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# Butenolide signaling molecules, SRB1 and SRB2, that induce lankacidin and lankamycin production in *Streptomyces rochei*

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## Abstract

Novel signaling molecule(s) that induce lankacidin and lankamycin production in *Streptomyces rochei* were extracted from the culture filtrate and purified by Sephadex LH20 and silica gel chromatography with the help of bioassay. Chiral HPLC and ESI-MS analyses indicated the presence of two active components, SRB1 and SRB2, and their molecular formulae were established to be $C_{15}H_{24}O_5$ and $C_{16}H_{26}O_5$, respectively. Based on extensive NMR analysis, SRB1 and SRB2 were determined to be 2-(1'-hydroxyl-6'-oxo-8'-methylnonyl)-3-methyl-4-hydroxybut-2-en-1,4-olide and 2-(1'-hydroxyl-6'-oxo-8'-methyldecyl)-3-methyl-4-hydroxybut-2-en-1,4-olide, respectively. These structures were finally confirmed by chemical synthesis and the absolute configuration of C-1' was determined to be *R*. The synthetic 1'*R*-isomers induced production of lankacidin and lankamycin at around 40 nM concentration. Thus, SRB1 and SRB2 are distinct from the well-known 2,3-disubstituted γ-butyrolactone molecules such as A-factor, virginia butanolide, and SCB1 and belong to the γ-butenolide family following avenolide which has been recently isolated from *Streptomyces avermitilis*.


## Introduction

The filamentous soil bacteria, *Streptomyces*, are characterized by the ability to produce a wide variety of secondary metabolites including antibiotics. In many *Streptomyces* species, small diffusible signaling molecules control antibiotic production and morphological differentiation.[1,2] The well-studied family of signaling molecules identified up to date have a 2,3-disubstituted γ-butyrolactone skeleton and are active at nanomolar concentrations.[3] The most extensively characterized molecule is A-factor

(2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) (Scheme 1A), which is responsible for streptomycin production and morphological differentiation in *Streptomyces griseus*.[4] When A-factor produced in a growth-dependent manner reaches a critical concentration, it binds to a specific receptor protein ArpA, and then the A-factor/ArpA complex dissociates from the promoter region of *adpA*, a global transcriptional activator gene. The derepressed gene product AdpA binds to its target genes to activate streptomycin production and morphological differentiation.[5] On the top of this regulatory cascade, the *afsA* gene encodes a key enzyme in A-factor biosynthesis.[6,7] Additional γ-butyrolactone-type signaling molecules and their specific receptors were identified in several *Streptomyces* species, including *Streptomyces coelicolor* A3(2),[8] *Streptomyces virginiae*,[9] and *Streptomyces lavendulae* FRI-5.[10] The γ-butyrolactone-type signaling molecules were classified into three major groups based on the reduction state and the stereochemistry of C-1' (Scheme 1A); (1) the 1'-keto type (e.g. A-factor in *S. griseus*), (2) the 1'*R*-hydroxyl type (SCB1 in *S. coelicolor* A3(2)), and (3) the 1'*S*-hydroxyl type (virginia butanolides in *S. virginiae*). Recently, novel signaling molecules were identified in *S. coelicolor* and *Streptomyces avermitilis*. A furan-type signaling molecule, methylenomycin furan (Scheme 1A), was shown to induce methylenomycin production in *S. coelicolor*.[11] A 4-monosubstituted butenolide named avenolide (Scheme 1A) induces avermectin production in *S. avermitilis* with a minimum inducing concentration of 4 nM.[12] Thus, various type of signaling molecules are involved in controlling antibiotic biosynthesis in *Streptomyces* species.

*Streptomyces rochei* strain 7434AN4 carries three linear plasmids (pSLA2-L, -M, and -S) and produces two structurally unrelated polyketide antibiotics, lankacidin and lankamycin (Figure S1).[13] Lankacidin and lankamycin inhibit peptide synthesis

synergistically by targeting neighboring sites in the ribosome.[14] The biosynthetic gene clusters of these antibiotics are located on the giant linear plasmid pSLA2-L.[15-17] In addition, many regulatory genes were identified on pSLA2-L, including a synthesis gene of the signaling molecule (*srrX*), six *tetR*-type repressor genes (*srrA-F*), and three SARP (*Streptomyces* antibiotic regulatory protein) genes (*srrY*, *srrZ*, and *srrW*). We revealed that *srrX* and *srrA* constitute the signaling molecule/receptor system in *S. rochei*.[18] Extensive mutational and transcriptional analyses revealed that the SARP gene *srrY* is a target of SrrA and that the signaling pathway goes from *srrX* through *srrA* to *srrY*, leading to lankacidin and lankamycin production.[19] Furthermore, it was shown that SrrY directly activates transcription of the second SARP gene *srrZ* for LM production.[20] In spite of these accumulated data on the regulatory cascade of antibiotic production in *S. rochei* 7434AN4, the signaling molecule(s) themselves have not been isolated. Here we report the isolation, structural elucidation, and biological activity of the signaling molecules termed "SRBs (*S*treptomyces *r*ochei butenolides)" that induce lankacidin and lankamycin production at a nanomolar concentration.

## Results

### Isolation of SRBs

*S. rochei* KA61, a disruptant of the SARP-family activator gene *srrY*, was used to isolate the SRB molecules. This strain does not produce lankacidin or lankamycin due to a shutdown of the regulatory cascade downstream of *srrY*, which improves the isolation efficiency of the SRB molecules without disturbance of antibiotics. A 160-liter culture of strain KA61 was grown in a 200-liter jar fermenter at 28°C for 43 h. The culture filtrate was passed through an Amberlite® XAD16 column to absorb

hydrophobic metabolites including SRBs. The column was extracted with ethyl acetate (EtOAc), and the resulting oil (18 g) was purified by silica gel chromatography with hexane-EtOAc (2:1 to 1:2, *v*/*v*). Each fraction was subjected to bioassay to check its antibiotic-inducing activity. The active fractions were combined and purified by Sephadex LH20 with methanol as eluent. Then, the active fractions were collected and further purified by series of silica gel chromatography with the following three solvent systems to give a single spot on TLC ($R_f$ = 0.20 in hexane-EtOAc=1:1, *v*/*v*); $CHCl_3$-methanol (50:1-30:1, *v*/*v*), toluene-EtOAc (2:1-1:1, *v*/*v*), and hexane-EtOAc (2:1-1:1, *v*/*v*). No other fractions showed any inducing activity, indicating that the purified component is an inducing factor of lankacidin and lankamycin in *S. rochei*.

The active component was further analyzed by chiral HPLC (TCI Chiral MB-S column; macroporous silica gel coated with optically active *N*-substituted polymaleimides) using 10% aqueous acetonitrile as mobile phase (flow rate; 1.0 $mLmin^{-1}$) with detection at 210 nm. In contrast to the results with HPLC and TLC, two peaks were detected at 9.1 min (SRB1) and 17.3 min (SRB2) (Figure 1A). However, due to a limiting amount of two compounds available (total ~250 μg; later estimated by UV absorbance), structural analysis was carried out as a mixture. ESI-MS analysis also indicated the presence of SRB1 and SRB2 in the ratio 1:1 (Figure 1B). Their molecular formulae were established to be $C_{15}H_{24}O_5$ for SRB1 and $C_{16}H_{26}O_5$ for SRB2, suggesting that SRB1 and SRB2 are homologs with one methylene difference. Concerning to the HPLC chromatogram of SRBs (Figure 1A), it is noteworthy that their retention times differed by 8.2 min. A similar relationship was observed in the *S. coelicolor* signaling molecules, SCB1 and SCB3 (Scheme 1A): SCB1 (*iso*-type $C_8$ side chain) eluted at 22-23 min, whereas SCB3 (*anteiso*-type $C_9$ side chain) eluted at 40-48

min.[21]

**Structural elucidation of SRBs**

The $^{13}C$ NMR and HMBC spectra of the mixture of SRB1 and SRB2 revealed two carbonyl groups (C-1 and C-6'; $\delta_C$ 170.7 and 213.1) and one C=C double bond (C-2 and C-3; $\delta_C$ 129.4 and 157.1) (Figure 1D). No olefinic protons were observed in the $^1H$ NMR spectrum (Figure 1C), suggesting that the C=C double bond was fully substituted. Taking account of the degree of unsaturation, the cyclic skeleton in SRBs was deduced. A strong absorbance at 1,750 $cm^{-1}$ in the IR spectrum indicated the presence of an α,β-unsaturated γ-lactone ring. The singlet H-5 protons at $\delta_H$ 2.08 were assigned to a methyl group at C-3 ($\delta_C$ 157.1) by HMBC of the H-5 protons to C-3 (Figure 1E-I). The H-5 protons showed long range HMBC correlations with the C-4 methine carbon ($\delta_C$ 98.7) and the C-2 quaternary carbon ($\delta_C$ 129.4). The C-4 carbon harbors a highly deshielded broad-singlet proton ($\delta_H$ 5.88), indicating that C-4 is hydroxylated. Furthermore, the broad-singlet H-4 proton showed a HMBC correlation with the C-1 carbonyl carbon ($\delta_C$ 170.7). The H-5 protons showed moderate NOEs on the deshielded H-1' proton ($\delta_H$ 4.50) of the alkyl side chain as well as on H-4. From these data, a 2,3-disubstituted γ-hydroxybutenolide ring was deduced as a partial structure, where C-3 was substituted with a methyl group and C-2 with an alkyl side chain (Figure 1E-I). The broad-singlet signal of H-4 implied that two compounds exist as mixtures of interconverting epimers at C-4. These assignments showed a good agreement with those of known 2,3-disubstituted γ-hydroxybutenolides (R = isopropyl or *sec*-butyl; Figure 1E-I), which were previously isolated from *Streptomyces antibioticus* Tü99.[22]

The partial structure of SRBs indicated that the alkyl side chains branched at C-2 have the formulae $C_{10}H_{19}O_2$ for SRB1 and $C_{11}H_{21}O_2$ for SRB2. The C-6' carbonyl

carbon at $\delta_C$ 213.1 was assigned to a ketone, which showed HMBC correlations with proton signals at $\delta_H$ 2.26 (doublet), 2.18 (double-doublet), and 2.37-2.40 (multiplet) (Figure 1D). These signals correspond to the methylene protons; $\delta_H$ 2.26 at $\delta_C$ 52.0 (C-7' of SRB1) (Figure 1E-II), $\delta_H$ 2.18 and 2.37 at $\delta_C$ 50.1 (C-7' of SRB2) (Figure 1E-III), and $\delta_H$ 2.40 at $\delta_C$ 42.6 (C-5' of SRB1 and SRB2) (Figures 1E-II and -III). These spectral data indicates that the methylene groups (C-5' and C-7') at both sides of C-6' have a different chemical environment. Concerning to SRB1, the doublet H-7' proton ($\delta_H$ 2.26) showed a $^1$H-$^1$H COSY correlation with proton at $\delta_H$ 2.10, which in turn connected to two methyl groups H-9' and H-9'' at $\delta_H$ 0.89-0.90. Thus, a partial structure of the alkyl chain of SRB1 was elucidated as shown in Figure 1E-II. Concerning to SRB2, the double-doublet H-7'a proton ($\delta_H$ 2.18) showed $^1$H-$^1$H COSY correlations with signals at $\delta_H$ 2.37 and $\delta_H$ 1.90, the former being assigned to a geminal H-7'b proton by HMQC experiment. The latter proton ($\delta_H$ 1.90) connected to C-8' showed a further $^1$H-$^1$H COSY correlation with the H-9'' methyl protons ($\delta_H$ 0.86) (Figure 1E-III). In addition, the C-8' carbon ($\delta_C$ 30.7) showed HMBC correlations with the double-doublet H-7'a proton and the H-10' methyl protons ($\delta_H$ 0.86), the latter of which showed a $^1$H-$^1$H COSY correlation with the H-9'' methylene protons ($\delta_H$ 1.2-1.3). These assignments led to a partial structure of the alkyl chain of SRB2 as illustrated in Figure 1E-III. Although further assignments of additional three methylene groups attached to C2'-C4' of SRB1 and SRB2 were difficult due to overlapping signals, SRB1 and SRB2 were assigned to be 2-(1'-hydroxyl-6'-oxo-8'-methylnonyl)-3-methyl-4-hydroxybut-2-en-1,4-olide (**1**) and 2-(1'-hydroxyl-6'-oxo-8'-methyldecyl)-3-methyl-4-hydroxybut-2-en-1,4-olide (**2**). Both compounds contain a 2,3-disubstituted γ-hydroxybutenolide skeleton, which is distinct

from that of the known γ-butyrolactone molecules such as A-factor, virginia butanolide, and SCB1 (Scheme 1).

**Synthesis of SRB molecules**

To confirm the proposed structures of **1** and **2** and determine their C-1' configurations, the 1'*R*-isomers (**1a** and **2a**) and the 1'*S*-isomers (**1b** and **2b**) were synthesized as shown in Scheme 2. The readily available 1-(benzyloxy)-6-hexanol (**3**)[23] was oxidized by pyridinium chlorochromate (PCC) to give aldehyde **4** in 81% yield. For the synthesis of the alkyl chains of **1a** and **1b** (Scheme 2A), the aldehyde **4** was coupled with the Grignard reagent isobutylmagnesium bromide to give a $C_{10}$ unit **5** in 81% yield, in which a newly generated hydroxyl group was oxidized by PCC to afford ketone **6** in 82% yield. Protection of the ketone group in **6** with ethylenedioxybis(trimethylsilane), deprotection of the benzyl group, and subsequent oxidation with PCC afforded aldehyde **7** in 64% yield (3 steps). Enantiomerically pure 3-methyl-4-(L-menthyloxy)but-2-en-1,4-olide (**8**) was prepared by condensation of propanal with glyoxylic acid and L-menthol, followed by two-stage recrystallizations from petroleum ether.[24,25] The key coupling reaction between the anion derived from **8** with lithium diisopropylamide (LDA) and the aldehyde **7** provided a diastereomeric mixture of **9a** and **9b** in the ratio 2:1 in 31% yield. The large protecting group (L-menthyl) at the C-4 hydroxyl of **8** prevented deprotonation of the more acidic H-5 proton,[26] leading to the production of a desired 2,3-disubstituted γ-(L-menthyloxy)butenolide. The diastereomeric mixture of **9a** and **9b** was separated by repeated runs of flash chromatography with hexane-ethyl acetate. Each of the purified compounds had 94% diastereomeric purity as judged from two well-separated hemiacetal H-4 proton signals in the $^1$H NMR spectra ($\delta_H$ 5.69 for **9a** and $\delta_H$ 5.71 for

**9b**). The C-1’ configurations of these compounds were determined from the Δδ ($\delta_S$ – $\delta_R$) values of their (*S*)- and (*R*)-α-methoxy-α-(trifluoromethyl)-α-phenyolacetate (MTPA) ester derivatives.[27] Characteristic H-4 and H-5 proton signals in the γ-butenolide moiety of **9a** showed distinct negative Δδ values (–25 and –98 Hz, respectively), while the H-2’ proton showed a slightly positive value (+5 Hz). Thus, the C’-1 configuration of **9a** was determined to be *R*, while that of **9b** is *S*. Finally, deprotection of the L-menthyl and 1,3-dioxolane groups in **9a** and **9b** was performed simultaneously by boron tribromide to afford SRB1a (**1a**) and SRB1b (**1b**) in 52% and 63% yields, respectively.

SRB2a (**2a**) and SRB2b (**2b**) were synthesized in a similar way for **1a** and **1b** but a different Grignard reagent, (*S*)-(2-methylbutyl)magnesium chloride, was used for construction of a $C_{11}$ unit **10** (Scheme 2B).

The retention times of SRB1 and SRB2 (9.1 and 17.3 min) on a chiral HPLC column (mobile phase; 10% aqueous acetonitrile containing 0.1% trifluoroacetic acid) at a flow rate of 1.0 $mLmin^{-1}$ were identical to those of the synthetic 1’*R*-isomers **1a** and **2a**, whereas the synthetic 1’*S*-isomers **1b** and **2b** eluted slightly earlier at 8.7 and 16.6 min (Figure 2A). Furthermore, the natural SRBs showed a positive optical rotation value ($[\alpha]_D^{22}$ = +17.5), which was in good agreement with that of the synthetic 1’*R*-isomers (+18.4 for **1a**, +22.4 for **2a**). The synthetic 1’*S*-isomers exhibited a negative value for the optical rotation (–9.32 for **1b**, –5.71 for **2b**). Consequently, the absolute C-1’ configuration of SRB1 and SRB2 were determined to be *R* (Scheme 1B). As shown in Figure 2B, the spectra of the natural SRBs showed a good agreement with those of the synthetic compounds **1a** and **2a**. In the $^1H$ and $^{13}C$ NMR spectra of the synthetic SRBs (**1a**, **1b**, **2a**, **2b**), small separate resonances (Δδ < 0.03 ppm in $^1H$ NMR

and $\Delta\delta$ < 0.3 ppm in $^{13}$C NMR) are observed due to the presence of interconverting epimers at C-4 (Table 1). These hemiacetalic epimers interconvert rapidly[28] and were inseparable even on the chiral HPLC.

**Biological activity of SRB molecules**

The minimum concentration of the synthetic SRBs necessary for antibiotic production was determined by bioassay. The 1'*R*-isomers **1a** and **2a** showed an inducing activity at 42 and 40 nM, while the 1'*S*-isomers **1b** and **2b** were active at 660 and 630 nM, respectively. The value of **1a** and **2a** corresponded to the level of the inducing activity of natural SRBs (approximately 50 nM). The 1'*S*-isomers (**1b** and **2b**) showed much lower activity (around 6%) compared with the 1'*R*-isomers, suggesting that the 1'*R* configuration is important for antibiotic-inducing activity. In this respect, we cannot exclude the possibility that the low inducing activity of **1b** and **2b** is due to a contamination of **1a** and **2a**. The average yield of SRBs in strain KA61 was around 6 μg per liter of the culture. The metabolic profiles of strain KA20, a *srrX-srrB* double mutant, separately supplemented with SRB1 and SRB2 were comparable (Figure 3), suggesting that both SRB1 and SRB2 play an equal role to induce lankacidin and lankamycin production in *S. rochei*.

## DISCUSSION

Two signaling molecules, SRB1 and SRB2, that induce production of lankacidin and lankamycin in *S. rochei* 7434AN4 were isolated, and structure-determined. The SRB molecules contain a 2,3-disubstituted γ-hydroxybutenolide skeleton. Structurally related butenolides have been isolated from *S. antibiotics* Tü99 as antibiotics, which exhibited a moderate antimicrobial activity against *Pseudomonas aeruginosa*.[22] About 60% of

*Streptomyces* species use γ-butyrolactone-type signaling molecules to induce antibiotic production,[29] and fourteen of this type molecules have been hitherto identified.[3,21] Recently, novel types of signaling molecules were isolated from several *Streptomyces* species. A furan-type signaling molecule, methylenomycin furan, was identified as an autoregulator for methylenomycin production in *S. coelicolor* A3(2).[11] In addition, avenolide, a novel 4-monosubstituted γ-butenolide-type molecule was identified as an inducing factor for avermectin production in *S. avermitilis*.[12] It is noteworthy that the *avaR* gene (*sav2269*) in *S. avermitilis*, which encodes a homolog of the AfsA-family proteins, is not involved in avenolide production. We have not determined the stereochemistry of C-8' of SRB2. Nevertheless the *S* configuration of C-8' in **2** is more plausible from the following evidences. 8-Methyldecanoyl-ACP, a possible biosynthetic intermediate of **2**, is synthesized by condensation of four malonyl-CoA units with a starter (*S*)-2-methylbutyryl-CoA, which is derived from L-isoleucine, in primary fatty acid biosynthesis. Hafner *et al*. reported that the branched-chain 2-oxo acid decarboxylase is specific to the (*S*)-isomer of 3-methyl-2-oxopentanoic acid for the synthesis of *anteiso*-type fatty acids and avermectins in *S. avermitilis*.[30]

The minimum concentrations of SRB1 (**1**=**1a**) and SRB2 (**2**=**2a**) to induce antibiotic production in *S. rochei* were 42 nM and 40 nM, respectively. These values were lower than that of SCB1 (128-256 nM) for actinorhodin and undecylprodigiosin production in *S. coelicolor*[31] and higher than those of A-factor (around 1 nM) for streptomycin in *S. griseus*[6] and virginia butanolides (around 3 nM) for virginiamycin in *S. virginiae*.[32] The synthetic 1'*S*-isomers **1b** and **2b** exhibited remarkably weaker activity than the 1'*R*-isomers **1a** and **2a**, indicating that the stereochemistry of C-1' is crucial for inducing activity. Our preliminary experiments demonstrated that 2,3-dihydro-SRB1,

which was prepared from **1a** via catalytic hydrogenation, showed no ability to induce antibiotic production in *S. rochei* at a concentration of 33.5 μM, indicating the importance of the C2-C3 double bond for activity. The function of the C-4 hemiacetal hydroxyl and the C-6' ketone groups together with the chain length should also be studied in due course. It was reported that higher concentrations of SCB1 (>1,000 nM) inhibited antibiotic production in *S. coelicolor*, but did not affect growth and morphological development.[31] A similar effect on antibiotic production was also observed for virginiae butanolides in *S. virginiae*.[33] In contrast, the inhibitory effect on the antibiotic production and morphological differentiation was not observed for SRBs in *S. rochei* even at the concentration of 6,700 nM. We previously observed a negative effect of *srrX* on spore formation on the basis of gene inactivation experiments.[18] However, the present work clearly indicates that SRBs themselves have no inhibitory effect on spore formation. Thus, it may be possible that the SrrX protein has a regulatory function in morphological differentiation.

Concerning the biosynthesis of SRB1 and SRB2, we speculated that *srrX* encodes a key enzyme like *afsA* in *S. griseus* based on the following evidences. (1) SRB1 and SRB2 resemble possible intermediates of the γ-butyrolactone signaling molecules. (2) The culture extract of the *srrX* mutant has no inducing activity of antibiotic production in *S. rochei*. (3) No other positive signals were detected in *S. rochei* DNA when *srrX* was used as a probe for hybridization. Consistent with these speculation, several possible genes for SRB biosynthesis were found around *srrX* (*orf85*) on pSLA2-L; an NAD-dependent dehydrogenase gene *srrG* (*orf81*), a phosphatase gene *srrP* (*orf83*), a P450 hydroxylase gene *srrO* (*orf84*), and a thioesterase gene *srrH* (*orf86*). By analogy to the biosynthesis of A-factor[7] and virginia butanolides,[34] SrrX may be responsible

for the coupling between a $C_3$ unit and a β-keto acid of $C_{12}$ or $C_{13}$ derived from fatty acid biosynthesis, followed by spontaneous intramolecular aldol condensation to form a butenolide skeleton. Then, the butenolide intermediate is further modified by biosynthetic enzymes including SrrG to synthesize SRBs. The SrrG protein is homologous to BarS1, which is involved in the reduction of the C-1' ketone group to produce virginia butanolides in *S. virginiae*.[35] Our preliminary data showed that a mutation of *srrG* abolished lankacidin and lankamycin production in *S. rochei* (unpublished result). Comprehensive analyses of SRB biosynthetic pathway by gene inactivation and feeding experiments are in progress in our laboratory.

## Experimental Section

**Strains and culture conditions:** *S. rochei* wild-type strain 7434AN4 and strain 51252 that carries only pSLA2-L were described previously.[13,15] Strain KA61, a disruptant of the SARP-family transcriptional activator gene *srrY*, was used as an SRB producer.[20] Strain KA20, a double mutant of *srrX* and the transcriptional repressor gene *srrB*, was used as an SRB indicator strain.[18] YM medium (0.4% yeast extract, 1.0% malt extract, and 0.4% D-glucose, pH 7.3) was used for SRB synthesis and bioassay.

**Spectroscopic instruments:** NMR spectra were recorded on a JEOL LA-500 spectrometer equipped with a field gradient accessory. $CDCl_3$ (99.8 atom% enriched; Acros) was used as a solvent. Chemical shifts were recorded as a δ value based on a resident solvent signal ($\delta_C$ 77.0), or an internal standard signal of tetramethylsilane ($\delta_H$ 0). Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI)

mass spectra were measured by a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, USA). Optical rotations were measured with a JASCO DIP-370 polarimeter. IR spectra were recorded on a JASCO FT/IR-5300 spectrometer. UV spectra were obtained on an Ultrospec 3300pro UV/visible spectrophotometer (Amersham Biosciences).

**Isolation of SRB molecules:** An inoculum of strain KA61 was grown using a two-stage seed culture system. The first-stage seed culture (50 mL) was used as an inoculum to 4 liter medium, which was grown at 28°C and 90 rpm for 28 h. The resulting second-stage seed culture was inoculated into 160 liter of YM medium, which was cultivated at 28°C and 110 rpm with an airflow rate of 0.5 vvm (air volume/liquid volume per minute) for 43 h. The culture supernatant was passed through a column of Amberlite® XAD16 resin (Sigma) (5 kg) at a flow rate of 10 $Lh^{-1}$ to absorb hydrophobic compounds including SRBs. The resin was extracted with equal volume of EtOAc three times. The combined organic phase was dried ($Na_2SO_4$), filtered, and concentrated to dryness. The resulting residue (18 g) was purified by silica gel chromatography with hexane-EtOAc (1:1 to 1:2, *v*/*v*). Each fraction was subjected to bioassay using strain KA20 as a test organism (see below). Active fractions were collected and purified by Sephadex LH-20 (GE Healthcare) with methanol, which was further purified by a series of silica gel column chromatography with three different solvent systems of $CHCl_3$-MeOH (50:1-30:1, *v*/*v*), toluene-EtOAc (2:1-1:1, *v*/*v*), and hexane-EtOAc (2:1-1:1, *v*/*v*). The purified sample was analyzed by ESI-MS and NMR (Figure 1). The $^{1}H$ and $^{13}C$ NMR assignments were supported by $^{1}H$-$^{1}H$ COSY, HMQC, and HMBC experiments (Supporting information).

Compound **1** (SRB1): high resolution ESI-MS; observed *m*/*z* 321.1671 $[M+Na]^+$ (calcd

for $C_{16}H_{26}O_5Na$, 321.1673).

Compound **2** (SRB2): high resolution ESI-MS; observed *m*/*z* 307.1513 $[M+Na]^+$ (calcd for $C_{15}H_{24}O_5Na$, 307.1516).

Optical rotation of natural SRBs: $[\alpha]_D^{22}$ = +17.5 (*c* = 0.012, $CHCl_3$).

**SRB assay:** The *srrX-srrB* double mutant KA20 was used as a highly sensitive SRB indicator, because this strain produces two antibiotics like the *srrB* mutant when SRBs are added. A seed culture of strain KA20 (100 μL) supplemented with an aliquot of fraction (10 μL) was grown in YM liquid medium (5 mL) at 28°C for 24 h. Antibiotic production was analyzed by high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) as follows. The crude extract was applied on a COSMOSIL 5C18-MS-II column (4.6 x 250 mm, Nakarai Tesque, Japan) and eluted with a mixture of acetonitrile-10 mM sodium phosphate buffer (pH 8.2) (3:7, *v*/*v*) at a flow rate of 1.0 $mL min^{-1}$. The eluate was monitored at 230 nm with a JASCO MD-2010 multiwavelength photodiode array detector. Lankacidin C and lankacidinol A were detected at 8.5 min and 16.9 min, respectively. TLC was developed with a mixture of $CHCl_3$-methanol (15:1, *v*/*v*) and baked after spraying with anisaldehyde-$H_2SO_4$.

**1-(Benzyloxy)-6-hexanal (4):** A mixture of 1-(benzyloxy)-6-hexanol (**3**)[23] (8.00 g, 38.4 mmol), pyridinium chlorochromate (PCC) (12.8 g, 59.5 mmol), and sodium acetate (1.40 g, 17.1 mmol) in $CH_2Cl_2$ (200 mL) was stirred at room temperature for 1.5 h. The mixture was diluted with ether (300 mL), and filtered through a pad of Celite. The filtrate and washings were concentrated *in vacuo*. The residue was purified over silica gel with hexane-EtOAc (7:1, *v*/*v*) to give **4** (6.45 g, 81%) as a colorless oil.

$^1H$ NMR ($CDCl_3$): δ = 1.40-1.45 (2H, m, H-3), 1.61-1.68 (4H, m, H-2 and H-4), 2.35 (1H, t, *J* = 7.4 Hz, H-5a), 2.43 (1H, dt, *J* = 1.8 and 7.4 Hz, H-5b), 3.47 (2H, t, *J* =

6.4 Hz, H-1), 4.50 (2H, s, Ph-C$H_2$), 7.25-7.37 (5H, m, *Ph*-$CH_2$), 9.75 (1H, t, *J* = 1.8 Hz, H-6); $^{13}$C NMR ($CDCl_3$): δ = 21.9 (C-3), 24.5 (C-4), 29.4 (C-2), 43.7 (C-5), 70.0 (C-1), 202.7 (C-6), benzyl resonances: 72.9 (Ph-*C*$H_2$), 127.5, 127.6, 128.4, 138.6; High resolution ESI-MS: observed *m*/*z* 229.1199 $[M+Na]^+$ (calcd for $C_{13}H_{18}O_2Na$, 229.1199) ; IR (neat): $\tilde{\nu}$ = 2938, 2861, 1725, 1454, 1364, 1101, 739, 698 $cm^{-1}$.

**1-(Benzyloxy)-8-methylnonan-6-ol (5):** To a suspension of magnesium (2.00 g, 82.3 mmol) and iodine (160 mg) in THF (40 mL) was added 1-bromo-2-methylpropane (9.00 mL, 83.4 mmol) at 0°C, and the mixture was stirred at 0°C for 2 h. A solution of aldehyde **4** (2.82 g, 13.7 mmol) in THF (10 mL) was added dropwise to the mixture at 0°C, and the mixture was stirred at room temperature for 2.5 h. Saturated aqueous $NH_4Cl$ (50 mL) was added, and the mixture was extracted with EtOAc twice. The combined organic phases were washed with brine, dried ($Na_2SO_4$), filtered, and concentrated to dryness. The residue was purified over silica gel with hexane-EtOAc (7:1-5:1, *v*/*v*) to give **5** (2.93 g, 81%) as a colorless oil.

Diastereomer mixture: $^1$H NMR ($CDCl_3$): δ = 0.91 (6H, t, *J* = 7.0 Hz, H-9 and H-9'), 1.22 (1H, m, H-7a), 1.32-1.46 (7H, m, H-3,4,5,7b), 1.63 (2H, t, *J* = 7.0 Hz, H-2), 1.76 (1H, m, H-8), 3.47 (2H, t, *J* = 6.7 Hz, H-1), 3.66 (1H, br, H-6), 4.50 (2H, s, Ph-C$H_2$), 7.26-7.34 (5H, m, *Ph*-$CH_2$); $^{13}$C NMR ($CDCl_3$): δ = 22.0 (C-9), 23.5 (C-9'), 24.6 (C-8), 25.4 (C-4), 26.3 (C-3), 29.7 (C-2), 38.0 (C-5), 46.8 (C-7), 69.9 (C-6), 70.3 (C-1), benzyl resonances: 72.8 (Ph-*C*$H_2$), 127.5, 127.6, 128.3, 138.6; High resolution ESI-MS: observed *m*/*z* 287.1981 $[M+Na]^+$ (calcd for $C_{17}H_{28}O_2Na$, 287.1982); IR (neat): $\tilde{\nu}$ = 3397, 2932, 2863, 1454, 1366, 1101, 735, 698 $cm^{-1}$.

**1-(Benzyloxy)-8-methylnonan-6-one (6):** A mixture of alcohol **5** (2.90 g, 11.0 mmol), PCC (3.75 g, 17.4 mmol), and sodium acetate (290 mg, 3.54 mmol) in $CH_2Cl_2$ (60 mL)

was stirred at room temperature for 2 h. The mixture was diluted with ether (100 mL), and filtered through a pad of Celite. The filtrate and washings were concentrated *in vacuo*. The residue was purified over silica gel with hexane-EtOAc (10:1, *v*/*v*) to give **6** (2.37 g, 82%) as a colorless oil.

$^{1}$H NMR ($CDCl_3$): δ = 0.90 (6H, d, *J* = 6.4 Hz, H-9 and H-9'), 1.34-1.40 (2H, m, H-3), 1.55-1.66 (4H, m, H-2 and H-4), 2.13 (1H, m, H-8), 2.26 (2H, d, *J* = 7.0 Hz, H-7), 2.37 (2H, t, *J* = 7.4 Hz, H-5), 3.46 (2H, t, *J* = 6.4 Hz, H-1), 4.49 (2H, s, Ph-C$H_2$), 7.25-7.35 (5H, m, *Ph*-$CH_2$); $^{13}$C NMR ($CDCl_3$): δ = 22.6 (C-9 and C-9'), 23.5 (C-4), 24.6 (C-8), 25.8 (C-3), 29.5 (C-2), 43.2 (C-5), 51.8 (C-7), 70.1 (C-1), 211.0 (C-6), benzyl resonances: 72.9 (Ph-*C*$H_2$), 127.5, 127.6, 128.3, 138.6; High resolution ESI-MS: observed *m*/*z* 285.1822 $[M+Na]^+$ (calcd for $C_{17}H_{26}O_2Na$, 285.1825); IR (neat): $\tilde{\nu}$ = 2936, 2866, 1711, 1454, 1366, 1103, 737, 698 $cm^{-1}$.

**5-(2'-Isobutyl-1',3'-dioxolan-2'-yl)pentanal (7):** To a solution of ketone **6** (2.30 g, 8.77 mmol) and ethylenedioxybis(trimethylsilane) (4.20 mL, 17.1 mmol) in $CH_2Cl_2$ (80 mL) was added trimethylsilyl trifluoromethanesulfonate (TMSOTf) (300 μL, 1.66 mmol) at –78°C, and the mixture was stirred at –78°C for 1 h and at room temperature for 7 h. Pyridine (1 mL) and water (10 mL) were added at 0°C, and the mixture was extracted with $CH_2Cl_2$ twice. The combined organic phases were washed with brine, dried ($Na_2SO_4$), filtered, and concentrated to dryness. The residue was purified over silica gel with hexane-EtOAc (20:1, *v*/*v*) to give the corresponding dioxolane derivative (2.43 g, 90%) as a colorless oil. A mixture of the dioxolane derivative (2.40 g, 7.83 mmol) and 10% Pd-C (750 mg) in EtOAc (60 mL) was stirred at room temperature for 24 h under hydrogen atmosphere. The mixture was passed through a pad of Celite. The filtrate and washings were concentrated *in vacuo*. The residue was purified over silica

gel with hexane-EtOAc (4:1-2:1, *v*/*v*) to give the deprotected alcohol (1.61 g, 95%) as a colorless oil. A mixture of this alcohol (1.61 g, 7.44 mmol), PCC (3.40 g, 15.8 mmol), and sodium acetate (280 mg, 3.41 mmol) in $CH_2Cl_2$ (60 mL) was stirred at room temperature for 4 h. The mixture was diluted with ether (50 mL), and filtered through a pad of Celite. The filtrate and washings were concentrated *in vacuo*. The residue was purified over silica gel with hexane-EtOAc (5:1, *v*/*v*) to give **7** (1.20 g, 75%) as a colorless oil. Overall yield; 64% (three steps).

$^1$H NMR ($CDCl_3$): δ = 0.94 (6H, d, *J* = 6.7 Hz, C*H*$_3$x2), 1.37-1.44 (2H, m, H-4), 1.51 (2H, m, iPr-C*H*$_2$), 1.60-1.67 (4H, m, H-3 and H-5), 1.75 (1H, m, Me$_2$C*H*), 2.43 (2H, m, H-2), 3.91 (4H, m, (C*H*$_2$O-)$_2$), 9.76 (1H, t, *J* = 1.9 Hz, H-1); $^{13}$C NMR ($CDCl_3$): δ = 22.3 (C-3), 23.4 (C-4), 24.0 (*Me*$_2$CH), 24.1 (Me$_2$*C*H), 37.1 (C-5), 43.9 (C-2), 45.2 (iPr-*C*H$_2$), 64.6 ((*C*H$_2$O-)$_2$), 111.7 (C-2'), 202.6 (C-1); High resolution APCI-MS: observed *m*/*z* 215.1642 $[M+H]^+$ (calcd for $C_{12}H_{23}O_3$, 215.1642); IR (neat): $\tilde{\nu}$ = 2953, 2872, 1711, 1466, 1366, 1090, 949 cm$^{-1}$.

**(1'*R*)-2-(1'-hydroxyl-5'-(2''-isobutyl-1'',3''-dioxolan-2''-yl)pentyl)-3-methyl-4-(L-menthyloxy)but-2-en-1,4-olide (9a) and (1'*S*)-2-(1'-hydroxyl-5'-(2''-isobutyl-1'',3''-dioxolan-2''-yl)pentyl)-3-methyl-4-(L-menthyloxy)but-2-en-1,4-olide (9b):** A solution of *n*-butyl lithium (2.80 mL, 1.65 M in hexane, 4.62 mmol) was added dropwise to a solution of diisopropylamine (640 μL, 4.55 mmol) in THF (10 mL) at 0°C. After 30 min of stirring, hexamethylphosphoric triamide (HMPA) (4 mL) was added dropwise to the mixture at 0°C. A solution of readily available 3-methyl-4-(L-menthyloxy)but-2-en-1,4-olide (**8**) (1.15 g, 4.55 mmol)[24,25] in THF (12 mL) was added dropwise at –78°C, and the mixture was further stirred at the same temperature for 30 min. Then a solution of aldehyde **7** (1.05 g, 4.90

mmol) in THF (10 mL) was added dropwise at –78°C within 10 min, and the mixture was further stirred at the same temperature for 1.5 h. Saturated aqueous $NH_4Cl$ (10 mL) was added to the mixture, and the mixture was extracted with $CH_2Cl_2$ twice. The combined organic phases were washed with brine, dried ($Na_2SO_4$), filtered, and concentrated to dryness. The residue was purified by silica gel chromatography with hexane-EtOAc (4:1, *v*/*v*) to give a 2:1 mixture of **9a** and **9b** (660 mg, 31%) as a colorless oil, which were further separated by repeated runs of flash chromatography. The absolute configuration at C-1' was established by the modified Mosher method.[27] The differences in chemical shifts ($\Delta\delta$) were obtained by subtracting the $\delta$ value of (*R*)-MTPA ester from that of (*S*)-MTPA ester ($\delta_S - \delta_R$).

Compound **9a**: $[\alpha]_D^{27}$ = –58.8 (*c* = 1.10, $CHCl_3$); $^1$H NMR ($CDCl_3$): $\delta$ = 0.91 (3H, d, *J* = 6.7 Hz, *Me*$_2$CH), 0.93 (3H, d, *J* = 6.8 Hz, *Me*$_2$CH), 1.28-1.42 (2H, m, C-3'), 1.50 (2H, d, *J* = 6.1 Hz, iPr-C*H*$_2$), 1.59-1.63 (5H, m, H-2'a,4',5'), 1.75 (1H, m, Me$_2$C*H*), 1.83 (1H, m, H-2'b), 1.97 (3H, s, H-5), 2.86 (1H, br, C1'-O*H*), 3.91 (4H, m, (C*H*$_2$O-)$_2$), 4.46 (1H, t, *J* = 6.7 Hz, H-1'), 5.69 (1H, s, H-4), menthyl resonances: 0.81 (3H, d, J = 6.8 Hz), 0.86 (1H, m), 0.88 (3H, d, *J* = 7.1 Hz), 0.96 (3H, d, *J* = 6.4 Hz), 1.02 (2H, m), 1.22-1.27 (1H, m), 1.28-1.42 (1H, m), 1.64-1.70 (2H, m), 2.08-2.14 (2H, m), 3.62 (1H, dt, *J* = 4.3 and 11 Hz); $^{13}$C NMR ($CDCl_3$): $\delta$ = 11.5 (C-5), 23.5 (C-4'), 24.0 (*Me*$_2$CH), 24.1 (Me$_2$*C*H), 25.7 (C-3'), 36.6 (C-2'), 37.2 (C-5'), 45.2 (iPr-*C*H$_2$), 64.6 ((*C*H$_2$O-)$_2$), 66.9 (C-1'), 100.9 (C-4), 111.9 (C-2''), 130.6 (C-2), 155.4 (C-3), 171.4 (C-1), menthyl resonances: 15.9, 20.8, 22.2, 23.2, 25.3, 31.4, 34.2, 40.5, 47.7, 79.6; High resolution ESI-MS: observed *m*/*z* 489.3182 $[M+Na]^+$ (calcd for $C_{27}H_{46}O_6Na$, 489.3187); IR (neat): $\tilde{\nu}$ = 2953, 2870, 1759, 1458, 1370, 1331, 1094, 947, 756 $cm^{-1}$.

Compound **9b**: $[\alpha]_D^{28}$ = –99.5 (*c* = 0.760, $CHCl_3$); $^1$H NMR ($CDCl_3$): $\delta$ = 0.91 (3H,

d, *J* = 6.4 Hz, *Me*$_2$CH), 0.93 (3H, d, *J* = 6.8 Hz, *Me*$_2$CH), 1.28-1.42 (2H, m, C-3'), 1.50 (2H, d, *J* = 6.1 Hz, iPr-C*H*$_2$), 1.59-1.72 (5H, m, H-2'a,4',5'), 1.75 (1H, m, Me$_2$C*H*), 1.83 (1H, m, H-2'b), 1.99 (3H, s, H-5), 3.91 (4H, m, (C*H*$_2$O-)$_2$), 4.48 (1H, t, *J* = 7.3 Hz, H-1'), 5.71 (1H, s, H-4), menthyl resonances: 0.80 (3H, d, J = 7.0 Hz), 0.86 (1H, m), 0.87 (3H, d, *J* = 7.1 Hz), 0.96 (3H, d, *J* = 6.7 Hz), 1.05 (2H, m), 1.22-1.27 (1H, m), 1.28-1.42 (1H, m), 1.59-1.72 (2H, m), 2.10-2.15 (2H, m), 3.62 (1H, dt, *J* = 4.3 and 11 Hz); $^{13}$C NMR ($CDCl_3$): δ = 11.5 (C-5), 23.5 (C-4'), 24.0 (*Me*$_2$CH), 24.1 (Me$_2$*C*H), 25.7 (C-3'), 36.4 (C-2'), 37.2 (C-5'), 45.1 (iPr-*C*H$_2$), 64.5 ((*C*H$_2$O-)$_2$), 66.7 (C-1'), 100.7 (C-4), 111.8 (C-2''), 130.7 (C-2), 155.4 (C-3), 171.4 (C-1), menthyl resonances: 15.7, 20.8, 22.2, 23.1, 25.1, 31.4, 34.2, 40.4, 47.7, 79.5; High resolution ESI-MS: observed *m*/*z* 489.3184 $[M+Na]^+$ (calcd for $C_{27}H_{46}O_6Na$, 489.3187); IR (neat): $\tilde{\nu}$ = 2953, 2870, 1759, 1456, 1370, 1333, 1096, 947 $cm^{-1}$.

**SRB1a (1a):** To a solution of **9a** (82 mg, 0.18 μmol) in $CH_2Cl_2$ (3.0 mL) was added 10% $BBr_3$ solution in $CH_2Cl_2$ (400 μL, 420 μmol) at –78°C, and the mixture was stirred at –78°C for 1.5 h. Saturated aqueous $NaHCO_3$ (2 mL) was carefully added, and the mixture was extracted with EtOAc twice. The combined organic phases were washed with brine, dried ($Na_2SO_4$), filtered, and concentrated to dryness. The residue was purified by silica gel chromatography with hexane-EtOAc (1:1, *v*/*v*) to give SRB1a (**1a**) (26 mg, 52%) as a colorless oil.

Mixture of C-4 epimers: $[\alpha]_D^{24}$ = +18.4 (*c* = 0.860, $CHCl_3$); $^1$H NMR and $^{13}$C NMR assignments were listed in Table 1; High resolution ESI-MS: observed *m*/*z* 307.1519 $[M+Na]^+$ (calcd for $C_{15}H_{24}O_5Na$, 307.1516); UV/Vis (MeOH): $\lambda_{max}$ (log ε) = 210 nm (3.98); IR (neat): $\tilde{\nu}$ = 3389, 2957, 2872, 1752, 1705, 1464, 1370, 1335, 1088, 955, 758 $cm^{-1}$.

**SRB1b (1b):** The compound **9b** (23 mg, 50 μmol) was treated in the same manner as described for the preparation of **1a** to give SRB1b (**1b**) (8.9 mg, 63%) as a colorless oil.

Mixture of C-4 epimers: $[\alpha]_D^{25}$ = –9.32 (*c* = 0.400, $CHCl_3$); $^1$H NMR ($CDCl_3$): δ = 0.89 (3H, d, *J* = 6.4 Hz, H-9' or H-9''), 0.90 (3H, d, *J* = 6.4 Hz, H-9'' or H-9'), 1.25 (1H, m, H-3'a), 1.36 (1H, m, H-3'b), 1.53 (2H, m, H-4'), 1.69-1.81 (2H, m, H-2'), 2.08/2.09 (3H, s, H-5), 2.09 (1H, m, H-8'), 2.26 (2H, d, *J* = 7.1 Hz, H-7'), 2.40 (2H, dt, *J* = 2.2 and 7.1 Hz, H-5'), 4.46 (1H, m, H-1'), 5.85 (1H, brs, H-4); $^{13}$C NMR ($CDCl_3$): δ = 11.5/11.6 (C-5), 22.5/22.6 (C-9' and C-9''), 22.9/23.2 (C-4'), 24.5/24.7 (C-8'), 24.7/24.8 (C-3'), 35.6/35.7 (C-2'), 42.6/42.8 (C-5'), 51.9/52.0 (C-7'), 66.4/66.6 (C-1'), 98.5/98.8 (C-4), 129.9/130.3 (C-2), 157.1/157.2 (C-3), 171.1/171.2 (C-1), 211.4/212.6 (C-6'); High resolution ESI-MS: observed *m*/*z* 307.1515 $[M+Na]^+$ (calcd for $C_{15}H_{24}O_5Na$, 307.1516); UV/Vis (MeOH): $\lambda_{max}$ (log ε) = 210 nm (4.02); IR (neat): $\tilde{\nu}$ = 3389, 2957, 2872, 1752, 1705, 1464, 1370, 1335, 1088, 955, 758 $cm^{-1}$.

**(8*S*)-1-(Benzyloxy)-8-methyldecan-6-ol (10):** To a suspension of magnesium (2.05 g, 84.3 mmol) and iodine (135 mg) in THF (35 mL) was added (*S*)-(+)-1-chloro-2-methylbutane (9.00 mL, 75.1 mmol) at room temperature, and the mixture was stirred at 70°C for 2 h. A solution of aldehyde **4** (2.78 g, 13.5 mmol) in THF (10 mL) was added dropwise to the mixture at 0°C, and the mixture was stirred at room temperature for 1 h. Saturated aqueous $NH_4Cl$ (30 mL) was added, and the mixture was extracted with EtOAc twice. The combined organic phases were washed with brine, dried ($Na_2SO_4$), filtered, and concentrated to dryness. The residue was purified over silica gel with hexane-EtOAc (7:1-5:1, *v*/*v*) to give **10** (2.55 g, 68%) as a colorless oil.

Diastereomer mixture: $^1$H NMR ($CDCl_3$): δ = 0.85-0.92 (6H, m, H-9' and H-10),

1.11 (1H, m, H-9a), 1.25-1.50 (10H, m, H-3,4,5,7,8,9b), 1.63 (2H, t, *J* = 6.7 Hz, H-2), 3.47 (2H, t, *J* = 6.7 Hz, H-1), 3.68 (1H, m, H-6), 4.50 (2H, s, Ph-C*H*$_2$), 7.25-7.34 (5H, m, *Ph*-CH$_2$); $^{13}$C NMR ($CDCl_3$): δ = 11.1/11.3 (C-10), 18.8/19.8 (C-9'), 25.3/25.5 (C-4), 26.3 (C-3), 29.0/30.4 (C-9), 29.7 (C-2), 30.8/31.1 (C-8), 37.7/38.3 (C-5), 44.6/44.8 (C-7), 69.6/69.9 (C-6), 70.3 (C-1), benzyl resonances: 72.8 (Ph-*C*H$_2$), 127.5, 127.6, 128.3, 138.7; High resolution ESI-MS: observed *m*/*z* 301.2142 $[M+Na]^+$ (calcd for $C_{18}H_{30}O_2Na$, 301.2138); IR (neat): $\tilde{\nu}$ = 3397, 2930, 2857, 1454, 1101, 735, 698 cm$^{-1}$.

**(8*S*)-1-(Benzyloxy)-8-methyldecan-6-one (11):** The alcohol **10** (2.50 g, 8.98 mmol) was treated in the same manner as described for the preparation of **6** to give **11** (1.53 g, 62%) as a colorless oil.

$[\alpha]_D^{26}$ = +2.40 (*c* = 0.500, $CHCl_3$); $^1$H NMR ($CDCl_3$): δ = 0.86 (3H, d, *J* = 6.7 Hz, H-9'), 0.87 (3H, t, *J* = 7.4 Hz, H-10), 1.18 (1H, m, H-9a), 1.30 (1H, m, H-9b), 1.37 (2H, m, H-3), 1.55-1.65 (4H, m, H-2 and H-4), 1.91 (1H, m, H-8), 2.18 (1H, dd, J = 8.0 and 16 Hz, H-7a), 2.35-2.42 (3H, m, H-5 and H-7b), 3.46 (2H, t, *J* = 6.7 Hz, H-1), 4.49 (2H, s, Ph-C*H*$_2$), 7.25-7.35 (5H, m, *Ph*-CH$_2$); $^{13}$C NMR ($CDCl_3$): δ = 11.3 (C-10), 19.4 (C-9'), 23.5 (C-4), 25.8 (C-3), 29.5 (C-9), 29.6 (C-2), 30.8 (C-8), 43.2 (C-5), 49.9 (C-7), 70.2 (C-1), 211.2 (C-6), benzyl resonances: 72.9 (Ph-*C*H$_2$), 127.5, 127.6, 128.3, 138.6; High resolution ESI-MS: observed *m*/*z* 299.1983 $[M+Na]^+$ (calcd for $C_{18}H_{28}O_2Na$, 299.1982); IR (neat): $\tilde{\nu}$ = 2936, 2861, 1713, 1456, 1366, 1103, 737, 698 cm$^{-1}$.

**5-(2'-((2"*S*)-2"-methylbutyl)-1',3'-dioxolan-2'-yl)pentanal (12):** The compound **11** (1.50 g, 5.43 mmol) was treated in the same manner as described for the preparation of **7** to give **12** (720 mg, 58% in three steps) as a colorless oil.

$[\alpha]_D^{27}$ = +5.92 (*c* = 1.09, $CHCl_3$); $^1$H NMR ($CDCl_3$): δ = 0.86 (3H, t, *J* = 7.4 Hz, *Me*CH$_2$), 0.93 (3H, d, *J* = 6.4 Hz, *Me*CH), 1.17 (1H, m, MeC*H*$_2$a), 1.36-1.44 (4H, m,

H-4 and MeCHC$H_2$), 1.51 (1H, m, MeC*H*), 1.60-1.67 (5H, m, H-3, 5, and MeC$H_2$b), 2.44 (2H, dt, *J* = 1.9 and 7.4 Hz, H-2), 3.91 (4H, m, (C$H_2$O-)$_2$), 9.76 (1H, t, *J* = 1.9 Hz, H-1); $^{13}$C NMR ($CDCl_3$): δ = 11.3 (*Me*$CH_2$), 20.5 (*Me*CH), 22.3 (C-3), 23.4 (C-4), 30.4 (Me*C*H), 30.6 (Me*C*$H_2$), 37.1 (C-5), 43.1 (MeCH*C*$H_2$), 43.9 (C-2), 64.6/64.7 ((*C*$H_2$O-)$_2$), 111.9 (C-2'), 202.6 (C-1); High resolution APCI-MS: observed *m*/*z* 229.1796 $[M+H]^+$ (calcd for $C_{13}H_{25}O_3$, 229.1798); IR (neat): $\tilde{\nu}$ = 2957, 2876, 1725, 1462, 1377, 1138, 1082, 949 $cm^{-1}$.

**(1'*R*)-2-(1'-hydroxyl-5'-(2"-((2"'*S*)-2'-methylbutyl-1",3"-dioxolan-2"-yl)pentyl)-3-methyl-4-(L-menthyloxy)but-2-en-1,4-olide (13a) and (1'*S*)-2-(1'-hydroxyl-5'-(2"-((2"'*S*)-2'-methylbutyl-1",3"-dioxolan-2"-yl)pentyl)-3-methyl-4-(L-menthyloxy)but-2-en-1,4-olide (13b):** The compound **12** (670 mg, 2.93 mmol) was treated in the same manner as described for the preparation of **9** to give a 2:1 mixture of **13a** and **13b** (656 mg, 54%) as a colorless oil, which was also further separated by flash chromatography.

Compound **13a**: $[\alpha]_D^{23}$ = –72.4 (*c* = 1.27, $CHCl_3$); $^1$H NMR ($CDCl_3$): δ = 0.86 (3H, t, *J* = 7.3 Hz, *Me*$CH_2$), 0.93 (3H, d, *J* = 6.8 Hz, *Me*CH), 1.14-1.20 (1H, m, MeC$H_2$a), 1.31-1.42 (6H, m, H-3', 4', MeC$H_2$b, and MeCHC$H_2$a), 1.50 (1H, m, MeC*H*), 1.59-1.63 (4H, m, H-2'a, 5', and MeCHC$H_2$b), 1.84 (1H, m, H-2'b), 1.97 (3H, s, H-5), 2.85 (1H, d, *J* = 9.2 Hz, C1'-O*H*), 3.91 (4H, m, (C$H_2$O-)$_2$), 4.46 (1H, q, *J* = 8.2 Hz, H-1'), 5.69 (1H, s, H-4), menthyl resonances: 0.81 (3H, d, J = 7.1 Hz), 0.86 (1H, m), 0.87 (3H, d, *J* = 7.3 Hz), 0.96 (3H, d, *J* = 6.7 Hz), 1.10 (2H, m), 1.25 (1H, m), 1.31-1.42 (1H, m), 1.64-1.71 (2H, m), 2.10 (2H, m), 3.62 (1H, dt, *J* = 4.3 and 11 Hz); $^{13}$C NMR ($CDCl_3$): δ = 11.3 (*Me*$CH_2$), 11.5 (C-5), 20.4 (*Me*CH), 23.5 (C-4'), 25.7 (C-3'), 30.3 (Me*C*H), 30.6 (Me*C*$H_2$), 36.6 (C-2'), 37.2 (C-5'), 43.1 (MeCH*C*$H_2$), 64.5/64.7

(($CH_2O$-)$_2$), 66.9 (C-1'), 100.9 (C-4), 112.0 (C-2''), 130.6 (C-2), 155.4 (C-3), 171.4 (C-1), menthyl resonances: 15.9, 20.8, 22.2, 23.2, 25.4, 31.5, 34.2, 40.5, 47.7, 79.6; High resolution ESI-MS: observed *m*/*z* 503.3339 $[M+Na]^+$ (calcd for $C_{28}H_{48}O_6Na$, 503.3343); IR (neat): $\tilde{\nu}$ = 2955, 2926, 1755, 1456, 1372, 1331, 1096, 949, 758 $cm^{-1}$.

Compound **13b**: $[\alpha]_D^{22}$ = –74.4 (*c* = 0.860, $CHCl_3$). $^1H$ NMR ($CDCl_3$): δ = 0.86 (3H, t, *J* = 7.3 Hz, *Me*$CH_2$), 0.93 (3H, d, *J* = 6.4 Hz, *Me*CH), 1.14-1.20 (1H, m, MeC*H*$_2$a), 1.31-1.42 (6H, m, H-3', 4', MeC*H*$_2$b, and MeCHC*H*$_2$a), 1.50 (1H, m, MeC*H*), 1.59-1.71 (4H, m, H-2'a, 5', and MeCHC*H*$_2$b), 1.82 (1H, m, H-2'b), 1.98 (3H, s, H-5), 2.39 (1H, m, C1'-O*H*), 3.91 (4H, m, (C*H*$_2$O-)$_2$), 4.47 (1H, t, *J* = 7.3 Hz, H-1'), 5.71 (1H, s, H-4), menthyl resonances: 0.80 (3H, d, J = 6.7 Hz), 0.86 (1H, m), 0.87 (3H, d, *J* = 7.1 Hz), 0.96 (3H, d, *J* = 6.7 Hz), 1.10 (2H, m), 1.26 (1H, m), 1.31-1.42 (1H, m), 1.59-1.71 (2H, m), 2.12 (2H, m), 3.62 (1H, dt, *J* = 4.3 and 11 Hz); $^{13}C$ NMR ($CDCl_3$): δ =11.3 (*Me*$CH_2$), 11.5 (C-5), 20.4 (*Me*CH), 23.5 (C-4'), 25.7 (C-3'), 30.3 (Me*C*H), 30.6 (Me*C*$H_2$), 36.4 (C-2'), 37.2 (C-5'), 43.1 (MeCH*C*$H_2$), 64.5/64.6 (($CH_2O$-)$_2$), 66.8 (C-1'), 100.7 (C-4), 112.0 (C-2''), 130.7 (C-2), 155.4 (C-3), 171.4 (C-1), menthyl resonances: 15.7, 20.8, 22.2, 23.1, 25.2, 31.4, 34.2, 40.4, 47.7, 79.5; High resolution ESI-MS: observed *m*/*z* 503.3341 $[M+Na]^+$ (calcd for $C_{28}H_{48}O_6Na$, 503.3343); IR (neat): $\tilde{\nu}$ = 2955, 2872, 1761, 1456, 1372, 1331, 1096, 949 $cm^{-1}$.

**SRB2a (2a):** The compound **13a** (20 mg, 42 μmol) was treated in the same manner as described for the preparation of **1a** to give SRB2a (**2a**) (8.4 mg, 70%) as a colorless oil.

Mixture of C-4 epimers: $[\alpha]_D^{22}$ = +22.4 (*c* = 0.800, $CHCl_3$); $^1H$ NMR and $^{13}C$ NMR assignments were listed in Table 1; High resolution ESI-MS: observed *m*/*z* 321.1676 $[M+Na]^+$ (calcd for $C_{16}H_{26}O_5Na$, 321.1673); UV/Vis (MeOH): $\lambda_{max}$ (log ε) = 210 nm (4.06); IR (neat): $\tilde{\nu}$ = 3397, 2959, 2932, 2876, 1750, 1705, 1460, 1381, 1337, 1090, 957,

756 $cm^{-1}$.

**SRB2b (2b):** The compound **13b** (20 mg, 42 μmol) was treated in the same manner as described for the preparation of **1a** to give SRB2b (**2b**) (6.8 mg, 55%) as a colorless oil.

Mixture of C-4 epimers: $[\alpha]_D^{24}$ = –5.71 (*c* = 0.700, $CHCl_3$); $^1H$ NMR ($CDCl_3$): δ = 0.86 (3H, d, *J* = 7.0 Hz, H-9''), 0.87 (3H, t, *J* = 6.7 Hz, H-10'), 1.15-1.20 (1H, m, H-9'a), 1.21-1.32 (2H, m, H-3'), 1.30-1.40 (1H, m, H-9'b), 1.51-1.59 (2H, m, H-4'), 1.65-1.80 (1H, m, H-2'a), 1.81-1.93 (2H, m, H-2'b and H-8'), 2.08/2.09 (3H, s, H-5), 2.17 (1H, d, *J* = 7.9 and 8.0 Hz, H-7'a), 2.35-2.42 (3H, m, H-5' and H-7'b), 3.03 (1H, br, C1'-O*H*), 4.49 (1H, m, H-1'), 5.85 (1H, brs, H-4); $^{13}C$ NMR ($CDCl_3$): δ = 11.3/11.3 (C-10'), 11.5/11.6 (C-5), 19.3/19.4 (C-9''), 23.0/23.2 (C-4'), 24.6/24.8 (C-3'), 29.5/29.5 (C-9'), 30.9 (C-8'), 35.6 (C-2'), 42.7/42.9 (C-5'), 50.0/50.1 (C-7'), 66.4/66.5 (C-1'), 98.7/98.9 (C-4), 129.9/130.1 (C-2), 157.4/157.5 (C-3), 171.3/171.4 (C-1), 211.7/212.8 (C-6'); High resolution ESI-MS: observed *m*/*z* 321.1676 $[M+Na]^+$ (calcd for $C_{16}H_{26}O_5Na$, 321.1673); UV/Vis (MeOH): $\lambda_{max}$ (log ε) = 210 nm (4.03); IR (neat): $\tilde{\nu}$ = 3397, 2959, 2932, 2876, 1750, 1705, 1460, 1381, 1337, 1090, 957, 756 $cm^{-1}$.

**Chiral HPLC analysis:** Natural SRB1 and SRB2 (**1** and **2**), synthetic **1a**, **1b**, **2a**, and **2b** were dissolved in acetonitrile (30 $\mu g mL^{-1}$), and an aliquot of each sample (10 μL) was analyzed by HPLC using a Chiral MB-S column (macroporous silica gel coated with optically active *N*-substituted polymaleimides, 4.6 x 250 mm, Tokyo Chemical Industry, Co., Ltd., Japan) with 10% aqueous acetonitrile containing 0.1% trifluoroacetic acid as solvent at a flow rate of 1.0 $mL min^{-1}$, and with detection at 210 nm. Natural SRB1 (**1**) and SRB2 (**2**) were eluted at 9.1 and 17.3 min. Synthetic **1a**, **1b**, **2a**, and **2b** were eluted at 9.1, 8.7, 17.3, and 16.6 min, respectively.

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**Table 1.** NMR data of **1a** and **2a** in $CDCl_3$.[a]

| | SRB1a (**1a**) | | SRB2a (**2a**) | |
|---|---|---|---|---|
| Position | $\delta_C$ (mult.)[b] | $\delta_H$ (mult., *J* [Hz])[b] | $\delta_C$ (mult.)[b] | $\delta_H$ (mult., *J* [Hz])[b] |
| 1 | 171.4/171.5 (s) | - | 171.4/171.5 (s) | - |
| 2 | 129.9/130.0 (s) | - | 129.9/130.0 (s) | - |
| 3 | 157.6/157.6 (d) | - | 157.6/157.7 (d) | - |
| 4 | 98.7/98.9 (d) | 5.85 (brs) | 98.7/98.9 (d) | 5.85 (brs) |
| 5 | 11.5/11.6 (d) | 2.07/2.09 (s) | 11.5/11.6 (q) | 2.08/2.09 (s) |
| 1’ | 66.4/66.5 (d) | 4.49 (m) | 66.4/66.5 (d) | 4.48 (m) |
| 2’ | 35.6/35.7 (t) | 1.70-1.85 (m) | 35.5/35.7 (t) | 1.70-1.85 (m) |
| 3’ | 24.7/24.8 (t) | 1.25, 1.36 (m) | 24.7/24.8 (t) | 1.22, 1.35 (m) |
| 4’ | 23.0/23.1 (t) | 1.53 (m) | 23.0/23.2 (t) | 1.53 (m) |
| 5’ | 42.7/42.9 (t) | 2.40 (dt, 3.4, 7.1) | 42.7/42.9 (t) | 2.40 (m) |
| 6’ | 211.6/212.5 (s) | - | 211.8/212.8 (s) | - |
| 7’ | 51.9/52.0 (t) | 2.26 (d, 7.1) | 50.0/50.1 (t) | 2.18 (dd, 7.9, 8.3), 2.37 (m) |
| 8’ | 24.7 (d) | 2.09 (m) | 30.9 (d) | 1.90 (m) |
| 9’ | 22.5/22.6 (d) | 0.89 (d, 6.8)[c] | 29.5 (t) | 1.18, 1.28 (m) |
| 10’ | - | - | 11.3 (q) | 0.87 (d, 6.7) |
| 9’’ | 22.5/22.6 (d) | 0.90 (d, 6.7)[c] | 19.3/19.4 (q) | 0.86 (d, 7.0) |

[a] Spectra were recorded at 500 MHz for $^1H$ and 125 MHz for $^{13}C$. [b] The residential solvent signal ($\delta_C$ 77) and internal standard tetramethylsilane ($\delta_H$ 0) were used as a reference. [c] Assignments are exchangeable.

Figure and Scheme Legends

**Figure 1.** Spectral analysis of natural SRBs. A) Chiral HPLC analysis. Elution profiles were monitored by UV absorbance at 210 nm. B) ESI-MS spectrum. C) $^{1}H$ NMR spectrum. D) HMBC spectrum and the key crosspeaks. E) $^{1}H$-$^{1}H$ COSY and HMBC correlations I) in the butenolide moiety of SRBs, II) in the $C_{10}$ alkyl chain moiety of SRB1, and III) in the $C_{11}$ alkyl chain moiety of SRB2. The $\delta_H$ and $\delta_C$ values were shown in underlined and italics, respectively.

**Figure 2.** Determination of the C-1' configuration of natural SRBs. A) Chiral HPLC analysis of I) natural SRBs (**1** and **2**), II) co-injection of natural SRBs and synthetic **1a**, III) co-injection of natural SRBs and synthetic **2a**, IV) synthetic **1a**, V) **1b**, VI) **2a**, and VII) **2b**. Elution profiles were monitored by UV absorbance at 210 nm. B) $^{1}H$ NMR spectra of I) natural SRBs, II) synthetic **1a**, and III) **2a** ($\delta_H$ 0.75-2.50).

**Figure 3.** Effect of SRB1 and SRB2 on lankacidin and lankamycin production. A) TLC analysis of metabolites. I) Parent 51252; II) strain KA20; III) strain KA20 supplemented with **1a** (840 nM); IV) strain KA20 supplemented with **2a** (840 nM). Lane LC is a standard sample of lankacidin C. B) HPLC analysis of metabolites. Elution profiles were monitored by UV absorbance at 230 nm. Closed diamonds and circle indicate the peaks of lankacidin C and lankacidinol A respectively.

**Scheme 1.** Structures of *Streptomyces* signaling molecules. A) The known signaling molecules. 2,3-disubstituted γ-butyrolactone-type; A-factor, SCB1, SCB3, virginia butanolide A. Furan-type; methylenomycin furan. 4-monosubstituted butenolide-type; avenolide. B) Novel 2,3-disubstituted butenolides SRB1 (**1**) and SRB2 (**2**) isolated from *S. rochei* 7434AN4.

**Scheme 2.** Synthesis of *S. rochei* butenolides A) SRB1 and B) SRB2. a) pyridinium chlorochromate, $CH_2Cl_2$; b) isobutylmagnesium bromide, THF; c) $TMSO(CH_2)_2OTMS$, TMSOTf, $CH_2Cl_2$; d) $H_2$, 10% Pd-C, EtOAc; e) LDA, THF-HMPA, and then **7**; f) $BBr_3$, $CH_2Cl_2$; g) (*S*)-(2-methylbutyl)magnesium chloride, THF; h) LDA, THF-HMPA, and then **12**.

## Table of Contents text

**Butenolide signaling molecules in *Streptomyces*:** Two signaling molecules, SRB1 and SRB2, that induce production of lankacidin and lankamycin in *Streptomyces rochei* 7434AN4 were isolated, and structure-determined. Both contain a 2,3-disubstituted γ-hydroxybutenolide skeleton, and the stereochemistry of C-1' is crucial for inducing activity.