Biosynthesis of tetrapetalones

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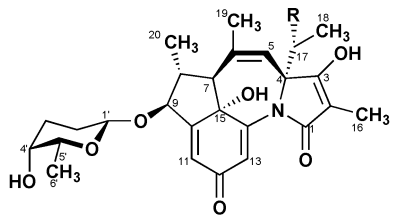
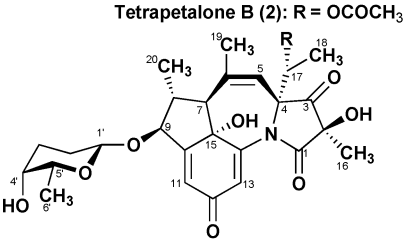
The biosynthesis of tetrapetalones (tetrapetalones A, B, C, and D) in *Streptomyces* sp. USF-4727 was studied by feeding experiments with [1-^{13}C] sodium propanoate, [1-^{13}C] sodium butanoate, [carbonyl-^{13}C] 3-amino-5-hydroxybenzoic acid (AHBA) hydrochloride, and [1-^{13}C] glucose, followed by analysis of the ^{13}C-NMR spectra. These feeding experiments revealed that the four tetrapetalones were polyketide compounds constructed from propanoate, butanoate, AHBA, and glucose. The tetrapetalone biosynthetic pathway was also suggested in this study. In this pathway, tetrapetalone A (**1**) is synthesized by polyketide synthase (PKS) using AHBA as a starter unit, then the side chain of **1** is subjected to acetoxylation to produce tetrapetalone B (**2**). Additionally, **1** is oxidized and transformed into tetrapetalone C (**3**). In a similar way, **2** is converted to tetrapetalone D (**4**). Therefore, the biosynthetic relationship of the four tetrapetalones was indicated.

Introduction

We are investigating the discovery of new bioactive compounds from microorganisms by using a lipoxygenase assay.[1] Human lipoxygenase and cyclooxygenase catalyze the first step in the arachidonic pathway, resulting in the production of some important signaling molecules that may be involved in a variety of human diseases.[2–4] Soybean lipoxygenase is used for our screening assay. This enzyme shows 15-lipoxygenase activity in arachinonate.

Tetrapetalones A (**1**), B (**2**), C (**3**), and D (**4**), were isolated from the culture broth of *Streptomyces* sp. USF-4727 as inhibitors of lipoxygenase.[5–7] Their chemical structures were elucidated by using spectroscopic methods to show that these compounds are constructed with a characteristic tetracyclic skeleton and a sugar moiety. Because the tetracyclic skeleton of these tetrapetalones was novel, we investigated the biosynthetic precursors and the biosynthetic pathway to these tetrapetalones.





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Results and discussion

To explore the biosynthetic origins of tetrapetalones, we added ^{13}C-labeled compounds to the culture of the tetrapetalone-producing strain, *Streptomyces* sp. USF-4727. After isolation of each tetrapetalone from this culture, the incorporation ratio of the ^{13}C-labeled compounds was evaluated.

In our feeding experiment of ^{13}C-labeled compounds, we identified that [1-^{13}C] propanoate, [1-^{13}C] butanoate, [1-^{13}C] glucose and [carbonyl-^{13}C] AHBA are efficiently incorporated into tetrapetalone A (**1**), and that the incorporation rate of [1-^{13}C] and [2-^{13}C] acetate into **1** was relatively low level in this experiment (Table 1).

As shown in Table 1, the peaks of C-1, -5, and -7 were highly enriched (each enrichment ratio >30.0) in the experiments with [1-^{13}C] propanoate. The carbons C-3 and C-9 were also remarkably enriched (each enrichment ratio >45.0) in the experiments with [1-^{13}C] butanoate and [carbonyl-^{13}C] AHBA, respectively.

These results indicated that the aglycon of **1** is constructed from three molecules of propanoate, one butanoate, and one AHBA, as shown in Fig. 1.

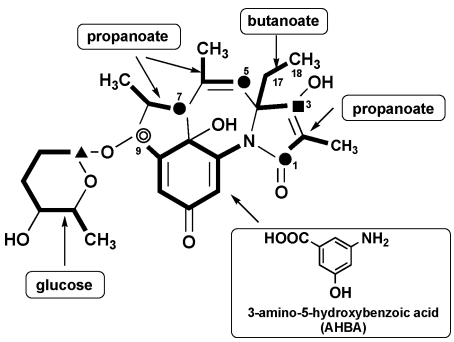

Fig. 1 Biosynthetic precursors for tetrapetalone A.

Table 1 Incorporation of ^{13}C-labeled compounds[a]

Position	Enrichment ratio (tetrapetalone A)						Enrichment ratio (tetrapetalone B)
	[1-^{13}C]AA	[2-^{13}C]AA	[1-^{13}C]PA	[1-^{13}C]BA	[1-^{13}C]G	^{13}C-AHBA	[1-^{13}C]AA
1	*3.4*[b]	*3.0*	**39.0**	*3.2*	Nd[c]	1.5	1.0
2	1.4	0.9	1.1	0.7	1.7	1.4	0.7
3	*3.2*	1.5	0.9	**45.1**	1.3	1.7	1.5
4	0.6	*3.1*	0.5	0.5	1.1	1.0	1.0
5	*3.8*	*2.5*	**37.5**	5.4	1.3	1.5	1.7
6	1.3	*2.5*	0.3	0.6	1.2	0.7	1.3
7	*3.5*	*2.9*	**34.4**	0.6	1.0	1.0	*2.1*
8	1.3	*3.6*	0.5	0.7	1.1	0.5	1.4
9	1.2	0.8	0.9	0.8	0.7	**58.3**	1.3
10	1.2	0.6	1.1	1.1	0.9	0.6	0.9
11	0.9	1.0	1.1	1.0	1.5	1.2	0.6
12	0.8	0.6	1.1	0.5	0.6	0.7	1.0
13	0.8	0.8	1.1	0.7	0.8	1.3	1.0
14	0.7	0.6	0.9	0.6	0.7	0.8	1.0
15	0.6	0.9	0.4	0.7	1.3	0.7	1.0
16	1.1	0.6	1.0	0.7	1.7	1.5	0.9
17	*6.3*	0.9	1.1	1.0	0.9	1.5	*2.8*
18	0.7	*3.0*	0.8	0.7	0.9	0.9	0.9
19	1.1	*3.3*	0.7	0.9	1.3	1.2	0.8
20	1.1	*3.2*	1.2	1.0	1.3	1.2	0.7
1′	0.9	1.5	0.9	0.8	**2.1**	1.0	0.7
2′	0.9	1.0	1.0	0.9	0.9	0.9	0.9
3′	1.5	1.2	1.2	1.4	1.2	1.4	1.1
4′	1.3	1.0	1.2	1.2	0.9	1.2	1.0
5′	1.8	1.4	1.2	1.4	1.3	1.5	1.2
6′	1.0	1.0	1.0	1.0	1.0	1.0	1.0
17-O**C**OCH$_3$	—	—	—	—	—	—	**4.4**
17-OCO**C**H$_3$	—	—	—	—	—	—	0.8

[a] [1-^{13}C]AA: CH$_3$^{13}COONa, [2-^{13}C]AA: ^{13}CH$_3$COONa, [1-^{13}C]PA: CH$_3$CH$_2$^{13}COONa, [1-^{13}C]BA: CH$_3$CH$_2$CH$_2$^{13}COONa, [1-^{13}C]G: [1-^{13}C] glucose, ^{13}C-AHBA: [carbonyl-^{13}C] 3-amino-5-hydroxybenzoic acid. [b] Enrichment ratio was calculated from the relative intensity of C-6′ as 1.0. [c] Not detected.

However, the relatively low incorporation rate of [1-^{13}C] and [2-^{13}C] acetate into **1** suggested that acetate was not directly incorporated into **1**. In fatty acid biosynthesis and in macrolide antibiotic biosynthesis, two molecules of acetyl-CoA could be joined in a Claisen condensation to form an acetoacetyl-CoA.[8,9] This condensation might be observed in our ^{13}C-labeled sodium acetate feeding experiment to produce **1**. The four carbons C-3, -4, -17, and -18 were shown to be derived from butanoate described above. However, C-3 and C-17 were also enriched in the [1-^{13}C] sodium acetate feeding experiment. The [2-^{13}C] sodium acetate feeding experiment lead to the enhancement of the signal intensity at C-4 and C-18 in the ^{13}C-NMR spectrum, showing that this C_4 unit was formed with two molecules of acetate in a head-to-tail pattern. Other relatively low enrichment ratios in the ^{13}C-labeled acetate feeding experiment also suggested that acetate was used in forming the C_3 unit (*i.e.* methylmalonyl-CoA) derived from propanoate, then incorporated into tetrapetalone A.

In the [1-^{13}C] glucose feeding experiment, the enrichment ratio of C-1′ was 2.1, indicating that the 2,3,6-trideoxy-D-galactosyl moiety of **1** is derived from glucose. This relatively low enrichment ratio (2.1) was explained as follows: because glucose is a major carbon source for microorganisms, [1-^{13}C] glucose fed to the culture broth of USF-4727 is not consumed in the tetrapetalone biosynthetic system alone, but is also distributed to many other metabolic and biosynthetic systems. Therefore, only a part of the labeled glucose was incorporated into **1** to give the relatively low enrichment ratio at the C-1′ position in the ^{13}C-NMR spectrum. Similar low enrichment ratios are often observed in other ^{13}C-labeled glucose feeding experiments.[10–12]

The origin of the carbon atoms in tetrapetalone B (**2**), except for the acetoxy group joining to the carbon at C-17, was estimated by the result of the feeding experiments for tetrapetalone A (**1**) described above. In this study, it was also revealed that the acetoxy group in **2** is derived from an acetate unit.

As shown in Table 1, the carbonyl carbon of the acetoxy group joined to C-17 in **2** was enriched (enrichment ratio: 4.4) by feeding with [1-^{13}C] sodium acetate. This result suggests that the labeled acetate was used for constructing the C-17 side chain of **2**. Moreover, [1,2-^{13}C$_2$] the sodium acetate feeding experiment supported this suggestion. In the feeding experiment with [1,2-^{13}C$_2$] sodium acetate, we observed a $J_{C\text{-}C}$ coupling (coupling constant: 59 Hz) only between the carbonyl carbon and the acetoxy methyl carbon in the ^{13}C-NMR spectrum of **2**. This observation indicated that these two carbons are constructed with the [1,2-^{13}C$_2$] acetate and that the side chain at C-17 in **2** is derived from an acetate unit.

Subsequently, we proposed a transformational scheme for tetrapetalones (A, B, C and D) by the results of these feeding experiments and the oxidation experiment of **1** described below.

To explore the chemical properties of **1**, we treated **1** with H_2O_2 (*aq.*) in 0.2 M borate buffer (pH 9.0). The reaction mixture was analyzed by HPLC every 30 minutes. In the reaction mixture

of **1** with H_2O_2 (*aq.*), the concentration of **1** was gradually decreased, while an unknown compound emerged in the HPLC chromatogram as shown in Fig. 2. In the control test, using H_2O instead of H_2O_2, no emergence of the unknown compound was observed. This observation showed that **1** is transformed to another compound by treatment with H_2O_2. We also observed a moderate reduction in the concentration of **1** in the control test of this experiment, showing that **1** is not completely stable under these conditions.

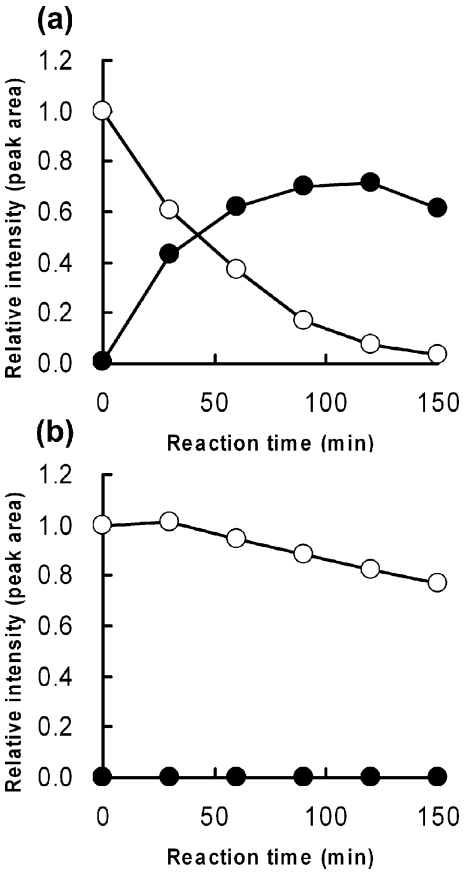

Fig. 2 Reaction of tetrapetalone A with H_2O_2: (a) treatment with H_2O_2 (*aq.*), (b) treatment with H_2O (control), ○: tetrapetalone A, ●: tetrapetalone C.

For the structure elucidation of the unknown compound, we treated 65 mg of **1** with H_2O_2 (*aq.*). After purification of the reaction mixture, we obtained 30 mg of the reaction product. The ^{1}H- and ^{13}C-NMR spectral data, HPLC retention time and the optical rotation $[\alpha]_D$ of this product were completely consistent with those of tetrapetalone C (**3**), thereby identifying this reaction product as **3**. These results indicated that **1** was subject to oxidation under these *in vitro* conditions, and was then transformed to **3**. In a similar way, **2** is implied to be converted to tetrapetalone D (**4**), indicating that **1** and **2** might be converted to **3** and **4**, respectively, in the culture broth of the tetrapetalone-producing strain.

The 3-amino-5-hydroxybenzoic acid (AHBA) unit is suggested as the biological starter unit for the *meta*-C_7N units of the aromatic chromophores in several ansamycin antibiotics.[13,14] In this report, it is revealed that the tetrapetalones are polyketide compounds constructed by AHBA, propanoate, butanoate, and glucose, indicating that tetrapetalones are biologically synthesized in a similar scheme to the ansamycin antibiotics. The ansamycin antibiotics have a common structural feature which is the "ansa" bridge system of polyketide origin initiated from AHBA as a starter unit. In the tetrapetalone biosynthetic pathway, a compound which has an "ansa" bridge system in its chemical structure should be synthesized as an intermediate compound, and then this bridge should be modified to form the tetracyclic structure. The details of the formation process of the tetracyclic skeleton are now under investigation.

The transformational scheme of tetrapetalones proposed due to this study is shown in Fig. 3. At first, tetrapetalone A (**1**) is synthesized by polyketide synthase using AHBA as a starter unit. After receiving an oxygen atom to give the hydroxy group at C-17 in **1**, the side chain of **1** should be subjected to acetoxylation, then form tetrapetalone B (**2**). On the other hand, **1** and **2** should be oxidized and transformed to tetrapetalones C (**3**) and D (**4**), respectively, in the culture broth of *Streptomyces* sp. USF-4727.

In this study, we were able to reveal the origins of tetrapetalones A, B, C, and D and indicate their proposed transformational scheme.

Experimental

Chemicals

[1-^{13}C] and [2-^{13}C] sodium acetate, [1-^{13}C] sodium butanoate, and [1-^{13}C] glucose were purchased from Sigma Co. (St. Louis, MO, USA). [1-^{13}C] sodium propanoate, and [carbonyl-^{13}C] benzoic acid were obtained commercially from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). [Carbonyl-^{13}C] 3-amino-5-hydroxybenzoic acid (AHBA) hydrochloride was prepared in our laboratory as described below.

Instruments

Spectroscopic measurements were taken with the following instruments: NMR, JEOL Alpha-400 spectrometer (tetramethylsilane as internal reference at 0 ppm for ^{1}H- and ^{13}C-NMR); FAB-MS, JEOL JMS-700 spectrometer; UV and visible spectra, Shimadzu UV-160A spectrometer; melting point, Yanagimoto MP-J3; IR, Jasco FT/IR-550; HPLC for analysis, Jasco PU-2080 Plus, Jasco UV 2077 Plus, Jasco MX-2080-32, and HPLC for preparation; Jasco 880-PU, Jasco UV-970.

Cultivation of a *Streptomyces* sp USF-4727 strain

The strain USF-4727 was inoculated into 800 mL of the medium (0.4% glucose, 0.4% yeast extract, 1.0% malt extract, pH 7.3) in a 2 litre Erlenmeyer flask and cultivated at 30 °C for 10–12 days on a rotary shaker (130 rpm).

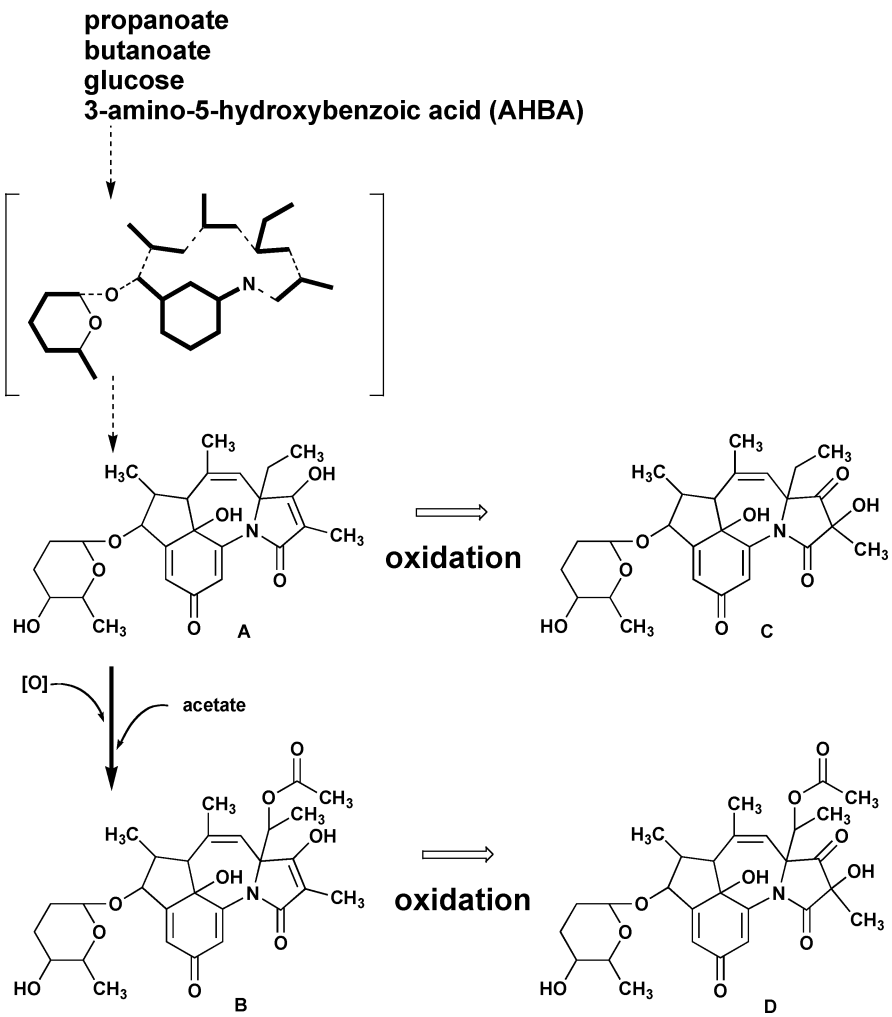

Fig. 3 Proposed transformational scheme for tetrapetalones.

Isolation of tetrapetalones A (1), B (2), C (3), and D (4)

The culture filtrate of *Streptomyces* sp. USF-4727 strain was purified by chromatography on a Diaion HP-20 column, silica gel column, Sephadex LH-20 (MeOH), and preparative HPLC to yield **1**, **2**, **3**, and **4**. The details of the isolation of the four tetrapetalones are described in our previous papers.[5,6]

Tetrapetalone A (1). Pale yellow amorphous powder; melting point, 190 °C. HRFAB MS $[M + H]^+$, m/z 472.2354 (472.2335 calcd. for $C_{26}H_{34}NO_7$). UV–VIS λ_{max} (MeOH): 219 nm (ε 18300), 245 nm (sh. ε 13300), 340 nm (ε 11700). IR λ_{max} (KBr) cm^{-1}: 3400, 1670, 1380, 1300, 1250, 1170, 1060, 1020. The ^{1}H- and ^{13}C-NMR data in CD_3OD are shown in our previous paper.[5]

Tetrapetalone B (2). Pale yellow amorphous powder; melting point, 191–193 °C. HRFAB MS $[M + H]^+$, m/z 530.2407 (530.2390 calcd. for $C_{28}H_{36}NO_9$). UV–VIS λ_{max} (MeOH): 217 nm (ε 9310), 336 nm (ε 4190). IR ν_{max} (KBr) cm^{-1}: 3420, 1580, 1570, 1560, 1540, 1250. The ^{1}H- and ^{13}C-NMR data in CD_3OD are shown in our previous paper.[6]

Tetrapetalone C (3). Colorless amorphous powder; melting point, 154–157 °C. HRFAB MS $[M + H]^+$, m/z 488.2286 (488.2284 calcd. for $C_{26}H_{34}NO_8$). UV–VIS λ_{max} (MeOH): 206 nm (ε 10350), 250 nm (sh. ε 5890), 311 nm (ε 3190), 350 nm (sh. ε 2900). IR ν_{max} (KBr) cm^{-1}: 3400, 1720, 1630, 1380, 1280, 1170, 1060, 1020. The ^{1}H- and ^{13}C-NMR data in CD_3OD are shown in our previous paper.[6]

Tetrapetalone D (4). Colorless amorphous powder; melting point, 148–151 °C. HRFAB MS $[M + H]^+$, m/z 546.2352 (546.2339 calcd. for $C_{28}H_{36}NO_{10}$). UV–VIS λ_{max} (MeOH): 208 nm (sh. ε 9920), 247 nm (sh. ε 4470), 312 nm (ε 1720), 335 nm (sh. ε 1440). IR ν_{max} (KBr) cm^{-1}: 3420, 1720, 1630, 1380, 1240, 1160, 1120, 1060, 1020. The ^{1}H- and ^{13}C-NMR data in CD_3OD are shown in our previous paper.[6]

Feeding experiments with [1-^{13}C] and [2-^{13}C] sodium acetate, and [1-^{13}C]sodium propanoate

[1-^{13}C] Sodium acetate (800 mg), [2-^{13}C] sodium acetate (800 mg), and [1-^{13}C] sodium propanoate (400 mg) were added to the culture of *Streptomyces* sp. USF-4727 strain in 800 mL of medium (0.4% glucose, 0.4% yeast extract, 1.0% malt extract, pH 7.3) on day 5–7 from the beginning of the incubation. At day 10–12, each culture was filtered and the tetrapetalones in these cultures were isolated and submitted to further investigation.

Feeding experiment with [1,2-$^{13}C_2$] sodium acetate

[1,2-$^{13}C_2$] Sodium acetate was added to two of the cultivative Erlenmeyer flasks (each 300 mg) containing 800 mL of culture at day 7 with non-labeled sodium propanoate (800 mg) and non-labeled sodium butanoate (250 mg). On day 10, the culture broth was filtered and each tetrapetalone was isolated and submitted to further investigation.

Feeding experiment with [1-^{13}C] sodium butanoate

[1-^{13}C] Sodium butanoate (250 mg) was added to the culture (800 mL) with non-labeled sodium propanoate (800 mg) on day 7 from the beginning of incubation. On day 10, the culture was filtered, and each tetrapetalone in the culture was isolated and used for further investigation.

Feeding experiment with [1-^{13}C] glucose

The culture medium of this feeding experiment was different from other feeding experiments. The strain USF-4727 was inoculated to the "glucose-less" medium (0.4% yeast extract, 1.0% malt extract, pH 7.3). The cultivation was done in a similar way to other feeding experiments. On day 5 after the inoculation of the tetrapetalone-producing strain, [1-^{13}C] glucose (100 mg) was added to the culture with non-labeled sodium propanoate (800 mg). On day 10, the culture was filtered, and each tetrapetalone in the culture was isolated in a similar way to the other feeding experiments.

Preparation of [carbonyl-^{13}C] 3-amino-5-hydoxybenzoic acid (AHBA) hydrochloride

First, we prepared non-labeled AHBA hydrochloride on the basis of the method by Becker *et al.*[15] After structure elucidation of this non-labeled compound by spectroscopic methods, we referred to the spectral data for the structure elucidation of [carbonyl-^{13}C] AHBA hydrochloride. In the preparation of non-labeled AHBA hydrochloride, benzoic acid was used as the starting compound. First, benzoic acid was treated with fuming sulfuric acid to form 3,5-disulfonicbenzoic acid. The reactant was neutralized with barium carbonate. This barium salt was reacted with sodium and potassium hydroxides. After dissolving in water, the reactant was extracted with diethyl ether (pH 3.0) to give 3,5-dihydroxybenzoic acid.[16] Then, this 3,5-dihydroxybenzoic acid was converted to AHBA hydrochloride by reaction with NH_4Cl and 28% *aq.* NH_3 in a steel bomb at 180 °C for 40 h.[8] Finally, we obtained AHBA hydrochloride (1.8 g, 58%) by using benzoic acid (2.0 g) as a starting compound. ^{1}H-NMR of non-labeled AHBA (400 MHz; CD_3OD; Me_4Si) δ_H: 7.05 (1 H, t, *J* 2.3 Hz), 7.49 (1 H, m), 7.52 (1 H, m); ^{13}C-NMR of 3-amino-5-hydroxybenzoic acid (100 MHz; CD_3OD) δ_C: 115.4 (d), 115.7 (d), 118.0 (d), 133.2 (s), 135.6 (s), 160.5 (s), and 168.0 (s); HRFAB MS (glycerol) $[M + H]^+$, *m/z* 154.0509 (154.0505 calcd. for $C_7H_8NO_3$). In the preparation of [carbonyl-^{13}C] AHBA hydrochloride, [carbonyl-^{13}C] benzoic acid was used as the starting compound. Other procedures were similar to those for non-labeled AHBA hydrochloride preparation, then we obtained [carbonyl-^{13}C] AHBA hydrochloride (1.2 g, 38%) by using [carbonyl-^{13}C] benzoic acid (2.0 g). In the structure elucidation of [carbonyl-^{13}C] AHBA hydrochloride, the ^{1}H-NMR spectrum (CD_3OD, 400 MHz) was consistent with that of non-labeled AHBA. However, in the ^{13}C-NMR spectrum (CD_3OD, 100 MHz) of [carbonyl-^{13}C] AHBA, the carbon signal at 168.0 ppm was extremely enriched, at least 100 times higher than that of non-labeled 3-amino-5-hydroxybenzoic acid.

Feeding experiment with [carbonyl-^{13}C] 3-amino-5-hydroxybenzoic acid (AHBA) hydrochloride

[Carbonyl-^{13}C] AHBA hydrochloride (200 mg) was added to the culture with non-labeled sodium propanoate (800 mg) and non-labeled sodium butanoate (250 mg) on day 7 from the beginning of incubation. On day 10, the culture was filtered, and each tetrapetalone in the culture was isolated and used for further investigation.

Evaluation of the incorporation of ^{13}C-labeled precursors

We evaluated the incorporation ratio of each ^{13}C-labeled compound by measurement of the ^{13}C-NMR spectrum. Tetrapetalones A (**1**) and B (**2**) isolated from the each culture broth to which were added the ^{13}C-labeled compounds were subject to measurement of the ^{13}C-NMR spectrum. The signal intensity of each peak in this spectrum was measured to evaluate the signal enrichment. The enrichment ratio was given from each peak height compared to the standard peak height (C-6′) in each ^{13}C-NMR spectrum.

Treatment of tetrapetalone A (1) with H_2O_2

First, we treated **1** with H_2O_2 solution for HPLC analysis. Two hundred microlitres of 30% H_2O_2 (*aq.*) were added to 0.2 mg of **1** in 0.5 mL of 0.2 M borate buffer (pH 9.0) to start the reaction. The mixture was analyzed by HPLC (25% CH_3CN–10 mM phosphate buffer (pH 2.6), Capcell Pak C18 SG120, ϕ 4.6 × 250 mm, UV 254 nm) every 30 minutes. Then we treated **1** with H_2O_2 for the HPLC preparation. Six millilitres of 30% H_2O_2 (*aq.*) were added to the 65 mg of tetrapetalone A in 10 mL of 0.2 M borate buffer (pH 9.0), standing for 180 min. The mixture was applied to the Diaion HP-20 (100 g) column. After being washed with water, the reaction product was eluted with MeOH. The MeOH fraction was purified by preparative HPLC (25% CH_3CN–10 mM phosphate buffer (pH 2.6), Capcell Pak C18 SG120, ϕ 15 × 250 mm, UV 254 nm) to yield 30 mg of the reaction product. The chemical structure of this compound was elucidated by spectroscopic methods.

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