-phase high-performance liquid chromatography using an L-column2 ODS column developed with 55% aqueous MeOH containing 0.1% formic acid to give **1** (1.94 mg; retention time 22.2 min).

[1]Biomedicinal Information Research Center (BIRC), Japan Biological Informatics Consortium (JBIC), Tokyo, Japan and [2]Biomedicinal Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST), Tokyo, Japan
Correspondence: Dr M Takagi, Biomedicinal Information Research Center (BIRC), Japan Biological Informatics Consortium (JBIC), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan.
E-mail: motoki-takagi@aist.go.jp
or Dr K Shin-ya, Biomedicinal Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan.
E-mail: k-shinya@aist.go.jp
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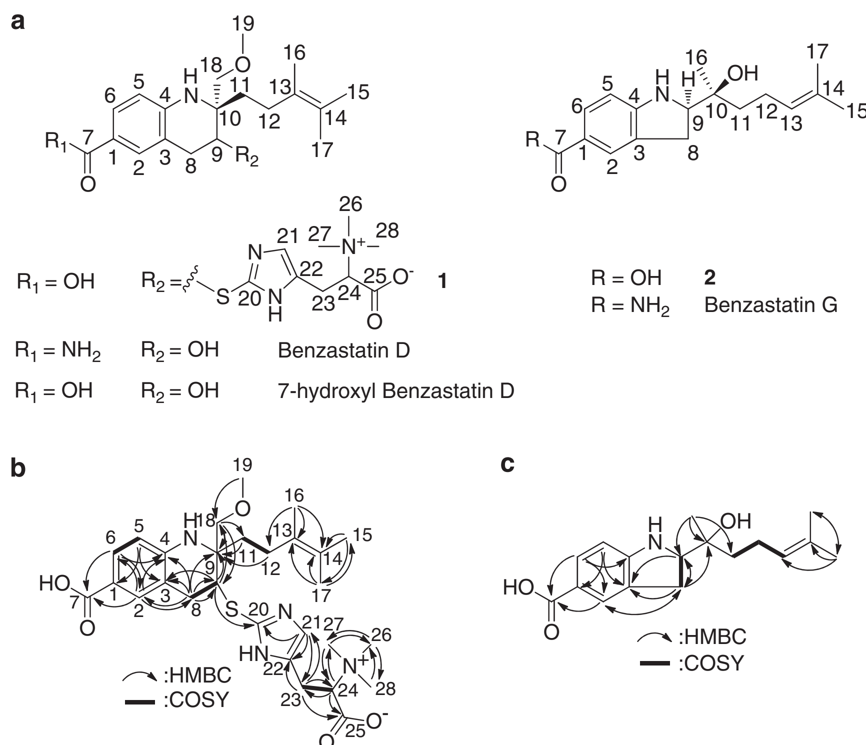


**Figure 1** (**a**) Structures of JBIR-73 (**1**) and JBIR-67 (**2**). (**b**) Key correlations observed in **1** by $^1$H–$^1$H COSY (bold lines) and HMBC (solid arrows). (**c**) Key correlations observed in **2** by $^1$H–$^1$H COSY (bold lines) and HMBC (solid arrows).

Compound **1** was isolated as a colorless oil ($[\alpha]_D$ +12.0, c 0.1, MeOH) that gave an $[M+H]^+$ ion at *m/z* 545.2797 on HR-ESI-MS. This spectrum was consistent with a molecular formula of $C_{28}H_{40}N_4O_5S$ (calcd for $C_{28}H_{41}N_4O_5S$, 545.2798). Compound **1** displayed the following IR and UV spectra: IR (KBr) $v_{max}$=3300 and 1670 cm$^{-1}$; UV $\lambda_{max}$ ($\varepsilon$)=306 nm (14 900) in MeOH. The $^1$H and $^{13}$C NMR spectral data for **1** are shown in Table 1. The structure of **1** was elucidated by a series of 2D NMR analyses, including HSQC, field-gradient $^1$H–$^1$H COSY and HMBC (Figure 1b). In the HMBC spectrum, a singlet methyl proton H-16 ($\delta_H$ 1.50) was long-range coupled to a methylene carbon C-12 ($\delta_C$ 28.1), olefinic quaternary carbons C-13 ($\delta_C$ 127.4) and C-14 ($\delta_C$ 124.4), which, in turn, was coupled from singlet methyl protons H-15 ($\delta_H$ 1.51) and H-17 ($\delta_H$ 1.50). The singlet methyl protons H-15 and H-17 were $^1$H–$^{13}$C long-range coupled to each other. Thus, the assignments of these three methyl signals were established, although the chemical shifts of H-16 and H-17 overlapped. In addition to these HMBC correlations, the spin coupling between methylene protons H-11 ($\delta_H$ 1.53) and H-12 ($\delta_H$ 2.08 and 1.95) observed in the $^1$H–$^1$H COSY spectrum revealed a 2,3-dimethylpent-2-ene moiety.

An *ortho*-coupling between the aromatic protons H-5 ($\delta_H$ 6.66) and H-6 ($\delta_H$ 7.53), which *meta*-coupled to the aromatic proton H-2, indicated the presence of a 1,3,4-trisubstituted benzene ring moiety. In addition, $^1$H–$^{13}$C long-range couplings from H-2 to aromatic quaternary carbons C-4 ($\delta_C$ 147.6), C-6 ($\delta_C$ 129.8) and the carbonyl carbon C-7 ($\delta_C$ 168.0), from H-5 to quaternary carbons C-1 ($\delta_C$ 118.8) and C-3 ($\delta_C$ 115.8), and from H-6 to C-2 ($\delta_C$ 132.3), C-4 and C-7 established the assignments of the benzene ring, and revealed that the substitution in the carbonyl functional group was at C-1.

An oxymethylene proton H-18 ($\delta_H$ 3.52, 3.40) was $^1$H–$^{13}$C long-range coupled to a methylene carbon C-11 ($\delta_C$ 34.1), a quaternary carbon C-10 ($\delta_C$ 57.3) and a methylene carbon C-9 ($\delta_C$ 49.0). The correlation between the methylene protons H-8 ($\delta_H$ 3.21 and 2.80) and the methine proton H-9 ($\delta_H$ 4.08) was observed in the $^1$H–$^1$H COSY spectrum. The HMBC correlation between the methoxyl proton H-19 ($\delta_H$ 3.24) and the methylene carbon C-18 ($\delta_C$ 74.4) revealed that the methoxyl group was substituted at C-18. $^1$H–$^{13}$C long-range couplings from H-2 to C-8 ($\delta_C$ 31.4) and from H-8 to C-2, C-3 and C-4 proved the connectivity of these partial structures.

The correlation between methylene protons H-23 ($\delta_H$ 3.56 and 3.29) and a methine proton H-24 ($\delta_H$ 4.35) was determined by $^1$H–$^1$H COSY. $^1$H–$^{13}$C long-range coupling from equivalent singlet methyl protons H-26, H-27 and H-28 ($\delta_H$ 3.25) to each other ($\delta_C$ 52.6) and the methine carbon C-24 ($\delta_C$ 73.0) was observed. H-24 was long-range coupled to the methyl carbons C-26, C-27 and C-28. Although the methyl carbons C-26, C-27 and C-28, and the methine carbon C-24 are thought to be connected through an oxygen atom according to the $^{13}$C chemical shift values of C-26, C-27 and C-28 ($\delta_C$ 52.6), and of C-24 ($\delta_C$ 73.0), these carbons are connected through an ammonium ion because C-26, C-27 and C-28 are equivalent. A trimethyl ammonium functional group with identical $^{13}$C chemical shift values was also found in ergothioneine[6] and clithioneine.[7] Both H-23 and H-24 were long-range coupled to a carbonyl carbon C-25 ($\delta_C$ 168.1), suggesting the existence of an amino-acid derivative. Moreover, the methylene proton H-23 was $^1$H–$^{13}$C long-range coupled to the aromatic carbons C-22 ($\delta_C$ 129.5) and C-21 ($\delta_C$ 121.0), while the proton H-21 ($\delta_H$ 7.58) was long-range coupled to aromatic carbons C-20 ($\delta_C$ 140.3) and C-22. These correlations indicated the presence of a hercynine moiety in **1**. The $^1$H–$^{13}$C long-range coupling from H-9 to C-20 established the connectivity between two partial structures. The remaining nitrogen and sulfur atoms were assigned according to the $^{13}$C chemical shift values of C-4, C-10, C-9 and C-20 as shown in Figure 1b. Thus, the planar structure of **1** was determined to be a

**Table 1 $^{1}H$ and $^{13}C$ NMR spectral data for JBIR-73 (1) and JBIR-67 (2)**

| | **1**[a] | | **2**[b] | |
|---|---|---|---|---|
| *Position* | *$^{13}C$* | *$^{1}H$ (J in Hz)* | *$^{13}C$* | *$^{1}H$ (J in Hz)* |
| 1 | 118.8 | | 119.1 | |
| 2 | 132.3 | 7.51, s | 126.4 | 7.63, s |
| 3 | 115.8 | | 128.6 | |
| 4 | 147.6 | | 156.2 | |
| 5 | 114.2 | 6.66, d (8.4) | 107.4 | 6.49, d (8.4) |
| 6 | 129.8 | 7.53, d (8.4) | 131.3 | 7.69, d (8.4) |
| 7 | 168.0 | | 169.8 | |
| 8 | 31.4 | 3.21, 2.80, dd (17.3, 4.6) | 30.2 | 2.98, m, 2.94, m |
| 9 | 49.0 | 4.08, t (4.6) | 68.1 | 3.91, t (9.1) |
| 10 | 57.3 | | 73.6 | |
| 11 | 34.1 | 1.53 | 37.1 | 1.51, m, 1.38, m |
| 12 | 28.1 | 2.08, m, 1.95, m | 22.2 | 2.03, m |
| 13 | 127.4 | | 124.4 | 5.05, t (7.0) |
| 14 | 124.4 | | 131.9 | |
| 15 | 20.9 | 1.51, s | 25.6 | 1.62, s |
| 16 | 18.9 | 1.50, s | 23.4 | 1.16, s |
| 17 | 20.2 | 1.50, s | 17.5 | 1.56, s |
| 18 | 74.4 | 3.52, d (9.6), 3.40, d (9.6) | | |
| 19 | 58.4 | 3.24, s | | |
| 20 | 140.3 | | | |
| 21 | 121.0 | 7.58, s | | |
| 22 | 129.5 | | | |
| 23 | 23.2 | 3.56, dd (14.8, 3.8), 3.29 | | |
| 24 | 73.0 | 4.35, dd (11.6, 3.8) | | |
| 25 | 168.1 | | | |
| 26 | 52.6 | 3.25, s | | |
| 27 | 52.6 | 3.25, s | | |
| 28 | 52.6 | 3.25, s | | |

$^{13}C$ (150 MHz) and $^{1}H$ (600 MHz) NMR spectra were taken on an NMR System 600 NB CL (Varian, Palo Alto, CA, USA).
[a]In DMSO-$d_6$ containing a small amount of trifluoroacetic acid. The solvent peak was used as an internal standard ($\delta_C$ 39.5, $\delta_H$ 2.50).
[b]In $CDCl_3$–$CD_3OD$ 1:1. The solvent peak was used as an internal standard ($\delta_C$ 49.0, $\delta_H$ 3.35).

derivative of 7-hydroxyl benzastatin D,[5] in which the hydroxyl residue at C-9 is replaced by an ergothioneine moiety.

Compound **2** was obtained as a colorless oil ($[\alpha]_D$ +19.0, *c* 0.1, MeOH) and displayed the following IR and UV spectra: IR (KBr) $\nu_{max}$=1720 $cm^{-1}$; UV $\lambda_{max}$ ($\varepsilon$)=302 (8500) in MeOH. The molecular formula of **2** was determined to be $C_{17}H_{23}NO_3$ by HR-ESI-MS (*m/z* 290.1763 $[M+H]^+$, calcd for $C_{17}H_{24}NO_3$, 290.1756). The structural information on **2** was obtained by a series of 2D NMR analyses, including HSQC, field-gradient $^{1}H$–$^{1}H$ COSY and HMBC (Figure 1c). The NMR data for **2** were similar to those of benzastatin G[8] (Table 1). On the basis of the differences between the molecular formulae of **2** ($C_{17}H_{23}NO_3$) and benzastatin G ($C_{17}H_{23}N_2O_2$), we conclude that **2** is a carboxylic acid form of benzastatin G, with the substitution at position of C-7 (1-carboxyl benzastatin G). In addition, the specific rotation value of **2** is closely similar to the specific rotation value ($[\alpha]_D$ +23.0 *c* 0.1, MeOH)[8] reported for benzastatin G. Thus, the absolute configuration of **2** was deduced to be the same as that of benzastatin G.

We evaluated the cytotoxic and antimicrobial activities of **1** and **2**, and found that these compounds were weakly cytotoxic to human cervical carcinoma HeLa cell lines ($IC_{50} > 50\,\mu M$), but did not show antimicrobial activity against *Candida albicans*, *Micrococcus luteus* or *Escherichia coli*.

In conclusion, we isolated two novel benzastatin derivatives, **1** and **2**, from a culture of *Streptomyces* sp. RI18 selected with the MF method. The structures of **1** and **2** were found to be structurally related to benzastatins isolated from *Streptomyces* spp.[5,8,9] In addition, although a compound possessing an *S*-hercynine moiety, like the one found in **1**, has been isolated from *Clitocybe acromelalga*,[7] there have been no reports of a compound that, like **1**, possesses both a benzastatin skeleton and an *S*-hercynine moiety. We anticipate that this study will convince chemists that *Actinobacteria* isolated using the MF method may be a source of new compounds containing unique skeletal structures, and encourage others to investigate new methods of isolating *Actinobacteria*.

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