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Isolation, structural elucidation, and biosynthesis of 15-norlankamycin derivatives produced by a type-II thioesterase disruptant of *Streptomyces rochei*

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ABSTRACT

Lankamycin, a 14-membered macrolide antibiotic, contains a 3-hydroxy-2-butyl side chain at C-13. To analyze the function of *lkmE*, which encodes type-II thioesterase in the lankamycin cluster, we carried out a gene disruption experiment. Disruption of *lkmE* resulted in a 70% decrease of lankamycin production concomitant with an accumulation of novel lankamycin derivatives (LM-NS01A and LM-NS01B), in which the C-13 side chain is replaced by a 1-carboxyethyl group. The biosynthetic origin of 1-carboxyethyl group was confirmed by incorporation of deuterium in [3-^{2}H]3-methyl-2-oxobutyrate into the C-14 position. These results indicate that the biosynthesis of LM-NS01A and LM-NS01B starts from isobutyryl CoA in place of (*S*)-2-methylbutyryl CoA and LkmE removes the aberrantly loaded starter unit and restores lankamycin production.

1. Introduction

Complex polyketides including macrolides are an important family of antibiotics that possess a wide range of biological activities. Macrolides are assembled by type-I polyketide synthases (PKSs) that contain sets of catalytic domains in giant polypeptides.[1] Each module is responsible for one round of condensation and reduction reactions in polyketide biosynthesis. During polyketide assembly, acyl building substrates are tethered to acyl carrier proteins (ACPs) as thioesters. After the completion of polyketide extention, the polyketide chain bound to PKS is released and cyclized by a thioesterase domain (TE-I) that is usually integrated into the final PKS module.

Additional thioesterases called type-II thioesterases (TE-IIs) are coded as discrete proteins in many polyketide biosynthetic clusters. They are thought to have an editing function to remove the aberrantly loaded acyl groups from the PKS and NRPS system.[2–4] Disruption of TE-II genes resulted in a decrease of the overall titer of tylosin (*tylO*),[5] rifamycin (*rifR*),[6] erythromycin (*ery-ORF5*),[7] and FR008/candicidin (*fscTE*).[8] While, disruption of *pikAV* showed no affect on picromycin production in *Streptomyces venezuelae*.[9] On the other hand, TE-IIs function for a release of the polyketide chain from the PKS module in several polyether antibiotic biosynthesis.[10,11] Thus, the function of TE-II is different from species to species and should be studied further.

Streptomyces rochei strain 7434AN4 carries three linear plasmid pSLA2-L, -M, and -S and produces two structurally unrelated polyketide antibiotics, lankamycin (**1**; Fig. 1) and lankacidin.[12] Lankamycin is a 14-membered macrolide antibiotic containing two deoxysugars, D-chalcose and 4″-*O*-acetyl-L-arcanose.[13] The complete nucleotide sequencing of the largest plasmid pSLA2-L (210,614 bp) has revealed that the lankamycin biosynthetic gene (*lkm*) cluster (*orf24-orf53*) is located on this plasmid.[14] We previously determined the origin of a starter unit and the order of the post-PKS modification steps including two hydroxylation and two glycosylation steps in lankamycin biosynthesis.[15,16] It was suggested that L-isoleucine is oxidized to (*S*)-3-methyl-2-oxopentanoic acid, which in turn is decarboxylated to (*S*)-2-methylbutyrate. Its CoA ester, (*S*)-2-methylbutyryl CoA, is loaded on the loading ACP domain (ACP_L) in LkmAI, and then condensed with six extender units of methylmalonyl CoA by three PKSs encoded by *lkmAI-lkmAIII* (*orf35-orf33*) to synthesize 8,15-dideoxylankanolide. This aglycon then receives the following modifications to afford **1**; C-15 hydroxylation by LkmK, attachment of L-arcanose at C-3 hydroxyl by LkmL, C-8 hydroxylation by LkmF, and attachment of D-chalcose at C-5 hydroxyl by LkmI.

The *lkm* cluster contains a TE-II gene, *lkmE* (*orf25*), which might be involved in lankamycin biosynthesis. To reveal the function of *lkmE*, we disrupted the *lkmE* gene, analyzed novel metabolites produced by a disruptant, and carried out a feeding experiment, the results of which are described in this paper.

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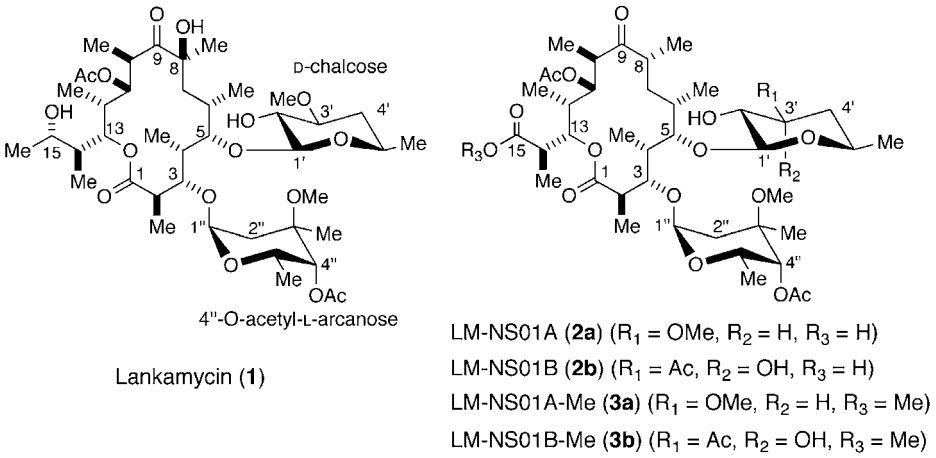

Fig. 1. Chemical structures of lankamycin (**1**), LM-NS01A (**2a**), LM-NS01B (**2b**), LM-NS01A-Me (**3a**), and LM-NS01B-Me (**3b**). Me, methyl; Ac, acetyl.

2. Results and discussion

2.1. Disruption of a type-II thioesterase gene *lkmE*

The *lkm* cluster comprises 30 open reading frames (ORFs) (*orf24-orf53*), in which the type-II thioesterase (TE-II) gene *lkmE* (*orf25*) is located on the left end. LkmE shows considerable similarities to TE-II, such as SACE0729 (Gene Bank accession number, CAM00070; 57% identity and 66% similarity) for erythromycin biosynthesis and TylO (AAA21345; 51% and 59%) for tylosin biosynthesis. Partial amino acid sequences of LkmE and other bacterial TE-IIs are aligned and compared in Fig. 2A. All of them contain the conserved GxSxG motif (x=any amino acid) characteristic of acyltransferases and thioesterases, in which the serine residue functions as an active-site center. A second conserved amino acid residue, which might be involved in catalysis, is the histidine residue indicated in a bold letter in Fig. 2A.

To reveal the function of LkmE in lankamycin biosynthesis, its gene disruptant was constructed from a strain KA07, a lankamycin high producer prepared by the disruption of the transcriptional repressor gene *srrB*.[17] The 424-bp *Apa*I fragment containing the conserved motif mentioned above was deleted from *lkmE* (Fig. 2B). As shown in Fig. 2C, the 4.6-kb and 1.0-kb *Bsi*WI fragments in the parent KA07 were replaced by a 5.2-kb *Bsi*WI fragment in the mutant NS01, which confirmed the gene disruption.

2.2. Metabolites from the *lkmE* disruptant NS01

Metabolites of the *lkmE* disruptant NS01 were compared with those of the parent KA07 by thin-layer chromatography (TLC)

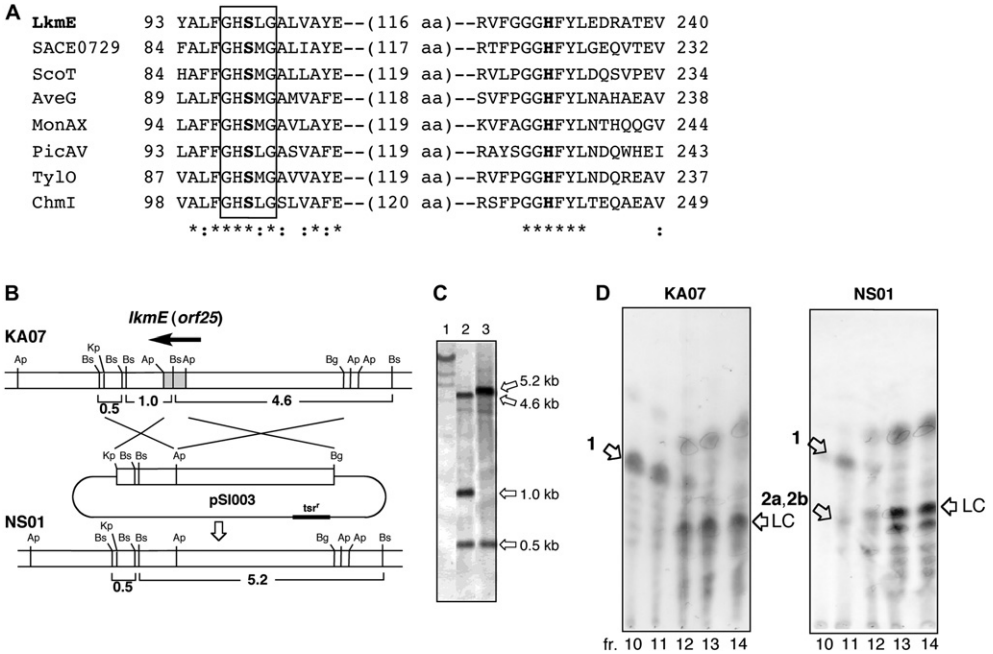

Fig. 2. Function of type-II thioesterase (TE-II) LkmE. (A) Partial amino acid alignment of TE-IIs among bacterial PKSs. Conserved motif was boxed. Bold letters indicate catalytic serine and histidine residues. LkmE (Accession number BAC76483, *S. rochei* 7434AN4); SACE0729 (Ery-orf5) (CAM00070, *Saccharopolyspora erythraea* NRRL2338); ScoT (CAC37888, *Streptomyces coelicolor* A3(2)); AveG (BAC68663, *Streptomyces avermitilis* MA-4680); MonAX (AAO65810, *Streptomyces cinnamonensis*); PicAV (AAC69333, *S. venezuelae*); TylO (AAA21345, *Streptomyces fradiae*); ChmI (AAS79448, *Streptomyces bikiniensis*). Conserved amino acid residues were marked as asterisk (identical) and colon (well conserved). (B) Construction of the *lkmE* disruptant NS01 by homologous recombination. Ap, *Apa*I; Bs, *Bsi*WI; Bg, *Bgl*II; Kp, *Kpn*I. (C) Southern blot analysis of total DNA. Lane 1, λ/*Hin*dIII; lane 2, strain KA07 (parent)/*Bsi*WI; lane 3, strain NS01 (Δ*lkmE*)/*Bsi*WI. (D) TLC analysis of metabolites partially purified by Sephadex LH-20 chromatography. Metabolites were passed through Sephadex LH-20 chromatography (1.5×40 cm) with methanol. Fractions were collected as 60 drops. TLC plates were developed with $CHCl_3$/MeOH=20:1. LC, lankacidin C.

(Fig. 2D). Strain NS01 produced 30% lankamycin (**1**) (4.5 mg/l) compared with the parent strain KA07 (15.5 mg/l). In addition, strain NS01 accumulated two novel lankamycin derivatives LM-NS01A (**2a**) and LM-NS01B (**2b**) (1.2 mg/l), which were not produced by the parent strain KA07. Compounds **2a** and **2b** were obtained as inseparable mixture in the ratio of 3:2 (R_f=0.25 in $CHCl_3$/MeOH=20:1). The molecular ion peaks of **2a** and **2b** ($[M+Na]^+$) were observed at 839 and 867, respectively.

To separate **2a** and **2b** and elucidate their structures, the mixtures were methylated with diazomethane. Methyl derivatives **3a** and **3b** were separable on TLC (R_f=0.3 for **3a** and 0.4 for **3b** in hexane/EtOAc=1:1). The molecular ion peaks of **3a** and **3b** ($[M+Na]^+$) were observed at 853 and 881, respectively, which suggested the presence of one carboxyl group in **2a** and **2b**.

The major ester LM-NS01A-Me (**3a**) was subjected to NMR and ESI-MS analyses. The ^1H and ^{13}C NMR spectral data were summarized in Table 1. The molecular formula of **3a** was determined by high resolution ESI-MS to be $C_{42}H_{70}O_{16}$. In its ^{13}C NMR, 42 carbons were classified into 15 methyl, 3 methylene, 18 methine, and 6 quaternary carbons. The quaternary carbon corresponding to the C-15 carboxyl group (δ_C=173.7) showed long range HMBC correlations with C-13 methine, C-14 methyl, and C-15 methoxy protons, therefore, the C-13 side chain in **3a** was determined to be (1-methoxycarbonyl)ethyl. In addition, the C-8 quaternary carbon in **1** was changed to a methine carbon (δ_C=44.3) in **3a**. This methine proton signal (δ_H=2.84) showed HMBC correlations with C-9 carbonyl and C-8 methyl carbons, indicating the absence of a hydroxyl group at C-8 in **3a**. Other signals of **3a** were almost identical to

Table 1
^1H and ^{13}C NMR data of lankamycin and its derivatives

No.	Lankamycin (**1**)		LM-NS01A-Me (**3a**)		LM-NS01B-Me (**3b**)	
	δ_C[a]	δ_H[b]	δ_C[a]	δ_H[b]	δ_C[a]	δ_H[b]
1	176.7 (s)	—	174.9 (s)	—	174.8 (s)	—
2	44.8 (d)	2.80 (br)	44.7 (d)	2.86 (m)	44.7 (d)	2.86 (m)
3	77.8 (d)	3.95 (d, 4.9)[e]	79.3 (d)	3.78 (d, 8.8)	79.6 (d)	3.76 (m)
4	44.1 (d)	1.83 (m)	43.2 (d)	1.78 (m)	43.0 (d)	1.80 (m)
5	84.5 (d)	3.51 (m)	84.4 (d)	3.49 (m)	84.8 (d)	3.53 (d, 8.5)[f]
6	33.9 (d)	2.21 (br)	36.1 (d)	2.00 (br)	36.2 (d)	2.00 (br)
7	39.4 (t)	1.93[d]	33.4 (t)	1.34,1.80 (m)	33.5 (t)	1.33,1.82 (m)
8	80.2 (s)	—	44.3 (d)	2.84 (m)	43.0 (d)	2.85 (m)
9	214.4 (s)	—	214.8 (s)	—	214.7 (s)	—
10	38.2 (d)	3.15 (q, 6.7)[g]	41.4 (d)	3.00 (m)	41.5 (d)	2.98 (m)
11	71.0 (d)	4.87 (d, 8.9)[g]	72.2 (d)	4.86 (dd, 9.5, 1.6)	72.2 (d)	4.88 (d, 9.8, 2.0)
12	39.4 (d)	1.93[d]	38.8 (d)	1.84 (m)	38.8 (d)	1.88 (m)
13	73.0 (d)	4.83 (d, 6.4)[h]	73.5 (d)	5.15 (d, 10.1)[h]	73.5 (d)	5.15 (d, 10.4)[h]
14	42.7 (d)	1.82 (m)	42.5 (d)	2.78 (m)	42.5 (d)	2.80 (m)
15	69.1 (d)	3.71 (m)	173.7 (s)	—	173.7 (s)	—
1′	102.5 (d)	4.33 (d, 7.7)	102.9 (d)	4.24 (d, 7.6)	102.1 (d)	4.53 (d, 7.7)
2′	75.3 (d)	3.34 (dd, 7.7,8.9)	75.5 (d)	3.31 (m)	72.4 (d)	3.74 (d, 7.6)
3′	80.2 (d)	3.25 (m)	80.1 (d)	3.23 (m)	80.6 (s)	—
4′	37.1 (t)	1.25,2.07 (m)	37.1 (t)	1.26,2.03 (m)	41.3 (t)	1.55,1.73 (m)
5′	67.3 (d)	3.51 (m)	67.4 (d)	3.47 (m)	66.9 (d)	3.94 (m)
6′	21.0 (q)[c]	1.22 (d, 6.1)	20.8 (q)[c]	1.21 (d, 6.1)	20.9 (q)[c]	1.18 (d, 7.0)
1″	96.6 (d)	5.05 (d, 4.6)	97.9 (d)	4.97 (d, 4.6)	97.9 (d)	4.96 (d, 4.6)
2″	30.6 (t)	1.67,2.09 (m)	31.1 (t)	1.70,2.07 (m)	31.6 (t)	1.69,2.07 (m)
3″	72.6 (s)	—	72.4 (s)	—	72.4 (s)	—
4″	73.9 (d)	4.67 (s)[i]	73.7 (d)	4.69 (s)[i]	73.5 (d)	4.72 (s)[i]
5″	62.6 (d)	4.47 (q, 6.6)[i]	62.6 (d)	4.47 (q, 6.7)[i]	62.8 (d)	4.49 (q, 6.8)[i]
6″	16.8 (q)	1.08[d]	16.9 (q)	1.10[d]	16.8 (q)	1.12 (d, 6.8)
3″-CH_3	21.0 (q)[c]	1.05 (s)	20.7 (q)[c]	1.06 (s)	20.7 (q)[c]	1.06 (s)
3′-OCH_3	56.9 (q)	3.44 (s)	56.9 (q)	3.44 (s)	—	—
3″-OCH_3	49.3 (q)	3.30 (s)	49.3 (q)	3.28 (s)	49.3 (q)	3.27 (s)
15-OCH_3	—	—	52.0 (q)	3.71 (s)	52.0 (q)	3.71 (s)
2-CH_3	20.7 (q)	1.17 (d, 7.4)	15.3 (q)	1.20 (d, 7.0)	15.2 (q)	1.19 (d, 7.3)
4-CH_3	20.9 (q)[c]	1.06[d]	9.9 (q)[c]	1.12[d]	9.8 (q)	1.10 (d, 7.1)
6-CH_3	14.4 (q)	1.16 (d, 6.8)	18.5 (q)	1.15 (d, 6.7)	18.3 (q)	1.16 (d, 7.0)
8-CH_3	27.0 (q)	1.34 (s)	18.9 (q)	1.13[d]	18.8 (q)	1.13[d]
10-CH_3	10.1 (q)	1.10[d]	9.2 (q)	0.97 (d, 7.1)	9.2 (q)	0.97 (d, 7.1)
12-CH_3	9.8 (q)	1.02[d]	9.8 (q)[c]	1.00 (d, 7.0)	9.7 (q)	0.99 (d, 7.9)
14-CH_3	11.2 (q)	0.83 (d, 7.0)	14.6 (q)	1.13[d]	14.6 (q)	1.13[d]
15-CH_3	19.7 (q)	1.15 (d, 6.4)	—	—	—	—
11-OC(=O)*C*H_3	20.9 (q)[c]	2.08 (s)	20.9 (q)[c]	2.06 (s)	20.7 (q)[c]	2.06 (s)
11-O*C*(=O)CH_3	170.1 (s)	—	169.9 (s)	—	169.9 (s)	—
4″-OC(=O)*C*H_3	21.0 (q)[c]	2.13 (s)	21.0 (q)[c]	2.15 (s)	20.9 (q)[c]	2.14 (s)
4″-O*C*(=O)CH_3	170.7 (s)	—	—	—	170.5 (s)	—
3′-C(=O)*C*H_3	—	—	—	—	23.9 (q)	2.31 (s)
3′-*C*(=O)CH_3	—	—	—	—	209.5 (s)	—

[a] Multiplicity is shown in parenthesis.
[b] Multiplicity and *J* value in Hz are shown in parenthesis.
[c] Assignments are exchangeable.
[d] Obscured by overlapping.
[e] Vicinal proton coupling constant $J_{3,4}$ is ~0 Hz.
[f] Vicinal proton coupling constant $J_{5,6}$ is ~0 Hz.
[g] Vicinal proton coupling constant $J_{10,11}$ is ~0 Hz.
[h] Vicinal proton coupling constant $J_{12,13}$ is ~0 Hz.
[i] Vicinal proton coupling constant $J_{4'',5''}$ is ~0 Hz.

those of **1**. From these data, compound **3a** was determined to be 8-deoxylankamycin analog, which contained a (1-methoxycarbonyl) ethyl side chain at C-13 in place of a 3-hydroxy-2-butyl side chain (Fig. 1).

The molecular formula of LM-NS01B-Me (**3b**) was established to be $C_{43}H_{70}O_{17}$. The ^{1}H and ^{13}C assignments of **3b** (Table 1) were almost identical to those of **3a**, except for the signals corresponding to D-chalcose attached to the C-5 hydroxyl. The methoxy group at the C-3′ in **3a** disappeared, while one acetyl moiety (δ_C=209.5 for a carbonyl and δ_C=23.9 for a methyl group) appeared in **3b**. The C-3′ methine carbon in **3a** was changed to a quaternary carbon (δ_C=80.6) in **3b**. This quaternary carbon showed HMBC correlations with methylene protons at C-4′ (δ_H=1.55 and 1.73) and a methyl proton of the newly appeared acetyl group (δ_H=2.31). These spectral data allowed us to assign the structure of **3b**, which contained a novel deoxysugar, 4′,6′-dideoxy-3′-*C*-acetyl-D-*ribo*-hexopyranose, instead of D-chalcose in **3a** (Fig. 1). The configuration of the new asymmetric center at C-3′ of **3b** was established to be *R* by differential NOE experiments. The axial–axial orientations of H-1′ (δ_H=4.53) and H-2′ (δ_H=3.74) were confirmed by their coupling constant of 7.7 Hz. This axial proton H-2′ (δ_H=3.74) showed a moderate NOE (5%) on the methyl proton of the acetyl group (δ_H=2.31), suggesting the equatorial orientation of the acetyl branched-chain at C-3′. This assignment was further strengthened by the following biosynthetic consideration; 4′,6′-dideoxy-3′-*C*-acetyl-D-*ribo*-hexopyranose is a possible biosynthetic intermediate of D-aldgarose (3,1′-*O*-carbo-4,6-dideoxy-3-C-[1′-hydroxyethyl]-D-*ribo*-hexopyranose), the configuration of whose branched-chain is also *R*, in the biosynthesis of a neutral macrolide antibiotic aldgamycin E.[18–20]

The spectroscopic analysis revealed that the *lkmE* disruptant accumulated novel 15-norlankamycin derivatives **2a** and **2b**, both of which harbored a 1-carboxyethyl group at C-13 instead of a 3-hydroxy-2-butyl group in **1**. Compounds **2a** and **2b** lack a hydroxyl group at C-8, suggesting that the 15-nor derivatives are poor substrates of the P450 hydroxylase LkmF. To our surprise, compound **2b** contains a novel branched-chain sugar, 4′,6′-dideoxy-3′-*C*-acetyl-D-*ribo*-hexapyranose, at the C-5 hydroxyl instead of D-chalcose in **2a**. It is noteworthy that *Streptomyces* sp. KMA001 produced two 16-membered macrolides, chalcomycin and aldgamycin I, which contain D-chalcose and decarboxylated D-aldgarose, respectively.[21] Isolation of the lankamycin derivatives with altered sugar components suggests that glycosyltransferase LkmI has a broad substrate specificity.

Deletion of the TE-II genes generally leads to a drastic reduction of polyketide production. Gene inactivation of *tylO* in *Streptomyces fradiae* resulted in a 85% loss of tylosin production, which was restored by introduction of the intact *tylO* into the mutant.[5] Removal of *rifR* from *Amycolatopsis mediterranei* resulted in a 60% decrease in rifamycin yield.[6] Disruption of *ery-ORF5* gene (*SACE0729*) in *S. erythraea* caused a severe loss of erythromycin production and an accumulation of 15-norerythromycin, which is synthesized from an aberrant acetate starter unit instead of propionate.[7] Similarly, inactivation of the *lkmE* gene resulted in a 70% loss of lankamycin production and an accumulation of 15-norlankamycin derivatives **2a** and **2b**. This result suggests that the LkmE protein controls specific incorporation of a starter unit.

The antimicrobial activities of 15-norlankamycin derivatives were determined by the agar dilution methods against *Micrococcus luteus*. The mixture of **2a** and **2b** exhibited moderate antimicrobial activity with a MIC value of 4.0 μg/ml, which was four-fold less than that of lankamycin **1** (MIC value of 1.0 μg/ml). In addition, methyl ester derivatives **3a** and **3b** showed antimicrobial activities with the MIC values of 4.0 μg/ml and 16 μg/ml, respectively. Together with previous results of deoxylankamycins (50% relative activity for 8-deoxylankamycin, 18% for 15-deoxylankamycin, 2.5% for 8,15-dideoxylankamycin compared with **1**),[15] these results show the importance of hydroxyl groups for antimicrobial activity of lankamycin. The MIC values of **3a** and **3b** indicated that D-chalcose is a better component than 4′,6′-dideoxy-3′-*C*-acetyl-D-*ribo*-hexapyranose for antimicrobial activity.

2.3. Biosynthesis of LM-NS01A and LM-NS01B

The biosynthetic origin of a starter unit for **2a** and **2b** may be L-valine. L-Valine is oxidized to 3-methyl-2-oxobutyric acid, and then decarboxylated to isobutyrate. To analyze the origin of the starter unit for **2a** and **2b**, we carried out a feeding experiment of deuterium labeled 3-methyl-2-oxobutyric acid. Deuterium labeled compound, [3-^{2}H]3-methyl-2-oxobutyric acid (**5**), was synthesized as shown in Fig. 3A. Hydrogen atom at C-3 of 3-methyl-2-oxobutyric acid (**4**) was replaced with deuterium atom in the presence of deuterium oxide and pyridine. The ^{2}H NMR of **2a** and **2b** obtained by feeding of **5** showed a distinct signal at 2.8 ppm, which corresponds to the C-14 proton (Fig. 3B). This result indicates that valine, a precursor of 3-methyl-2-oxobutyric acid, is a biosynthetic origin of the C-13 side chain of **2a** and **2b**.

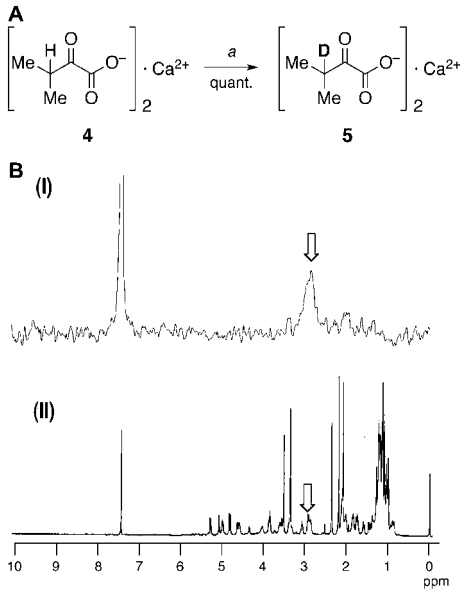

Fig. 3. Analysis of starter unit biosynthesis of **2a** and **2b**. (A) Synthetic scheme of [3-^{2}H]3-methyl-2-oxobutyrate (**5**). Reagents: *a*, D_2O, pyridine. Me, methyl. (B) ^{2}H NMR spectrum of labeled **2a** and **2b** obtained by feeding of compound **5** (I), and ^{1}H NMR spectrum of unlabeled **2a** and **2b** (II).

During the biosynthesis of **2a** and **2b**, either of the two methyl groups in valine is oxidized to carboxylate. To assign the C-14 configuration in **2a** and **2b**, we carried out feeding experiments of stereospecifically labeled isobutyrate. As shown in Fig. 4A, the synthesis of (2*R*)-[3-^{2}H]isobutyrate **9** was started from the commercially available methyl (*S*)-(+)-3-hydroxyisobutyrate **6**. The hydroxyl of **6** was protected with a tetrahydropyranyl (THP) group, and then reduction of the methyl ester with lithium aluminum hydride gave alcohol **7** in 99% yield. Tosylate of **7**, obtained by treatment with *p*-toluenesulfonyl chloride, was treated with $LiAl^{2}H_4$ to afford the deuterium labeled compound **8** in 68% yield (two steps). Finally, compound **8** was converted to (2*R*)-[3-^{2}H]isobutyrate **9** by acidic hydrolysis, Jones' oxidation, and neutralization with NaOH. Compound (2*S*)-[3-^{2}H]isovalerate **13** was obtained from methyl (*R*)-(−)-3-hydroxyisobutyrate **10** in the same manner for **9**.

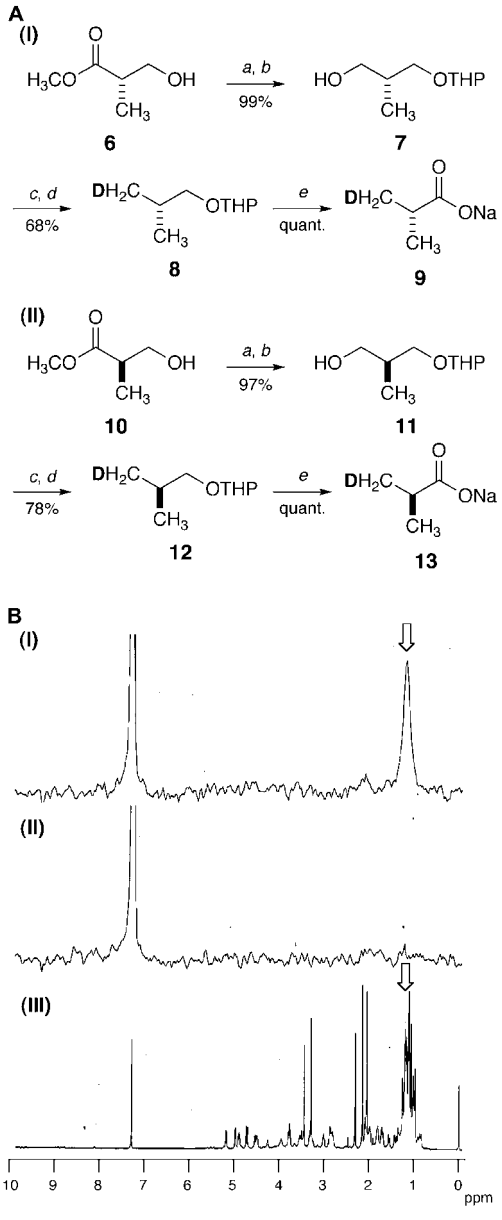

Fig. 4. Determination of stereochemistry at C-14 position in **2a** and **2b**. (A) Synthetic scheme of (2*R*)-[3-^{2}H]isobutyrate (**9**) and (2*S*)-[3-^{2}H]isobutyrate (**13**). Reagents: *a*, 3,4-dihydro-2*H*-pyran, *p*-toluenesulfonic acid, CH_2Cl_2; *b*, lithium aluminum hydride, THF; *c*, *p*-toluenesulfonyl chloride, Et_3N, 4-dimethylaminopyridine, CH_2Cl_2; *d*, lithium aluminum deuteride, THF; *e*, Jones' reagent, acetone, and then NaOH. THP, tetrahydropyranyl. (B) ^{2}H NMR spectrum of labeled **2a** and **2b** obtained by feeding of compounds **9** (I), **13** (II), and ^{1}H NMR spectrum of unlabeled **2a** and **2b** (III).

The synthesized (2*R*)-[3-^{2}H]isobutyrate **9** and (2*S*)-[3-^{2}H]isobutyrate **13** was fed to the cell culture of strain NS01. Analysis of the mixture **2a** and **2b** by ^{2}H NMR showed that the deuterium atom of **9** was specifically incorporated into the C-14 methyl of **2a** and **2b**. On the other hand, deuterium atom of **13** was not incorporated at all. These results demonstrated that a pro-*S* methyl group of isobutyrate moiety in 8,15-dideoxy-15-norlankanolide (Fig. 5) was stereospecifically oxidized to a carboxylate group during the biosynthesis of **2a** and **2b**.

3. Conclusion

We have analyzed the function of the TE-II enzyme LkmE in lankamycin biosynthesis. The *lkcE* disruptant produced lankamycin (**1**) in 30% yield compared with the parent, and in addition accumulated the 15-norlankamycin derivatives **2a** and **2b**, both of which harbored a 1-carboxyethyl group at C-13 instead of a 3-hydroxy-2-butyl group in **1**. Based on these results, we propose the early biosynthetic pathway of lankamycin including the function of LkmE in Fig. 5. During lankamycin biosynthesis, LkmE hydrolyzes the aberrantly loaded isobutyryl CoA starter unit from ACP domain of the loading module in LkmAI. Then the vacant ACP domain accepts the specific starter unit, (*S*)-2-methylbutyryl CoA, to afford **1**. Disruption of *lkmE* caused an accumulation of **2a** and **2b** in some extent by utilization of an isobutyryl CoA instead of (*S*)-2-methylbutyryl CoA starter unit, and resulted in a severe decrease of lankamycin production compared with the parent strain. Thus, the main function of LkmE was shown to remove an aberrantly loaded starter unit(s) and restore the enzymatic activity of lankamycin PKSs that prefer to use (*S*)-2-methylbutyryl CoA.

4. Experimental

4.1. Strains and culture conditions

S. rochei wild type strain 7434AN4 and strain 51252 that carries only pSLA2-L were described previously.[12] Strain KA07, a disruptant of the transcriptional repressor gene *srrB*, was used as a parent strain.[17] Cosmid B10 (nt 3341-48,756 of pSLA2-L), which carries the *lkmE* gene, was constructed previously.[14] YM medium (0.4% yeast extract, 1.0% malt extract, and 0.4% D-glucose, pH 7.3) was used for antibiotic production. YEME liquid medium[22] was used for preparation of *Streptomyces* protoplasts. Protoplasts were regenerated on R1M solid medium.[23] *E*scherichia *coli* XL1-Blue was used for routine cloning and constructing of targeting plasmids. *E. coli* strains were grown in Luria Bertani (LB) medium supplemented with ampicillin (100 μg/ml). Lithium aluminum deuteride (98 atom% enriched) was purchased from Cambridge Isotope Laboratory Inc., USA. $CDCl_3$ (99.8 atom% enriched) and D_2O (99.8 atom% enriched) were purchased from Acros, USA.

4.2. Spectroscopic instruments

NMR spectra were recorded on a JEOL LA-500 spectrometer equipped with a field gradient accessory. For ^{1}H and ^{13}C NMR, $CDCl_3$ and D_2O were used as solvents. Chemical shifts were recorded as a δ value based on resident solvent signals (δ_C=77.0 in $CDCl_3$ and δ_H=4.65 in D_2O), or internal standard signals of tetramethylsilane (δ_H=0) or dioxane (δ_C=66.5). For ^{2}H NMR, $CHCl_3$ and H_2O were used as solvents and internal references. Electrospray ionization-mass (ESI-MS) spectra were measured by an 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-8000 spectrometer.

4.3. Construction of the *lkmE* disruptant NS01

The 5.0-kb *Kpn*I-*Bgl*II fragment from cosmid B10 was cloned into pUC19 predigested with *Kpn*I and *Bam*HI to give pSI001. The 424-bp *Apa*I fragment in the middle of the *lkmE* gene was eliminated from pSI001 to afford pSI002. The vector part of pSI002 was replaced with an *E. coli-Streptomyces* shuttle vector pRES18[24] to give a targeting plasmid pSI003.

This plasmid was transformed into protoplasts of *S. rochei* strain KA07. The transformant was grown in YEME liquid medium supplemented with thiostrepton to give a plasmid-integrated (single crossover) strain. The thiostrepton-resistant colonies were subjected to a sequential cultivation in YEME liquid medium in the absence of thiostrepton to give the double crossover strain. Gene disruption was confirmed by Southern hybridization using a DIG

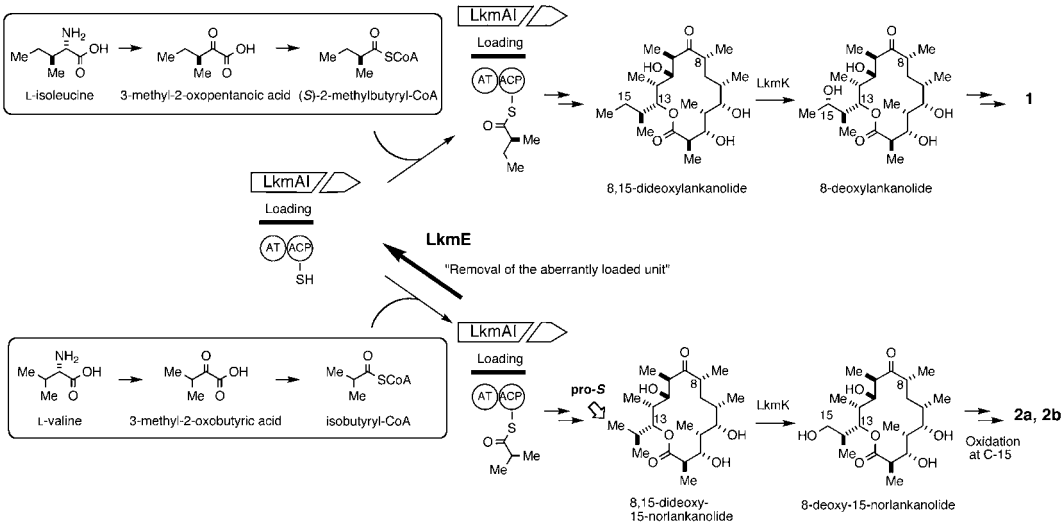

Fig. 5. Proposed biosynthetic pathway of **1**, **2a**, and **2b**. Upper panel shows a loading of (*S*)-2-methylbutyryl CoA, a metabolite of L-isoleucine, for the synthesis of **1**. Lower panel shows a loading of non-specific isobutyryl CoA, a metabolite of L-valine, for the synthesis of **2a** and **2b**. The isobutyryl CoA starter unit is hydrolyzed by LkmE to reload the specific (*S*)-2-methylbutyryl CoA.

DNA Labeling and Detection Kit (Roche Diagnostics, Germany) according to the manufacture's protocol.

4.4. Isolation of metabolites

The strains were incubated in YM liquid medium at 28 °C for 2 days. The culture broth was extracted with equal volume of ethyl acetate twice. The combined organic phase was dried (Na_2SO_4), filtered, and concentrated to dryness. The resulting residue was purified by Sephadex LH-20 (GE Healthcare) with methanol. The fractions containing **2a** and **2b** were combined, and further purified by silica gel chromatography with $CHCl_3$/methanol=50:1–10:1. Yields in strain KA07 (per liter): **1**, 15.5 mg; lankacidin C, 25.2 mg. Yields in strain NS01 (per liter): **1**, 4.5 mg; **2a** and **2b**, 1.2 mg; lankacidin C, 22.7 mg.

4.5. Esterification of LM-NS01

To a solution of **2a** and **2b** (12 mg) in ether (8 ml) was added an ethereal solution of diazomethane at 0 °C until the mixture turns yellow. The mixture was stirred at room temperature for 1 h, and concentrated in vacuo. The residue was purified by silica gel chromatography with hexane/EtOAc (2:1–1:1, v/v) to give LM-NS01A-Me (**3a**) (4.5 mg; R_f=0.3 in hexane/EtOAc=1:1) and LM-NS01B-Me (**3b**) (3.3 mg; R_f=0.4 in hexane/EtOAc=1:1). The ^1H and ^{13}C NMR spectral data of **1**, **3a**, and **3b** are summarized in Table 1.

Compound **3a**: high resolution ESI-MS; observed *m*/*z* 853.4537 $[M+Na]^+$ (calcd for $C_{42}H_{70}O_{16}Na$, 853.4556). $[\alpha]_{D^{25}}$ −74.6 (*c* 0.932, $CHCl_3$).

Compound **3b**: high resolution ESI-MS; observed *m*/*z* 881.4482 $[M+Na]^+$ (calcd for $C_{43}H_{70}O_{17}Na$, 881.4505). $[\alpha]_{D^{25}}$ −80.5 (*c* 0.950, $CHCl_3$).

4.6. Antimicrobial activity

The antimicrobial activities of compounds **2a**, **2b**, **3a**, and **3b** were measured by the agar dilution methods. The growth of test microorganism *Micrococcus luteus* was judged after 24 h incubation at 28 °C.

4.7. Synthesis of the labeled compounds

*4.7.1. [3-^{2}H]3-methyl-2-oxobutyric acid, calcium salt (**5**).* A solution of calcium 3-methyl-2-oxobutyrate (**4**) (1.0 g) in pyridine (20 ml) and deuterium oxide (10 ml) was stirred at room temperature for 1 day. The mixture was concentrated in vacuo, and this cycle was repeated further three times. More than 90% deuterium atom was incorporated at the C-3 position in **5**.

^1H NMR (D_2O) δ 1.00 (6H, s), 2.91 (<0.1H, m, trace of *CH*Me_2); ^{13}C NMR (D_2O) δ 17.0, 37.7 (t, *J*=18.5 Hz), 172.8, 212.1; ^2H NMR (H_2O) δ 2.85; High resolution ESI-MS: observed *m*/*z* 116.0463 $[M-H]^-$ (calcd for $C_5H_6{}^2HO_3$, 116.0463).

*4.7.2. (2R)-2-Methyl-3-(tetrahydro-2H-pyran-2-yloxy)propan-1-ol (**7**).* A solution of methyl (*S*)-(+)-3-hydroxyisobutyrate **6** (980 mg, 8.30 mmol), 3,4-dihydro-2*H*-pyran (2.00 ml, 21.9 mmol), and *p*-toluenesulfonic acid (18 mg) in CH_2Cl_2 (25 ml) was stirred at room temperature for 2 h. Saturated aqueous $NaHCO_3$ (15 ml) was added, and the mixture was extracted with EtOAc twice. The combined organic phase was washed with brine, dried (Na_2SO_4), filtered, and concentrated to dryness. The residue was purified over silica gel with hexane/EtOAc (10:1, v/v) to give tetrahydropyranyl ether (1.68 g, quant.) as an oil. This compound (1.68 g, 8.30 mmol) in THF (25 ml) was treated with lithium aluminum hydride (730 mg, 19.2 mmol) at 0 °C for 12 h. The mixture was quenched by successive addition of water (800 μl), 5 N NaOH (800 μl), and water (2.4 ml). The resulting mixture was filtered through a pad of Celite with ether, and the filtrate and washings were concentrated in vacuo. The residue was purified over silica gel with hexane/EtOAc (2:1, v/v) to give alcohol **7** (1.45 g, quant.) as a colorless oil.

Diastereomer mixture: ^1H NMR ($CDCl_3$) δ 0.90 (1.5H, d, *J*=6.8 Hz), 0.91 (1.5H, d, *J*=6.8 Hz), 1.52–1.60 (4H, m), 1.70–1.82 (2H, m), 2.01–2.08 (1H, m), 2.62 (0.5H, dd, *J*=5.2 and 6.1 Hz), 2.67 (0.5H, dd, *J*=5.2 and 6.4 Hz), 3.35 (0.5H, dd, *J*=7.3 and 9.5 Hz), 3.48–3.70 (4H, m), 3.84–3.90 (1.5H, m), 4.58 (1H, br); ^{13}C NMR ($CDCl_3$) δ 13.4, 13.5, 19.6, 25.3, 25.3, 30.5, 30.6, 35.4, 35.6, 62.5, 62.6, 67.4, 67.5, 72.1, 72.2, 99.1, 99.4; High resolution ESI-MS: observed *m*/*z* 197.1146 $[M+Na]^+$ (calcd for $C_9H_{18}O_3Na$, 197.1148).

*4.7.3. 2-[(2R)-[3-^{2}H]Isobutoxy]tetrahydro-2H-pyran (**8**).* *p*-Toluenesulfonyl chloride (2.68 g, 14.1 mmol) was added to a solution of **7** (1.38 g, 7.92 mmol), Et_3N (5.0 ml, 36.1 mmol), and

4-dimethylaminopyridine (30 mg) in CH_2Cl_2 (30 ml) at 0 °C, and the mixture was stirred at room temperature overnight. Water (20 ml) was added, and the mixture was extracted with EtOAc twice. The combined organic phase was washed with brine, dried (Na_2SO_4), filtered, and concentrated to dryness. The residue was purified over silica gel with hexane/EtOAc (4:1, v/v) to give tosylate (2.59 g, quant.) as an oil. A mixture of tosylate (2.60 g, 7.92 mmol) and lithium aluminum deuteride (500 mg, 11.9 mmol) in THF (35 ml) was stirred at 0 °C for 12 h. The mixture was quenched by successive addition of water (800 μl), 5 N NaOH (800 μl), and water (2.4 ml). The resulting suspension was filtered through a pad of Celite with ether, and the filtrate and washings were concentrated in vacuo. The residue was chromatographed over silica gel with hexane/EtOAc (9:1, v/v) to give the labeled compound **8** (862 mg, 68%) as a colorless oil.

Diastereomer mixture: ^{1}H NMR ($CDCl_3$) δ 0.87–0.91 (2H, m), 0.91 (1.5H, d, J=6.8 Hz), 0.93 (1.5H, d, J=6.8 Hz), 1.51–1.62 (4H, m), 1.68–1.74 (1H, m), 1.81–1.90 (2H, m), 3.15 (1H, dd, J=6.4 and 9.5 Hz), 3.50 (1H, dd, J=7.1 and 9.2 Hz), 3.51 (1H, m), 3.85 (0.5H, dd, J=3.7 and 8.3 Hz), 3.88 (0.5H, dd, J=3.1 and 8.0 Hz), 4.58 (1H, t, J=4.3 Hz); ^{13}C NMR ($CDCl_3$) δ 19.1 (dt, J=7.3 and 19.6 Hz), 19.4 (d, J=7.3 Hz), 19.6, 25.5, 28.4, 30.7, 62.1, 74.3, 98.9; ^{2}H NMR ($CHCl_3$) δ 0.93; High resolution ESI-MS: observed m/z 182.1260 $[M+Na]^+$ (calcd for $C_9H_{17}{}^2HO_2Na$, 182.1262).

*4.7.4. Sodium (2R)-[3-^{2}H]isobutyrate (**9**).* Jones' reagent (3 ml) was dropwisely added to a solution of **8** (576 mg, 3.62 mmol) in acetone (30 ml) at 0 °C, and the mixture was stirred at room temperature for 2 h. Excess oxidant was destroyed by addition of 2-propanol at 0 °C, and the resulting greenish solution was passed through a pad of Celite while washing with ether. The filtrate and washings were concentrated in vacuo. The residue was dissolved in water, and titrated by 1 N NaOH. The solution was washed with ether, and the aqueous phase was lyophilized to give sodium (2*R*)-[3-^{2}H]isobutyrate **9** (400 mg, quant.) as a white powder.

^{1}H NMR (D_2O) δ 0.90 (2H, m), 0.92 (3H, d, J=7.0 Hz), 2.26 (1H, m); ^{13}C NMR (D_2O) δ 20.0 (t, J=18.5 Hz), 20.3, 37.7, 184.1; ^{2}H NMR (H_2O) δ 0.87; High resolution ESI-MS: observed m/z 88.0516 $[M-H]^-$ (calcd for $C_4H_6{}^2HO_2$, 88.0514).

*4.7.5. (2S)-2-Methyl-3-(tetrahydro-2H-pyran-2-yloxy)propan-1-ol (**11**).* Methyl (*R*)-(−)-3-hydroxyisobutyrate **10** (1.09 g, 9.26 mmol) was treated in the same manner as described for the preparation of **7** to give **11** (1.70 g, 97%) as a colorless oil.

Diastereomer mixture: ^{1}H NMR ($CDCl_3$) δ 0.90 (1.5H, d, J=7.1 Hz), 0.91 (1.5H, d, J=7.1 Hz), 1.53–1.59 (4H, m), 1.67–1.82 (2H, m), 2.01–2.07 (1H, m), 2.60 (0.5H, br), 2.64 (0.5H, br), 3.35 (0.5H, dd, J=7.4 and 10.3 Hz), 3.48–3.70 (4H, m), 3.84–3.87 (1.5H, m), 4.58 (1H, br); ^{13}C NMR ($CDCl_3$) δ 13.4, 13.5, 19.6, 25.3, 25.3, 30.5, 30.6, 35.4, 35.6, 62.5, 62.5, 67.4, 67.5, 72.1, 72.2, 99.1, 99.4; High resolution ESI-MS: observed m/z 197.1146 $[M+Na]^+$ (calcd for $C_9H_{18}O_3Na$, 197.1148).

*4.7.6. 2-[(2S)-[3-^{2}H]Isobutoxy]tetrahydro-2H-pyran (**12**).* Compound **11** (1.14 g, 6.54 mmol) was treated in the same manner as described for the preparation of **8** to give **12** (770 mg, 78%) as a colorless oil.

Diastereomer mixture: ^{1}H NMR ($CDCl_3$) δ 0.89–0.92 (2H, m), 0.91 (1.5H, d, J=6.8 Hz), 0.93 (1.5H, d, J=6.8 Hz), 1.51–1.63 (4H, m), 1.68–1.74 (1H, m), 1.81–1.90 (2H, m), 3.16 (1H, dd, J=6.4 and 9.5 Hz), 3.50 (1H, dd, J=7.1 and 9.5 Hz), 3.50 (1H, m), 3.85 (0.5H, dd, J=3.4 and 8.3 Hz), 3.88 (0.5H, dd, J=2.8 and 7.7 Hz), 4.58 (1H, t, J=4.3 Hz); ^{13}C NMR ($CDCl_3$) δ 19.1 (dt, J=7.3 and 19.6 Hz), 19.4 (d, J=7.3 Hz), 19.6, 25.5, 28.4, 30.7, 62.1, 74.3, 98.9; ^{2}H NMR ($CHCl_3$) δ 0.93; High resolution ESI-MS: observed m/z 182.1260 $[M+Na]^+$ (calcd for $C_9H_{17}{}^2HO_2Na$, 182.1262).

*4.7.7. Sodium (2S)-[3-^{2}H]isobutyrate (**13**).* Compound **12** (590 mg, 3.71 mmol) was treated in the same manner as described for the preparation of **9** to give sodium (2*S*)-[3-^{2}H]isobutyrate **13** (410 mg, quant.) as a white powder.

^{1}H NMR (D_2O) δ 0.90 (2H, m), 0.92 (3H, d, J=7.0 Hz), 2.26 (1H, m); ^{13}C NMR (D_2O) δ 20.0 (t, J=18.5 Hz), 20.3, 37.7, 184.1; ^{2}H NMR (H_2O) δ 0.90; High resolution ESI-MS: observed m/z 88.0516 $[M-H]^-$ (calcd for $C_4H_6{}^2HO_2$, 88.0514).

4.8. Feeding of labeled compounds

One hundred milligram of the labeled compound (**5**, **9**, or **13**) was added at 8 h period to 1 L culture of strain NS01. The culture was incubated at 28 °C for additional 2 days and analyzed.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2011.05.047. These data include MOL files and InChiKeys of the most important compounds described in this article.

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