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S-Adenosyl-L-Methionine Analogues to Enhance the Production of Actinorhodin

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Abstract It is known that overexpression of *S*-adenosyl-L-methionine (SAM) synthetase or exogenous addition of SAM enhances the production of actinorhodin, one of pigmented antibiotics found from *Streptomyces coelicolor*. In order to discover a novel compound as a signal molecule to produce actinorhodin instead of SAM, several compounds were synthesized based on the relationships between structures of the SAM analogues and their actinorhodin productivities. Of these, a few compounds showed better productivities of actinorhodin than SAM.

Key words: Actinorhodin, *Streptomyces coelicolor*, *S*-adenosyl-L-methionine, *S*-adenosyl-L-homocysteine

Polyketides are secondary metabolites biosynthesized from plants, animals, and microorganisms [1]. They have structural diversity as well as a broad range of biological activities such as antibiotics, antifungals, antiparasitics, insecticides, and so on. Actinorhodin belongs to the aromatic polyketides. Like lithmus, its color changes by pH; that is, blue in basic pH and red in acidic pH. When *Streptomyces lividans* and *S. coelicolor* grow in liquid culture, despite that many proteins participating in actinorhodin biosynthesis are known, they are not usually expressed. Overexpression of activator protein is required for actinorhodin production, which is ActII-ORF4. A limitation of nutrients can trigger secondary metabolism. It has been reported that *S*-adenosyl-L-methionine (SAM) synthesized by SAM synthetase is important in actinorhodin production, where SAM functions as a signal molecule. Overexpression of SAM synthetase or exogenous addition of SAM enhances actinorhodin production.

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The first protein influencing actinorhodin production is AfsK, which belongs to the serine/threonine kinases. It consists of 799 amino acids and has an ability of autophosphorylation on Ser and Thr, and phosphorylation of AfsR, which functions as one of the AfsK substrates. Disruption of AfsK inhibits actinorhodin production but does not affect morphogenesis. AfsR is consisted of 993 amino acids and its disruption causes decrement of actinorhodin production. Because AfsR is not related with ACTII-ORF directly, the existence of another gene was suspected, which was AfsS. Even though AfsR is silent, overexpression of AfsS enhances actinorhodin production. It stimulates transcription of ActII-ORF, which results in the production of actinorhodin. Therefore, actinorhodin production is regulated by the AfsK, AfsR, AfsS, and ACTII-ORF systems [7]. Here, as a signal molecule, SAM can trigger this system.

Because actinorhodin is useful as an antibiotic, its production is of interest. Actinorhodin production by a signal molecule was observed in *S. lividans* as well as *S. coelicolor* [3, 6]. Until now, the target protein that the signal molecule binds directly is not known, but the addition of the molecule results in the enhancement of actinorhodin production. Therefore, we can screen the signal molecules based on the phenomenon mentioned above. We tried to discover novel compounds to be used as a signal molecule instead of SAM.

Before the introduction of quantitative structure-activity relationships (QSAR), the discovery of a novel ligand for a target protein was dependent upon intuition. Nowadays, however, rational ligand design based on QSAR methods can help us find novel compounds. Even though the target protein is not known, the relationships between the structures of the ligands and their activities give information about the structural data of the candidate showing high activity. We tested the production of actinorhodin for 30 ligands including

S-adenosyl-L-homocysteine (SAH) on *S. coelicolor* M145 and, based on their structure-activity relationships, novel ligands were predicted. Because their theoretical actinorhodin production values were expected to be higher than those obtained from the known ligand compounds, several candidates were synthesized chemically.

Even though the compounds have an adenosine moiety, the compounds with more phosphates or sulfur at the 5'-position of the sugar moiety showed high production. In order to find the relationships between the structures of the compounds and their actinorhdin production, we have performed a three-dimensional QSAR study by Comparative Molecular Field Analysis (CoMFA) with a set of 30 commercially available adenosine analogues. The QSAR result indicated that SAM/SAH analogues with bulky substituents at the C6 position of the adenine ring would show good biological activity. Furthermore, according to the QSAR study, the end of the long 5'-alkyl chain of adenosine

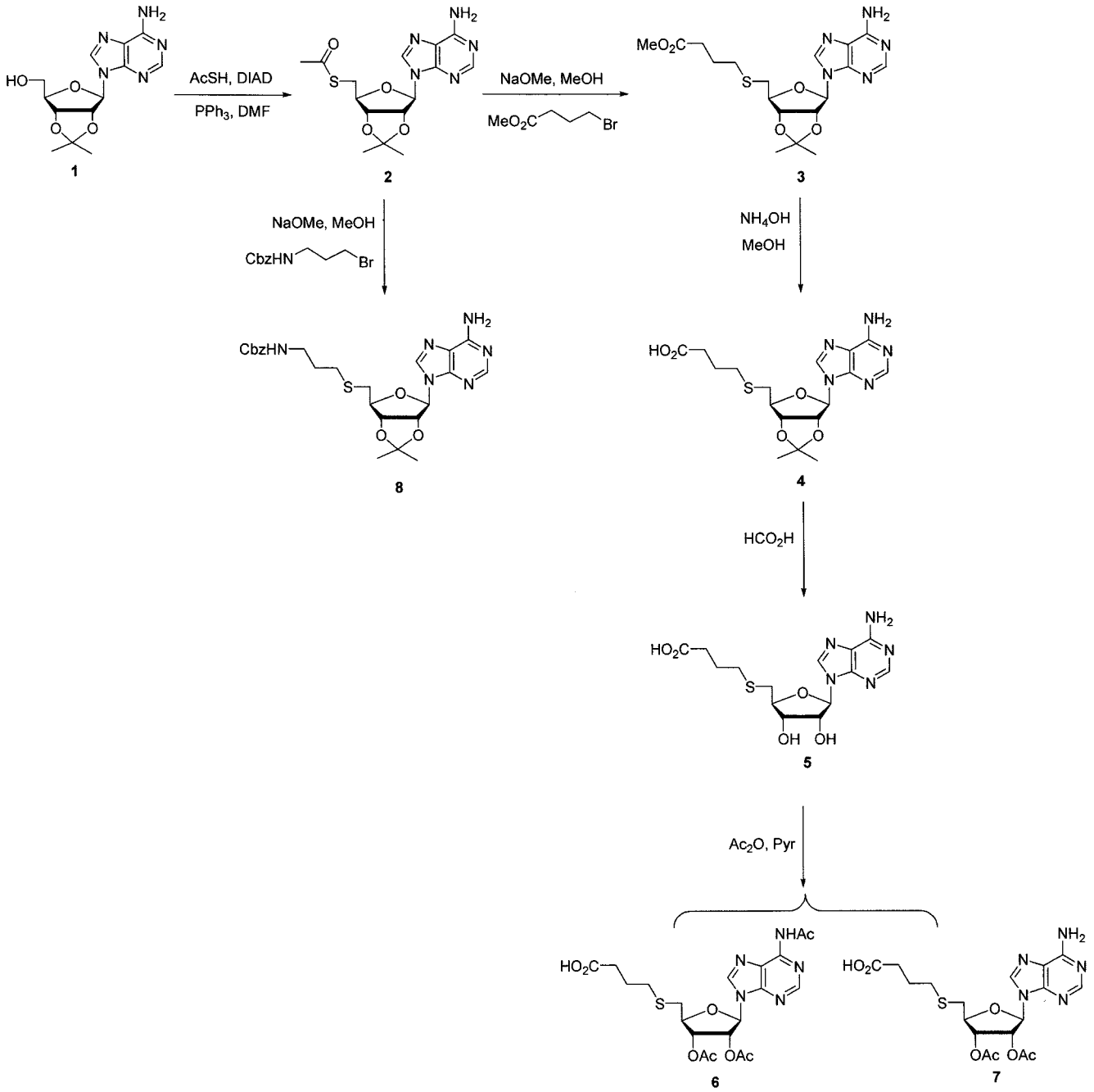

Fig. 1. Synthesis of SAM/SAH analogues with different side chains.

should be substituted with either electronegative or electropositive groups that are capable of electrostatic interactions with the biological target. Thus, for the sake of elucidating the structural determinant for SAM/SAH analogue binding of the biological target and easy preparation, we designed terminal deamino- as well as decarboxylated-SAM/SAH analogues.

On the other hand, we investigated the crystal structures of 120 SAM/SAH binding proteins available from the protein database in order to find the characteristic binding modes of SAM/SAH to the target enzyme. Even though there is no evidence that our biological target has a SAM/SAH binding pocket, we speculated that the biological activity of SAM comes from correct recognition of SAM by the target. Interestingly, a common structural feature among the 120 SAM/SAH binding proteins was found, in which a wide open space was observed right below the 2'- and 3'-hydroxyl groups of the ribose ring of SAM/SAH. The cavity appeared to be a water binding pocket, which might be bridging the 2',3'-hydroxyl groups of the SAM molecule with the enzyme residues by hydrogen bonding. Thus, even though it was not clearly indicated by our QSAR study, substitution at the 2'- and/or 3'- positions of SAM might be tolerated by many SAM/SAH binding proteins and presumably by our biological target. Taken together, we designed several SAM/SAH analogues carrying different deletions in the amino acid portion (deamino and decarboxylated) but with common substituents at N^6, *O*-2' or *O*-3' positions (Fig. 1).

Prediction of the activities of the candidate compounds based on our QSAR model is given in Table 1. Compared with SAM, the deaminated (**5**) SAM/SAH analogues were expected to have comparable biological activities. It is also worthwhile to note that *O*-2', *O*-3'-isopropylidene substitution (**3**, **4**, and **8**) appeared to have favorable influence on the activity of the compounds.

Owing to the biological importance and the variety of the *S*-adenosyl-L-methionine metabolism, a great number of synthetic strategies were developed. Among those, an efficient synthetic route leading to this class of compounds developed by Cohen *et al.* [2], was adopted in this study (Fig. 1). The key step of the synthesis is the direct formation of the thionucleoside **2** from commercially available, protected adenosine derivative **1** and thioacetic acid under standard Mitsunobu conditions, without previous protection of the amino group of the adenine ring and activation of the hydroxy group (Fig. 1). Coupling of the protected thionucleoside **2** with methyl 4-bromobutyrate was achieved after *in situ* cleavage of the acetylthio function with $NaOCH_3$ in MeOH (Fig. 1) to give the protected SAM/SAH analogue **3** in 65% yield. Hydrolysis of the methyl ester **3** was accomplished with a mixture of aqueous ammonia and methanol to yield **4**. Deaminated SAM (**5**) was then obtained quantitatively by hydrolysis of the isopropylidene acetal **4** with aqueous formic acid. We attempted to install acetyl groups at the various positions of **5** by treatment with acetic anhydride in pyridine. The reaction gave three isolable spots on TLC, but it was found that the acetyl group was both acid and base labile. Thus, the crude reaction mixture slowly decomposed upon purification by silica gel column chromatography to give a trace amount of the triacetate **6** and diacetate **7**, and the compound **7** was obtained as an inseparable 1:2 mixture with **5**. The versatility of the protected thionucleoside **2** as an intermediate in the synthesis of SAM/SAH analogues is readily demonstrated by the excellent coupling yields with 3-bromopropylcarbamic acid benzyl ester, which was prepared by protecting the 3-bromopropyl amine with benzyl chloroformate (CbzCl) (Fig. 1). The coupling product **8** was obtained in 95% yield. Purification and identification data of all synthesized compounds are not shown here but provided by supplementary materials.

S. coelicolor M145 was used for the screening. R2YE medium was used for actinorhodin production [4] and actinorhodin was determined photometrically based on the previous method published by Liao *et al.* [5]. Seven synthesized samples and SAM of 100 μM concentration were tested. The control without an addition of any signal molecules showed an absorbance of 0.041, and SAM was 0.089. The UV absorbance data of the samples except for derivative **6** were listed in Table 1, because the data of **6** were not obtained. All analogues except **2** were predicted to show good actinorhodin production. As expected, all analogues except for derivative **7** showed higher UV absorbance than that by SAM. Both the methylthiopropyl group substituted at the 5'-position of adenine and the hydrophobic groups at the 3',4'-position of adenine are important for high activity. Analogues **3**, **4**, and **8** fitted to the above conditions and their activities were 1.16, 1.29, 1.07, and 1.17 times the activity of SAM, respectively. Of these, the analogue **8** is a novel compound. As a result, the derivative **8**, benzyl 3-(((3aR,4S,6R,6aS)-6-(6-amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methylthio)

Table 1. Predicted values for UV absorbance by actinorhodin production of the candidate molecules and experimental values. The absorption of the control without the addition of any compounds is 0.041.

SAM/SAH analogues	Predicted values	Experimental values
2	0.065	0.059
3	0.103	0.103
4	0.097	0.115
5	0.099	0.095
6	0.098	ND*
7	0.096	0.075
8	0.092	0.104
SAM	0.100	0.089

*: No data observed.

propylcarbamate, is worthy of being a good signal molecule to trigger the biosynthetic pathway for actinorhodin production in *S. coelicolor*, instead of SAM.

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