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Genetically Engineered Production of 1,1'-bis-Valienamine and Validienamycin in *Streptomyces hygroscopicus* and Their Conversion to Valienamine

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Abstract

The antifungal agent validamycin A is an important crop protectant and the source of valienamine, the precursor of the antidiabetic drug voglibose. Inactivation of the *valN* gene in the validamycin A producer, *Streptomyces hygroscopicus* subsp. *jinggangensis* 5008, resulted in a mutant strain that produces new secondary metabolites 1,1'-bis-valienamine and validienamycin. The chemical structures of 1,1'-bis-valienamine and validienamycin were elucidated by 1D and 2D NMR spectroscopy in conjunction with mass spectrometry and bioconversion employing a glycosyltransferase enzyme, ValG. 1,1'-bis-Valienamine and validienamycin exhibit a moderate antifungal activity against *Pellicularia sasakii*. Chemical degradation of 1,1'-bis-valienamine using *N*-bromosuccinimide followed by purification of the products with ion-exchange column chromatography only resulted in valienamine, whereas parallel treatments of validoxylamine A, the aglycon of validamycin A, resulted in an approximately 1:1 mixture of valienamine and validamine, underscoring the advantage of 1,1'-bis-valienamine over validoxylamine A as a commercial source of valienamine.

Keywords

aminocyclitol; antifungal; biosynthesis; validamycin; valienamine

Introduction

The antifungal antibiotic validamycin A (**1**) (Figure 1) is a commercially important compound due to its wide application in controlling fungal infections of rice plants caused by *Rhizoctonia solani* (= *Pellicularia sasakii*) (Shibata et al. 1982). Structurally, validamycin A consists of the unsaturated aminocyclitol unit valienamine, the saturated aminocyclitol unit validamine, and glucose. The valienamine moiety is believed to be the pharmacophore of this compound and is also present in a number of other important aminocyclitols, such as the α -glucosidase inhibitors acarbose, the adiposins, the amylostatis, and the trestatis. In addition, the valienamine unit is an important synthetic precursor of the clinically used antidiabetic agent voglibose (Horie 1993). Due to its high commercial value, efforts have been made by many

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groups to obtain valienamine by chemical synthesis (Chang et al. 2005, Kok et al. 2001, Mahmud 2003). During the past twenty years more than a dozen enantiospecific syntheses have been described for valienamine, however, its commercial supply still relies on natural product degradation processes (Asano et al. 1984).

As part of our efforts to use biosynthetic approaches to generate novel aminocyclitol analogs, we have identified the biosynthetic gene cluster of **1** in *S. hygroscopicus* subsp. *jinggangensis* 5008 (Bai et al. 2006). A 45 kb sequence of DNA fragment containing the gene cluster revealed 16 structural genes, two regulatory genes, five genes related to transport, transposition/integration, tellurium resistance, and another four genes with no obvious identity. One of the essential genes for validamycin A biosynthesis is *valN*, which encodes protein homologous with the zinc-dependent sorbitol dehydrogenase from *Geobacillus thermodenitrificans* NG80-2 (28% identity, 45% similarity) and the alcohol dehydrogenase from *Prosthecochloris vibrioformis* DSM 265 (29% identity, 42% similarity). Both sorbitol and alcohol dehydrogenases are NAD⁺-dependent enzymes that catalyze the oxidation of an alcohol to a ketone. ValN also shares conserved domains with other dehydrogenase proteins, such as the shikimate 5-dehydrogenases and the ketopantoate reductases, which catalyze the conversions of shikimate to 5-dehydroshikimate and (*R*)-pantoate to 2-dehydropantoate, respectively. To investigate the function of *valN* in validamycin A biosynthesis, we inactivated the *valN* gene in *S. hygroscopicus* 5008 and analyzed the metabolite profile of the mutant. We discovered that the mutant strain lacks the ability to produce validamycin A, but instead produces two new secondary metabolites, which can be used as alternative sources of valienamine. Herein we report the construction of the *valN* mutant, isolation and structure elucidation of the new metabolites, their antifungal activity, and their efficient conversion to valienamine.

Materials and Methods

Inactivation of *valN* by ReDirect Technology

The inactivation of *valN* in *Streptomyces hygroscopicus* subsp. *jinggangensis* 5008 was carried out using ReDirect Technology (Gust et al. 2003). A 1449-bp replacement fragment was obtained by amplifying the *aac(3)IV-oriT* cassette from pIJ773 using primers ValN-PCR-F (5'-

GTGACTCTGGAGGAGGGCGGGCCCCGTCTGCACCGCTCGATTCCGGGGATCCGT
CG ACC-3') and ValN-PCR-R (5'-

TCAGAAGGGTTCGGGGTGGACAACGATCTTGCCGGGTCCTGTAGGCTGGAGCTG
CTT C-3') (homologous sequences for recombination are underlined). A 7096-bp *Bam*HI

fragment from cosmid 17F2 containing *valN* was cloned into *Bam*HI digested pHZ1358, to generate pJTU751. Plasmid pJTU751 was then transferred into *E. coli* BW25113 harboring the λ RED plasmid pIJ790. *ValN* in pJTU751 was replaced by the above PCR product to generate plasmid pJTU753, which has 3924-bp upstream and 2244-bp downstream sequences flanking the *valN* gene for double-crossover recombination and was transformed into *S.*

hygroscopicus 5008 by conjugation. Replacement of *valN* in the resulting XH-2 mutant was confirmed by PCR amplification with the primers of ValN-det-F (5'-TGCTTCCGCTGCTTCTAC-3') and ValN-det-R (5'-GTTGCTGTCACGCTCCC-3').

Production and purification of 1,1'-bis-valienamine (3) and validienamycin (4)

Mutant strain XH-2 was cultured at 37 °C in YMG (yeast extract, malt extract, and glucose) for 7 days. 1,1'-bis-Valienamine (20 mg) and validienamycin (8 mg) were isolated from fermentation broth (1L) through a two-column chromatography system, using Dowex 50Wx8 (H⁺ form) (100 g) and Dowex 1x8 (OH⁻ form) (80 g), adopting the same procedures reported previously (Dong et al. 2001).

1,1'-bis-Valienamine (**3**): $[\alpha]_D^{21} +143.9$ ($c = 0.2$ in MeOH) {lit. (Shing et al. 2004) $[\alpha]_D^{20} +47.1$ ($c = 1.0$ in MeOH)}*; IR (thin film): $\nu = 3349, 2920, 1656, 1408, 1310, 1097, 1012$ cm^{-1} . ^1H NMR (300 MHz, D_2O , 23 °C, DSS): $\delta = 3.56$ (dd, $J = 4.7, 4.8$ Hz, 2H; H-1 and H-1'), 3.66 (dd, $J = 4.7, 10.0$ Hz, 2H; H-2 and H-2'), 3.73 (dd, $J = 6.5, 10.0$ Hz, 2H; H-3 and H-3'), 4.07 (d, $J = 6.5$ Hz, 2H; H-4 and H-4'), 4.12 (d, $J = 13.9$ Hz, 2H; H-7a and H-7'a), 4.24 (d, $J = 13.9$ Hz, 2H; H-7b and H-7'b), 5.95 (brd, $J = 4.8$ Hz, 2H; H-6 and H-6'). ^{13}C NMR (75 MHz, D_2O , 23 °C, DSS): $\delta = 55.9$ (C-1 and C-1'), 64.3 (C-7 and C-7'), 72.5 (C-2 and C-2'), 74.2 (C-4 and C-4'), 75.8 (C-3 and C-3'), 126.3 (C-6 and C-6'), 142.1 (C-5 and C-5'). ESI(+)-MS: m/z : 334.13 $[\text{M}+\text{H}]^+$; HRMS (ESI+): m/z : 356.1341 (calcd for $\text{C}_{14}\text{H}_{23}\text{NO}_8\text{Na}$: 356.1321). *To address the discrepancy between our $[\alpha]_D$ value with the reported value (Shing et al. 2004), the experiments were repeated three times in two different polarimeters. However, our $[\alpha]_D$ reading was consistently measured around +144 in all of the experiments. Subsequently, **3** was converted to its peracetate derivative and its optical rotation was measured. The $[\alpha]_D$ value of the product is +107.3, which is consistent with that reported by Ogawa ($[\alpha]_D +109$) (Ogawa et al. 1993) and somewhat similar to that reported by Shing ($[\alpha]_D +92$) (Shing et al. 2004).

LC-MS analysis

LC-MS analysis was performed on a ThermoFinnigan LCQ Advantage LC/MS system (ThermoElectron), equipped with an autosampler and a photodiode array detector, and controlled by a PC running Xcalibur 1.3 software. Separation was done on a C_{18} column (AtlantisTM C_{18} 5 mm column, Waters) with $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (3:97) as mobile phase. The flow rate was 0.6 mL/min and the effluent was monitored at 214 nm. The retention times of 1,1'-bis-valienamine and validienamycin were 7.6 min and 8.5 min, respectively. Positive mode electrospray ionization was used for MS detection.

Biotransformation of 1,1'-bis-valienamine (**3**) to validienamycin (**4**)

1,1'-bis-Valienamine (4.2 mg, 0.013 mmol) was incubated with recombinant ValG (0.45 mg), MgCl_2 (1 mM), and UDP-glucose (10 mg, 0.018 mmol) in Tris-buffer (1.2 mL, 40 mM, pH 7.6) at 30 °C for 3 h. Recombinant ValG was prepared according to the method reported previously (Bai et al. 2006). The reaction was quenched by addition of methanol (1.2 mL) and the mixture was centrifuged at 12,000 rpm for 10 min. The supernatant was transferred and the organic solvent was evaporated *in vacuo*. The aqueous solution was lyophilized and dissolved in water (0.5 mL). The solution was subjected to Dowex-50Wx8 (H^+ form) and Dowex 1x8 (OH^- form) columns to give pure validienamycin (5.6 mg, 0.011 mmol, 85% yield). Validienamycin (**4**): $[\alpha]_D^{21} +124.6^\circ$ ($c = 1.0$ in MeOH); IR (thin film): $\nu = 3353, 2918, 1653, 1419, 1075$ cm^{-1} . ^1H NMR (300 MHz, D_2O , 23 °C, DSS): $\delta = 3.33$ (dd, $J = 8.0, 9.2$ Hz, 1H; H-2''), 3.41 (dd, $J = 9.4, 9.2$ Hz, 1H; H-4''), 3.50 (d, $J = 9.2$ Hz, 1H; H-3''), 3.51 (m, 1H; H-5''), 3.55 (m, 2H; H-1', H-1), 3.67 (m, 1H; H-2), 3.69 (d, $J = 6.5$ Hz, 1H; H-4), 3.74 (m, 1H; H-6''), 3.76 (dd, $J = 5.1, 9.5$ Hz, 1H; H-2'), 3.94 (dd, $J = 2.0, 12.4$ Hz, 1H; H-6''), 4.01 (dd, $J = 5.9, 9.5$ Hz, 1H; H-3'), 4.07 (d, $J = 6.5$ Hz, 1H; H-3), 4.14 (brt, $J = 13.9$ Hz, 2H; H-7), 4.22 (m, 1H; H-4'), 4.27 (brt, $J = 14.1$ Hz, 2H; H-7'), 4.64 (d, $J = 8.0$ Hz, 1H; H-1''), 5.96 (brd, $J = 5.0$ Hz, 1H; H-6), 6.02 (brd, $J = 4.6$ Hz, 1H; H-6'). ^{13}C NMR (75 MHz, D_2O , 23 °C, DSS): $\delta = 55.5$ (C-1'), 55.9 (C-1'), 63.4 (C-6''), 64.3 (C-7), 64.5 (C-7'), 72.0 (C-2'), 72.3 (C-4''), 72.5 (C-2), 74.2 (C-3), 74.3 (C-3'), 75.9 (C-4), 76.1 (C-2''), 78.4 (C-5''), 78.7 (C-3''), 84.4 (C-4'), 106.3 (C-1''), 126.3 (C-6), 128.5 (C-6'), 140.0 (C-5'), 142.2 (C-5). ESI(+)-MS: m/z : 496.13 $[\text{M}+\text{H}]^+$; HRMS (ESI+): m/z : 518.1850 (calcd for $\text{C}_{20}\text{H}_{33}\text{NO}_{13}\text{Na}$: 518.1850).

Fungal growth inhibitory assay of 1,1'-bis-valienamine (**3**) and validienamycin (**4**)

1% agar (14 mL) was mixed with the compounds (dissolved in 1 mL H_2O) and then plated into Petri dishes. Discs of PDA agar with mycelia of *Pellicularia sasakii* were placed in the

center of the above plates as indicator strain for bioassay of the compounds. The plates were incubated at 30 °C for 2 days.

Cleavage of 1,1'-bis-valienamine with NBS to produce valienamine

To a solution of 1,1'-bis-valienamine (13 mg, 0.039 mmol) in DMF/H₂O (4:1, 0.4 mL) NBS (10.4 mg, 0.0585 mmol) was added and the reaction mixture was stirred for 12 h at rt. The reaction mixture was diluted with H₂O (5 mL) and CH₂Cl₂ (5 mL) and the aqueous layer was collected and lyophilized. The crude residue was redissolved in H₂O (1 mL) and subjected to Dowex 50Wx2 (H⁺ form) column chromatography. The column was washed with H₂O (75 mL) and the compounds were eluted with 0.5 M NH₄OH solution (75 mL) and lyophilized. The residue was then subjected to a Dowex 1x8 (OH⁻ form) column and eluted with H₂O to give valienamine and a trace amount of the starting material. The pure product was then obtained by silica gel column chromatography (CHCl₃:MeOH:aq. 5% NH₄OH (15 M) = 3:6:1). ¹H NMR (300 MHz, D₂O): δDSS = 3.54 (m, 1H; H-1), 3.72 (m, 2H; H-2, H-3), 4.13 (m, 1H; H-4), 4.15 (d, *J* = 13.6, 1H; H-7a), 4.27 (d, *J* = 13.5, 1H; H-7b), 5.84 (m, 1H; H-6).

Cleavage of validoxylamine A (2) with NBS to produce valienamine and validamine

To a solution of validoxylamine A (2) (20 mg, 0.060 mmol) in DMF/H₂O (4:1, 0.5 mL) NBS (15.9 mg, 0.0895 mmol) was added and the reaction mixture was stirred for 8 h at rt. The reaction mixture was diluted with H₂O (5 mL) and CH₂Cl₂ (5 mL) and the aqueous layer was collected and lyophilized. The residue was dissolved in H₂O (1 mL) and subjected to Dowex 50Wx2 (H⁺ form) column chromatography. The column was washed with H₂O (100 mL) and the compounds were eluted with 0.5 M NH₄OH solution (100 mL) and lyophilized. The residue was chromatographed on Dowex 1x8 (OH⁻ form) with H₂O as eluent to give a 1:1 mixture of valienamine and validamine (based on ¹H-NMR integration).

Results

Inactivation of *valN*

To inactivate *valN* in *S. hygroscopicus* 5008, the gene in the genome was replaced by an *aac* (3)*IV-oriT* cassette (Figure 2a) (Gust et al. 2003). A pHZ1358-derived plasmid (pJTU753) which contains an *aac*(3)*IV-oriT* cassette flanked with sequences of 3924-bp upstream and 2244-bp downstream of *valN* was obtained by ReDirect Technology in *E. coli* BW25113 (pIJ790) (Gust et al. 2003). The plasmid pJTU753 was introduced into strain 5008 by conjugation from *E. coli* ET12567 (pUZ8002) and the apramycin-resistant phenotype was screened to get the *valN*-inactivated mutant, XH-2. Total DNA was extracted from the mutant and the wild-type of *S. hygroscopicus* 5008 as template for PCR amplification. The mutant gave a 2.30-kb PCR product and the wild-type gave a 1.86-kb PCR product (Figure 2b), which confirmed that a 918-bp DNA fragment of *valN* has been replaced by the 1371-bp *aac*(3)*IV-oriT* cassette.

Isolation and structure elucidation of 1,1'-bis-valienamine and validienamycin

Mutant strain XH-2 was cultured at 37 °C in YMG medium for 7 days and the culture broth was directly checked by TLC and LC-MS. Neither validamycin A (1) (MW=497) nor validoxylamine A (2) (MW=335) were found in the culture broth of XH-2, confirming the involvement of ValN in 1 biosynthesis. However, two new peaks were observed in the mass spectra at *m/z* 496 and 334. As ValN was originally thought to be a dehydrogenase that is responsible for the reduction of the C-1 keto group of valienone 7-phosphate to 1-*epi*-valienol 7-phosphate (Bai et al. 2006, Zhang et al. 2002), the production of the two new metabolites by the XH-2 mutant was rather unexpected. Therefore, a 2.5 L culture of XH-2 was fermented for 7 days and the culture broth was subjected to Dowex 50Wx8 (H⁺ form) and Dowex 1x8

(OH⁻ form) column chromatography. Guided by TLC and MS analyses, the two compounds (20 mg/L of that with m/z 334 and 8 mg/L of that with m/z 496) were isolated.

The chemical structure of the major metabolite was first elucidated on the basis of its 1D- and 2D NMR and MS data. In spite of having a molecular mass of 333, which is only two atomic mass units less than that of **2**, the compound showed much simpler ¹H- and ¹³C NMR spectra (Figure 3). Only seven carbon signals were observed in its ¹³C NMR spectrum (Figure 3f). The presence of an olefinic proton signal at 5.95 ppm (brd, $J = 4.8$ Hz) typical for an unsaturated cyclitol moiety suggested that it contains a valienamine moiety. Taking into account the molecular mass of the compound, it was proposed that the compound is a symmetrical dimer of valienamine (1,1'-bis-valienamine, **3**).

Glycosylation of 1,1'-bis-valienamine

To confirm the chemical structure of 1,1'-bis-valienamine, the compound was then subjected to an enzymatic desymmetrization by converting it to its glycosylated product using the glycosyltransferase ValG in the presence of Mg²⁺ and UDP-glucose. The product was purified using ion-exchange column chromatography and analyzed by NMR and MS. The ¹H NMR spectrum of the product revealed the presence of two olefinic protons [5.96 (brd, $J = 5.0$ Hz), 6.02 (brd, $J = 4.6$ Hz)] and an anomeric proton [4.64 (d, $J = 8.0$ Hz)], in addition to other protons designated to the core aminocyclitol and glucose (Figure 3e). The ¹³C NMR spectrum showed twenty carbon signals (Figure 3g). Among them are four olefinic carbon signals [126.3 (C-6), 128.5 (C-6'), 140.0 (C-5'), 142.2 (C-5)] and an anomeric carbon [106.3 (C-1'')], confirming the nature of the product as a pseudotrisaccharide, which consists of two valienamine moieties – coupled to each other via a nitrogen bridge – and glucose. This proposed structure is consistent with the ms data (m/z 496 [M+H]⁺). 2D NMR analysis confirmed the glucose attachment at the C-4' position, which is consistent with the regioselectivity of ValG (Bai et al. 2006). The enzyme product is identical with the second metabolite isolated from the mutant, which was identified as a new compound, validienamycin (**4**).

Antifungal activity of 1,1'-bis-valienamine and validienamycin

Antifungal activity assays of **3** and **4** were tested against *Pellicularia sasakii* (= *R. solani*) using an agar plug assay (Minagawa et al. 2007). Agar plugs with diameter of 5 mm and containing *P. sasakii* mycelia were placed in the center of agar plates that contain each compound and the growth of the pathogen was monitored after two days. Active compounds were determined based on their ability to inhibit the expansion of the fungal mycelia on the Petri dishes. The results revealed that at 0.2 mM concentration, **3** and **4** demonstrated a comparable fungistatic activity with **1** and **2**. However, at a lower concentration (0.02 mM), **1** turns out to be the most active compound among those tested (Figure 4).

Conversion of 1,1'-bis-valienamine and validienamycin to valienamine

Taking advantage of our ability to produce 1,1'-bis-valienamine and validienamycin by fermentation, we developed an efficient methodology for the production and purification of valienamine. 1,1'-bis-Valienamine contains two identical unsaturated cyclitol units and its efficient conversion to valienamine would provide an alternative avenue to this important precursor of antidiabetic drugs (Figure 5). The glucoside validienamycin can be hydrolyzed to 1,1'-bis-valienamine by refluxing the compound in 1N H₂SO₄/AcOH (1:1) for 48 h. Such procedure has previously been reported for validamycin A to give validoxylamine A, which can be subsequently cleaved by using *N*-bromosuccinimide (NBS) to give the corresponding aminocyclitols and ketones (Ogawa et al. 1989, Ogawa et al. 1991). In this study, a similar procedure was also used for the chemical cleavage of 1,1'-bis-valienamine. However, in contrast to the cleavage of validoxylamine A that gave multiple products, treatment of 1,1'-bis-valienamine with NBS is predicted to give only valienamine and its corresponding ketone,

which can be easily separated by ion exchange column chromatography. As shown in Figure 6, cleavage of validoxylamine A with NBS and purification with Dowex-50Wx8 (H⁺ form) and Dowex 1x8 (OH⁻ form) columns gave an approximately 1:1 mixture of valienamine and validamine, whereas similar treatments of 1,1'-bis-valienamine gave only valienamine as a product with a trace amount of unreacted starting material, which can be removed by silica gel column chromatography to give pure valienamine (Figure 6b).

Discussion

Due to the importance of the C₇N-aminocyclitols the validamycins as biologically active natural products, the biosynthesis of this class of compounds has been extensively studied. Preliminary studies were carried out by the groups of Rinehart, Floss, and Mahmud (Dong et al. 2001, Mahmud et al. 2001a, Mahmud et al. 2001b, Toyokuni et al. 1987), mainly by feeding experiments with isotopically labeled precursors, resulting in the identification of a number of cyclitols believed to be involved in the biosynthesis of validamycin A. The active pharmacophores of validamycin A, valienamine and validamine, are derived from 2-*epi*-5-*epi*-valiolone, the product of an intramolecular aldol cyclization of sedoheptulose 7-phosphate (Dong et al. 2001, Yu et al. 2005). The same compound is also involved in the biosynthesis of acarbose, pyralomicin, cetoniacytone, BE-40644, and salbostatin (Mahmud et al. 2007, Mahmud et al. 1999, Wu et al. 2007). Based on the feeding studies, it was proposed that in validamycin biosynthesis the conversion from 2-*epi*-5-*epi*-valiolone to valienone involves subsequent epimerization at the C-2 stereocenter and dehydration between C-5 and C-6. Valienone then plays a dual role as an intermediate, where it is specifically incorporated into the unsaturated cyclitol moiety directly and incorporated into the saturated one *via* validone. The two compounds are connected through a nitrogen bridge to give validoxylamine A, the core structure of validamycin A. However, the timing and mode of introduction of the bridging nitrogen atom are not completely understood. Therefore, further studies on the genetics and biochemistry of validamycin biosynthesis may provide significant insights into the mode of formation of this antibiotic. Recently, we have identified the biosynthetic gene cluster of validamycin in *S. hygroscopicus* subsp. *jinggangensis* 5008 (Bai et al. 2006, Yu et al. 2005), and have characterized a number of new types of enzymes within the pathway with interesting reaction mechanisms. Among those believed to be essential for the synthesis of validamycin A in *S. hygroscopicus* 5008 are the 2-*epi*-5-*epi*-valiolone synthase (ValA), the nucleotidyltransferase (ValB), the cyclitol kinase (ValC), the cyclitol epimerase (ValD), the glycosyltransferase (ValG), the epimerase/dehydratase (ValK), the validoxylamine A 7'-phosphate synthase (ValL), the aminotransferase (ValM), and the cyclitol reductase (ValN).

The cyclitol reductase ValN is homologous with the zinc-dependent sorbitol dehydrogenase from *G. thermodenitrificans* NG80-2 and the alcohol dehydrogenase from *P. vibrioformis* DSM 265, both of which are NAD⁺-dependent enzymes that catalyze the oxidation of an alcohol to a ketone. ValN also shares conserved domains with the shikimate 5-dehydrogenases and the ketopantoate reductases, which catalyze the conversions of shikimate to 5-dehydroshikimate and (*R*)-pantoate to 2-dehydropantoate, respectively. Therefore, it was anticipated that ValN would catalyze the reduction of the C-1 ketone of one of the early intermediates in the validamycin pathway to its corresponding alcohol and thus inactivation of the cyclitol reductase (*valN*) gene in the validamycin producer would abolish the production of validamycin A. However, to our surprise, the mutant strain produces the unsaturated analogs, 1,1'-bis-valienamine (**3**) and validienamycin (**4**), indicating that ValN is responsible for the reduction of the C-5/C-6 double bond, rather than the C-1 ketone. This finding may lead to a new type of Zn-binding NADH/NADPH-dependent reductase. However, efforts to characterize this enzyme *in vitro* using recombinant ValN produced in *E. coli* have not been successful.

1,1'-bis-Valienamine (**3**) was previously reported as a synthetic compound, derived from (–)-quinic acid in 14 steps, and shown to be a potent trehalase inhibitor (Shing et al. 2004). The fact that **3** can now be produced by fermentation is noteworthy, as it can be used as an alternative source of valienamine. While it has been proposed that valienamine is not directly involved in **1** biosynthesis, the production of **3** and **4** also provides new insights into the possibility of producing valienamine in a genetically engineered microorganism.

Commercially used valienamine is derived from validoxylamine A, the aglycon of validamycin A, via chemical or enzymatic degradations (Horii 1993). A number of methodologies have been reported for the chemical degradation of validoxylamine A. The most robust method involves the use of NBS as a cleavage reagent (Ogawa et al. 1989, Ogawa et al. 1991). However, the non-specific nature of this cleavage resulted in numerous reaction products, such as valienamine, validamine, valienone, validone, and other side products, which requires expensive purification processes. A number of other methods using acid/base conditions such as hydrolysis with TFA or NaOH solutions have also been reported in the patent literature. However, we found that such reactions were relatively slow and the overall conversion yields were low (unpublished data). In this study, both validoxylamine A and 1,1'-bis-valienamine could be efficiently cleaved by NBS and the amino products trapped by ion-exchange resins. The fact that chemical degradation of 1,1'-bis-valienamine followed by purification with ion-exchange columns solely gave valienamine suggests that 1,1'-bis-valienamine is a better starting material for valienamine production than validoxylamine A.

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Figure 1.
Chemical structures of validamycin A and its analogs.

Figure 2.

Inactivation of *valN* in *S. hygroscopicus* 5008. (A) Schematic representation of the replacement of a 918-bp fragment of *valN* with the 1371-bp *aac(3)IV-oriT* cassette. In shuttle plasmid pJTU753, *aac(3)IV-oriT* was inserted between the 3924-bp and 2244-bp genomic fragments originally flanking the 918-bp region. While wild-type 5008 should give a 1860-bp PCR-amplified product, mutant XH-2 should yield a 2313-bp product using primers ValN-det-F and ValN-det-R. (B) PCR analysis of wild-type 5008 and mutant XH-2. PCR products were run on an agarose gel.

Figure 3.
 ^1H - and ^{13}C NMR spectra of validamycin analogs

Figure 4. Fungal growth inhibitory assays of validamycin analogs. The fungus was inoculated as an agar plug placed in the center of the plates.

Figure 5.
Chemical degradation of validoxylamine A and 1,1'-bis-valienamine

Figure 6.

¹H NMR spectra of 1,1'-bis-valienamine (A), degradation products of 1,1'-bis-valienamine treated with NBS and purified by ion exchange and silica gel columns (B), valienamine standard (C); validamine standard (D); degradation products of validoxylamine A treated with NBS and purified by ion exchange columns (E), and validoxylamine A (F).