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# *N*-Acetylglycine Side Chain is Critical for the Antimicrobial Activity of Xanthostatin

**KIM, SI-KWAN[1], MAKOTO UBUKATA[2]*, AND KIYOSHI ISONO[3]**

[1]*Department of Life Science, College of Natural Science, Konkuk University, Chungju Chungbuk 380-701, Korea*
[2]*Division of Biofunctional Chemistry, Biotechnology Research Center, Toyama Prefecture University, Kosugi Toyama 939-0398, Japan*
[3]*The Institute of Physics & Chemistry Research (RIKEN), Wako, Saitama 351-0198, Japan*

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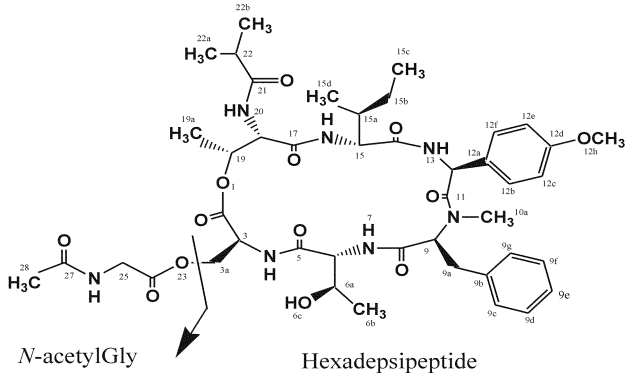
**Abstract** This study was carried out to elucidate the mode of bacteriostatic property of xanthostatin (XS), a novel depsipeptide antibiotic with an *N*-acetylglycine side chain and selective antimicrobial activity against *Xanthomonas* spp. Two biotransformed XSs were isolated by the treatment of XS with the cell lysate of *Xanthomonas campestris* pv. *citri*, a solvent partition, preparative TLC, and HPLC. Structure determination of those two biotransformed XSs demonstrated deletion of the *N*-acetylglycine side chain. Noteworthily, they showed no antimicrobial activity against *Xanthomonas* spp. This result suggests that the *N*-acetylglycine side chain plays a critical role in the antimicrobial activity of XS, and that the bacteriostatic property of XS is due to susceptibility of the ester bond between the hexadepsipeptide nucleus and the *N*-acetylglycine side chain to hydrolytic enzyme(s) produced by *Xanthomonas* spp.

**Key words:** Structures of biotransformed xanthostatin, inactivation, mode of bacteriostatic action, *Xanthomonas* sp.

Crop protection from phytopathogen plays an important role in agrobusiness, and antibiotics for crop protection cannot be underestimated in terms of their market size. For the sake of pest control, studies to discover new antibiotics for agricultural use have also been attempted in Korea [3, 5]. Canker, a bacterial disease affecting citrus plants by causing necrotic spots on citrus fruits, leaves, and stems, is one of the plant diseases that is difficult to control. It is induced by *Xanthomonas campestris* pv. *citri*. Control measures include windbreaks of trees or netting, pruning of diseased shoots, and chemical sprays. Six to seven sprays of copper are necessary to protect new growth from infection [7]. However, the control of canker on susceptible or highly susceptible trees has not yet been adequate for commercial production [9]. Only a few antibiotics that are active against *Xanthomonas* spp. have been discovered to date and xanthostatin (XS, **1**) is one of them [1, 2]. XS is a




**1**

novel depsipeptide antibiotic produced by *Streptomyces spiroverticillatus* [2]. Absolute structure was determined previously [6]. XS demonstrates a selective inhibitory activity against *Xanthomonas* sp. However, the activity is not bactericidal but bacteriostatic. In this study, the characterization of the bacteriostatic property of XS against *Xanthomonas* sp. was investigated.

Xanthostatin {m.p. 157–160°C (dec), $[\alpha]_D^{25}$ –76° (*c* 0.5, MeOH), FAB-MS *m/z* 896 $(M+H)^+$, *m/z* 918 $(M+Na)^+$} was isolated from a culture broth of *Streptomyces spiroverticillatus* as described previously [2]. It was further purified by recrystallization in a solvent mixture of EtOAc-MeOH-acetone-EtOH-hexane-benzene (1:1:1:1:1:1, vol/vol) as described previously [6]. $^1$H-$^1$H and $^1$H-$^{13}$C COSY, and heteronuclear multiple-bond correlation (HMBC) spectra of biotransformed XS (TXS) were measured by a Jeol GX-400 NMR spectrophotometer in $CDCl_3$. Molecular weights of TXSs were determined by FAB-MS (Jeol JMS HX-110).

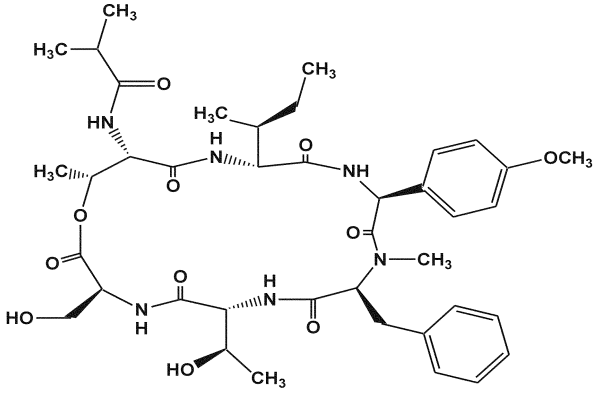
The treatment of XS with *Xanthomonas campestris* pv. *citri* was investigated according to the model of the nucleoside

*Corresponding author
Phone: 81-776-56-7500; Fax: 81-766-56-2498;
E-mail: ubukata@pu-toyama.ac.jp

antibiotic, ascamycin {9-β-[(5'-*O*-*N*-L-alanyl)sulphamoyl-D-ribofuranosyl]-2-chloroadenine}, which has a selective antibacterial activity against *Xanthomonas* spp. When ascamycin is dealanylated, the dealanylascamycin shows a broader antibacterial activity against various Gram positive and negative bacteria. *Xanthomonas campestris* pv. *citri* (XC) is susceptible to ascamycin because in its envelope it contains the L-prolyl-peptide hydrolase, an XC aminopeptidase, which catalyzes the dealanylation of ascamycin [8].

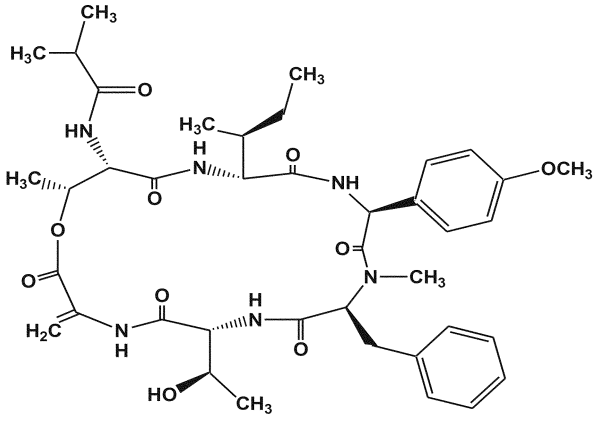
Likewise, XS was treated with the cell lysate of *Xanthomonas campestris* pv. *citri*. *Xanthomonas campestris* pv. *citri* was cultured in a potato sucrose broth (400 ml) for 48 h and the cells were harvested by centrifugation at 10,000 rpm for 30 min. The cell pellet was resuspended in the Tris-HCl buffer (pH 7.8, 200 ml) and subjected to sonication according to the method of Osada and Isono [8]. Xanthostatin (40 mg) dissolved in 10 ml MeOH was added to the cell lysate and incubated at 27°C for 90 min. The cell lysate containing XS was then partitioned with EtOAc and subjected to preparative TLC (silica gel) with $CHCl_3$-EtOAc-MeOH-$NH_4OH$ (5:2:1:0.01). It was further purified by HPLC (ODS, 230 nm) with 70% MeOH to obtain the intact XS (15 mg) and two biotransformed xanthostations; TXS-1 (10 mg) and -2 (3.6 mg).

The structures of these compounds were determined by NMR analyses of the HMQC and HMBC spectra. TXS-1: FAB-MS, *m/z* 797 $(M+H)^+$; $^1H$ NMR ($CDCl_3$, 400 MHz), δ 4.62 (1H, br, 2-H), 3.99 and 4.2 (each 1H, each m, H-3a), 4.22 (1H, dd, H-6), 4.3 (1H, m, H-6a), 1.57 (3H, d, H-6b), 5.13 (1H, br, H-6c), 7.06 (1H, d, H-7), 3.71 (1H, dd, H-9), 3.15 (1H, dd, H-a1), 3.54 (1H, dd, H-9a2), 6.6 (2H, br. d, H-9cg), 6.87 (2H, br. t, H-9df), 7.05 (1H, br. t, H-9e), 2.74 (3H, s, 10a), 5.16 (1H, br. s, H-12), 7.35 (2H, br. d, H-12bf), 7.06 (2H, br. d, H-12ce), 3.86 (3H, s, H-12h), 4.3 (1H, m, H-15), 1.64 (1H, m, H-15a), 1.23 (1H, m, H-15b1), 1.31 (1H, m, H-15b2), 0.88 (3H, t, H-15c), 0.8 (3H, d, 15d), 4.84 (1H, br. d, 18), 5.69 (1H, m, H-19), 1.29 (3H, d, H-19a), 7.65 (1H, br. d, H-20), 2.72 (1H, m, H-22), 1.2 (3H, d, H-22a), 1.23 (3H, d, H-22b). TXS-1 (TXS-1) was found to be *N*-acetylGly-eliminated XS (**2**).



**2**

TXS-2: FAB-MS, *m/z* 779 $(M+H)^+$; $^1H$ NMR ($CDCl_3$, 400 MHz), δ 6.8 and 5.65 (each 1H, each s, H-3a), 4.35 (1H, dd, H-6), 4.27 (1H, m, H-6a), 1.56 (3H, d, H-6b), 5.28 (1H, br. s, H-6c), 3.76 (1H, dd, H-9), 3.35 (1H, dd, H-9a1), 3.49 (1H, dd, H-9a2), 6.73 (2H, br. d, H-9cg), 6.92 (2H, br. t, H-9df), 7.06 (1H, br. t, H-9e), 2.74 (3H, s, 10a), 5.16 (1H, br. s, H-12), 7.3 (2H, br. d, H-12bf), 6.79 (2H, br. d, H-12ce), 3.89 (3H, s, H-12h), 4.76 (1H, dd, H-15), 1.6 (1H, m, H-15a), 1.12 (1H, m, H-15b1), 1.32 (1H, m, H-15b2), 0.87 (3H, t, H-15c), 0.79 (3H, d, 15d), 6.64 (1H, d, H-16), 4.83 (1H, dd, H-18), 5.55 (1H, dq, H-19), 1.32 (3H, d, H-19a), 7.38 (1H, d, H-20), 2.71 (1H, m, H-22), 1.22 (3H, d, H-22a), 1.24 (3H, d, H-22b). TXS-2 was determined to be dehydrated TXS-1 with a double bond between 3 and 3a (**3**).



**3**

Antimicrobial activities of TXS-1 and -2 were examined using the conventional paper disc agar diffusion method with a potato sucrose agar medium but none of them showed inhibitory activity against *Xanthomonas campestris* pv. *citri* at the dose of 75 μg/disc. From this antimicrobial assay, it can be surmised that the *N*-acetylGly moiety in the XS structure is essential for its inhibitory activity against *Xanthomonas* sp. It is noteworthy that the ester bond of the *N*-acetylGly moiety was selectively cleaved by the cell lysate. This observation suggests that XS is transformed to TXS-1 by such an α-amino acid ester hydrolase as reported by Kato *et al*. [4] and the TXS-1 further to TXS-2 by an unknown hydratase produced by XC.

In the previous paper [6], the unique conformation of the *N*-acetylGly moiety folding over the hexadepsipeptide was determined by an X-ray analysis. It has been suspected that the inward orientation of the moiety plays an important role in the antimicrobial activity of XS. In fact, NH signals derived from peptide bond appear in the vicinity of $\delta_H$ 6.56 (16)~7.84 (20) as in XS [6]. In this respect, an unusually low-field chemical shift of the NH signal (H-13, $\delta_H$ 8.82) in the $^1H$ NMR spectrum suggested the presence of a hydrogen bond between the O (27a) and H (13) of XS in solution. This idea was confirmed by the fact that the NH

signals in TXS-1 and -2 devoid of *N*-acetylGly moiety shift to the aromatic region as usual. Although the amide proton in xanthostatin is coupled with the methine proton at H (12) ($J$=3.1 Hz), possibly because of the fixed conformation, the NH protons in both TXS-1 and -2 are not coupled with the corresponding H (12). These results indicate that *N*-acetylGly moiety orients the peptide ring inward, not only in a crystal state but also in solution ($CDCl_3$).

From these results, it can definitely be said that the *N*-acetylGly moiety is essential for the antimicrobial activity of XS against *Xanthomonas* spp. In addition, the inactivation mode of XS by XC, supporting the bacteriostatic action of XS against *Xanthomonas* spp., was elucidated. Study on the substitution of *N*-acetylGly with different chemical moieties is underway to develop new semisynthetic antibiotics for the control of citrus canker.

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