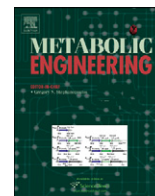




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Hybrid antibiotics with the nikkomycin nucleoside and polyoxin peptidyl moieties

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ABSTRACT

Acting as competitive inhibitors of chitin synthase, nikkomycins and polyoxins are potent antibiotics against pathogenic fungi. Taking advantage of the structural similarities between these two peptidyl nucleoside antibiotics, genes required for the biosynthesis of the dipeptidyl moiety of polyoxin from *Streptomyces cacaoi* were introduced into a *Streptomyces ansiochromogenes* mutant producing the nucleoside moiety of nikkomycin X. Two hybrid antibiotics were generated. One of them was identified as polyoxin N, and the other, a novel compound, was named polynik A. The hybrid antibiotics exhibited merits from both parents: they had better inhibitory activity against phytopathogenic fungi than polyoxin B, and were more stable under different pH and temperature conditions than nikkomycin X. This study demonstrates the use of the combinatorial biosynthetic approach to produce valuable and novel hybrid antibiotics with improved properties.

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1. Introduction

Nikkomycins and polyoxins are peptidyl nucleoside antibiotics (Bormann et al., 1985; Suzuki et al., 1965). A variety of nikkomycins have been isolated from the culture filtrates of *Streptomyces tendae* and *Streptomyces ansiochromogenes* (Bormann et al., 1989; Chen et al., 2000), and some components of polyoxins are produced by *Streptomyces cacaoi* (Isono et al., 1969). With structural similarity to UDP-*N*-acetylglucosamine, both nikkomycins and polyoxins act as competitive inhibitors of chitin synthases in pathogenic fungi and insects (Endo et al., 1970; Kim et al., 2002). Because of their non-toxic and easily degradable properties, nikkomycins and polyoxins are potent antifungal agents and insecticides in agriculture and human therapy (Becker et al., 1983; Brillinger, 1979).

Both nikkomycins and polyoxins consist of peptidyl and nucleoside moieties which are synthesized separately and then linked together by peptide bonds (Decker et al., 1991; Funayama and Isono, 1977). The nucleoside moieties of nikkomycin Z and X, two major components of nikkomycins in *S. ansiochromogenes* (Liao et al., 2009), are composed of 5-aminohexuronic acid *N*-glycosidically bound to uracil or 4-formyl-4-imidazoline-2-one, respectively (Chen et al., 2002), while the peptidyl moieties consist of hydroxyppyridylhomothreonine (HPHT) which is derived from *L*-lysine (Bruntnet et al., 1999; Niu et al., 2006). Like nikkomycins,

polyoxins contain 5-aminohexuronic acid *N*-glycosidically bound to uracil or its analogs as the nucleoside moieties (Isono and Suzuki, 1968), while polyoximic acid (POIA) and carbamoylpolyoxamic acid (CPOAA), which originate from *L*-isoleucine and *L*-glutamate, respectively, are used as the peptidyl moieties (Funayama and Isono, 1977). The biosynthetic gene cluster for nikkomycin contains one pathway-specific regulatory gene and 21 structural genes (Bormann et al., 1996; Liu et al., 2005), while the polyoxin biosynthetic gene cluster contains 20 genes, five of which (*polL-P*) are supposed to be involved in the biosynthesis of the peptidyl moiety of CPOAA (Chen et al., 2009).

In order to improve the production and widen the inhibitory spectra of nikkomycins and polyoxins, it is desirable to make new derivatives. In addition to isolating new components from natural resources and mutated strains (Delzer et al., 1984; Olano et al., 2008), novel analogs were also obtained by chemical synthesis (Plant et al., 2009). However, the production of new derivatives by combinatorial biosynthesis, to our knowledge, has not been reported so far.

Combinatorial biosynthesis has become an important approach to generate chemical diversity by genetic manipulation (Zhang and Tang, 2008). As a supplement to total synthesis and semi-synthesis, it has been applied widely to achieve new derivatives related to polyketides, peptides, coumarins, indolecarbazoles and other types of antibiotics (Baltz, 2006; Heide et al., 2008; Miao et al., 2006; Sanchez et al., 2009). Some of the derivatives gained improved pharmacological properties, and became promising agents for clinical therapy (Baig et al., 2008; Brautaset et al., 2008). However, most

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work focused on the production of modified antibiotics with alternative groups linked to the skeleton of parent molecules (Floss, 2006). Moreover, the production of hybrid antibiotics with changes on the backbone was limited in the polyketide and nonribosomal peptide antibiotics (Staunton and Wilkinson, 2001).

Base on the fact that nikkomycins and polyoxins are similar molecules assembled from nucleoside and peptidyl moieties (Chen et al., 2009; Lauer et al., 2001), it appeared feasible to generate hybrid antibiotics by combining the nucleoside and peptidyl moieties from these two antibiotics. We set out to assemble the nikkomycin X nucleoside (nikkomycin Cx, with 4-formyl-4-imidazole-2-one as the base) and the polyoxin dipeptidyl moiety (CPOAA) to result in desirable hybrid compound(s) with better bioactivity and stability from both parent antibiotics. The study was also expected to provide information about the enzyme(s) catalyzing the formation of the peptide bond between the nucleoside and peptidyl moieties.

We obtained two hybrid compounds, and they indeed have improved features. The results showed that the enzyme(s) responsible for condensing the nucleoside and peptidyl moieties in nikkomycin biosynthesis, even though not identified *in vitro*, can catalyze the condensation of the nucleoside moiety of nikkomycin X and the peptidyl moiety of polyoxin.

2. Materials and methods

2.1. Strains and growth conditions

S. ansochromogenes 7100 (wild type), *S. ansochromogenes sanN* disruption mutant and *S. cacaoi* were grown in liquid YEME for genomic DNA extraction. For spore formation, they were grown on minimal medium (MM, with mannitol as carbon source) or mannitol/soya medium (MS) at 28 °C (Kieser et al., 2000). For antibiotics production, spore suspensions were inoculated in liquid YEME and incubated for 36 h as seed culture, and then 1 ml seed culture was transferred to 100 ml fresh SP medium (Liu et al., 2005) and further incubated for an additional 5 days before the fermentation broth was collected. *E. coli* DH5 α was used as a host for propagating plasmids. *E. coli* BW25113 (pJ790) was used for construction of recombinant plasmids via λ -Red-mediated recombination technology (Gust et al., 2003). *E. coli* ET12567 (pUZ8002) was used as a host for transferring DNA from *E. coli* to *Streptomyces* by intergeneric conjugation (Kieser et al., 2000). Filamentous fungi including *Pellicularia sasakii*, *Alternaria kikuchiana*, *Rhizoctonia solani*, *Mucor hiemalis*, *Botrytis cinerea* and yeast including *Pichia farinose*, *Yarrowia lipolytica* and *Candida albicans* were used as the indicator strains for detecting bioactivity of the hybrid antibiotics. Except for *C. albicans*, which was preserved in our lab, all other strains were purchased from China General Microbiological Culture Collection Center (CGMCC).

2.2. Construction of the recombinant strain

For the biosynthesis of the polyoxin dipeptidyl moiety, pPOL2 was constructed by truncation of pPOL, a pSET152-derived recombinant plasmid containing the polyoxin biosynthetic gene cluster and 14 flanking genes (Tan et al., unpublished). Genes unrelated to the biosynthesis of the polyoxin dipeptidyl moiety were replaced by antibiotic resistance cassettes obtained by PCR amplification with primers listed in Table S1. The kanamycin resistance gene cassette (*kan^R*) was firstly amplified from pUC119::*kan^R* with the primers AF2 and AR2. Then the PCR amplification was performed for a second round with primers AF1 and AR1, which contain sequences overlapping with AF2 and AR2. The resulting *kan^R* cassette (C/K-Kan^R) contained 49-nt and 56-nt gene-specific sequences complementary to *polC* and *polK*, respectively. The same strategy was applied to replace a region from *polQ1* to *orf14* with a streptomycin/spectinomycin

resistance cassette (*str/spe^R*), which was amplified from pJ778 (Gust et al., 2003) with primer pairs BF2/BR2 and BF1/BR1. The resulting *str/spe^R* cassette (Q1/14-*str/spe^R*) contained 58-nt homologous sequences at both ends for targeted recombination. C/K-Kan^R and Q1/14-*str/spe^R* were sequentially used to replace regions from *polC* to *polK* and from *polQ1* to *orf14* by λ -Red-mediated recombination. They had the same orientation as the *polC* promoter. The resulting pPOL2 was introduced into *S. ansochromogenes sanN* disruption mutant by intergeneric conjugation, and then the recombinant strain, Δ *sanN*/pPOL2, was obtained.

2.3. Purification of antibiotics

The active compounds produced by the recombinant strains were purified from the culture broth. Growth inhibition of *C. albicans* was used to indicate the bioactivity of compounds. The protocol was summarized as follows: 5 l of fermentation broth were centrifuged, and the pH of the supernatant was adjusted to 4.5 with acetic acid. Then it was chromatographed on a macroporous absorption resin HP-20 column (Mitsubishi). Because no antifungal compounds in the broth bound to the resin, the flow-through was subjected to a Dowex[®] 50WX2 (H⁺, 100–200 mesh, Sigma) column, and the column was eluted with 0.4 N ammonia solution. The samples with antifungal activity were concentrated to a small volume *in vacuo* (below 40 °C) and immediately adjusted pH to 4.5 with acetic acid. Then, 6 volumes of ethanol were added. After leaving the mixture at 4 °C overnight, the precipitate was collected by centrifugation. The dried powder was subsequently dissolved in water and analyzed by HPLC. Separation of the active compounds was achieved with Agilent 1100 HPLC system and ZORBAX SB-Aq column (5 μ m, 4.6 \times 250 mm). HPLC conditions were as follows: 90% H₂O (0.1% TFA), 10% methanol, flow rate 0.3 ml/min, UV=260 and 290 nm. Two active fractions (compounds **1** and **2**) were separately collected and lyophilized for further analysis.

Nikkomycin X used as control was purified from *S. ansochromogenes* fermentation broth as described previously except for the HPLC conditions (Bormann et al., 1999). The separation of nikkomycins by HPLC was carried out via a semi-preparative column (ZORBAX SB-C18, 5 μ m, 9.4 \times 250 mm), using a mobile phase of 95% H₂O (0.1% TFA) and 5% methanol with a flow rate of 1 ml/min. Nikkomycin X was detected at 290 nm absorption. Polyoxin B as another control was also purified from the crude powder by HPLC using the same conditions as for nikkomycin X except that the detection wavelength was 260 nm.

2.4. Structural analysis of hybrid antibiotics

The UV spectra of compounds were recorded by a photodiode array detector during HPLC. MS analysis and tandem mass spectrometry analysis were carried out on LCQ Deca XP Plus (Thermo-Finnigan) with the electrospray ionization source in positive mode. Samples were infused directly into the source at 5 μ l/min, and the transfer capillary temperature and spray voltage were set at 275 °C and 5.5 kV, respectively. The sheath gas flow rate was set to 12 arbitrary units and the tube lens offset was set to 25 V. The high resolution MS analysis was performed on an accurate mass quadrupole time-of-flight (Q-TOF) LC/MS spectrometer (Agilent). NMR spectra were recorded on a Bruker spectrometer (AV600 MHz for compound **1** and AV400 MHz for compound **2**). Compounds, purified by HPLC, were dissolved in D₂O and analyzed at room temperature with the water peak suppressed. To deduce the chemical structure of the compounds, one dimensional ¹H and ¹³C spectra were recorded. Two

dimensional COSY and DEPT were also used for compound **1**, and COSY, HMBC and HMQC for compound **2**.

2.5. Determination of bioactivity and stability

Bioassay of fermentation broths against *A. kikuchiana* and *C. albicans* was performed by a disk diffusion method as described previously (Liu et al., 2005). Relative titer of hybrid antibiotics in fermentation broth was determined by bioassay method using *A. kikuchiana* as indicator strain.

The minimal inhibitory concentrations of nikkomycin X, polyoxin B, polyoxin N and polynik A were determined using solutions ranging from 0.1 to 100 µg/ml. 10 µl of each solution was added to 100 µl PDA agar in 96 well cell plates, then the medium was kept for 24 h at 4 °C to allow the even diffusion of antibiotics into the agar. The fungi were then incubated in overlaid agar medium at 26 °C for 48 h to observe growth inhibition.

To study their stability under various pH conditions, the above four antibiotics were dissolved in buffers with different pH values: 0.2 M CH₃COONa buffer, adjusted to pH 4.0 or 5.5 by acetic acid; 0.05 M KH₂PO₄ buffer, adjusted to pH 7.0 by NaOH; 0.05 M Tris buffer, adjusted to pH 8.5 by HCl; 0.025 M Na₂CO₃ buffer, adjusted to pH 10.0 by NaOH. All samples were incubated at 25 °C for up to 30 days. To study the effect of temperature on stability, antibiotics were dissolved in 0.05 M KH₂PO₄ buffer (pH 6.0) and incubated, respectively, at 10, 20, 30, 40 or 50 °C for up to 15 days. Residual antibiotics were quantified by the peak areas in Agilent 1100 HPLC system and ZORBAX SB-C18 column. The mobile phase was composed of 90% H₂O (0.1% TFA) and 10% methanol, the flow rate was 1 ml/min. Nikkomycin X, polyoxin N and polynik A were detected at 290 nm, while polyoxin B was detected at 260 nm.

3. Results

3.1. Construction of gene cluster for the biosynthesis of CPOAA

Nikkomycins and polyoxins have similar chemical structures (Fig. 1). To generate novel hybrid compounds by combining the nucleoside moiety from nikkomycin X and the dipeptidyl moiety from polyoxins, we first constructed plasmid pPOL2, which

harbors the genes required for the biosynthesis of the polyoxin dipeptidyl moiety CPOAA. Genes unrelated to the biosynthesis of CPOAA were deleted by replacing them with antibiotic resistance cassettes (Fig. 2). The genes deleted from the polyoxin biosynthetic gene cluster include *polA*, *B*, *D*, *H*, *I*, *J* and *K* involved in nucleoside skeleton biosynthesis and modification (Chen et al., 2009), *polG* encoding a putative amide synthetase that may be responsible for the assembly of the whole polyoxin structure, and *polQ1* and *Q2*, which have been proposed to function in the transport of polyoxins. Since tripeptidyl polyoxins are less effective and less stable than dipeptides (Krainer et al., 1991), genes (*polC*, *E* and *F*) likely responsible for the biosynthesis of tripeptidyl polyoxins were also deleted. The resulting plasmid pPOL2 contained the *polC* operon with five structural genes (*polL*, *M*, *N*, *O* and *P*) and two regulatory genes (*polY* and *polR*) required for activation of the *polC* operon (Li et al., 2009, 2010).

3.2. Generation of hybrid antibiotics from nikkomycin and polyoxin

Previous results showed that *sanN* was involved in the biosynthesis of the peptidyl moiety of nikkomycins in *S. ansochromogenes*, and the resulting *sanN* disruption mutant abolished production of nikkomycins and antifungal activity (Ling et al., 2008). However, the mutant was still expected to accumulate the nucleoside moiety. After the introduction of pPOL2 into a *sanN* disruption mutant of *S. ansochromogenes*, the resulting recombinant strains (Δ *sanN*/pPOL2) regained antifungal activity (Fig. 3). As pPOL2 did not contain a *sanN* homolog, it was reasonable to suppose that the compounds with antifungal activity were produced from the co-expression of genes related to the biosynthesis of the nikkomycin nucleoside and polyoxin peptidyl moieties.

In order to characterize the compounds responsible for the antifungal activity, we isolated and purified them from the fermentation broth of the recombinant strains. Two active compounds (compounds **1** and **2**) were obtained. They were white powders, soluble in water but insoluble in ethanol, ether, ethyl acetate and acetone. When the compounds were dissolved in 0.1% trifluoroacetic acid (TFA) solution, they both showed maximum ultraviolet absorption at 287 nm, like nikkomycin X (Fig. S1A), indicating that both compounds probably contain the nikkomycin X nucleoside moiety. When reacted with the aldehyde reagent barbituric acid, which can be used to distinguish the heterocyclic

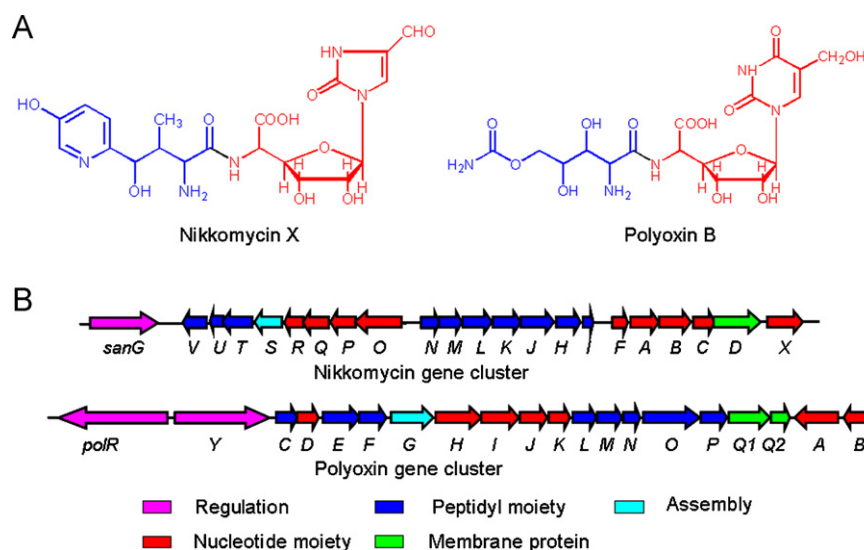


Fig. 1. Structures of nikkomycin X, polyoxin B and their biosynthetic gene clusters: (A) structures of nikkomycin X and polyoxin B and (B) organization of nikkomycin and polyoxin biosynthetic gene clusters.

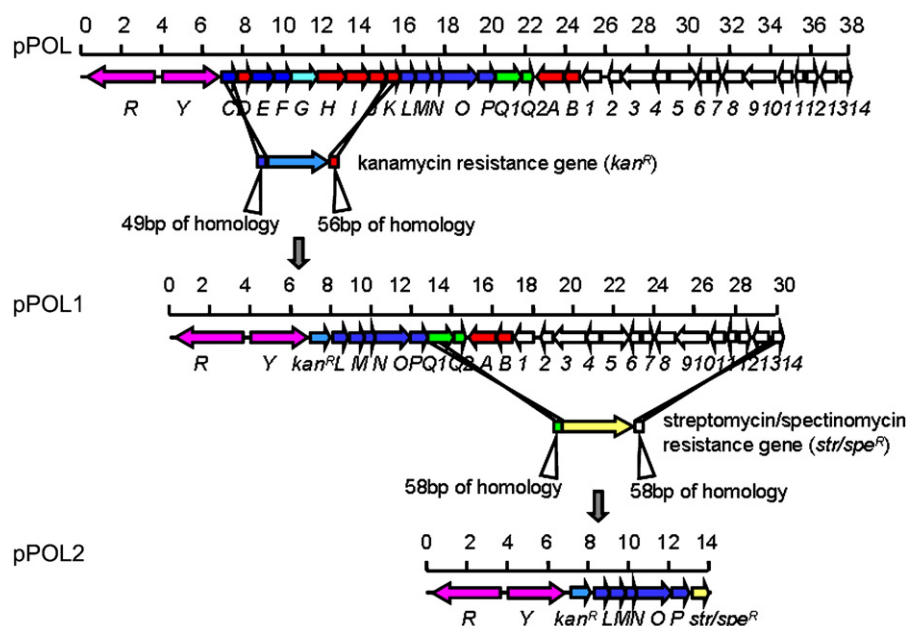


Fig. 2. The construction of pPOL2 by λ -Red-mediated recombination. The regions from *polC* to *polK* and from *polQ1* to *orf14* were replaced by a kanamycin resistance cassette and a spectinomycin/streptomycin resistance cassette, respectively. The resulting plasmid pPOL2 contains regulatory genes *polY* and *polR*, and structural genes responsible for the biosynthesis of the dipeptidyl moiety of polyoxin.

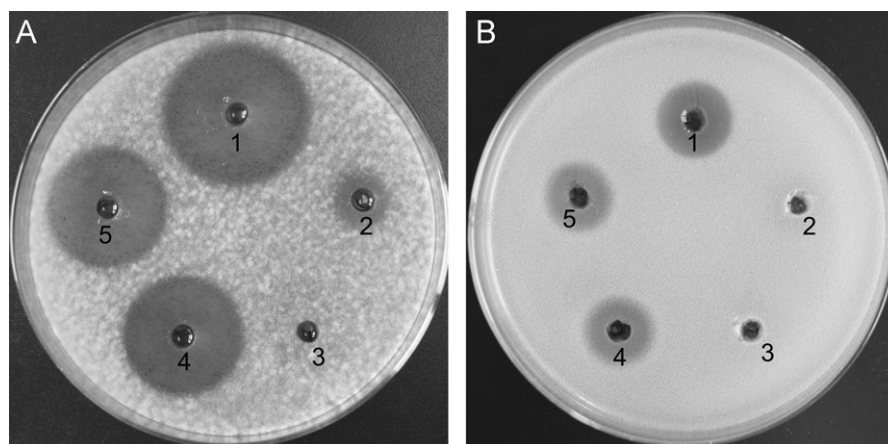


Fig. 3. Bioassay of the fermentation broth against *A. kikuchiana* and *C. albicans*: (A) *A. kikuchiana* was used as the indicator strain and (B) *C. albicans* was used as the indicator strain. Sources of broths were: (1) *S. ansiochromogenes* 7100; (2) *S. cacaioi*; (3) *S. ansiochromogenes sanN* disruption mutant; (4) and (5) recombinant strain Δ *sanN*/pPOL2.

bases of nikkomycins (Delzer et al., 1984), both compounds gave an orange color similar to nikkomycin X, while polyoxin B did not display the orange color (Fig. S1B). This result supported the hypothesis that the two compounds contained 4-formyl-4-imidazole-2-one as the base in the nucleoside moiety.

The compounds were further analyzed by electrospray ionization mass spectrometry (ESI-MS) and high resolution electrospray ionization mass spectrometry (HR-ESI-MS). HR-ESI-MS analysis revealed compound **1** with an $[M+H]^+$ ion at m/z 478.1383, consistent with the molecular formula $C_{16}H_{23}N_5O_{12}$ (478.1421 calculated) and compound **2** with an $[M+H]^+$ ion at m/z 462.1459, consistent with the molecular formula $C_{16}H_{23}N_5O_{11}$ (462.1472 calculated). Moreover, ESI-MS/MS indicated that they both contained fragments (m/z 288, 176) identical with the fragments of nucleoside residue from nikkomycin X. Compound **1** also had the same fragmentation pattern as the peptidyl residue of polyoxin B, suggesting that it contained CPOAA as the peptidyl moiety. Compound **2**, 16 amu less than Compound **1**, appeared to lack a hydroxyl group on the peptidyl residue (Fig. S1C).

Compounds **1** and **2** were both subjected to NMR analysis (Fig. S2 and S3 and Tables S2 and S3). The results confirmed that compound **1** contained CPOAA as the peptidyl moiety and 4-formyl-4-imidazole-2-one as the base of the nucleoside moiety. This structure was identical with that of polyoxin N, which was isolated and identified previously (Uramoto et al., 1981). For compound **2**, the chemical shifts of 1H and ^{13}C were similar to those of polyoxin N, except for the alteration at C-3'' position: from 69.6 to 34.0 ppm (^{13}C) and 4.06 to 1.6 ppm (1H), respectively. These data indicated that compound **2** shared a similar structure to polyoxin N, but lacked the hydroxyl group attached to the C-3'' position of the peptidyl moiety (Fig. 4). To our knowledge, this structure has not been reported before, and it was designated as polynik A. According to the proposed biosynthetic pathways of nikkomycins and polyoxins (Chen et al., 2009; Lauer et al., 2000), we speculate that the peptidyl moiety (CPOAA and its 3''-dehydroxyl analog) and nucleoside moiety (nikkomycin Cx) of the hybrid molecules were produced independently, then linked together by a peptide bond (Fig. 5). The generation of hybrid antibiotics suggested that pPOL2 carried all the

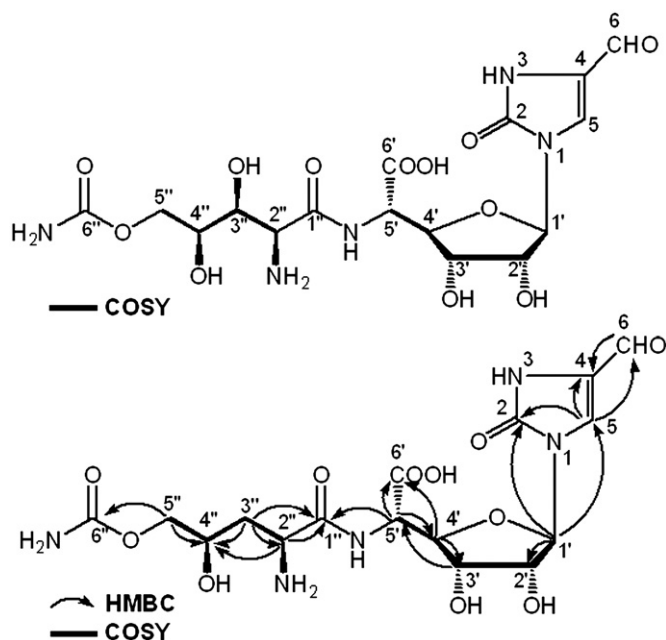


Fig. 4. Structures of polyoxin N and polynik A. COSY was used for polyoxin N; COSY and HMBC were used for polynik A.

genes required for the production of polyoxin peptidyl moiety, and that the putative synthetase responsible for the peptide bond formation between the peptidyl and nucleoside moieties has broad substrate tolerance. This is of great importance for generating hybrid peptidyl nucleoside antibiotics by combinatorial biosynthesis.

3.3. Bioactive detection of polyoxin N and polynik A

The antifungal activities of polyoxin N and polynik A were compared with those of their parent antibiotics nikkomycin X and polyoxin B. Both polyoxin N and polynik A exhibited inhibitory activity against most phytopathogenic fungi and human pathogens tested.

The titers of hybrid compounds were determined by using nikkomycin X as control. The inhibitory activity of 1 mg nikkomycin X against *A. kikuchiana* was defined as one unit (U). Results showed that the titers of hybrid antibiotics in crude samples were 69 U/L, while the titers of nikkomycins in the fermentation broth of *S. ansochromogenes* were 390 U/L, but the titers of polyoxins in the broth of *S. caocai* were 11 U/L.

The minimum inhibitory concentrations (MICs) against various organisms were generally lower than those of polyoxin B, but higher than those of nikkomycin X (Table 1). Compared to polyoxin B, which had weak inhibitory activity against the human pathogen *C. albicans*, both polyoxin N and polynik A showed strong inhibitory activity, similar to nikkomycin X. They also displayed the same inhibitory activity as nikkomycin X against *B. cinerea*, but different from that of polyoxin B. For most organisms tested, polyoxin N and polynik A exhibited the same inhibitory activity, the only exception being that polynik A was slightly less active than polyoxin N against *P. sasakii*.

3.4. pH and temperature stability of polyoxin N and polynik A

The pH and temperature stability of polyoxin N and polynik A was compared with those of polyoxin B and nikkomycin X. In general, they resembled polyoxin B, and were much more stable

than nikkomycin X in different pH buffers (Fig. 6A). All four antibiotics investigated were more stable under acid conditions than under neutral and alkaline conditions. At pH 4.0 and pH 5.5, after incubation for 30 days, polyoxin B, polyoxin N and polynik A remained more than 80% intact, while only about 50% of nikkomycin X remained intact at pH 4.0, and 20% at pH 5.5. At pH 7.0, like polyoxin B, both polyoxin N and polynik A remained about 70% intact after 15 days, whereas nikkomycin X was almost completely degraded. At pH 8.5, about 40–60% polyoxin N and polynik A remained intact after 10 days (pH 8.5), whereas nikkomycin X was almost completely hydrolyzed. At pH 10.0, all the antibiotics were quickly hydrolyzed except polynik A.

No obvious instability of the four compounds was observed at 10 °C, but all of them became more unstable with increasing temperature (Fig. 6B), nikkomycin X being markedly less temperature-stable than the other three. Thus, replacing the HPHT by COPAA gave rise to significantly higher stability under various pH and temperature conditions.

4. Discussion

In this paper, we describe the generation of two hybrid antibiotics by introducing genes required for CPOAA biosynthesis into a *sanN* disruption mutant of *S. ansochromogenes*. This suggests that pPOL2 contains all the genes required for the production of the dipeptidyl moiety of polyoxins. Previous studies showed that PolR and PolY are two transcriptional regulators required for polyoxin biosynthesis (Li et al., 2009, 2010). PolO has been proved to catalyze the conversion of α -amino- δ -hydroxyvaleric acid (AHV) to α -amino- δ -carbamoylhydroxyvaleric acid (ACV) in the biosynthetic pathway of the polyoxin peptide moiety (Chen et al., 2009). Our results support the assumption that PolL, PolM, PolN and PolP are involved in the biosynthesis of CPOAA. The inefficient conversion of ACV to CPOAA by PolL is likely to be the reason why the hybrid strain generates both polynik A, lacking a hydroxyl group at the peptidyl moiety, and polyoxin N.

Although it is known that the nucleoside and peptidyl moieties of nikkomycins and polyoxins are produced independently and then linked together via a peptide bond (Bruntnner et al., 1999; Funayama and Isono, 1977), the mechanism of the peptide bond formation remains unclear. It has been suggested that PolG in *S. cacaoi* might be responsible for the peptide bond formation in the biosynthesis of polyoxins (Chen et al., 2009), but *polG* was not included in pPOL2. The equivalent nikkomycin synthetase is therefore possibly responsible for forming the peptide bond between CPOAA and nikkomycin Cx. SanS, the homolog of PolG in *S. ansochromogenes*, is the obvious candidate. The ability of the putative synthetase to utilize a broad range of substrates opens up new insights for generating more hybrid antibiotics by exchanging the nucleoside or peptidyl moieties among nikkomycin and other nucleoside peptidyl antibiotics, such as blasticidin and mildiomycin (Cone et al., 2003; Li et al., 2008).

With the nucleoside moiety from nikkomycin X, the antifungal activity of polyoxin N and polynik A is much higher than polyoxin B; while, with the peptidyl moiety from polyoxins, polyoxin N and polynik A show much better stability than nikkomycin X. Thus, the hybrid antibiotics may have advantages that could be exploited therapeutically. We propose two possible reasons for the different antifungal activities and different stability between nikkomycins and the hybrid antibiotics: (1) the structure of CPOAA may have a lower affinity than HPHT for chitin synthase, but it has better stability; (2) the rate of transporting the antibiotics with CPOAA as the peptidyl moiety may be lower than that with HPHT. Polyoxin N and polynik A have similar

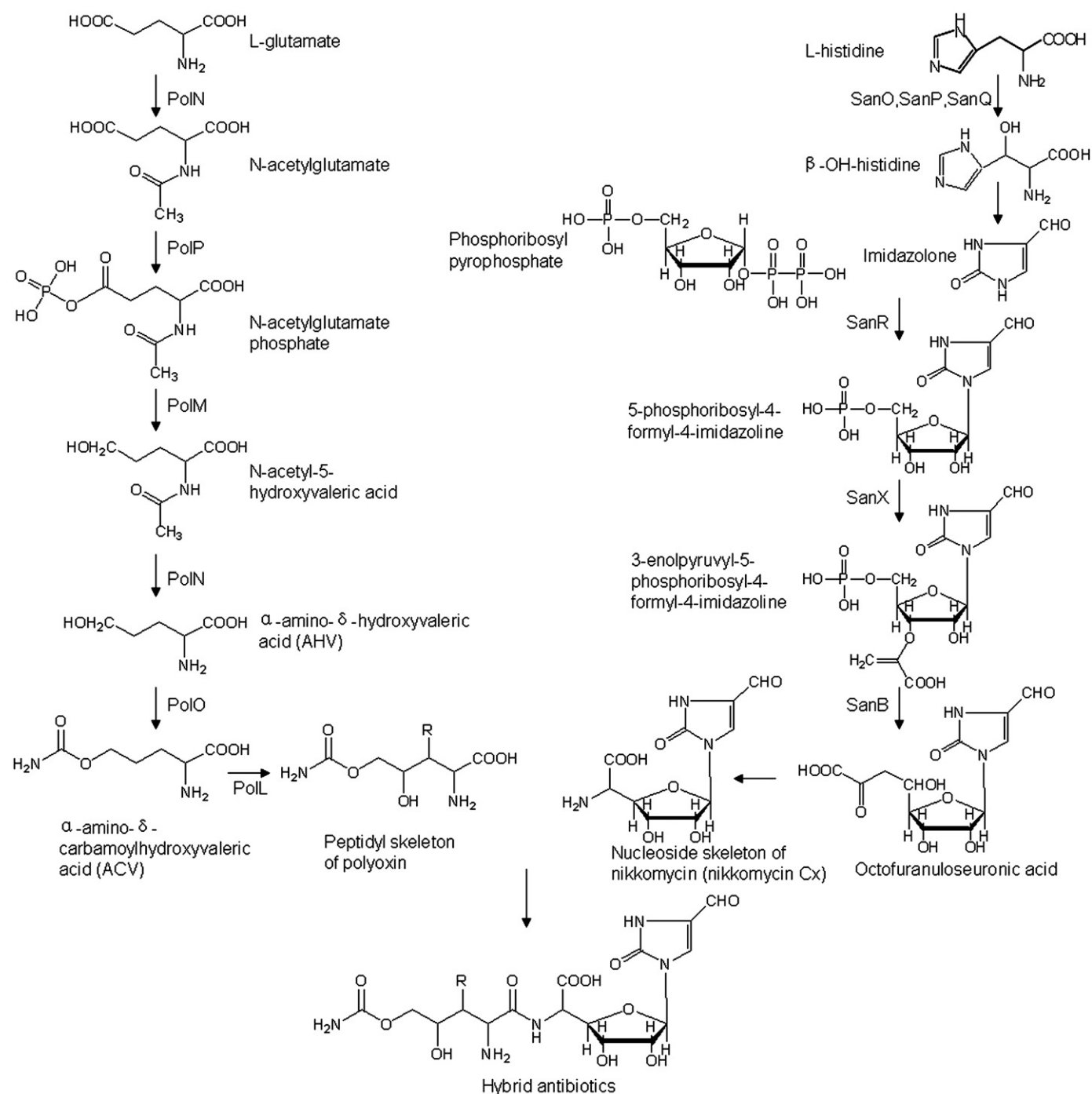


Fig. 5. Proposed biosynthetic pathway of polyoxin N and polynik A in the recombinant strain. The nucleoside moieties were proposed from the nikkomycin biosynthetic pathway, while the peptidyl moieties were deduced from the polyoxin biosynthetic pathway. R=OH in polyoxin N; R=H in polynik A.

Table 1
Minimal inhibitory concentrations (MICs) of antibiotics against fungi and yeast ($\mu\text{g/ml}$).

Test organisms	Nikkomycin X	Polyoxin N	Polynik A	Polyoxin B
<i>Pellicularia sasakii</i>	2	8	10	25
<i>Alternaria kikuchiana</i>	2	6	6	25
<i>Rhizoctonia solani</i>	> 100	> 100	> 100	> 100
<i>Mucor hiemalis</i>	0.6	2	2	10
<i>Botrytis cinerea</i>	10	10	10	25
<i>Candida albicans</i>	2	6	6	50
<i>Pichia farinosa</i>	> 100	> 100	> 100	> 100
<i>Yarrowia lipolytica</i>	0.5	4	4	10

structures except for a hydroxyl group present in polyoxin N but absent in polynik A. Since polynik A exhibited better stability at alkaline conditions than polyoxin N, and showed weak inhibitory activity against *P. sasakii*, it means that the hydroxyl group linked to the C-3' position of the peptidyl moiety could affect the bioactivity and the stability.

Polyoxin B has been used as an antifungal agent against phytopathogenic fungi for decades (Hori et al., 1974). Polyoxin N and polynik A, which have stability similar to polyoxin B, but have much higher titer than polyoxins, are promising new antifungal agents in agriculture. In addition, fungal infections can be fatal for patients with cancer or immune deficiency. The

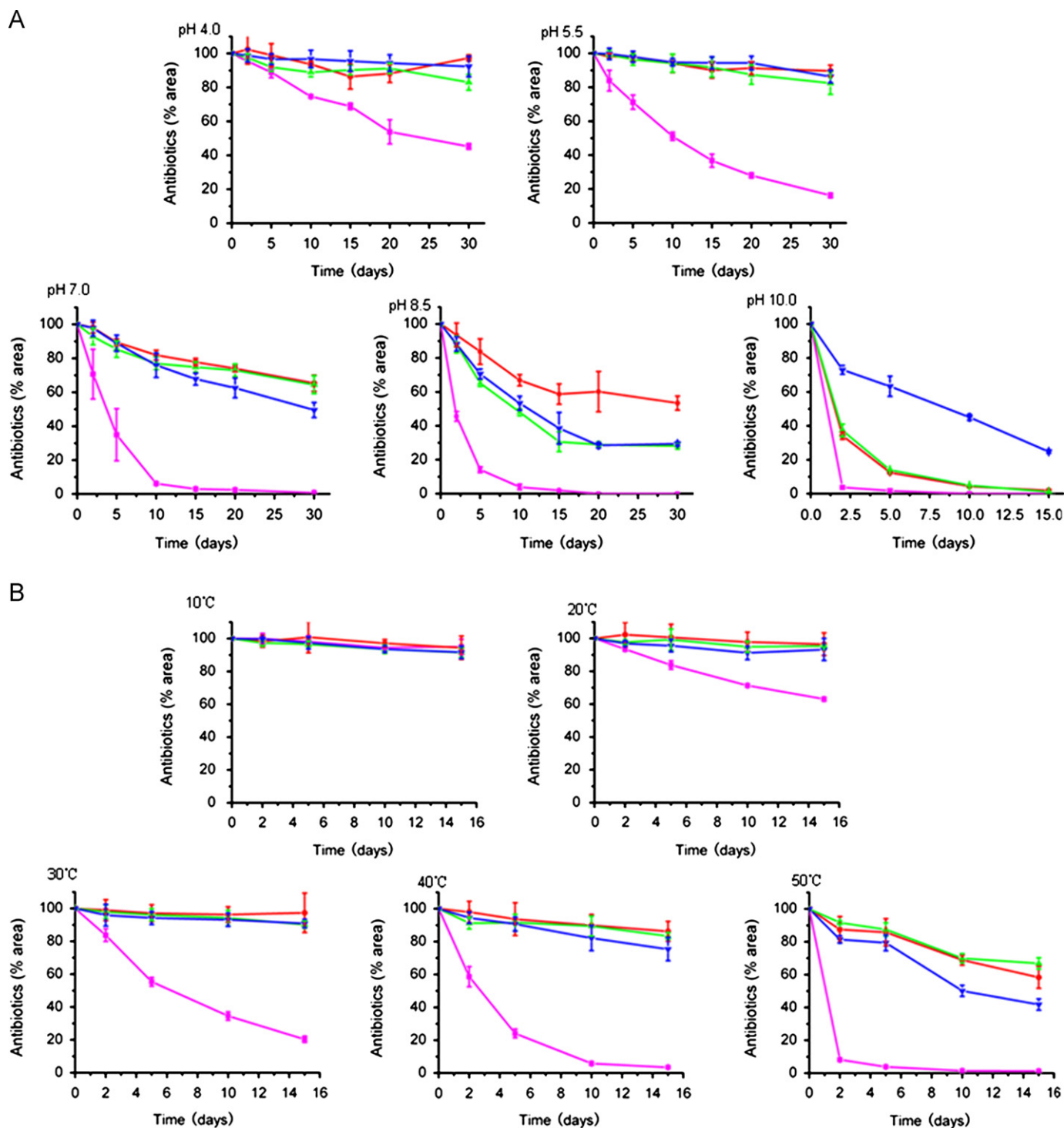


Fig. 6. Stability of nikkomycin X (■), polyoxin N (▲), polynik A (▼) and polyoxin B (●) under different pH and temperature conditions: (A) stability of purified four antibiotics under different pH conditions (in various sterilized buffers) at 25 °C and (B) stability of the four antibiotics at different temperatures. All samples were analyzed by HPLC and quantified according to the areas of peaks.

demand for new drugs is growing with the increase of antibiotic-resistant pathogens. Nucleoside peptidyl antibiotics represent a class of novel antifungal drugs different from the traditional antifungal agents like azoles (Kauffman and Carver, 1997), echinocandins (Zaas and Alexander, 2005) and polyene macrolides (Brautaset et al., 2008). A component of nikkomycins, nikkomycin Z, has been under development as an antifungal drug, used independently or in combination with other fungicidal drugs

(Brun et al., 2007; Ganesan et al., 2004; Nix et al., 2009). However, nikkomycin X or nikkomycin Z is unstable under neutral and alkaline conditions and easily loses its antifungal activity (Bormann et al., 1999). Even though the titers of polyoxin N and polynik A are much lower than nikkomycin X, they have much better tolerance for neutral and alkaline conditions. Therefore, polyoxin N and polynik A may be developed as antifungal agents for clinical therapy in future.

Combinatorial biosynthesis has been applied widely to increase the diversity of natural products, typically generating derivatives with skeletons entirely from one parent and one or more tailoring steps from the other. The approach used to generate hybrid skeletons in this study opens up insights for making new drug candidates.

5. Conclusions

Nikkomycins and polyoxins are important peptidyl nucleoside antibiotics used as antifungal agents and insecticides in agriculture or in human therapy. Here we demonstrate the generation of two hybrid antibiotics with improved properties by genetic manipulation of the nikkomycin and polyoxin biosynthetic gene clusters. One of them, a novel compound designed as polynik A, was identified as a potent antifungal agent. The other was a naturally existing compound, polyoxin N. Our finding implied that the synthetase in nikkomycin biosynthesis has flexible substrate tolerance. This is important for production of novel hybrid compounds involved in peptidyl nucleoside antibiotics by combinatorial biosynthesis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2011.01.002.

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