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Absolute stereochemistry and solution conformation of promothiocins

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Abstract—Two members of thiopeptide class antibiotics, promothiocins A and B, were previously isolated from the mycelial extract of *Streptomyces* sp. SF2741 as *tipA* promoter inducing substances. Promothiocins are unique 26-membered thiopeptides composed of valine, 2-aminomethyl-5-methyloxazole-4-carboxylic acid, 2-(1-aminoethyl)thiazole-4-carboxylic acid, 2-(2-(1-aminoethyl)-5-methyloxazolyl)-3-(4-carboxythiazolyl)pyridine-6-carboxylic acid and dehydroalanine(s). The absolute stereochemistry and solution conformation of promothiocins have been investigated by a combination of the degradation works and molecular modeling experiments. These compounds contain characteristic dehydroalanine side chains in their structures that are closely related to promoter inducing activity, but are not essential. © 2001 Elsevier Science Ltd. All rights reserved.

1. Introduction

The thiopeptide class of antibiotics is distinguished by structural features containing thiazole(s) and/or oxazole(s) with highly modified amino acids in mono-, di- or tri-cyclic peptide ring(s). Several members of this class such as thiostrepton, nosiheptide, berninamycin, micrococcin, thiopeptin and sulfomycin induced the biosynthesis of many proteins of unknown function in *Streptomyces lividans*.[1] One of these proteins (*tipA*) was purified and its corresponding gene containing its promoter region (ptipA) was cloned and sequenced. *TipA* proved to be a regulatory protein which autonomously activates transcription of its own promoter after interacting with thiostrepton or other related thiopeptide antibiotics.[2]

This powerful inducible promoter (ptipA) has been incorporated into a series of vectors to allow regulated expression of genes in *Streptomyces*. In addition, ptipA has been employed in a very sensitive and specific microbiological disc assay to screen for compounds inducing its transcription.[3–5] During our screening program to find *tipA* promoter inducing compounds of microbial origin, unique 26-membered thiopeptides, promothiocins A (**1**) and B (**2**) (Fig. 1), were isolated from the mycelial cake of *Streptomyces* sp. SF2741, and their planar structures were assigned by the phase-sensitive ^{13}C-decoupled HMBC, a NMR spectroscopic technique capable of detecting four- and five-bonded $^{13}C-^{1}H$ long-range correlation.[5]

In spite of its simplest structure among *tipA* promoter inducers, **2** showed the most strong *tipA* promoter inducing activity as compared with other inducers. **1** and **2** also differed from other promoter inducers in having no dehydroalanine (Deala) residue in the macrocyclic core. In this paper, we report the absolute configuration and solution conformation of promothiocins, which have been established by the chiral HPLC analyses of an acid hydrolysate and a molecular modeling experiment using the DADAS90 program in combination with NMR constraints. In addition, the relationships of the Deala side chain and activity to induce *tipA* promoter are described.

2. Results and discussions

The producing-strain *Streptomyces* sp. SF2741 was cultured as outlined in Section 3. Using a bioassay-guided fractionation, promothiocins A and B were obtained from the fermentation broth. The acetone extract of the mycelial cake was partitioned between ethyl acetate and water, and the concentrate of EtOAc-soluble portion was fractionated by silica gel column chromatography. The biologically active fraction was further purified by Sephadex LH-20

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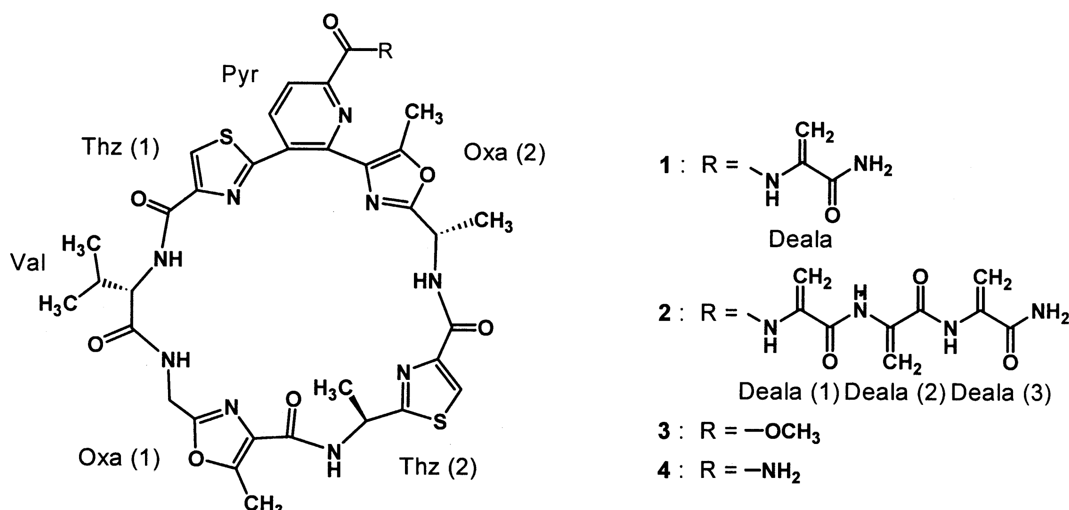
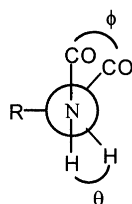


Figure 1. Structures of promothiocin A (**1**), B (**2**), and its methanolysis products **3** and **4**.

Table 1. Dihedral angles used for Monte Carlo conformational searches for promothiocin B



Position	$^3J_{HN-\alpha H}$ (Hz)	θ (°)	ϕ (°)
Val	9.0	0, 154	60, −146, −94
Oxa (1)	7.2	24, 143	84, 36, −157, −83
	4.2	46, 126	106, 14, −174, −66
Thz (2)	8.6	6, 151	66, 54, −149, −91
Oxa (2)	7.5	20, 145	80, 40, −155, −85

column chromatography, followed by preparative TLC to afford pure promothiocins A and B.

Promothiocins have three chiral centers in their structures. We first examined the degradation products of **1** and **2**. Acid hydrolyses of **1** and **2** gave a mixture of amino acids, the amino acid analyses of which revealed the presence of one mole each of Gly, Ala and Val. The chiral HPLC analysis established the configuration of Ala and Val to be L. In addition, the isolation of 2-(1-aminoethyl)thiazole-4-carboxylic acid (Thz (2)) from the hydrolysates implied that L-alanine originated from the masked alanine in 2-(1-aminoethyl)-5-methyloxazole. Therefore chiralities of Val and Oxa (2) were readily assigned as *S*. The stereochemistry Thz (2), however, remained unknown, since the ORD spectrum and chiral HPLC analysis revealed complete racemization of the isolated alaninylthiazole. Thiazole amino acids are known to racemize very easily during acid hydrolysis.[6] Thus the configuration of thiazole amino acids is generally determined by ozonolysis of the aromatic ring for preservation of the chiral center followed by acid hydrolysis.[7,8] Promothiocin was thus ozonized and hydrolyzed; an amino acid analysis of the acid hydrolysate provided a ratio of Gly/Ala/Val (1:2:2). This molar ratio was not consistent with the component amino acids of **1** and **2**. Consequently the ozonolysis–acid hydrolysis sequence turned out not to be a method of choice for stereostructure determination of alaninylthiazole in promothiocin.[9] Therefore, we next tried to determine the configuration of the alaninylthiazole by a molecular modeling experiment which was expected to provide conformational information of the 26-membered macrocyclic core as well.

Conformational study was carried out by the DADAS90 program on an SGI Indy computer. Stereochemistries of Val and Oxa (2) were fixed as *S* and Deala side chain was excluded from this experiment because of its flexibility. The

Table 2. Interproton distance constraints of promothiocin B for DADAS90 calculations

Atom pair		Upper distance constraint (Å)	Atom pair		Upper distance constraint (Å)
Atom 1	Atom 2		Atom 1	Atom 2	
Pyr H-4	Val NH	4.5	Val αCH	Oxa (1) NH	2.5
Thz (1) H-5	Val NH	3.5	Val αCH	Oxa (1) CH_2	5.0
Thz (1) H-5	Thz (2) αCH	6.0	Val γCH_3	Oxa (2) NH	5.0
Thz (1) H-5	Thz (2) βCH_3	7.0	Oxa (1) CH_2	Oxa (1) NH	3.5
Thz (1) H-5	Oxa (2) NH	4.0	Thz (2) NH	Thz (2) αCH	2.5
Val NH	Val γCH_3	3.5	Thz (2) NH	Thz (2) βCH_3	3.5
Val NH	Val αCH	2.5	Oxa (2) αCH	Oxa (2) NH	2.5
Val αCH	Val γCH_3	3.5			

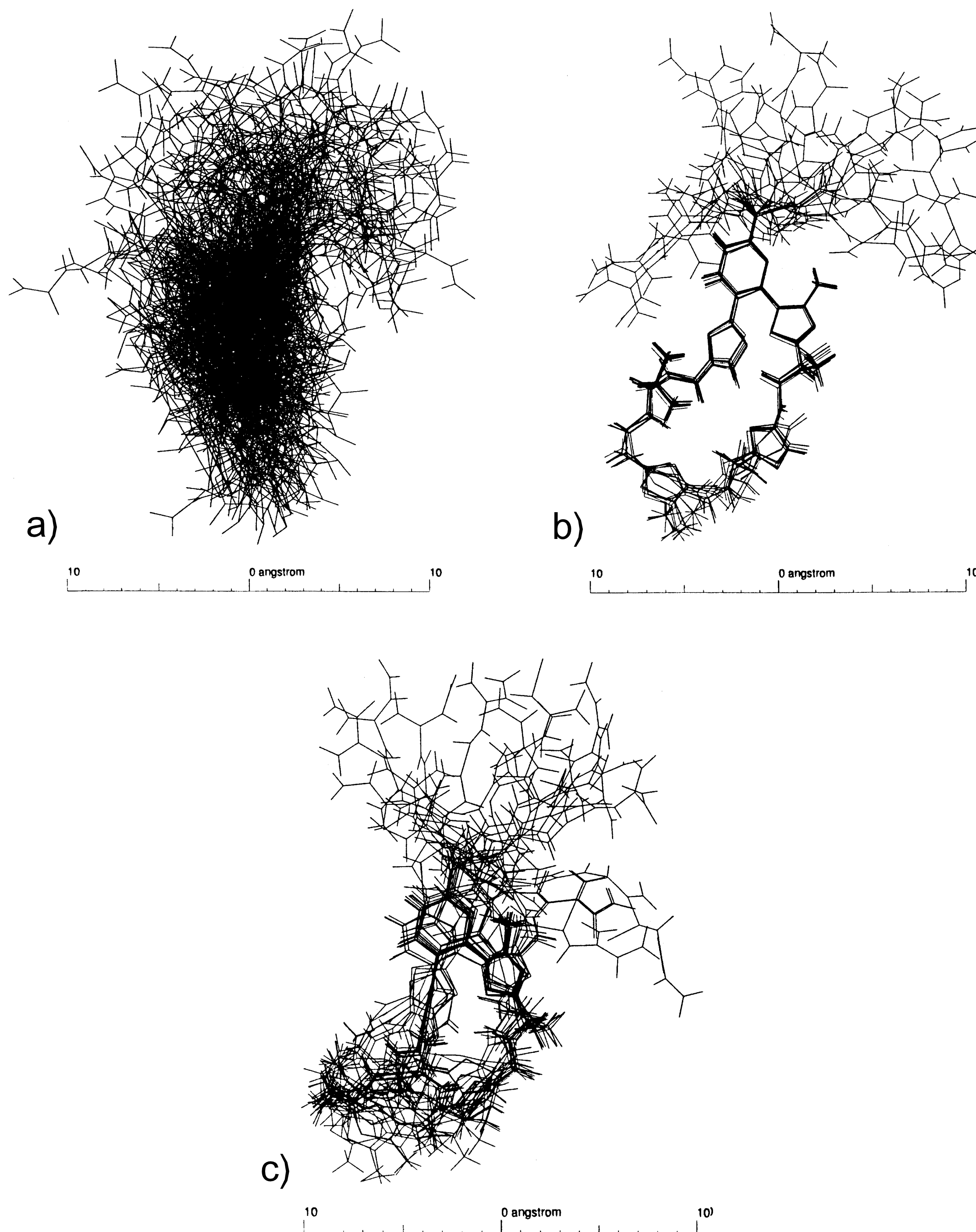


Figure 2. Superposition of 20 final structures of promothiocin A in DMSO-d_6 as determined by NMR restraints in conjunction with the Monte Carlo conformational search method. (a) Randomly generated 100 initial structures. (b) Superposition of 20 best fit structures for *S*-alaninylthiazole. (c) Superposition of 20 best fit structures for *R*-alaninylthiazole.

NMR spectral data of **1** and **2** were extremely similar, suggesting that both compounds had the same configuration and conformation, and thus major component **2** was selected for conformational analysis. We used dihedral angle information obtained by ^{1}H NMR coupling constants, amide proton temperature coefficients and detailed NOEs for distance between protons as the restrictions. Vicinal proton coupling constants show a Karplus-type dihedral angular dependence and thereby provide conformational information. The dihedral angles between α-methine protons and amide protons were calculated by ^{1}H NMR coupling constants ($^3J_{NH-CH^{\alpha}}$) in DMSO-d_6 using the modified Karplus equation,[10,11] as shown in Table 1.

The temperature dependence of the amide proton chemical shifts gives information about their chemical environment.

Generally temperature coefficient exceeding 4 ppb/°C are considered to be an evidence for the exposure of the NH group, while values smaller than 2 ppb/°C are indicative of intramolecular hydrogen bonding.[12] The temperature coefficients of amide protons of **2** are −4.6 ppb/°C for Oxa (1) NH, −5.7 ppb/°C for Oxa (2) NH, −5.6 ppb/°C for Thz (2) NH and −2.6 ppb/°C for Val NH. The amide protons except Val showed significant upfield shifts over the range 25–60°C dependent on increasing temperature, suggesting the external NH orientation. Also the temperature coefficients exhibited no intermolecular hydrogen-bond in **2**. In order to determine interproton distances, NOESY data were collected with a mixing time of 300 ms at 500 MHz. Important NOESY correlations[13] observed are as follows: Thz (1) 5-CH↔Thz (2) αCH; Thz (1) 5-CH↔Oxa (2) NH; Val NH↔Oxa (1) NH; Val γCH_3↔Thz (2) NH, Oxa (2) NH. In order to obtain convergent conformations, we needed to use the interproton distance constraints in the calculations. The interproton distance constraints are listed in Table 2.

Starting with 100 randomly generated initial structures for the *R*- and *S*-alaninylthiazole, respectively, each 20 final structures with an allowable error in the target function values and pseudo-energy values which are regarded as variations of the conformational-energy function values were obtained as a result of a series of optimization of the function.[14] The 20 structures for the *R* and *S* configurations, respectively, were superimposed for the best fit of backbone atoms of the macrocycle of the molecule, as shown in Fig. 2.

A survey of these structures revealed that the *S*-alaninylthiazole structures showing small rmsd values are in more good fit than *R* structures, suggesting the *S* configuration for Thz (2). Recently, promothiocin A has been synthesized by assuming *S* configurations for three chiral centers and the synthetic material was proposed to take the same stereostructure with the natural product on the basis of the comparison of their specific rotation values and NMR spectra.[15] Results of our chemical degradation and the molecular modeling experiments are well compatible with the chemical synthetic data. Therefore the stereochemistry of Thz (2) is determined to be *S*.

Thiopeptides with *tipA* promoter inducing activity have a characteristic side chain composed of Deala residue(s) but the non-inducible thiopeptides such as cyclothiazomycin[16] and GE2270[17] lack this functional group. For the relationship of promoter inducing activity and the Deala side chain, **3** and **4** lacking the Deala side chain were prepared by methanolysis of **2**. Bioassay revealed that minimum concentrations of **1**, **2**, **3** and **4** for the *tipA* promoter induction were 19.5, 0.6, 2,500 and 2,500 ng/mL, respectively. Although **1** and **2** have the identical core structure, **2** with two additional Deala units in the side chain exhibited about 20 times higher activity than **1**, and **3** and **4** showed about 4200 times lower activity than their parent compound **2**. These results imply that a longer Deala side chain is necessary for higher activity, and the moderate activity of **3** and **4** indicates that macrocyclic peptide core is essential for the *tipA* promoter induction.

3. Experimental

3.1. General experimental procedures

^{1}H NMR spectra were obtained on a JEOL JNM-A500 spectrometer at 500 MHz. Chemical shifts are given in ppm using TMS as internal standard. All NMR experiments were performed on 30 and 45 mg of promothiocins A and B, respectively, dissolved in 0.8 mL of DMSO-d_6. TLC was carried out on precoated Merck plates of silica gel with a florescent indicator (F_{254}). Amino acid analysis were done on a Beckman 120C amino acid analyzer.

3.2. Fermentation, extraction and isolation procedures

An inoculum was prepared by cultivating the producing organism for 48 h at 28°C in a 30 L jar fermentor containing 20 L of the medium consisting of starch 2.0%, glucose 1.0%, polypeptone 0.5%, oatmeal 0.6%, yeast extract 0.3%, soybean meal 0.2% and $CaCO_3$ 0.2%. The fermentation was carried out in a 2000-L jar fermentor for 96 h at 28°C containing 1000 L of the medium consisting of starch 1.0%, glucose 2.0%, soybean meal 1.5%, oatmeal 0.8%, polypeptone 0.1%, NaCl 0.1%, $ZnSO_4$ 0.001% and $CaCO_3$ 0.2%. The pH of the medium was adjusted to 7.0 before sterilization.

The mycelial cake filtered with a filter press from the harvested culture was extracted with acetone. After concentration in vacuo, the resulting aqueous solution was adjusted to pH 4.0 and extracted with ethylacetate. The solvent layer was dried over anhydrous Na_2SO_4, concentrated in vacuo, and the residue was subjected to silica gel column chromatography. After washing with $CHCl_3$–MeOH (50:1), active substances were eluted with $CHCl_3$–MeOH (20:1). The active eluate concentrated in vacuo was applied to a Sephadex LH-20 column developed with $CHCl_3$–MeOH (1:2). After concentration in vacuo, the active fraction was purified by preparative TLC using $CHCl_3$–Me_2CO (1:1) and two zones of R_f 0.28 and 0.22 were extracted from the absorbent with $CHCl_3$–MeOH (10:1) to give promothiocins A and B, respectively, as pure crystalline solids (30 and 225 mg, respectively).

3.3. Acid hydrolysis of promothiocins

A solution of **2** (10 mg) in 6N HCl (5 mL) was refluxed for 20 h under an inert atmosphere. After removal of traces of HCl by repeated evaporation in vacuo, the hydrolysate was suspended in 1 mL H_2O. A small volume (100 μL) was used for amino acid analysis and the remainder was purified by preparative TLC with BuOH–MeOH–H_20 (2:1:1) for amino acid configuration. Three zones with R_f 0.48, 0.36 and 0.10 for valine, alanine and 2-(1-aminoethyl)thiazole-4-carboxylic acid (**5**), respectively, giving positive responses to ninhydrin were resolved and scraped off TLC plates for chiral-HPLC analyses. Compound **5** was further purified by reversed-phase HPLC (Capcell-pak, C_{18}) eluted with H_2O.

3.3.1. 2-(1-Aminoethyl)thiazole-4-carboxylic acid (5). White crystalline; HRFAB-MS: MH^+, *m/z* 173.0425. $C_6H_9N_2O_2S$ required 173.0385; ninhydrin reagent: yellow; ^{1}H NMR(D_2O) 7.87 (1H, s), 4.65 (overlapped with solvent

peak), 1.54 (3H, d, J=7.0 Hz); ^{13}C NMR(D_2O) 169.0, 168.2, 152.7, 126.0, 48.4, 19.8.

3.4. Ozonolysis and continued hydrolysis

A stream of O_3 was bubbled into a 10 mL CH_2Cl_2 solution of **2** (3 mg) in a threaded bomb at room temperature for 10 min. After removal of the O_3 under a stream of N_2, CH_2Cl_2 was evaporated in vacuo and the residue was subjected to hydrolysis in 3 mL of 6N HCl under a nitrogen atmosphere in a sealed bomb at 110°C for 24 h. After removal of HCl by repeated evaporation in vacuo, the hydrolysate was analyzed with chiral-GC.

3.5. Methanolysis of promothiocin B under mild conditions

To a solution of promothiocin B (70 mg) in 3 mL of methanol, dried Amberlyst 15 (1 g) was added and refluxed for 24 h under N_2. The reaction mixture was taken to dryness under reduced pressure. The residue dissolved in 2 mL of $CHCl_3$–MeOH (5:1) was applied on preparative-TLC plates developed with $CHCl_3$–MeOH (15:1). The two zones with R_f 0.59 and 0.51 were scrapped off the plates, and each fraction was further purified by preparative-HPLC using a YMC packed C_{18} column (20×250 mm^2) eluted with a mixture of CH_3CN–H_2O (38:62) and a mixture of CH_3CN–H_2O (33:67), respectively, at a flow rate of 18 mL/min to give methanolysis products **3** and **4**.

3.5.1. Methanolysis product 3. White crystalline; HRFAB-MS: MH^+, m/z 762.2175. $C_{34}H_{36}N_9O_8S_2$ required 762.2128; ^1H NMR ($CDCl_3$–CD_3OD (9:1)) 8.55 (1H, d, J=7.9 Hz), 8.20 (1H, d, J=8.2 Hz), 8.07 (1H, s), 8.01 (1H, s), 5.38 (1H, q, J=7.0 Hz), 5.30 (1H, q, J=7.0 Hz), 4.70 (1H, d, J=16.2 Hz), 4.35 (1H, d, J=6.4 Hz), 4.12 (1H, d, J=16.2 Hz), 4.00 (3H, s), 2.53 (3H, s), 2.51 (3H, s), 2.22 (1H, m), 1.62 (3H, d, J=7.0 Hz), 1.54 (3H, d, J=7.0 Hz), 1.01 (6H, d, J=6.7 Hz).

3.5.2. Methanolysis products 4. White crystalline; HRFAB-MS: MH^+, m/z 747.2086. $C_{33}H_{35}N_{10}O_7S_2$ required 747.2132; ^1H NMR ($CDCl_3$–CD_3OD (9:1)) 8.44 (1H, d, J=8.2 Hz), 8.21 (1H, d, J=8.2 Hz), 8.07 (1H, s), 8.03 (1H, s), 5.39 (1H, q, J=7.0 Hz), 5.34 (1H, q, J=7.0 Hz), 4.65 (1H, d, J=16.2 Hz), 4.37 (1H, d, J=6.1 Hz), 4.15 (1H, d, J=16.2 Hz), 2.52 (3H, s), 2.39 (3H, s), 2.24 (1H, m), 1.62 (3H, d, J=6.7 Hz), 1.60 (3H, d, J=7.0 Hz), 1.01 (3H, d, J=6.7 Hz), 1.00 (3H, d, J=7.0 Hz).

3.6. Conformational analysis

Conformational search was executed by minimizing the target function values in DADAS90 calculation. The target function was defined as the following pseudo-energy (V_O). $V_O = W_D V_{NOE} + W_V V_{VDW} + W_J V_{ANG} V_{NOE}$: a sum of squares of differences between a distance constraint and a distance between the same pair of atoms in a computer-generated conformation, V_{VDW}: an interatomic distance derived from van der Waals radius was used instead of the interproton distance constraint, V_{ANG}: an angle constraint and the corresponding dihedral angle in a computer-generated conformation were adopted instead of the interatomic distance constraint (V_{NOE}). W_D: weight for V_{NOE}, W_V: weight for V_{VDW}, W_J: weight for V_{ANG}.

3.7. *TipA* promoter inducing activity

Minimum induction concentrations for *TipA* promoter were determined by agar dilution method with the serial dilutions. A spore suspension of *S. lividans* (pAK114) was spread on nutrient agar supplemented with 5 μg/mL kanamycin and a series of two-fold diluted compounds. After incubation for 24 h at 30°C, the plate was scored for growth of spores spread on agar medium.

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