

Effect of Different Biosynthetic Precursors on the Production of Nargenicin A₁ from Metabolically Engineered *Nocardia* sp. CS682

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Nargenicin A₁ is a 28-membered polyketide macrolide, with antibacterial activity against methicillin-resistant *Staphylococcus aureus*, produced by *Nocardia* sp. CS682. In this study, the production of nargenicin A₁ was improved by enhancing the supply of different biosynthetic precursors. In *Nocardia* sp. CS682 (KCTC11297BP), this improvement was ~4.62-fold with the supplementation of 30 mM methyl oleate, 4.25-fold with supplementation of 15 mM sodium propionate, and 2.81-fold with supplementation of 15 mM sodium acetate. In *Nocardia* sp. metK18 and *Nocardia* sp. CS682 expressing *S*-adenosylmethionine synthetase (MetK), the production of nargenicin A₁ was improved by ~5.57-fold by supplementation with 30 mM methyl oleate, 5.01-fold by supplementation with 15 mM sodium propionate, and 3.64-fold by supplementation with 15 mM sodium acetate. Furthermore, supplementing the culture broth of *Nocardia* sp. ACC18 and *Nocardia* sp. CS682 expressing the acetyl-CoA carboxylase complex (AccA2 and AccBE) with 30 mM methyl oleate, 15 mM sodium propionate, or 15 mM sodium acetate resulted in ~6.99-, 6.46-, and 5.58-fold increases, respectively, in nargenicin A₁ production. Our overall results showed that among the supplements, methyl oleate was the most effective precursor supporting the highest titers of nargenicin A₁ in *Nocardia* sp. CS682, *Nocardia* sp. metK18, and *Nocardia* sp. ACC18.

Keywords: Methyl oleate, *Nocardia* sp. CS682, nargenicin A₁, polyketide, precursor

Nargenicin A₁ is a member of a group of naturally occurring reduced alicyclic polyketides containing a characteristic *cis*-fused octalin ring system. It was first isolated as antibioticum CP-47,444 from cultures of *Nocardia argentinensis*

in 1977 [2]. Recently, nargenicin A₁ was isolated and purified from *Nocardia* sp. CS682 (KCTC11297BP) from soil in Jeonnam, Korea, and its molecular structure was determined as depicted in Fig. 1. Nargenicin A₁ is effective against Gram-positive bacteria and shows strong antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA). It has stronger anti-MRSA activity than erythromycin, spiramycin, and vancomycin, and its cytotoxicity is remarkably lower than those of erythromycin and spiramycin. Nargenicin A₁ is also reported to have antioxidant activity [27]. It also inhibits cell proliferation and induces HL-60 cell differentiation when administered in combination with 1,25-dihydroxyvitamin D₃ or all-*trans* retinoic acid, and enhanced leukemia cell differentiation via PKCβ1/MAPK pathways [11]. Because of its wide range of pharmacologically important activities, immense interest is directed toward nargenicin A₁. However, the low level of nargenicin A₁ production from the wild-type strain indicates a need for strain improvement to increase the nargenicin A₁ titer.

The rising numbers of many multidrug-resistant Gram-positive bacterial pathogens are a major concern. To reduce the future clinical impact of resistant pathogens such as MRSA and vancomycin-resistant enterococci, the development of new antimicrobial agents or the improvement of existing drugs is crucial. However, discovery of novel antibiotics active against such drug-resistant Gram-positive pathogens is difficult. Thus, overproduction of existing drugs is a promising approach to fulfill the commercial requirement [22]. One of the methods to increase the productivity of microorganisms is metabolic engineering where primary metabolic fluxes are redirected by the introduction of genetic modifications through recombinant DNA technology, so as to increase production of secondary metabolites. The enhanced production of various secondary metabolites has been previously reported from different *Streptomyces* species by heterologous expression of the positive regulator *S*-adenosylmethionine synthetase (*metK1*)

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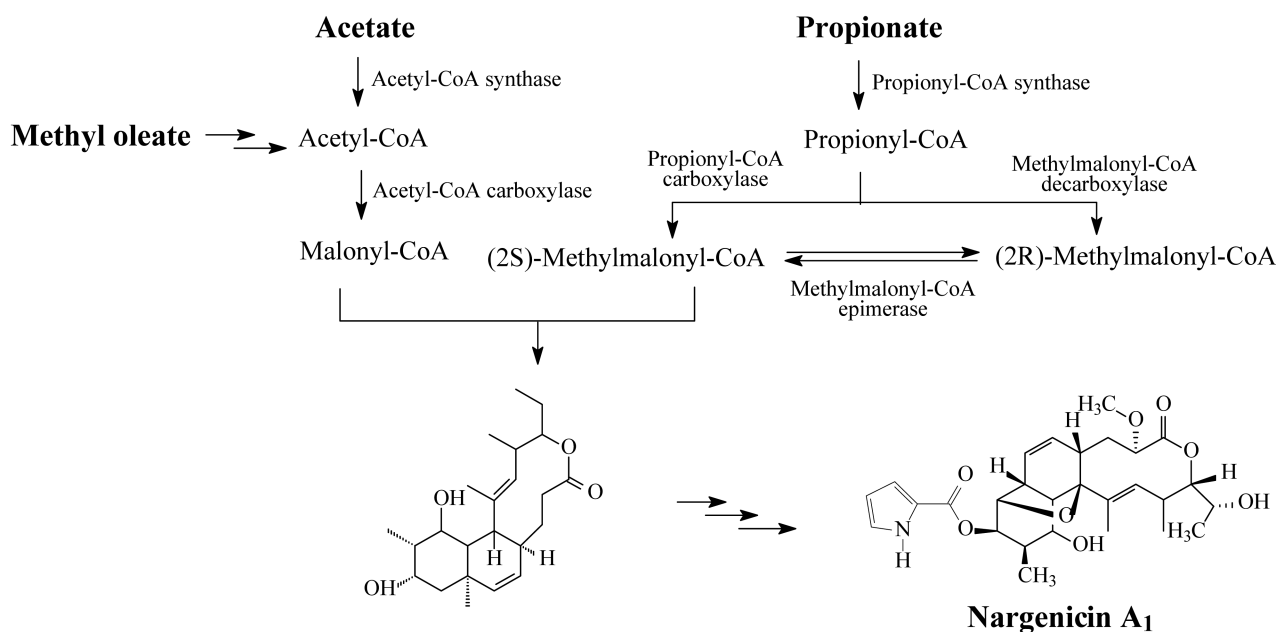


Fig. 1. Diagrammatic sketch showing various approaches for the enhancement of nargenicin A₁ production.

and a global regulatory gene, *afsR*, [10, 12, 16, 17, 21, 28, 29, 30].

In microorganisms, the polyketides are assembled by a series of decarboxylative condensations of simple carboxylic acid precursors catalyzed by polyketide synthase complexes, following a mechanism similar to that utilized in fatty acid synthesis [6]. As polyketides are assembled using several common biosynthetic precursors, which include malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA, regardless of their structural diversity [3, 6, 9] one of the strategies is to increase the respective precursor pool for enhancing the production of polyketides. There has been considerable interest in enhancing the yield of polyketides by increasing the supply of precursors, because intracellular availability of biosynthetic precursors is a key factor determining the productivity of secondary metabolites. [8, 13, 18–20, 24–26].

Nocardia species are partially acid-fast stainable and their complex cell walls cannot easily be disrupted by standard chaotropic solutions used for the rapid lysis of other bacteria [5]. Even though genetic manipulation in *Nocardia* species has been limited by available microbiological methods, in our previous study, we successfully carried out the metabolic engineering of *Nocardia* sp. CS682 and showed that transcriptional activator genes and precursor genes from *Streptomyces* strains could be expressed to enhance the production of antibiotics in *Nocardia* species. To enhance the production of nargenicin A₁, we separately overexpressed *S*-adenosylmethionine synthetase (MetK1-sp) from *S. peucetius* and the acetyl-CoA carboxylase complex (AccA2 and AccBE) from *S. coelicolor* A3 (2) in *Nocardia* sp. CS682 [15].

Nargenicin A₁ aglycon has been shown to be derived from common precursors (acetate and propionate) by a series of incorporation experiments employing [1-¹³C]-, [2-¹³C]-, and [1,2-¹³C₂]-acetate and [1-¹³C]- and [2-¹³C]-propionate [1]. In the present study, we investigated the influence of different biosynthetic precursors, including sodium acetate, sodium propionate, and methyl oleate on nargenicin A₁ biosynthesis by *Nocardia* sp. CS682 and its metabolically engineered strains. When the optimal concentrations of these precursors were determined, the effect of each precursor concentration was further observed. Our study showed that the production of nargenicin A₁ in *Nocardia* strains was enhanced to different levels by increasing the intracellular pools of different biosynthetic precursors. To our best knowledge, this is the first study to investigate the influence of precursors of short-chain fatty acids on the biosynthesis of nargenicin A₁ in *Nocardia* strains.

MATERIALS AND METHODS

Chemicals and Reagents

Methyl oleate (Acros Organics), sodium propionate (Sigma), and sodium acetate, anhydrous (Amresco) were used for feeding experiments. All the other chemicals and reagents used were of analytical grade.

Bacterial Strains and Growth Conditions

Nocardia sp. CS682 and its metabolically engineered strains, *Nocardia* sp. metK18 and *Nocardia* sp. ACC18, were employed to study the influence of different biosynthetic precursors on nargenicin A₁ biosynthesis by *Nocardia* strains. *Nocardia* sp. NV18 was used

as the control [15]. All strains were seeded in Brain Heart Infusion (BHI) broth at 37°C for 5 days. For production of nargenicin A₁, oatmeal medium containing 1% maltose, 0.3% oatmeal, 0.3% yeast extract, 0.3% soybean meal, and 0.1% CaCO₃ (pH 7) was used as the production medium. The production culture of each strain was incubated at 37°C for 9 days. The medium was supplemented with an appropriate amount of neomycin (500 µg/ml) when necessary.

Determination of Optimal Precursor Concentration

Nocardia sp. CS682 and its recombinant strains were seeded in 50 ml of BHI medium and incubated in a shaking incubator at 37°C for 5 days. A 500 µl aliquot of seed culture of each strain was inoculated into 80 ml of oatmeal medium and incubated under similar conditions. After 50 h of incubation, each culture broth was supplemented with varied concentrations of methyl oleate (10 to 40 mM), sodium propionate (5 to 20 mM), and sodium acetate (5 to 20 mM), and incubation was continued until 9 days.

Analysis of Nargenicin A₁ Production

For analysis of nargenicin A₁ production, the cell pellets were removed from each sample by centrifugation at 6,000 ×g, and the supernatant was extracted with a double volume of ethyl acetate. The ethyl acetate extract was evaporated and then concentrated with 1.0 ml of methanol. A 15 µl aliquot of the extract was analyzed by HPLC using a reverse-phase C-18 column (Mytil RP-18) with buffer A (0.05% TFA in water) and buffer B (100% acetonitrile) at a flow rate of 1.0 ml/min. Detection was carried out with a UV absorbance detector monitoring at 236 nm [15]. The major peaks corresponding to nargenicin A₁ were confirmed by liquid chromatography/mass spectrometry (LC/MS) analysis. The amount of nargenicin A₁ produced by each strain was quantified and compared. Nargenicin A₁ standard was used as a reference. A calibration curve for nargenicin was used to quantify nargenicin A₁ [4]. The experimental data were averaged from three different extractions.

RESULTS AND DISCUSSION

Nargenicin A₁ is a 28-membered macrolide antibiotic, with strong antibacterial activity against a methicillin-resistant *Staphylococcus aureus*, produced by *Nocardia* sp. CS682.

In 1984, David E. Cane and colleagues showed that nargenicin A₁ aglycon is derived from the common precursors acetate and propionate [1]. Moreover, methyl oleate, which is the probable source of acyl-CoA, has been used in fermentation broth to improve the production levels of different polyketides [8, 18, 19, 23]. The enhancing effect of exogenous feeding of methyl oleate has also been reported earlier, where a 20-fold increase in antibiotic production was observed [19]. Studies have reported that methyl oleate may protect the mycelium from toxic antibiotics that limit its growth [7]. Thus, in this study, we conducted the feeding of different biosynthetic precursors including sodium acetate, sodium propionate, and methyl oleate using *Nocardia* sp. CS682 and its metabolically engineered strains, *Nocardia* sp. metK18 and *Nocardia* sp. ACC18, with the aim of enhancing production of nargenicin A₁.

Optimization of Concentration of Different Biosynthetic Precursors for Feeding Experiments

The effects of short-chain fatty acids, including sodium propionate, sodium acetate, and methyl oleate, on nargenicin A₁ production were studied by separately adding each of these precursors to *Nocardia* sp. CS682 grown in the oatmeal medium. To determine the optimal concentrations of methyl oleate, sodium propionate, and sodium acetate for the production of nargenicin A₁, the concentrations of methyl oleate (10 to 40 mM), sodium propionate (5 to 20 mM), and sodium acetate (5 to 20 mM) added to the medium were varied. Extraction and analyses of nargenicin A₁ production by HPLC showed that the highest level of nargenicin A₁ production was obtained with an initial methyl oleate concentration of 30 mM; whereas 40 mM methyl oleate reduced the level of nargenicin A₁ production in *Nocardia* sp. CS682 (Fig. 3A).

Similarly, with an initial concentration of 15 mM sodium propionate (Fig. 4A) or 15 mM sodium acetate (Fig. 5A), the highest level of nargenicin A₁ production

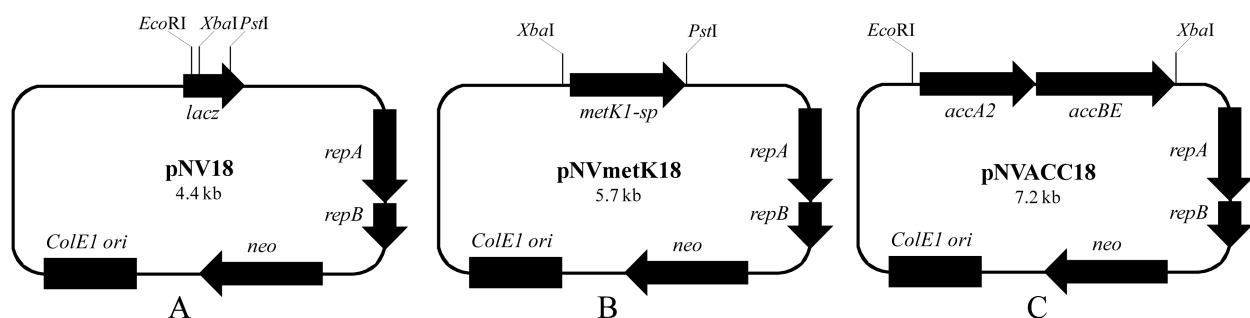


Fig. 2. Vector maps of pNV18 and recombinant plasmids.

(A) pNV18 as *Nocardia*-*E. coli* shuttle vector. (B) pNVmetK18 was generated to clone the *metK1-sp* gene from *S. peucetius* ATCC 27952 into *EcoRI*-*PstI* sites of pNV18. (C) The *accA2*, *accB*, and *accE* from *S. coelicolor* were excised from pACC152 and cloned into *XbaI*-*EcoRI* sites of pNV18 to generate pNVACC18.

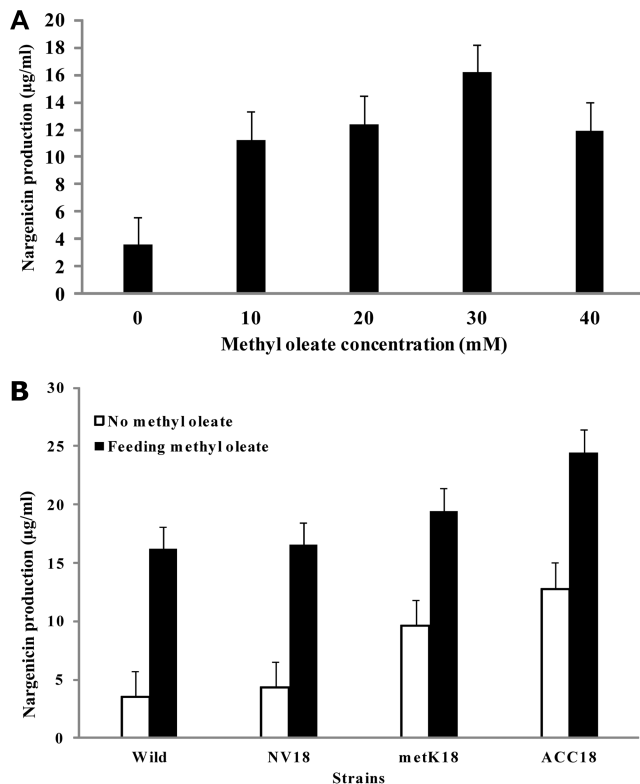


Fig. 3. (A) Nargenicin A_1 production in *Nocardia* sp. CS682 when feeding various concentrations of methyl oleate and (B) comparison of nargenicin A_1 production in *Nocardia* sp. CS682 and its recombinant strains: *Nocardia* sp. NV18, *Nocardia* sp. metK18, and *Nocardia* sp. ACC18 when feeding 30 mM methyl oleate.

was obtained in the *Nocardia* sp. CS682, whereas 20 mM sodium propionate or sodium acetate reduced the level of nargenicin A_1 production in all strains.

Effect of Methyl Oleate on Nargenicin A_1 Production

To study the effect of methyl oleate on nargenicin A_1 production in *Nocardia* sp. CS682 and its metabolically engineered strains *Nocardia* sp. metK18 and *Nocardia* sp. ACC18, after 50 h of incubation in the oatmeal medium, each culture broth was supplemented with 30 mM methyl oleate. The production of nargenicin A_1 in each strain was monitored after a 9 day incubation period with and without 30 mM methyl oleate supplementation. *Nocardia* sp. NV18 was used as the control.

When methyl oleate was added, the levels of nargenicin A_1 production were ~4.62-fold higher in *Nocardia* sp. CS682, 5.57-fold higher in *Nocardia* sp. metK18, and 6.99-fold higher in *Nocardia* sp. ACC18, than those of nonsupplemented fermentations (Fig. 3B). The results strongly suggest that methyl oleate promotes the production of nargenicin A_1 in *Nocardia* sp. CS682 and its metabolically

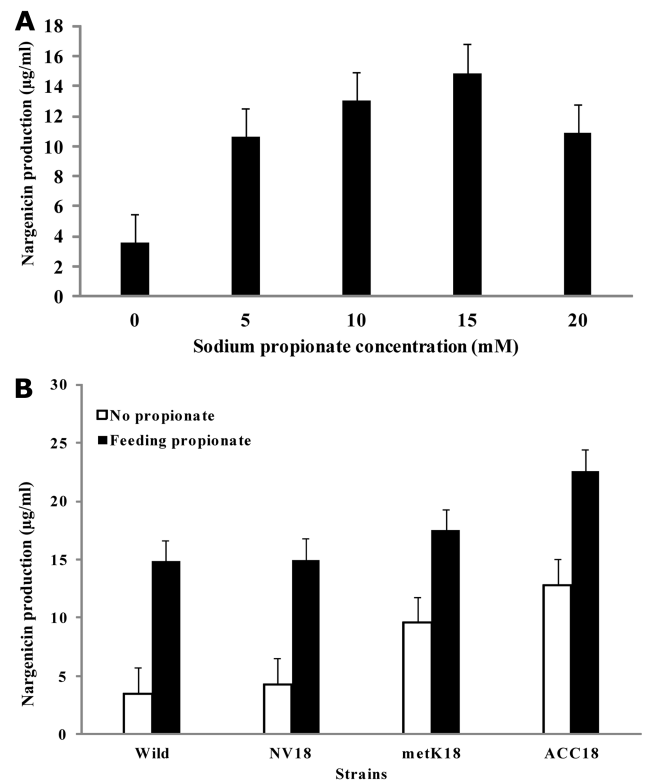


Fig. 4. (A) Nargenicin A_1 production in *Nocardia* sp. CS682 when feeding various concentrations of sodium propionate and (B) comparison of nargenicin A_1 production in *Nocardia* sp. CS682 and its recombinant strains: *Nocardia* sp. NV18, *Nocardia* sp. metK18, and *Nocardia* sp. ACC18 when feeding 15 mM propionate.

engineered strains, *Nocardia* sp. metK18 and *Nocardia* sp. ACC18.

Effect of Sodium Propionate on Nargenicin A_1 Production

To study the effect of sodium propionate on nargenicin A_1 production in *Nocardia* sp. CS682 and its metabolically engineered strains *Nocardia* sp. metK18 and *Nocardia* sp. ACC18, after 50 h of incubation in oatmeal medium, each culture broth was supplemented with 15 mM sodium propionate. The production of nargenicin A_1 in each strain was monitored after a 9 day incubation period with and without 15 mM sodium propionate supplementation. *Nocardia* sp. NV18 was used as the control.

When sodium propionate was added, the levels of nargenicin A_1 production were ~4.25-fold higher in *Nocardia* sp. CS682, 5.01-fold higher in *Nocardia* sp. metK18, and 6.46-fold higher in *Nocardia* sp. ACC18 than those of nonsupplemented fermentations (Fig. 4B). The results strongly suggest that sodium propionate promotes the production of nargenicin A_1 in *Nocardia* sp. CS682 and its metabolically engineered strains, *Nocardia* sp. metK18

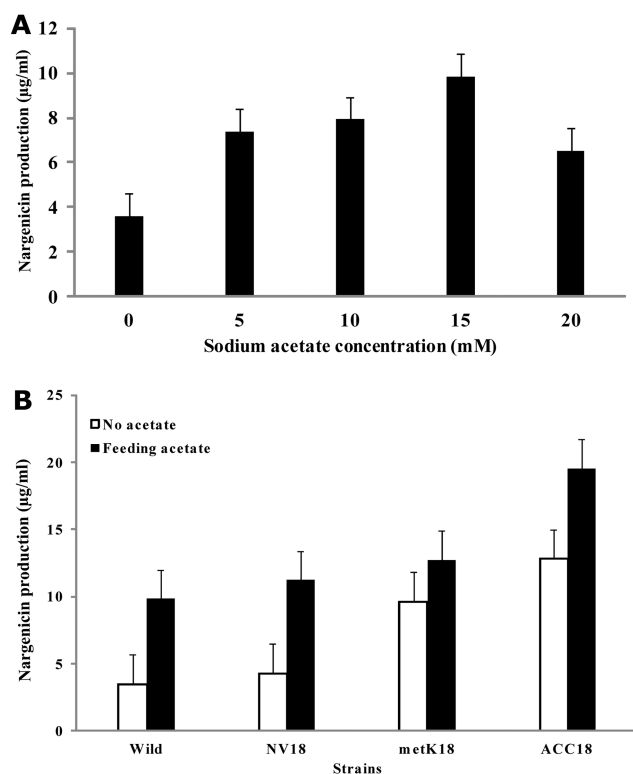


Fig. 5. (A) Nargenicin A₁ production in *Nocardia* sp. CS682 when feeding various concentrations of sodium acetate and (B) comparison of nargenicin A₁ production in *Nocardia* sp. CS682 and its recombinant strains: *Nocardia* sp. NV18, *Nocardia* sp. metK18, and *Nocardia* sp. ACC18 when feeding 15 mM acetate.

and *Nocardia* sp. ACC18. Similar results were observed in a study conducted to evaluate the effect of propionate on streptolydigin production by *Streptomyces lydicus* [14] and enhancement of pradimicin production in *Actinomadura hibisca* P157-2 [23].

Effect of Sodium Acetate on Nargenicin A₁ Production

Similarly, to study the effect of sodium acetate on nargenicin A₁ production in *Nocardia* sp. CS682 and its metabolically engineered strains, *Nocardia* sp. metK18 and *Nocardia* sp. ACC18, after 50 h of incubation in oatmeal medium, each culture broth was supplemented with 15 mM sodium acetate. The production of nargenicin A₁ in each strain was monitored after a 9 day incubation period with and without 15 mM sodium acetate supplementation. *Nocardia* sp. NV18 was used as the control.

When sodium acetate was added, the levels of nargenicin A₁ production were ~2.81-fold higher in *Nocardia* sp. CS682, 3.64-fold higher in *Nocardia* sp. metK18, and 5.58-fold higher in *Nocardia* sp. ACC18, than those of nonsupplemented fermentations (Fig. 5B). In this case also, the results strongly suggest that sodium acetate promotes the production of nargenicin A₁ in *Nocardia* sp.

CS682 and its metabolically engineered strains, *Nocardia* sp. metK18 and *Nocardia* sp. ACC18. A similar result was observed for the enhancement of pradimicin production in *Actinomadura hibisca* P157-2 [23].

In our previous study, we applied a metabolic engineering approach to boost the production of nargenicin A₁ from *Nocardia* sp. CS682. Heterologous expression of *S*-adenosylmethionine synthetase (MetK1-sp) from *S. peuceetius* and the acetyl-CoA carboxylase complex from *S. coelicolor* A3(2) in *Nocardia* sp. CS682 improved production of nargenicin A₁ by ~2.8-fold in *Nocardia* sp. metK18 and 3.8-fold in *Nocardia* sp. ACC18. In our present study, to enhance the production of nargenicin A₁ in *Nocardia* strains to the greatest extent, we supplemented organism cultures with methyl oleate, sodium propionate, or sodium acetate to promote the intracellular pools of biosynthetic precursors. Our results showed that supplementation with these biosynthetic precursors improved the production of nargenicin A₁ from wild-type *Nocardia* sp. CS682 and its metabolically engineered strains, *Nocardia* sp. metK18 and *Nocardia* sp. ACC18, to different extents. Our overall results showed that among the three biosynthetic precursors supplied, methyl oleate, which is the probable source of acyl-CoA, was the most effective precursor and supported the highest titers of nargenicin A₁ in *Nocardia* sp. CS682, *Nocardia* sp. metK18, and *Nocardia* sp. ACC18.

Thus, in this study, the production of nargenicin A₁ was improved in *Nocardia* sp. CS682, wild type, and its metabolically engineered strains, *Nocardia* sp. metK18 and *Nocardia* sp. ACC18, by promoting the intracellular pool of biosynthetic precursors *via* supplementing the culture broth of these strains with methyl oleate, sodium propionate, or sodium acetate.

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