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Generation of Novel Pikromycin Antibiotic Products Through Mutasynthesis

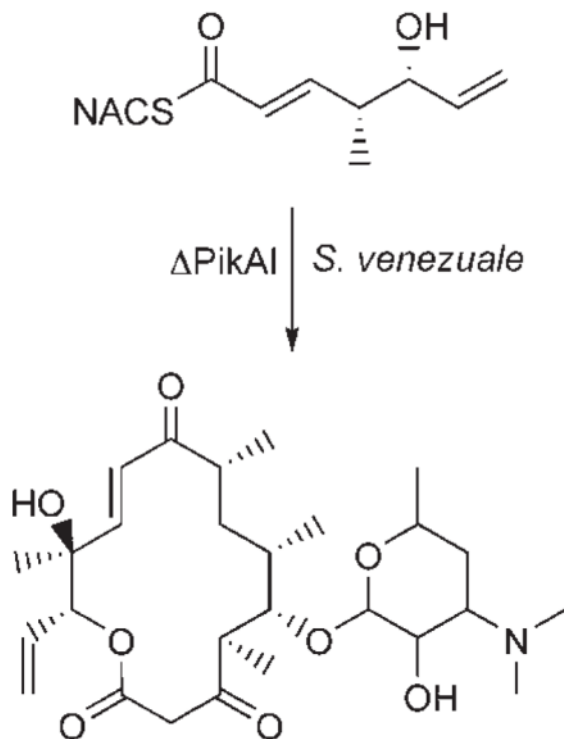
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Abstract

Mutasynthesis in pikromycin PKS: The amenability of pikromycin polyketide synthase to mutational biosynthesis has been demonstrated. A natural triketide and its analogues, activated as *N*-acetyl-cysteamine thioesters, were synthesized and fed to a *pikAI*-deleted strain; this led to the production of new antibiotics. A vinyl analogue was found to have better antibacterial activity than pikromycin.



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The pikromycin polyketide synthase (PKS) of *S. venezuelae*, which consists of one loading module and six extension modules, is responsible for the formation of the hexaketide narbonolide, a key intermediate in the biosynthesis of the antibiotic pikromycin. *S. venezuelae* strains in which PikAI, which houses the loading domain and first two modules of the PKS, is either absent or catalytically inactive, produce no pikromycin product. When these strains are grown in the presence of a synthetically prepared triketide product, activated as the *N*-acetylcysteamine thioester, pikromycin yields are restored to as much as 11 % of that seen in the wild-type strain. Feeding analogues of the triketide intermediate provides pikromycin analogues bearing different alkyl substituents at C13 and C14. One of these analogues, $\Delta^{15,16}$ -dehydropikromycin, exhibits improved antimicrobial activity relative to pikromycin.

Keywords

biosynthesis; natural products; pikromycin; polyketides; triketides

Introduction

Polyketides constitute a large and diverse group of natural products that possess important anticancer, antibiotic, immunosuppressive, and other biological activities[1–3] Many of them are biosynthesized by modular type I polyketide synthases (PKSs), which are giant multifunctional enzymes consisting of several discrete modules, each responsible for one cycle of polyketide acyl chain elongation. Each module contains three basic domains for each biosynthetic step; an acyltransferase (AT), an acyl carrier protein (ACP), and a ketosynthase (KS). The extent of modification within each module of the β -ketoacyl thioester formed depends on the presence or absence of ketoreductase (KR), dehydratase (DH), or enoyl reductase (ER) domains.[4] The pikromycin (Pik) PKS of *S. venezuelae* comprises four separate polypeptides (PikAI-PikAIV) that house six such extension modules, a loading module, and a thioesterase domain (TE) responsible for priming and terminating the polyketide biosynthetic process, respectively (Figure 1).[5] Full extension through this Pik PKS gives rise to a heptaketide product, which is then cyclized to the 14-membered aglycone narbonolide. Modification by the DesVII–DesVIII glycosyltransferase and PikC (a P450 hydroxylase) gives the biologically active pikromycin product (**1a**).[5] Termination of the chain-elongation process at the hexaketide intermediate (without extension by module 6) has been shown to give rise to the 12-membered aglycone 10-deoxymethynolide, which is then converted by DesVII and PikC to methymycin.[5]

It has been shown through both in vivo and in vitro experiments that PKS intermediates activated as *N*-acetylcysteamine (NAC) thioesters can be loaded onto the KS domains of elongation modules.[6–11] Such loading presumably accounts for the observation that polyketide production can be achieved by feeding diketide and triketide intermediates to bacterial strains in which either the priming or initial elongation steps have been abrogated. [6–11] Feeding of isomers of presumptive natural intermediates in such systems has proven to be a useful tool for determining unresolved stereochemical questions, as shown recently for diketides involved in ansamitocin,[9] rifamycin,[12] and phoslactomycin biosynthesis.[8] In all of these cases, feeding of the diketide intermediate led to only partial restoration of the final polyketide product. In the case of ansamitocin feeding, the presumed triketide intermediate gave no restoration.[9] These observations have led to the suggestion that loading polyketide intermediates onto the cognate PKS modules is inefficient.[9]

There are a few in vivo examples in which feeding analogues of polyketide intermediates leads to the generation of novel polyketide products. This mutasynthetic approach[13,14] with type I PKSs has been used to generate new epothilones[11] and erythromycins.[6,7,15] In the latter

case, a 6-deoxyerythronolide B synthase (DEBS) in which module 1 was made catalytically inactive has been used successfully to produce many new structures with unique physicochemical properties and new functional groups.[15] Yields of the new natural products in these cases were very low, presumably as a result of inefficient loading onto the cognate module, and slower conversion of the unnatural intermediate by the downstream PKS modules and post-PKS modification enzymes. A number of approaches have been taken to improve the efficiency of this process. In the case of erythromycin biosynthesis, it has been shown that natural product yields improve as much as tenfold if diketide intermediates are fed to strains in which the loading domain and module 1 of the PKS are deleted, rather than made catalytically inactive through mutation.[10]

To date there have been no reports of the generation of novel pikromycins by the same type of mutasynthetic approaches as for erythromycins. A number of observations suggest potentially significant barriers to the success of such an approach. Previous incorporation experiments with dual-labeled diketide and triketide intermediates in the wild-type *Streptomyces venezuelae* were shown initially to lead to no intact incorporation into a polyketide product. [16] Refinement of the fermentation conditions and timing of addition of the intermediates led to successful but extremely low incorporation levels (0.15–0.5 % of the polyketide product was generated from the intact intermediate), potentially indicating poor loading onto the cognate PKS modules. Recent in vivo and in vitro analyses of modules 3, 5, and 6 of the pikromycin PKS have also demonstrated that they have significant substrate specificity[17, 18] and thus might not efficiently process unnatural pathway intermediates.

We report here a series of mutasynthetic experiments with a *pikAI* deletion mutant of *S. venezuelae* (BB138) (Figure 1), in which PikAI, which houses the loading domain and first two modules, is absent. Feeding the *N*-acetyl cysteamine thioester of the triketide intermediate restores the yield of pikromycin to approximately 11% of production levels of the wild-type strain. Expression of a catalytically inactive PikAI in BB138 does not alter the efficiency of this process. A series of triketide analogues was synthesized and shown to be processed by the BB138 strain, and four were shown to be processed into new biologically active 14-membered macrolide products. The levels of production of these new macrolides varied, but in all cases were at least tenfold lower than seen for pikromycin production from the natural triketide. Preliminary analysis of one new product, $\Delta^{15,16}$ -dehydropikromycin, indicated slightly improved antibacterial activity. These data show for the first time that the Pik PKS system is amenable to mutasynthesis and can be used to generate new and potentially useful antibiotics. However, we observed incorporation inefficiencies similar to those encountered in other type I PKSs.

Results and Discussion

Synthesis of natural triketide and its analogues

The natural triketide **2a** and its analogues **2b–f** were prepared as shown in Scheme 1. The aldol intermediates **3a–f** were generated by stereoselective aldol coupling reaction between an Evans' acyl oxazolidinone and various commercially available aldehydes. Silylation of the hydroxyl groups of **3a–f** followed by replacement of the chiral auxiliary with a benzyl group gave intermediates **4a–f**. Subsequent reduction of the benzyl ester with DIBAL-H gave alcohols **5a–f**. (Attempts to generate alcohols **5a–f** by direct reductive removal of the chiral auxiliary by using various reducing agents resulted in poor yields and decomposition products.) The alcohols were oxidized by using the Dess–Martin periodinane, and the resulting crude aldehydes were immediately treated with the respective Wittig salts. The resulting unsaturated esters were hydrolyzed under mild basic conditions to generate the corresponding carboxylic acids **6a–f**. Treatment of the acids with diphenylphosphoryl azide and triethylamine followed

by addition of *N*-acetylcysteamine afforded the protected NAC-thioesters, which were treated with aqueous HF to afford the final triketide NAC thioesters **2a–f**.

The synthesis of the triketide analogues **2g–i** was troublesome due to difficulty in removing the chiral auxiliary. Thus, for these triketides, a different route, based on modifications of a literature procedure,[19] was used to prepare the aldehyde intermediate (Scheme 2). Briefly, treatment of the aldol products **3g–i** with *N,O*-dimethylhydroxyl amine and trimethyl aluminum gave the corresponding Weinreb amides, which were protected to give the silyl ethers. Reduction with DIBAL-H in THF gave the corresponding aldehydes in a much higher yield and purity compared to the strategy employed in Scheme 1. The aldehydes were converted to the desired triketide NAC thioesters **2g–i** by using the same approach described above (steps f–i, Scheme 1).

Feeding of unsaturated triketide **2a** to *S. venezuelae* BB138 mutant

Fermentations of the BB138 in 10 mL please define SGGP (SGGP) media[20] were supplemented after 8 h of incubation with 1 mM of the unsaturated triketide **2a**. After incubation for additional 3 days at 30 °C and 220 rpm, the culture was centrifuged, and the broth was extracted twice with ethyl acetate. The extract was concentrated, redissolved in a small volume of methanol, and analyzed by LC/MS. Feeding of the natural triketide **2a** to BB138 resulted in the production of natural pikromycin in 10–12 % relative yield compared to wild-type *S. venezuelae* (Table 1). We also observed the formation of the 12-membered macrolides methymycin and neomethymycin in BB138 supplemented with **2a**, also at about 10–12 % of that seen for the wild-type strain under the same growth conditions. There was no significant difference in the production level of pikromycin when the triketide concentration was increased to 2 or 5 mM (Figure 2). However, the levels of pikromycin decreased dramatically when lower (<0.5 mM) concentrations were used; this suggests that, under these conditions, priming of module 3 of the Pik PKS in BB138 was limited by the availability of **2a**.

The efficiency of precursor-directed biosynthesis of erythromycins by using diketides has been shown to be improved by removal of the loading domain and module 1 of DEBS.[10] While the reasons for this observation remain undetermined, it seems likely that these preceding modules can interfere with the transfer of the diketide onto the active-site cysteine of module 2 of DEBS. Experiments were carried out to determine if the modules of PikAI interfered with loading of the natural triketide **2a** onto module 3. A plasmid (pBK51) that expresses the entire PikAI with a Cys–Ala mutation in the active site of module 1, and pDHS722 (which expresses the natural PikAI) were introduced into BB138 (Figure 1). Pikromycin production was observed in BB138/pDHS722, but not in BB138/pBK51 or BB138/pSG1 (plasmid control). These observations were consistent with expression of an active PikAI from pDHS722 and an inactive PikAI from pBK51. The levels of pikromycin production obtained by growing BB138/pBK51 and BB138/pSG1 in the presence of a range of concentrations of the natural triketide **2a** were determined (Figure 3). In both cases the same significant drop in pikromycin production was observed when triketide concentrations were less than 0.5 mM. However, there was no significant difference in pikromycin production for BB138/pBK51 and BB138/pSG1 at each triketide concentration. A feeding study with the natural diketide resulted in pikromycin production in BB138/pBK51, thus unequivocally demonstrating the presence of soluble PikAI with an active module 2 and inactive module 1 (data not shown). These experiments suggest that the PikAI protein does not affect loading of the triketide onto module 3 of the Pik PKS, and contrast the observations made with DEBS.[10] In the DEBS case, however, the experiments were carried out with diketides, and the preceding modules (loading domain and module 1) were housed on the same polypeptide. In the case of triketide feeding for the Pik

PKS, the preceding modules are housed on a separate polypeptide and are therefore arguably less likely to present a significant barrier to priming of the module (Table 1).

A number of factors could account for the observation that maximal yields of pikromycin production from feeding triketide to BB138 were only about 10 % of that seen in the wild-type strain. One potential limiting factor is the loading of **2a** onto module 3 of the Pik PKS. Indeed, previous in vitro work with individual modules of DEBS has shown that the catalytic efficiency for formation of a triketide lactone increases more than 100-fold if the priming diketide substrate is presented attached to the cognate ACP rather than as an *N*-acetylcysteamine.[21] In the normal Pik PKS process, the docking domains of PikAI and PikAII presumably facilitate efficient passage of the triketide intermediate from ACP3 of PikAI to the KS4 of PikAII.[22] The efficiency of this process coupled with the poor loading of **2a** on to KS3 likely accounts for previous observations of extremely low intact incorporation of labeled triketide into pikromycin in the wild-type *S. venezuelae*. [16]

Production of novel pikromycin analogues by mutational biosynthesis in strain BB138

NAC thioester analogues of the natural triketide **2a** were added to fermentations of BB138 to determine which could be processed into novel pikromycin-related products. All triketide analogues were fed at a final concentration of 1 mM, and the resulting fermentation products were analyzed by LC-MS. As detailed in Figure 4, four of these triketides (**2b**, **2c**, **2h**, and **2i**) were processed to the corresponding 14-membered analogues of pikromycin. In none of the triketide experiments were 12-membered products (analogues of methymycin, neomethymycin, and 10-deoxymethynolide) observed. The reason for this observation was unclear, but might simply reflect the fact that they were produced at levels harder to detect (the 14-membered pikromycin predominated in BB138 grown in the presence of **2a**).

Replacement of the ethyl group of the natural triketide with propyl (**2b**), isopropyl (**2c**), or ethylene (**2i**) resulted in detectable levels of fully elongated polyketide products (**1b**, **1c**, and **1e**, respectively). Replacement of the ethyl group with the cyclohexyl group in **2d**, did not give the corresponding product, potentially indicating an inability of the Pik PKS to process a bulky substituent at the end of the polyketide chain. Surprisingly, the triketide analogue **2e**, in which the ethyl group was shortened to a methyl was also not processed. Thus, there appears to be an upper and lower limit to the size of the substituent used to replace the ethyl group.

Replacement of the methyl substituent at the γ -position of the triketide with an ethyl in **2h** successfully led to the new fully elongated product **1d**, while replacement with a hydrogen in **2g** did not. The same negative result was obtained with **2f**, when the α -hydrogen in the natural triketide was replaced with a methyl group.

These experiments demonstrate clearly that the subunits PikAII–PikAIV of the Pik PKS have sufficient flexibility to process some analogues of the natural triketide in which the structural components introduced by the loading domain and module 1 of PikAI have been varied. In all cases the yields were extremely poor. In the case of triketides **2b**, **2c**, and **2h**, production of the pikromycin analogue was <1 % of the normal pikromycin production (and thus more than a tenfold decrease over yields of pikromycin made by feeding the natural triketide **2a** to strain BB138, Table 1). The vinyl triketide analogue **2i** was utilized more efficiently than other unnatural analogues (~1 % relative yield, calculated by using a standard curve for pikromycin, Table 1). We cannot discount the possibility that the triketide analogues are either taken up more poorly or degraded faster than the natural triketide. Nonetheless, the compounds are very similar in structure, and we hypothesize that the low yields seen with all triketide analogues relative to the natural triketide are consistent with slower loading and elongation of these analogues due to the specificity of the Pik PKS modules. Presumably triketide analogues that

did not result in detectable production of new products were either not loaded and processed by the Pik PKS, or did so very slowly.

In the cases of the triketides **2a–c**, **h**, and **i**, analogues **7a–e** (Figure 4) of narbomycin (pikromycin lacking the C-13 hydroxyl substituent) were also observed in levels higher than their hydroxylated counterparts, as indicated by LC-MS analyses (the low titers precluded an accurate determination of this ratio). Compounds **7b**, **c**, and **e** were purified, and accurate mass analyses (Table 2) were shown to be consistent with the proposed structures (Figure 4). These observations clearly demonstrate that PikC is able to process analogues of the natural substrate, but likely does so with reduced catalytic efficiency. For none of these four triketide analogues were there detectable levels of the 14-membered aglycone products. This observation demonstrated that DesVII–DesVIII,[23] a protein complex that catalyzes attachment of D-desosamine, can tolerate structural variations in the C13–C14 region of the aglycone structure. The lack of aglycone products also indicates that the low levels of pikromycin products made from these triketides is not a result of poor hydroxylation and glycosylation, and more likely poor processing by the Pik PKS.

Antibacterial activity of $\Delta^{15,16}$ -dehydropikromycin

A small quantity of $\Delta^{15,16}$ -dehydropikromycin (**1e**) was isolated and its antibacterial activity against three different bacteria was compared to that of natural pikromycin (Table 3). In this preliminary analysis a twofold improvement in the MIC₉₉ was observed. Directed biosynthesis of erythromycin analogues bearing an azido group at C15 has recently been reported.[15] In this case, a twofold improvement in activity against the Gram-negative respiratory pathogen *H. influenzae* was observed relative to erythromycin (this difference was not observed with other bacteria). These two studies show that directed-biosynthesis can be used to generate pikromycin and erythromycin analogues bearing new C14 substituents that either do not impact biological activity, or lead to a modest improvement. The method also allows vinyl and ethylamino groups to be added, thereby providing chemical handles for additional modification steps.

In conclusion, the data show for the first time that the Pik PKS system is amenable to directed biosynthesis and that analogues of the natural triketide intermediate can be used to generate new and potentially useful antibiotics.

Experimental Section

All reactions were carried out with dry solvents under anhydrous conditions (under nitrogen), unless otherwise noted. All solvents and reagents were purchased from Aldrich. Normal-phase flash column chromatography was carried out on Davisil[®] silica gel (100–200 mesh, Fisher). Preparative thin-layer chromatography (PTLC) separations were carried out on 1 or 2 mm Merck silica gel plates (60F–254). ¹H NMR spectra were recorded on Tecmag Libra-modified NM-500 MHz or Bruker AC-F 300 MHz spectrometers and calibrated by using residual undeuterated solvent as an internal reference. ¹³C NMR spectra were recorded on Bruker AMX-400 MHz or Bruker AC-F 300 MHz NMR spectrometers. High-resolution mass spectra were recorded on a Micromass LCT Electrospray mass spectrometer at the Mass Spectrometry & Proteomics Facility (Ohio State University).

HPLC and LC-MS analysis

HPLC was performed on a system equipped with Waters 600 pump connected to Waters 2487 Dual Absorbance. Products were analyzed by using a 5 μ m Discovery HS C18 reversed-phase column (4.6 \times 250 mm, Supelco) with an elution gradient from 20 to 80 % acetonitrile in ammonium acetate buffer (10 mM) at flow rate of 1 mL min^{−1}. LC/MS analysis was performed

by using the same gradient solvent system as described for HPLC analysis at a flow rate of 0.3 mL min^{-1} on a Surveyor HPLC system (ThermoFinnigan) connected to diode ray detector equipped with a $2.1 \mu\text{m}$ Discovery HS C18 reversed-phase column ($4.6 \times 250 \text{ mm}$, Supelco). Mass spectra were collected on an LCQ quadrupole ion trap (ThermoFinnigan) mass spectrometer equipped with an electrospray ion source operating in positive mode.

Strains

The construction of *PikAI* deletion mutant (BB138) used for the feeding studies has been described previously.[24] The plasmid SG1 was constructed from pDHS702[5] by digestion with BamHI and BglII and religation by using a Roche Ligation kit. For the construction of pBK51, a BamHI–BglII fragment of the *pikAI* complementation plasmid pDHS722[24] was subcloned, and the desired mutation was introduced by using a QuickChange kit and the primers 5'-GTGGACACGGCCGCTAGCTCGTCGCTG-3' and GTCCAC 5' CAGCGACGAGCTAGCGGCCGT. The resulting mutated fragment was then subcloned back into pDHS722 to give pBK51 according the methodologies previously described.[25] *S. venezuelae* transformants were selected on R2YE agar plates by overlaying with thiostrepton (1 mL , $500 \mu\text{g mL}^{-1}$). SGGP liquid medium was used for propagation of all the mutants of *Streptomyces*.

Feeding experiments

A loop full of spores of BB138 was inoculated in SGGP medium (10 mL) in a 50 mL flask and grown for 16 h at 30°C and 220 rpm . This seed culture ($100 \mu\text{L}$) was then used to inoculate fresh SGGP medium (10 mL) in a 50 mL flask. The culture was grown for 8 h at 30°C and 220 rpm , after which triketide SNACs (1 mM) were added to it, and the culture was grown for an additional 3 days . At the end of the fermentation period the mycelia were removed by centrifugation, the pH of the supernatant was adjusted to 9.5 (1 N NaOH), and the supernatant was extracted with ethyl acetate ($2 \times 20 \text{ mL}$). The organic extract was concentrated by rotary evaporation, redissolved in methanol, and analyzed by HPLC and LC/MS. The feeding of compounds **2 a–i** was performed in triplicate.

Quantification of pikromycin analogues

Pikromycin was purified from *S. venezuelae* (wild-type) by HPLC on a C-18 column. Specific amounts of pikromycin were injected into the chromatograph, and its UV absorption was determined. A standard plot of concentration versus area under the peak (UV absorption) was obtained and used to quantify the production levels of other pikromycin analogues.

Determination of MIC₉₉

Minimum inhibitory concentrations were determined in triplicate according to the broth microdilution method.[26] *E. coli* TolC, *S. aureus* NorA, and *Bacillus subtilis* (stock solutions in glycerol) were grown overnight at 37°C in LB, tryptic broth, and nutrient broth media respectively. The cells were diluted to $\sim 0.001 \text{ OD}_{600}$ with the respective media. The assay was performed in a 96-well plate. Stock solutions of compounds **1a** and **1e** in ethanol introduced into a 96-well plate were diluted with medium to produce the desired concentration. The plate was incubated for 6 h at 37°C for *E. coli* TolC and *S. aureus* NorA, and over-night for *Bacillus subtilis*. MIC₉₉ values are reported as the minimum concentration at which there was no visible growth. The OD_{600} was used as a measure of cell growth.

Chemical synthesis

General procedure for aldol coupling: Diisopropylethylamine (25.8 mmol) and dibutylboron triflate (1.0 M in CH_2Cl_2 , 25.8 mmol) were added to a stirred solution of Evans' acyl-oxazolidinone (25.8 mmol) in CH_2Cl_2 (0.3 M) at 0°C . The resulting reaction mixture was

stirred at 0 °C for 30 min and then cooled to -78 °C. Propionaldehyde (17.2 mmol) in CH₂Cl₂ (0.5 M) was added, and the mixture was stirred at -78 °C for 1 h and then allowed to warm to 0 °C. After the mixture had been stirred for 2 h at this temperature, the reaction was quenched by addition of phosphate buffer pH 7 (20 mL). The reaction mixture was poured into a flask containing MeOH (85 mL) at 0 °C, treated with precooled 30% H₂O₂ (107 mL), and stirred at 0 °C for 1 h. MeOH was removed by rotary evaporation, saturated aqueous NaHCO₃ was added, and the resultant aqueous layer was extracted with CH₂Cl₂ (3 × 100 mL) and purified by flash silica gel chromatography to afford aldols **3**.

General procedure for protection of aldol **3:** 2,6-Lutidine (14.9 mmol) was added to a solution of aldols **3** (4.9 mmol) in CH₂Cl₂ (0.2 M) at 0 °C. After the mixture had been stirred for 5 min at that temperature, *tert*-butyldimethylsilyltrifluoromethane sulfonate (7.5 mmol) was added dropwise, and the reaction mixture was stirred at 0 °C for 20 min, after which time no starting material was detected by TLC. Saturated aqueous NH₄Cl was then added. The organic phase was separated, and the aqueous layer was extracted with CH₂Cl₂ (3 × 30 mL). The combined organic extracts were dried over anhydrous Na₂SO₄, concentrated, and purified by flash silica gel chromatography.

General procedure for benzylation: *n*BuLi (1.6 M in hexanes, 6.4 mmol) was added to a solution of benzyl alcohol (10.4 mmol) in THF (0.5 M) at 0 °C. The resulting solution was stirred at 0 °C for 30 min, and a solution of the protected aldols **3** (4.9 mmol) in THF (0.8 M) was added. The reaction mixture was stirred at this temperature for 5 h and then quenched by addition of saturated aqueous NH₄Cl. The organic phase was separated, and the aqueous layer was extracted with diethyl ether (3 × 20 mL). The combined organic extracts were washed with water, dried over anhydrous Na₂SO₄, concentrated, and purified by flash silica gel chromatography to afford the benzyl esters **4**.

General procedure for DIBAL-H reduction: DIBAL-H (1 M in hexanes, 8.9 mmol) was added to a solution of **4** (4.1 mmol) in CH₂Cl₂ (0.6 M) at -78 °C. The mixture was stirred at -78 °C for 30 min and then warmed up to 0 °C. After the mixture had been stirred at 0 °C for 30 min, the reaction was quenched with MeOH (0.2 mL), and the mixture was diluted with CH₂Cl₂ (14 mL). A saturated aqueous solution of Rochelle salt (14 mL) was added, and the mixture was stirred at room temperature until there were two clear layers. The organic layer was separated, and the aqueous layer was extracted with diethyl ether (3 × 20 mL). The combined organic extracts were washed with brine, dried over anhydrous Na₂SO₄, concentrated, and purified by flash silica gel chromatography to afford alcohols **5**.

General procedure for Wittig olefination: Dess–Martin periodinane (3.1 mmol) was added to a solution of **5** (2.6 mmol) in CH₂Cl₂ (0.2 M) at 0 °C. The resulting mixture was stirred at room temperature for 30 min, after which time it was subjected to silica gel column chromatography. The column was eluted with 10 % EtOAc in hexane to obtain the corresponding aldehyde as colorless oil, which was used directly in the next step. Methyl (triphenylphosphoranylidene) acetate was added to a solution of aldehyde (2.6 mmol) in THF (0.06 M). The mixture was heated under reflux for 24 h, after which time no starting material was detected by TLC. The reaction mixture was applied to a silica gel column, and the unsaturated esters were eluted with 1% EtOAc in hexane.

General procedure for hydrolysis of unsaturated esters: Potassium carbonate (3.5 mmol) was added to a solution of the unsaturated esters (0.69 mmol) in methanol (0.08 M) and water (0.25 M). The reaction mixture was heated under reflux for 3 h, after which methanol was removed by rotary evaporation, and the aqueous layer was acidified to pH 2 with concentrated HCl. The mixture was saturated with solid NaCl and extracted with diethyl ether (3 × 15 mL). The combined organic extracts were washed with saturated aqueous NaHCO₃, dried over

anhydrous Na₂SO₄, concentrated, and purified by flash silica gel chromatography to afford the unsaturated acid **6**.

General procedure for the preparation of protected NAC: Thioesters: Triethylamine (1.1 mmol) and diphenylphosphorylazide (0.8 mmol) were added to a solution of **6** (0.5 mmol) in DMF (0.2 M) at 0 °C. The reaction mixture was stirred at 0 °C for 2 h. *N*-acetylcysteamine (0.6 mmol) was added, and the mixture was stirred at room temperature for 2 h after which water (2.6 mL) was added. The organic layer was separated, and the aqueous layer was extracted with EtOAc (3 × 2.6 mL). The combined organic extracts were dried over anhydrous Na₂SO₄, concentrated, and purified by flash silica gel chromatography.

General procedure for the deprotection and preparation of final NAC thioesters: Hydrofluoric acid (48 % wt in H₂O) was added to a solution of the protected NAC thioester (0.4 mmol) in acetonitrile (0.1 M) and water (0.6 M). After being stirred for 2 h at room temperature the reaction mixture was cooled to 0°C and the pH was adjusted to 7.5 with saturated aqueous NaHCO₃. Acetonitrile was removed by rotary evaporation, and the resultant aqueous layer was extracted with EtOAc (3 × 10 mL). The combined organic extracts were dried over anhydrous Na₂SO₄, concentrated, and purified by flash silica gel chromatography to afford the final Triketide NAC thioesters **2a–f**.

General procedure for Weinreb amides: AlMe₃ (2.0M in heptane, 0.9 mmol) was added to a suspension of *N,O*-dimethylhydroxylamine hydrochloride (0.9 mmol) in THF (1.9 M) at 0° C. The solution was stirred at room temperature for 30 min and then cooled to –15°C. A solution of **3g–i** (0.3 mmol) in THF (0.7 M) was added, and the mixture was allowed to warm to 0 °C. After stirring at 0°C for 2.5 h, CH₂Cl₂ (0.1 M) and HCl (0.5 N, 0.06 M) were added to the mixture, and it was stirred at 0°C for an additional 1 h. The organic layer was separated, and the aqueous layer was extracted with CH₂Cl₂ (3 × 5 mL). The combined organic extracts were washed with saturated aqueous NaHCO₃, dried over anhydrous Na₂SO₄, concentrated, and purified by flash silica gel chromatography.

General procedure for protection of Weinreb amides: 2,6-Lutidine (15.3 mmol) was added to a solution of alcohol (5.1 mmol) in CH₂Cl₂ (0.2 M) at 0 °C. After stirring for 5 min at that temperature, *tert*-butyldimethylsilyltrifluoromethane sulfonate (7.7 mmol) was added dropwise to the reaction mixture, and it was stirred at 0°C for 20 min, after which time no starting material was detected by TLC. Saturated aqueous NH₄Cl was added. The organic phase was separated, and the aqueous layer was extracted with CH₂Cl₂ (3 × 30 mL). The combined organic extracts were dried over anhydrous Na₂SO₄, concentrated, and purified by flash silica gel chromatography.

General procedure for DIBAL-H reduction of Weinreb amides: To a solution of the protected Weinreb amide (3.3 mmol) in THF (0.08M) at –78°C was added DIBAL-H (1 M in hexanes, 6.6 mmol). The mixture was stirred at –78°C for 1 h, after which the reaction was quenched by the addition of acetone (0.3 mL). The solution was immediately poured into a vigorously stirring mixture of hexane (64 mL) and tartaric acid (0.5 M, 64 mL) at 0°C. The mixture was stirred at 0 °C for 30 min. The organic phase was separated, and the aqueous layer was extracted with dichloromethane (3 × 100 mL). The combined organic extracts were washed with water and brine, dried over anhydrous Na₂SO₄, concentrated, and purified by flash silica gel chromatography.

Analytical data for final triketide NAC thioesters **2a–i**

Compound 2a: *R*_f = 0.18 (5 % MeOH/CH₂Cl₂); ¹H NMR (500 MHz, CDCl₃): δ = 6.94 (dd, *J* = 7.5, 15.5 Hz, 1H), 6.18 (dd, *J* = 1.0, 16.0 Hz, 1 H), 5.87 (br s *ok?*, 1H), 3.53 (m, 1 H), 3.48

(q, J = 6.0 Hz, 2 H), 3.11 (t, J = 6.0 Hz, 2 H), 2.45 (m, 1H), 1.98 (s, 3H), 1.63–1.53 (m, 1 H), 1.46–1.38 (m, 1 H), 1.12 (d, J = 7.0 Hz, 3H), 0.99 (t, J = 8.0 Hz, 3 H); ^{13}C NMR (100 MHz, CDCl_3): δ = 190.5, 170.5, 148.6, 128.4, 76.0, 42.4, 39.9, 28.6, 27.6, 23.4, 14.0, 10.5; HRMS calcd for $\text{C}_{12}\text{H}_{21}\text{NO}_3\text{S} + \text{Na}$: 282.1140; found 282.1118 [$M+\text{Na}$].

Compound 2b: R_f = 0.19 (5 % MeOH/ CH_2Cl_2); ^1H NMR (500 MHz, CDCl_3): δ = 6.93 (dd, J = 7.5, 15.5 Hz, 1 H), 6.15 (m, 2 H), 3.60 (br s, 1 H), 3.44 (q, J = 6.0 Hz, 2 H), 3.08 (t, J = 6.5 Hz, 2 H), 2.41 (m, 1 H), 2.06 (s, 1 H), 1.95 (s, 3 H), 1.41 (m, 4 H), 1.08 (d, J = 6.5 Hz, 3 H), 0.92 (t, J = 6.5 Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ = 190.5, 170.6, 148.7, 128.4, 74.3, 42.8, 39.9, 36.8, 28.6, 23.4, 19.4, 14.2, 14.0; HRMS calcd for $\text{C}_{13}\text{H}_{23}\text{NO}_3\text{S} + \text{Na}$: 296.1296; found 296.1278 [$M+\text{Na}$].

Compound 2c: R_f = 0.22 (5 % MeOH/ CH_2Cl_2); ^1H NMR (500 MHz, CDCl_3): δ = 6.90 (dd, J = 8.0, 15.5 Hz, 1H), 6.16 (d, J = 16.0 Hz, 1 H), 6.01 (br s, 1 H), 3.46 (q, J = 6.0 Hz, 2 H), 3.28 (m, 1 H), 3.09 (t, J = 6.5 Hz, 2H), 2.52 (m, 1 H), 1.97 (s, 3 H), 1.70 (m, 2 H), 1.11 (d, J = 6.0 Hz, 3H), 0.93 (d, J = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, CDCl_3): δ = 194.2, 170.2, 143.6, 135.5, 39.8, 39.4, 28.6, 27.9, 23.3, 15.1, 12.8, 10.2; HRMS calcd for $\text{C}_{13}\text{H}_{23}\text{NO}_3\text{S} + \text{Na}$: 296.1296; found 296.1296 [$M+\text{Na}$].

Compound 2d: R_f = 0.15 (5 % MeOH/ CH_2Cl_2); ^1H NMR (500 MHz, CDCl_3): δ = 6.94 (dd, J = 8.5, 16.5 Hz, 1H), 6.16 (d, J = 16.0 Hz, 1 H), 5.92 (br s, 1 H), 3.46 (q, J = 6.0 Hz, 2H), 3.32 (m, 1H), 3.11 (t, J = 6.5 Hz, 2H), 2.57 (m, 1H), 1.98 (s, 3 H), 1.88 (m, 1 H), 1.10 (d, J = 7.5 Hz, 3H), 0.93–1.79 (m, 1 H); ^{13}C NMR (100 MHz, CDCl_3): δ = 190.6, 170.4, 149.6, 128.1, 78.6, 41.1, 40.0, 39.3, 29.9, 28.6, 27.9, 26.5, 26.4, 26.1, 23.5, 13.3; HRMS calcd for $\text{C}_{16}\text{H}_{27}\text{NO}_3\text{S} + \text{Na}$: 336.1609; found 336.1605 [$M+\text{Na}$].

Compound 2e: R_f = 0.21 (5 % MeOH/ CH_2Cl_2); ^1H NMR (500 MHz, CDCl_3): δ = 6.78 (dd, J = 8.0, 16.0 Hz, 1H), 6.19 (dd, J = 1.0, 15.5 Hz, 1 H), 5.87 (br s, 1H), 3.83 (m, 1 H), 3.48 (q, J = 6.0 Hz, 2H), 3.12 (t, J = 6.5 Hz, 2 H), 2.42 (m, 1H), 1.44 (d, J = 5.0 Hz, 1 H), 1.20 (d, J = 6.5 Hz, 3H), 1.12 (d, J = 6.5 Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ = 190.3, 189.9, 147.6, 128.6, 70.6, 43.7, 39.8, 28.4, 23.3, 20.6, 14.3; HRMS calcd for $\text{C}_{11}\text{H}_{19}\text{NO}_3\text{S} + \text{Na}$: 268.0983; found 268.0989 [$M+\text{Na}$].

Compound 2f: R_f = 0.12 (5 % MeOH/ CH_2Cl_2); ^1H NMR (500 MHz, CDCl_3): δ = 6.64 (d, J = 10 Hz, 1 H), 5.89 (br s, 1H), 3.45 (m, 3 H), 3.08 (t, J = 6.5 Hz, 2 H), 2.67 (m, 1H), 1.98 (s, 3 H), 1.92 (s, 3 H), 1.56 (m, 1 H), 1.38 (m, 1H), 1.09 (m, J = 6.5 Hz, 3H), 0.98 (t, J = 7.5 Hz, 3 H); ^{13}C NMR (100 MHz, CDCl_3): δ = 194.2, 170.2, 143.6, 135.5, 76.5, 39.8, 39.4, 28., 27.9, 23.3, 15.1, 12.8, 10.2; HRMS calcd for $\text{C}_{13}\text{H}_{23}\text{NO}_3\text{S} + \text{Na}$: 296.1296; found 296.1296 [$M+\text{Na}$].

Compound 2g: R_f = 0.08 (5 % MeOH/ CH_2Cl_2); ^1H NMR (500 MHz, CDCl_3): δ = 6.97 (m, 1H), 6.22 (d, J = 15.5 Hz, 1 H), 5.91 (br s, 1 H), 3.73 (m, 1H), 3.48 (q, J = 6.0 Hz, 2H), 3.11 (t, J = 6.0 Hz), 2.43 (m, 1H), 2.34 (m, 1H), 1.98 (s, 3 H), 1.55 (m, 2 H), 0.98 (t, J = 7.5 Hz); ^{13}C NMR (100 MHz, CDCl_3): δ = 190.1, 170.2, 142.6, 130.5, 71.9, 39.8, 39.6, 30.1, 28.4, 23.2, 9.8; HRMS calcd for $\text{C}_{11}\text{H}_{19}\text{NO}_3\text{S} + \text{Na}$: 268.0983; found 268.0989 [$M+\text{Na}$].

Compound 2h: R_f = 0.16 (5 % MeOH/ CH_2Cl_2); ^1H NMR (500 MHz, CDCl_3): δ = 6.75 (dd, J = 9.5, 15.0 Hz, 1H), 6.16 (d, J = 15.5 Hz, 1 H), 6.0 (br s, 1H), 3.51 (m, 1H), 3.46 (q, J = 6.0 Hz, 2 H), 3.10 (t, J = 6.5 Hz, 2H), 2.16 (m, 1 H), 1.97 (s, 3 H), 1.76 (m, 2H), 1.55 (m, 1 H), 1.36 (m, 2 H), 0.97 (t, J = 8.0 Hz, 3 H), 0.87 (t, J = 7.5 Hz, 3 H); ^{13}C NMR (100 MHz, CDCl_3): δ = 190.2, 170.4, 146.9, 129.9, 75.3, 51.0, 39.8, 28.5, 27.7, 23.3, 22.6, 12.0, 10.3; HRMS calcd for $\text{C}_{13}\text{H}_{23}\text{NO}_3\text{S} + \text{Na}$: 296.1296; found 296.1299 [$M+\text{Na}$].

Compound 2i: R_f = 0.10 (5 % MeOH/CH₂Cl₂); ¹H NMR (300 MHz, CDCl₃): δ = 6.94 (dd, J = 15, 7.5 Hz, 1H), 6.16 (dd, J = 18, 1.2 Hz, 1 H), 5.95 (br s, 1 H), 5.83 (ddd, J = 18, 9, 4.5 Hz, 1 H), 5.17–5.32 (m, 2 H), 4.13 (dt, J = 9, 1.2 Hz, 1H) 3.46 (q, J = 15, 6 Hz, 2H), 3.09 (t, J = 6 Hz, 2 H), 2.54 (dsext, J = 6, 1.2 Hz, 1 H), 1.96 (s, 3 H), 1.79 (br d, J = 18.9 Hz, 1H), 1.09 (d, J = 6.3 Hz, 3 H); ¹³C NMR (75 MHz, CDCl₃): δ = 190.1, 170.4, 147.4, 137.9, 128.4, 116.6, 75.6, 42.2, 39.7, 28.4, 23.2, 14.2; HRMS calcd for C₁₂H₁₉NO₃S + Na: 280.0978; found 280.0990 [M +Na].

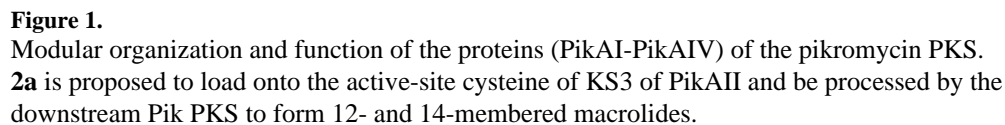
Acknowledgements

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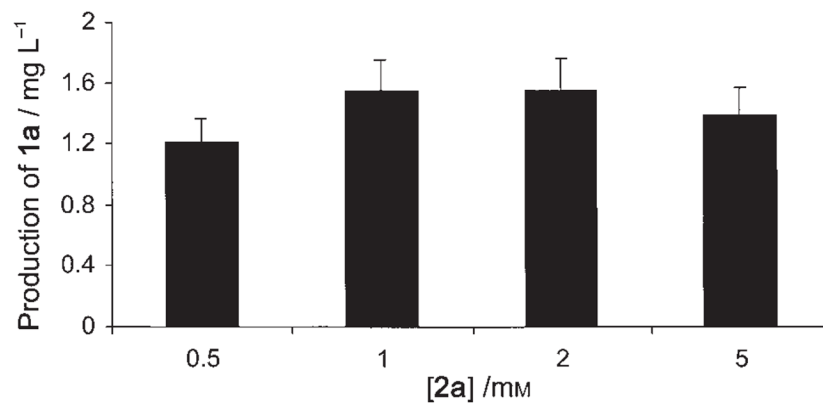


Figure 2. Pikromycin yields from growing *S. venezuelae* BB138 in the presence of varying concentrations of the triketide **2a**. Results are average (\pm SEM) of triplicate assays.

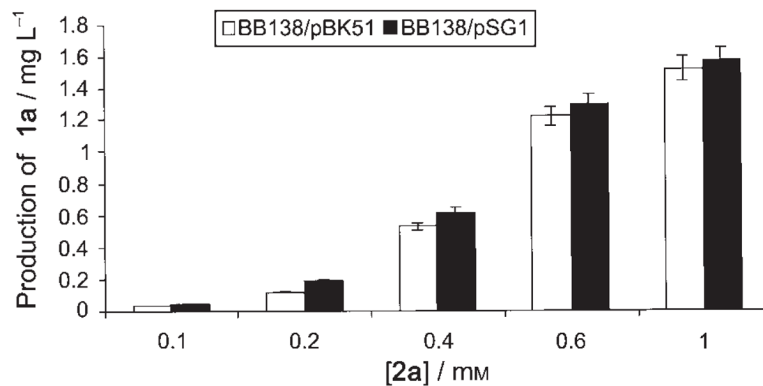
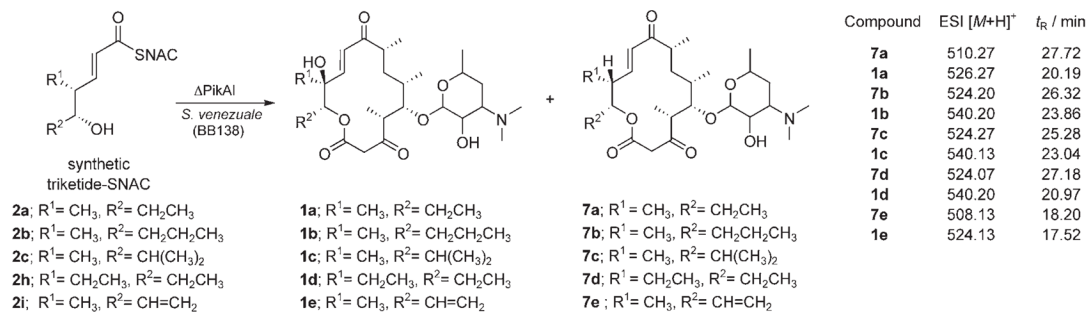
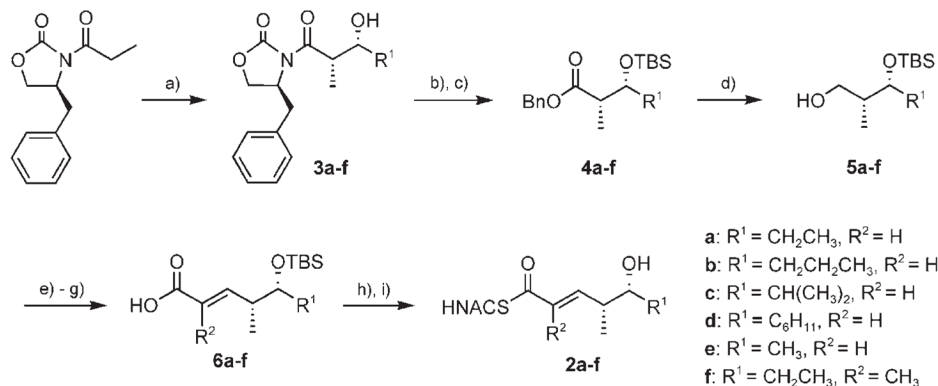


Figure 3.

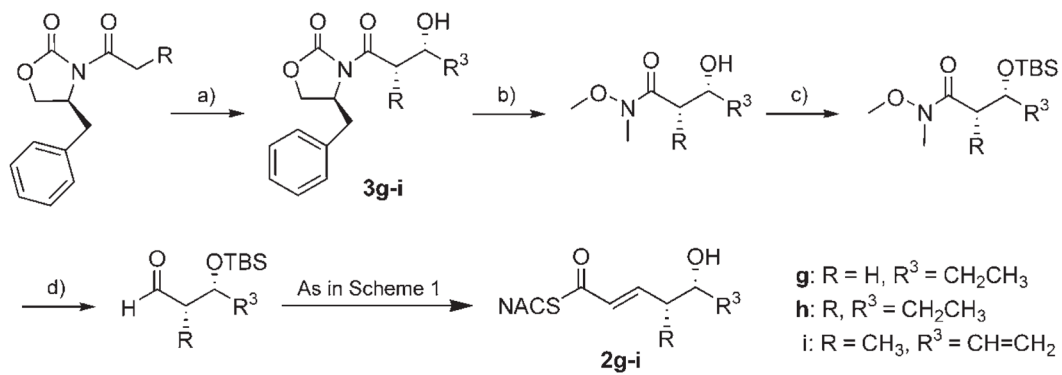
Pikromycin yields from growing *S. venezuelae* BB138/pBK51 and BB138pSG1 in the presence of varying concentrations of the triketide **2a**. Results are average (\pm SEM) of triplicate assays.

**Figure 4.**

Left: Analogues of pikromycin (**1a**) and narbomycin (**7a**) generated by growing *S. venezuelae* BB138 in the presence of analogues **2b–i** of the natural triketide **2a**. Right: ESI mass and HPLC retention times for **1a–e** and **7a–e**.

**Scheme 1.**

Synthesis of triketides **2a–f**. Reagents and conditions: a) DBBT, DIEA, CH_2Cl_2 , R^1CHO ; b) TBSOTf, 2,6-lutidine, CH_2Cl_2 ; c) $nBuLi$, BnOH, THF; d) DIBAL-H, CH_2Cl_2 ; e) Dess–Martin periodinane, CH_2Cl_2 ; f) Wittig salt, THF; g) K_2CO_3 , MeOH/ H_2O ; h) $(PhO)_2P(O)N_3$, Et_3N , $AcHN(CH_2)_2SH$, DMF; i) 48% HF, CH_3CN , H_2O . DBBT = dibutylboron triflate, DIEA = *N,N*-diisopropylethylamine.

**Scheme 2.**

Synthesis of triketides **2g–i**. Reagents and conditions: a) DBBT, DIEA, CH_2Cl_2 , R^3CHO ; b) AlMe_3 , $\text{MeO}(-\text{Me})\text{NH}\cdot\text{HCl}$, CH_2Cl_2 ; c) TBSOTf, 2,6-lutidine, CH_2Cl_2 ; d) DIBAL-H, CH_2Cl_2 .

Table 1

Production of natural pikromycin and its analogues by feeding substrates **2a–i** to BB138 mutant.^[a] Production yield relative to wild type *S. venezuelae*.

Substrate	Feeding product	Production [%] ^[a]
2a	Pikromycin (1a)	10–12
2b	1b	<1
2c	1c	<1
2h	1d	<1
2i	1e	~1

Table 2

HRMS data of compounds **7b**, **7c**, and **7e**.

Compound	Calculated [<i>M</i> + <i>H</i>]	Observed [<i>M</i> + <i>H</i>]
7b	524.3587	524.3563
7c	524.3587	524.3560
7e	508.3274	508.3250

Table 3

Antibacterial activity of **1a** and vinyl analogue **1e**.^[a] MIC₉₉ is the minimum concentration at which no visible growth was detected.

Test organism <i>ok?</i>	MIC ₉₉ [µm] ^[a]	
	1a	1e
<i>E. coli</i> To1C	50–60	20–30
<i>S. aureus</i> NorA	90–100	40–50
<i>Bacillus subtilis</i>	25–30	10–15