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Biosynthesis of phoslactomycins: cyclohexanecarboxylic acid as the starter unit

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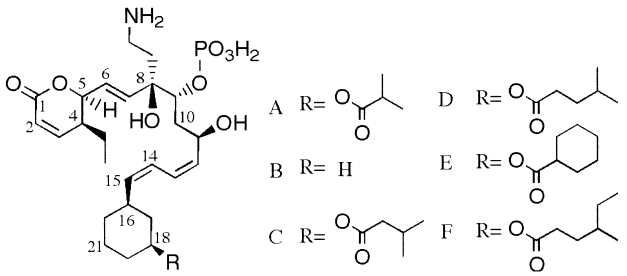
Abstract—Phoslactomycins (PLMs) A–F, produced by actinomycetes are polyketide-type antibiotics derived from a hydroxycyclohexanecarboxylic acid or a cyclohexanecarboxylic acid starter unit. Feeding experiments with [2-^{13}C]shikimic acid indicated that the C-18 carbon of PLMs comes from C-5 of shikimate. Further feeding studies of *cis* and *trans*-3-hydroxy[7-^{13}C]cyclohexanecarboxylic acid, [7-^{13}C]- and [$^{2}H_{11}$]cyclohexanecarboxylic acid have suggested that the starter unit in the PLM biosynthesis is not *cis*-3-hydroxycyclohexanecarboxylate but cyclohexanecarboxylate and that PLM-B is produced initially, and subsequently converted to other analogs by hydroxylation and acylation.

1. Introduction

Polyketides are a large and structurally diverse group of natural products, which are constructed via successive condensation of simple extender units such as malonyl CoA with various starter units such as activated short-chain fatty acids, alicyclic acids, aromatic acids, glycerol metabolites, and amino acids.[1] Natural products biosynthesized using a cyclohexanecarboxylic acid (CHC) derivative as the starter unit are found in microbial metabolites and exhibit important biological activities. For example, manumycin group antibiotics (e.g. askamycin),[2] and the immunosuppressive macrolides (e.g. rapamycin,[3] FK506[4]) were all isolated from cultures of actinomycetes. ω-Cyclohexyl fatty acids are found in thermoacidophilic bacterial membranes.[5] Phoslactomycins (PLMs) A–F, isolated from several *Streptomyces* strains including *S.* sp. HK-803[6–8] as antifungal antibiotics, belong to this group of compounds. Similar to the structurally-related compound, fostriesin,[9] PLMs exhibit type 2A serine/threonine protein phosphatase (PP2A) specific inhibitory activity.[10] Leustroducsins, which differ from PLMs only at the acyl moiety bound to the cyclohexane ring, and PLM-F were reported to be inducers of a colony stimulating factor in bone marrow stromal cells.[11] Most recently, the PP2A inhibition activity of PLMs has been shown to inhibit tumor metastasis through augmentation of natural killer cells.[12] Their structures are characterized as possessing an α,β-unsaturated δ-lactone, amino group, phosphate ester, cyclohexane ring and absolute configuration 4*S*,5*S*,8*R*,9*R*,11*R*,16*R*,18*S* (determined by Shibata et al.[13]).



Biosynthetically, PLMs can be considered as polyketides elongated from an unusual starter unit, hydroxycyclohexanecarboxylic acid or CHC (**1**). The biosynthetic studies of ansatrienin A,[14,15] ω-cyclohexyl fatty acids,[16] FK-520[17,18] and rapamycin[19,20] have revealed that these cyclohexane moiety was biosynthesized from shikimic acid (**2**) via a series of dehydrations and double bond reductions (Scheme 1). The CHC pathway and (1*R*,3*R*,4*R*)-3,4-dihydroxycyclohexanecarboxylic acid (DHCHC, **3**) pathway diverge at the first step, suggesting that these two related pathways evolved independently. Since PLM producers make two types of PLMs i.e. PLMs with a hydroxyl substituent at C-18, which is *cis* to C-16 alkyl substituent (PLM-A and PLMs C–F), and PLM-B, which has no hydroxyl substituent on the cyclohexane ring,

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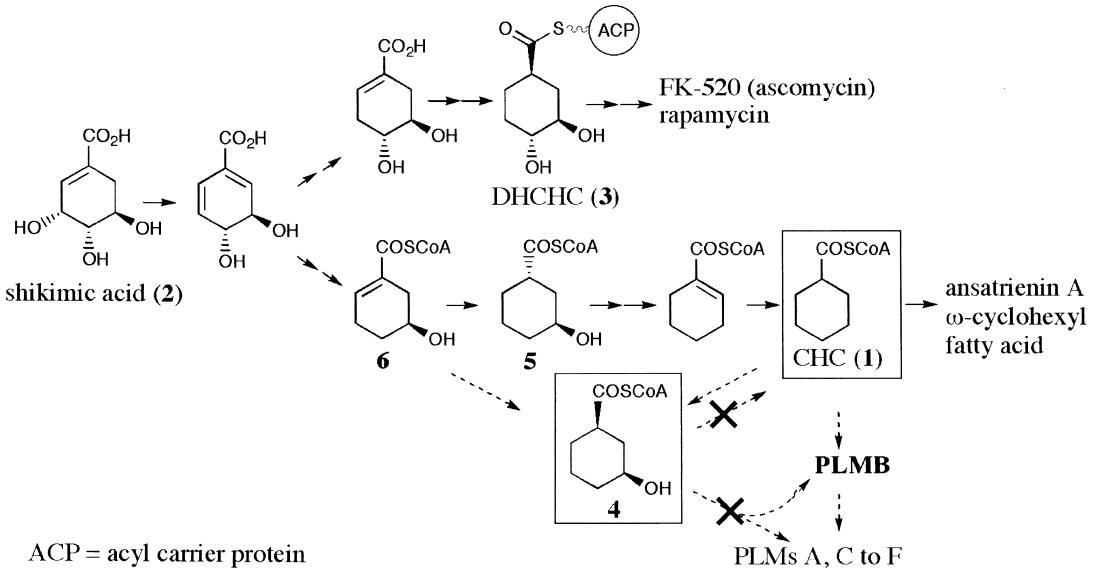


Scheme 1. Proposed conversion of shikimic acid (**2**) to cyclohexanecarboxylic acid derivatives and possible biosynthetic pathways of PLMs.

cis-3-hydroxycyclohexanecarboxylic acid (**4**) and/or **1** could be envisioned as starter units. Several possible biosynthetic routes can be considered in the biosynthesis of PLMs (Scheme 1). In this paper, we report the results of labeling studies with *Streptomyces* sp. HK-803, which led to the conclusion that **1** served as the starter unit in all cases, and that the resulting PLM-B is likely converted by hydroxylation and acylation to the other PLM analogs.

2. Results

2.1. Feeding experiment with [2-^{13}C]shikimic acid

We initiated feeding studies of shikimic acid (**2**) in order to both confirm its intermediacy and establish a biosynthetic correlation of shikimate carbon and cyclohexane carbon of PLMs. [2-^{13}C]**2** was synthesized according to the procedure described by Moore et al.[14] and fed to *Streptomyces* sp. HK-803. LC–MS analysis of the resulting PLM mixture showed that $(MH+1)^+$ peaks of PLM-A to F and $(MH+2)^+$peak of PLM-E were enhanced (Table 1), indicating that **2** was efficiently incorporated into PLMs. The major components, PLM-E and F were purified by HPLC and analyzed by ^{13}C NMR. ^{13}C NMR spectrum of PLM-E showed signals at δ 30.2 (C-3′ or 7′) and δ 33.1 (C-21), while that of PLM-F showed a signal at δ 33.1 (C-21) (>10 fold enrichment, consistent with the LC–MS analysis, Fig. 1(c) and (f)). In addition to demonstrating the shikimic acid origin of the cyclohexane moiety of PLMs, these results confirmed that C-21 of PLMs was originated from C-2 of **2**, thereby indicating that C-18 of PLMs was derived from C-5 of **2** (Scheme 2).

2.2. Feeding experiments with potential starter units

Since the majority of the PLMs produced in a fermentation have a hydroxyl substituent at C-18, which is *cis* to C-16 alkyl substituent, we next carried out an incorporation study with *cis*-3-hydroxycyclohexane carboxylic acid (**4**), and for comparison, the corresponding *trans* isomer **5**. The (±)-[7-^{13}C]**4** (containing 5% of *trans* isomer (±)-[7-^{13}C]**5**, estimated by integrating the methine ^{1}H NMR signals at

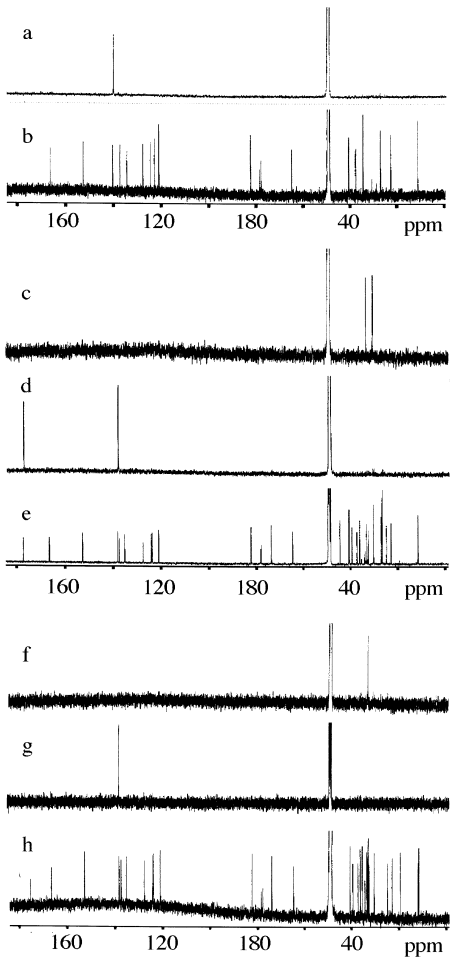


Figure 1. ^{13}C NMR spectra (125 MHz). (a) PLM-B derived from [7-^{13}C]**1**; (b) non-labeled PLM-B; (c) PLM-E derived from [2-^{13}C]**2**; (d) PLM-E derived from [7-^{13}C]**1**; (e) non-labeled PLM-E; (f) PLM-F derived from [2-^{13}C]**2**; (g) PLM-F derived from [7-^{13}C]**1**; (h) non-labeled PLM-F.

Table 1. Mass spectral analysis of PLMs obtained from a feeding experiment with [2-^{13}C]**2**[21]

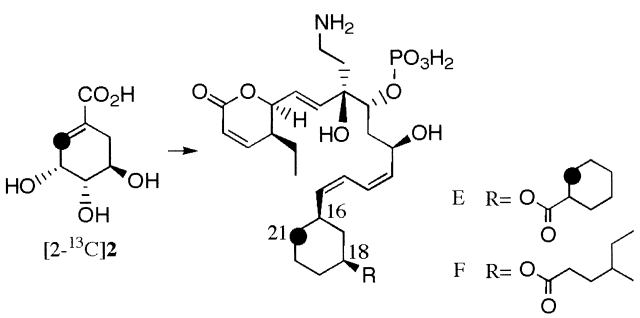
Molecular related ions	(*m*/*z*)	Unlabeled PLMs	Relative intensity obtained from [7-^{13}C]**2**
PLM-A	600	100	100
	601	29	48 (16%)
	602	10	16
PLM-B	514	100	100
	515	31	47 (14%)
	516	7	10
PLM-C	614	100	100
	615	35	61 (21%)
	616	9	18
PLM-D	628	100	100
	629	38	52 (12%)
	630	10	14
PLM-E	641	100	100
	642	35	87 (43%)
	643	11	35
	644	0	9
PLM-F	642	100	100
	643	38	66 (22%)
	644	10	17

C-3) was prepared by reduction of methyl 3-oxo[7-^{13}C]cyclohexanecarboxylate (**7**), which was obtained by cyanation of 2-cyclohexenone[22] with K^{13}CN, with $NaBH_4$.[23] The (±)-[7-^{13}C]**5** (containing 10% of *cis* isomer (±)-[7-^{13}C]**4**, estimated by integrating the methine ^{1}H NMR signals at C-3) was synthesized by reduction of **7** with K-selectride.[24] LC–MS analysis of the PLM mixture obtained from feeding of (±)-[7-^{13}C]**4** showed that **4** was poorly converted into PLMs. On the other hand, $(MH+1)^+$ peaks of PLMs A–F and $(MH+2)^+$peak of PLM-E obtained from (±)-[7-^{13}C]**5** were significantly enhanced (Table 2). The specific uptake of **5** indicated that the starter unit of PLMs is not **4**, but **1** biosynthesized from **5**. Support for this hypothesis was obtained by an incorporation study of **1**. The requisite substrate, [7-^{13}C]**1** was synthesized from cyclohexanone and K^{13}CN via cyclohexanecarbonitrile.[25] LC–MS analysis of the resulting PLM mixture showed remarkable incorporation of **1** into PLMs (Table 2). The ^{13}C NMR spectra of the purified PLM-B, E and F obtained from feeding [7-^{13}C]**1** showed enhanced signals at δ 139.9 (C-15), δ 138.2 (C-15) and 177.3 (C-1′), and δ 138.2 (C-15), respectively, (>20 fold enrichment, consistent with the LC–MS analysis, Fig. 1(a), (d), and (g)). The ^{13}C NMR spectra of PLM-B, E and F obtained from feeding [7-^{13}C]**5** were essentially identical to those from **1**. These results indicated that **1** and **5** were incorporated into the cyclohexane moiety of PLMs (Scheme 3).

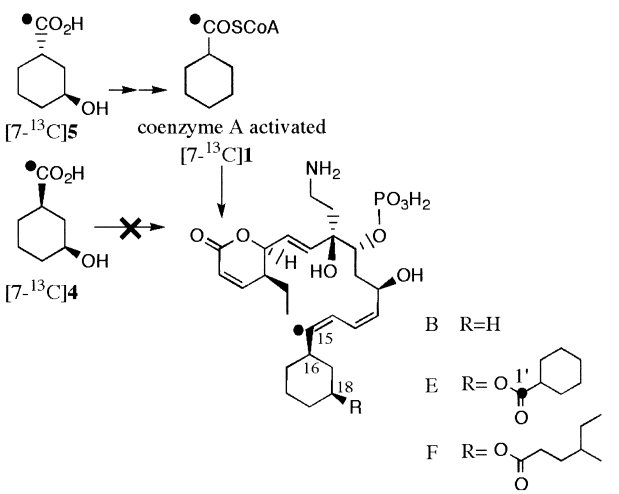
Finally, [$^2H_{11}$]**1**, (showing a deuterium distribution by MS of 10% D_9, 28% D_{10}, 62% D_{11} species) was synthesized from sodium benzoate by reduction with deuterated Raney nickel,[26] and fed to the PLM producer. Analysis of the resulting PLM mixture by LC–MS revealed the occurrence of the undecadeuterio species $(MH+11)^+$ of PLM-B, and decadeuterio species $(MH+10)^+$ of PLMs A, C–F, providing additional evidence that **1** was incorporated as such, and used as the starter unit (Table 3).

3. Discussion

PLM producers make two types of PLMs, i.e. PLMs with an acyloxy side chain at C-18, which is *cis* to C-16 alkyl substituent (PLM-A and PLMs C-F), and PLM-B, which has



Scheme 2. Incorporation of [2-^{13}C]**2** into PLMs E and F.



Scheme 3. Incorporation of [7-^{13}C]**4**, [7-^{13}C]**5** and [7-^{13}C]**1** into PLMs B, E and F.

Table 2. Mass spectral analysis of PLMs obtained from feeding experiments of [7-^{13}C]**4**, [7-^{13}C]**5** and [7-^{13}C]**1**[21]

Molecular related ions	(*m*/*z*)	Unlabeled PLMs	Relative intensity obtained from		
			(±)-[7-^{13}C]**4**	(±)-[7-^{13}C]**5**	[7-^{13}C]**1**
PLM-A	600	100	100	73	100
	601	29	38 (8%)	100 (52%)	68 (28%)
	602	10	8	28	19
PLM-B	514	100	100	93	80
	515	31	40 (8%)	100 (43%)	100 (48%)
	516	7	10	33	27
PLM-C	614	100	100	74	100
	615	35	43 (7%)	100 (50%)	63 (22%)
	616	9	10	29	20
PLM-D	628	100	100	72	100
	629	38	42 (4%)	100 (50%)	65 (21%)
	630	10	10	30	19
PLM-E	641	100	100	45	74
	642	35	46 (13%)	100 (78%)	100 (68%)
	643	11	15	76	91
	644	0	0	21	29
PLM-F	642	100	100	71	100
	643	38	42 (4%)	100 (51%)	63 (20%)
	644	10	9	31	18

Table 3. Mass spectral analysis of PLMs obtained from a feeding experiment with [$^{2}H_{11}$]**1**

Molecular related ions	(*m*/*z*)	Unlabeled PLMs	Relative intensity obtained from [$^{2}H_{11}$]**1**
PLM-A	600	100	100
	601	29	38
	602	10	9
	609		7
	610		16
	611		5
PLM-B	514	100	100
	515	31	31
	516	7	7
	524		13
	525		23
	526		7
PLM-C	614	100	100
	615	35	34
	616	9	8
	623		6
	624		12
	625		4
PLM-D	628	100	100
	629	38	37
	630	10	9
	637		10
	638		21
	639		6
PLM-E	641	100	100
	642	35	37
	643	11	9
	649		7
	650		12
	651		7
PLM-F	642	100	100
	643	38	37
	644	10	9
	651		7
	652		15
	653		5

no hydroxyl substituent on cyclohexane ring. *cis*-3-Hydroxycyclohexanecarboxylic acid (**4**) and CHC (**1**) can thus be considered as potential starter units. If **4** is utilized for PLM biosynthesis, PLM-B could be produced by dehydration and reduction of 18-hydroxylated precursor before acylation, or biosynthesized from **1** formed by dehydration and reduction of **4** (Scheme 1). In this case, **4** can be biosynthesized via reduction of 1,2-unsaturated intermediate (**6**) from 1-*Re* face, as in the biosynthesis of the DHCHC moiety of FK-520[17,18] and rapamysin,[19,20] or hydroxylation of **1** (Scheme 1). Alternatively, **1** could be used to initiate biosynthesis of all PLMs (Scheme 1). The product of the polyketide synthase might then be PLM-B, which could be converted to other analogs by hydroxylation and acylation (Scheme 1). The specific uptake of **1** and **5** indicated that the starter unit of PLMs is not **4**, but **1** biosynthesized from **5**. The moderate enrichment of PLMs observed in the feeding experiment of **4** can be attributed to contamination of **4** with 5% of **5** and/or inefficient dehydration of **4** by actynomycetes to produce **1** as shown in biosynthesis of ω-cyclohexyl fatty acids in *Alicyclobacillus acidocaldarius*.[16] The fact that all deuterium atoms of [$^2H_{11}$]**1** were incorporated into PLMs has confirmed that **1** was incorporated in an intact form and used as the starter unit directly. A pathway from shikimic acid (**2**) to the coenzyme A activated CHC has been delineated in the ansatrienin producer, *S. collinus*.[14–16] This pathway has been shown to have **5** as an intermediate in this process (Scheme 1). The labeling of PLMs by **1**, **4** and **5** demonstrated in this work is consistent with the same pathway in *Streptomyces* sp. HK-803. A homologous set of genes to those, which encode a CHC–CoA biosynthetic pathway in *S. collinus* might also be predicted.

In conclusion, we have demonstrated that CHC (**1**) is the starter unit in the biosynthesis of all PLMs. The resulting product from the polyketide synthase would then be hydroxylated, before or after conversion to PLM-B, to provide the other PLM analogs. Additional support for this hypothesis has recently come through the cloning and sequencing of the PLM biosynthetic gene cluster. Disruption of a cytochrome P450 monooxygenase gene within this cluster results in a strain, which generates only PLM-B.[27]

4. Experimental

4.1. General procedures and materials

NMR spectra were recorded on JEOL ECP-500 or EX-270 spectrometers and chemical shifts (δ) were referenced to the residual undeuterated solvents as internal standards. LC–MS spectra [positive turbo-ion spray ionization mode; HPLC: Hewlett–Packard Series 1100; column: 150×2.1 mm RP-18 3.5 μm column from Waters; mobile phase: 40:60 CH_3CN–water (containing 0.05% HCO_2H) at a flow rate 0.2 mL/min] were taken on a Perkin–Elmer SCIEX API 2000 pneumatically assisted electrospray triplequadrapole mass spectrometer. Melting points were measured by a Yanagimoto micro melting point apparatus, and were uncorrected. Analytical TLC was carried out on Merck silica gel 60F-254 plates (0.25 mm precoated). Merck silica gel (60, 70–230 mesh) was used for column chromatography. Flash column chromatography was performed on Merck silica gel (60, 230–400 mesh). All reactions were performed under argon atmosphere. Reagents and solvents were purchased at highest commercial quality and used without further purification. K^{13}CN (99% ^{13}C) was purchased from Sigma-Aldrich Co. D_2O (>99.8% D) was purchased from Merck Co.

4.1.1. [2-^{13}C]Shikimic acid ([2-^{13}C]2). This compound was prepared according to the procedure described by Moore et al.[14] ^{1}H NMR (500 MHz, CD_3OD) δ 2.18 (1H, dm, $^1J_{H-H}$=18.4 Hz), 2.69 (1H, dm, $^1J_{H-H}$=18.3 Hz), 3.63–3.71 (1H, m), 3.98 (1H, dd, J=12.4, 5.5 Hz), 4.32–4.40 (1H, m), 6.79 (1H, dm, $^1J_{C-H}$=161.3 Hz, H-2); ^{13}C NMR (125 MHz, CD_3OD) δ 31.6, 67.3 (d, $^1J_{C-C}$=43.9 Hz, C-3), 68.4 (d, $^3J_{C-C}$=1.9 Hz, C-5), 72.7, 130.7 (d, $^1J_{C-C}$=68.7 Hz, C-1), 138.8 (enhanced signal, C-2), 170.0.

4.1.2. (±)*cis*-3-Hydroxy[7-^{13}C]cyclohexanecarboxylic acid ([7-^{13}C]4). The mixture of 2-cyclohexenone (97.0%, 500 mg, 5.04 mmol), triethylamine hydrochloride (1.04 g, 7.55 mmol), K^{13}CN (412 mg, 6.23 mmol), MeOH (2.00 mL) and water (1.00 mL) was stirred under reflux at 70°C for 9 h. The reaction mixture was diluted with saturated aqueous $NaHCO_3$ and $CHCl_3$. The separated aqueous layer was repeatedly extracted with $CHCl_3$. The combined organic layer was dried over Na_2SO_4, and concentrated under reduced pressure. Column chromatography of the resulting crude product with hexane–ether (2:1) gave 3-oxo[7-^{13}C]cyclohexanecarbonitrile (**8**) (430 mg, 69%) as a colorless oil: ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.70–2.23 (4H, m), 2.30–2.48 (2H, m), 2.50–2.73 (2H, m), 2.93–3.12 (1H, m); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 23.8 (d, $^3J_{C-C}$=4.46 Hz, C-5), 28.1 (d, $^2J_{C-C}$=2.23 Hz, C-6), 28.6 (d, $^1J_{C-C}$=56.6 Hz, C-1), 40.7, 43.2 (d, $^2J_{C-C}$=2.23 Hz, C-2), 120.16 (enhanced signal, C-7), 205.18 (d, $^3J_{C-C}$=3.92 Hz, C-3).

The mixture of **8** (430 mg, 3.46 mmol), MeOH (16.0 mL) and H_2SO_4 (95.0%, 1.95 mL, 34.7 mmol) was stirred under reflux for 15 h. The reaction mixture was diluted with saturated aqueous $NaHCO_3$ and $CHCl_3$. The separated aqueous layer was repeatedly extracted with $CHCl_3$. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. Column chromatography of the resulting crude product with hexane–EtOAc (10:1) gave methyl 3-oxo[7-^{13}C]cyclohexanecarboxylate (**7**) (301 mg, 55%) as a colorless oil: ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.65–1.94 (2H, m), 1.99–2.18 (2H, m), 2.25–2.44 (2H, m), 2.55 (2H, dd, J_{H-H}=7.97 Hz, $^3J_{C-H}$=3.11 Hz), 2.71–2.89 (1H, m), 3.70 (3H, d, $^3J_{C-H}$=3.78 Hz); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 24.3 (d, $^3J_{C-C}$=5.00 Hz, C-5), 27.6 (d, $^2J_{C-C}$=1.62 Hz, C-6), 40.7, 42.88 (d, $^1J_{C-C}$=58.3 Hz, C-1), 42.93 (d, $^2J_{C-C}$=1.69 Hz, C-2), 51.9 (d, $^2J_{C-C}$=2.77 Hz, OMe), 174.1 (enhanced signal, C-7), 208.6 (d, $^3J_{C-C}$=4.46 Hz, C-3).

$NaBH_4$ (90.0%, 32 mg, 0.76 mmol) was added to a solution of **7** (301 mg, 1.92 mmol) in MeOH (7.68 mL) at 0°C. The reaction mixture was stirred at room temperature for 15 min and diluted with 2N HCl and ether. The separated aqueous layer was repeatedly extracted with ether. The combined organic layer was washed with saturated aqueous $NaHCO_3$

and brine, dried over Na_2SO_4, and concentrated under reduced pressure. Flash column chromatography of the resulting crude product with hexane–ether (4:1) gave methyl (±)-*cis*-3-hydroxy[7-^{13}C]cyclohexanecarboxylate (**9**) (181 mg, 59%). **9**: colorless oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.15–1.49 (4H, m), 1.70–2.01 (4H, m), 2.13–2.24 (1H, m), 2.29–2.45 (1H, m), 3.62 (1H, tt, J=9.90, 4.20 Hz), 3.67 (3H, d, $^3J_{C-H}$=3.90 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 23.1 (d, $^3J_{C-C}$=5.55 Hz, C-5), 28.0 (d, $^2J_{C-C}$=1.88 Hz, C-6), 34.9, 37.6 (d, $^2J_{C-C}$=1.88 Hz, C-2), 41.7 (d, $^1J_{C-C}$=58.1 Hz, C-1), 51.7, (d, $^2J_{C-C}$=2.48 Hz, OMe), 69.7 (d, $^3J_{C-C}$=4.95 Hz, C-3), 175.5 (enhanced signal, C-7).

Sodium hydroxide (97.0%, 175 mg, 4.24 mmol) was added to the solution of **9** (335 mg, 2.10 mmol) in H_2O (22.3 mL). The mixture was stirred at room temperature for 12 h and then continuously extracted with ether. The separated aqueous layer was acidified with 6N HCl and continuously extracted with ether. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to afford [7-^{13}C]**4** (214 mg, 70%) as a white solid; mp 129–130°C; ^{1}H NMR (500 MHz, acetone-d_6) δ 1.09–1.37 (4H, m), 1.42–1.60 (0.2H, m, for **5**), 1.73–1.93 (3H, m), 2.16 (1H, dm, J=12.4 Hz), 2.27–2.37 (1H, m), 2.70–2.78 (0.05H, m, H-1 of **5**), 3.54 (1H, tt, J=10.8, 4.13 Hz), 3.96–4.01 (0.05H, m, H-3 of **3**); ^{13}C NMR (125 MHz, acetone-d_6) δ 23.4 (d, $^3J_{C-C}$=4.76 Hz, C-5), 28.3, 35.2, 38.3, 41.5 (d, $^1J_{C-C}$=56.3 Hz, C-1), 69.0 (d, $^3J_{C-C}$=5.73 Hz, C-3), 175.6 (enhanced signal, C-7). Anal. calcd for $C_6^{13}CH_{12}O_3$: C+^{13}C, 57.92; H, 8.33. Found: C+^{13}C, 57.76; H, 8.32.

4.1.3. (±)*trans*-3-Hydroxy[7-^{13}C]cyclohexanecarboxylic acid ([7-^{13}C]5). K-selectride (7.64 mL, 1.0 M solution in THF, 7.64 mmol) was added dropwise to a solution of **7** (600 mg, 3.82 mmol) in THF (39.0 mL) at −20°C and the mixture was stirred at the same temperature for 30 min. The reaction mixture was diluted with saturated aqueous NH_4Cl and ether. The separated aqueous layer was repeatedly extracted with ether. The combined organic layer was dried over Na_2SO_4, and concentrated under reduced pressure. Flash column chromatography of the resulting crude product with hexane–ether (2:1) gave methyl (±)-*trans*-3-hydroxy[7-^{13}C]-cyclohexanecarboxylate (**10**) (682 mg, 56%) as a colorless oil: ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.35–1.94 (8H, m), 2.70–2.83 (1H, m), 4.02–4.13 (1H, m); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 20.4 (d, $^3J_{C-C}$=3.38 Hz, C-5), 28.6 (d, $^2J_{C-C}$=1.69 Hz, C-6), 33.4, 36.1 (d, $^2J_{C-C}$=1.69 Hz, C-2), 38.3 (d, $^1J_{C-C}$=57.2 Hz, C-1), 52.1, (d, $^2J_{C-C}$=2.77 Hz, OMe), 66.6 (d, $^3J_{C-C}$=3.31 Hz, C-3), 176.6 (enhanced signal, C-7).

The ester **10** (682 mg, 4.28 mmol) was hydrolyzed to [7-^{13}C]**5** (461 mg, 74%) in the same manner as described for [7-^{13}C]**4**. [7-^{13}C]**5**: white solids; mp 101–102°C; ^{1}H NMR (500 MHz, acetone-d_6) δ 1.14–1.42 (0.4H, m, for **4**), 1.47–1.64 (4H, m), 1.71–1.90 (4H, m), 2.18–2.24 (0.1H, H-2 of **4**), 2.32–2.42 (0.1H, m, H-1 of **4**), 2.75–2.84 (1H, m), 3.58 (0.1H, tt, J=11.0, 4.13 Hz, H-3 of **4**), 3.99–4.06 (1H, m); ^{13}C NMR (125 MHz, acetone-d_6) δ 19.8 (d, $^3J_{C-C}$=3.81 Hz, C-5), 28.4, 32.9, 35.9, 37.4 (d, $^1J_{C-C}$=56.3 Hz, C-1), 65.0 (d, $^3J_{C-C}$=2.86 Hz, C-3), 176.3 (enhanced signal, C-7); Anal. calcd for $C_6^{13}CH_{12}O_3$: C+^{13}C, 57.92; H, 8.33. Found: C+^{13}C, 57.65; H, 8.36.

4.1.4. [7-^{13}C]Cyclohexanecarboxylic acid ([7-^{13}C]1). Cyclohexanone (200 mg, 2.04 mmol) and 2,4,6-triisopropylbenzenesulfonyl hydrazide (761 mg, 2.55 mmol) were stirred together in MeOH (3.00 mL) solution at room temperature for 1.5 h. K^{13}CN (405 mg, 6.12 mmol) was then added and the reaction mixture was stirred under reflux for 4 h. The reaction mixture was diluted with saturated aqueous $NaHCO_3$ and CH_2Cl_2. The separated aqueous layer was repeatedly extracted with CH_2Cl_2. The combined organic layer was dried over Na_2SO_4, and concentrated under reduced pressure to give [7-^{13}C]cyclohexanecarbonitrile (**11**) (178 mg, 79%) as a pale yellow oil which was used for the next step without further purification: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.15–1.96 (10H, m), 2.56–2.71 (1H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.0 (d, $^3J_{C-C}$=3.08 Hz, C-3), 25.2, 27.9 (d, $^1J_{C-C}$=55.0 Hz, C-1), 29.4 (d, $^2J_{C-C}$=2.48 Hz, C-2), 122.6 (enhanced signal, C-7).

The mixture of **11** (325 mg, 2.95 mmol), EtOH (10.8 mL), KOH (85%, 1.95 g, 29.5 mmol) was stirred under reflux for 6 h. The mixture was diluted with ether and H_2O. The separated organic layer was repeatedly extracted with 15% aqueous NaOH. The combined aqueous layer was acidified with 6N HCl and extracted with ether. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Column chromatography of the resulting crude product with hexane–ether (5:1) afforded [7-^{13}C]**1** (204 mg, 54%) as a colorless oil: ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.24–2.00 (10H, m), 2.33 (1H, tdt, J_{H-H}=10.9 Hz, $^2J_{C-H}$=7.1 Hz, J_{H-H}=3.6 Hz), 11.4 (1H, bs); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 25.4, (d, $^3J_{C-C}$=3.85 Hz, C-3), 25.8, 28.8 (d, $^2J_{C-C}$=1.08 Hz, C-2), 43.0 ($^1J_{C-C}$=55.0 Hz, C-1), 182.6 (enhanced signal, C-7).

4.1.5. [$^2H_{11}$]Cyclohexanecarboxylic acid ([$^2H_{11}$]1). This compound was prepared according to the procedure described by Pojer.[26] ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.14–1.31 (0.3H, m), 1.41 (0.3H, bs), 1.70 (0.2H, bs), 1.88 (0.2H, bs), 2.30 (0.2H, bs), 11.52 (1H, bs); ^{13}C NMR (125 MHz, $CDCl_3$), δ 23.6–25.4 (m), 27.2–28.6 (m), 41.8–42.8 (m), 182.4; ^{2}H NMR (77 MHz, $CHCl_3$) δ 1.08–1.31 (3D), 1.39 (2D), 1.69 (2D), 1.87 (2D), 2.27 (1D); ESI MS (direct infusion, negative turbo-ion spray ionization mode, Perkin–Elmer SCIEX API 2000 pneumatically assisted electrospray triplequadrapole mass spectrometer) m/z 136 (16.7), 137 (45.1), 138 (100), 139 (8.1).

4.2. Fermentation

Streptomyces sp. HK-803 was maintained at 15°C on SY agar slants (0.1% yeast extract, 1.0% soluble starch, 0.1% N–Z amine type A, 1.5% agar, pH 7.0). A loopful of spore was inoculated into a 500 mL cylindrical flask containing 70 mL of the production medium (2.0% glucose, 0.1% beef extract, 1.0% soybean flour, 0.2% NaCl, 0.005% K_2HPO_4, 0.2% phenylalanine, pH 7.0). The inoculated cultures were incubated at 28°C in the dark for 96 h on a rotary shaker at 170 rpm.

4.3. Feeding experiments with labeled compounds

Labeled compounds were dissolved in EtOH and added directly to each flask 30 h after inoculation in the amount

indicated in parenthesis: [7-^{13}C]**1** (10 mg), [7-^{13}C]**2** (30 mg), [7-^{13}C]**4** (20 mg), [7-^{13}C]**5** (20 mg), [$^{2}H_{11}$]**1** (10 mg).

4.4. Isolation of PLMs

The cultures were worked up, as previously described.[7] Preparative HPLC was performed on a system equipped with a Waters™ 600 pump and a Waters 996 photodiode array detector using a following conditions: column, Senshu Pak PEGASIL ODS (20×250 mm); mobile phase: 40:60 CH_3CN–water (containing 0.05% HCO_2H) at a flow rate 9.0 mL/min; detection at UV 235 nm.

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