# The Biosynthesis of Protoanemonin in *Ranunculus glaber*. The Pivotal Biosynthetic Intermediate and the Stereospecific Hydrogen Elimination from the Intermediate

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The pivotal biosynthetic intermediate directly leading to protoanemonin was established to be 2-oxoglutarate on the basis of the labeling patterns in protoanemonin biosynthesized from sodium acetate-1-$^{14}C$, sodium malonate-2-$^{14}C$, sodium succinate-2,3-$^{14}C_2$, sodium glutamate-5-$^{14}C$, and sodium 2-oxoglutarate-5-$^{14}C$ by *Ranunculus glaber*. Also the $^{3}H/^{14}C$ ratio in protoanemonin biosynthesized from (3*R*)-2-oxoglutaric-3-$^{3}H_1$-3,4-$^{14}C_2$ and 2-oxoglutaric-3-$^{3}H_2$-3,4-$^{14}C_2$ acids demonstrated that the formation of the double bond at the 3-position of protoanemonin involves the stereospecific elimination of the *pro*-3*S* hydrogen atom from 2-oxoglutaric acid.

It has been well known that many of the plants belonging to Ranunculaceae family show antimicrobial activities and, in some cases, the bruised tissues of the plants cause an intense burn or blister to the skin.[1-5] Such properties of the plants are described to be owing to protoanemonin (**1**), which is probably enzymatically liberated from ranunculin (**2**) by crushing the tissues of the *Ranunculus* plants.[6] It is documented that many $C_5$ compounds similar to protoanemonin have been found in the form of a glycoside in *Ranunculus* species.[7] Although the biosynthetic pathway of such a simple lactone is of considerable interest, it has not been investigated yet. We have performed feeding experiments of the radioisotopically labeled compounds into protoanemonin (**1**) with the intact plant of *Ranunculus glaber* Makino for clarifying the pivotal biosynthetic intermediate directly leading to the formation of protoanemonin (**1**) and then the stereospecificity in the hydrogen elimination from the intermediate at the stage of the double bond formation in **1**. The findings of the feeding experiments have been partly outlined in preliminary communications.[8-10] We here wish to describe, *en bloc*, details of the results.

## Results and Discussion

*Biosynthetic Pathway of Protoanemonin (**1**) in* R. glaber. The feeding experiments were carried out on the sprigs of *R. glaber*. The radioisotopically labeled compounds used for the feeding experiments were sodium acetate-1-$^{14}C$, sodium malonate-2-$^{14}C$, sodium succinate-2,3-$^{14}C_2$, sodium glutamate-5-$^{14}C$, and sodium 2-oxoglutarate-5-$^{14}C$. After the incorporation of the respective precursors, the sprigs were subjected to steam-distillation to give protoanemonin (**1**). Since protoanemonin (**1**) is unstable, it was immediately converted to pentanoic acid by hydrogenation in the presence of $PtO_2$.[3] All the above-described labeled compounds were incorporated into protoanemonin (**1**) to the extent as shown in Table 1. This indicates that these labeled compounds are of the biosynthetic precursors for protoanemonin (**1**) in a wide sence.

In order to elucidate a pivotal biosynthetic intermediate for protoanemonin (**1**), the labeling pattern in protoanemonin (**1**) biosynthesized from the above-described labeled compounds was determined by subjecting the pentanoic acid derived from the labeled protoanemonin (**1**) to degradation to each carbon atom as follows. The labeled pentanoic acid on Schmidt's reaction[11] gave carbon dioxide and butylamine, which was further converted to butyric acid by potassium permanganate oxidation. Such procedures as described above were repeated in the same manner, but with the acid obtained in the just former procedure. The degradation involving the procedure as above gave carbon dioxide originating from each carbon atom of C-1—C-3 of the pentanoate. On the

Table 1. The incorporation of the radioisotopically labeled precursors into protoanemonin (**1**) in *R. glaber*

| Exptl No. | Precursors | Radioactivities of precursors used / mCi | Feeding time[a] / d | Sp. act. of **1**[b] / (dpm/mmol) × $10^{-4}$ | Incorp. / % |
|---|---|---|---|---|---|
| 1 | Sodium acetate-1-$^{14}C$ | 0.1 | 2 | 3.34±0.04 | 0.063 |
| 2 | Sodium malonate-2-$^{14}C$ | 0.015 | 1 | 7.10±0.15 | 0.37 |
| 3 | Sodium molonate-2-$^{14}C$ | 0.015 | 2 | 10.5 ±0.13 | 0.60 |
| 4 | Sodium succinate-2,3-$^{14}C_2$ | 0.05 | 1 | 8.85±0.21 | 0.48 |
| 5 | Sodium succinate-2,3-$^{14}C_2$ | 0.05 | 2 | 10.5 ±0.11 | 0.61 |
| 6 | Sodium 2-oxoglutarate-5-$^{14}C$ | 0.025 | 1 | 12.0 ±0.22 | 0.21 |
| 7 | Sodium 2-oxoglutarate-5-$^{14}C$ | 0.025 | 2 | 21.8 ±0.39 | 0.35 |
| 8 | Sodium glutamate-5-$^{14}C$ | 0.025 | 1 | 13.2 ±0.25 | 0.24 |
| 9 | Sodium glutamate-5-$^{14}C$ | 0.025 | 2 | 8.31±0.24 | 0.15 |

a) Metabolic period after uptake of the precursors. b) The specific radioactivity of **1** was determined on the basis of the radioactivity of pentanoic acid obtained from **1** by its hydrogenation.

Table 2. The distribution of radioactivity in protoanemonin (**1**) after uptake of the radioisotopically labeled precursors

| Exptl No.[a] | Precursors | The distribution of radioactivity on the carbon atoms originating from **1** (%) | | | | |
|---|---|---|---|---|---|---|
| | | C-1 | C-2 | C-3 | C-4 | C-5 |
| 1 | Sodium acetate-1-$^{14}C$ | | 5.9±0.4 | | 3.1±0.4 | 91.0±1.1 |
| 3 | Sodium malonate-2-$^{14}C$ | 11.4±0.5 | 12.2±0.5 | 12.0±0.7 | 60.8±3.1 | 3.6±0.1 |
| 5 | Sodium succinate-2,3-$^{14}C_2$ | 21.2±0.8 | 30.2±0.1 | 34.1±1.3 | 7.4±0.3 | 7.1±0.2 |
| 6 | Sodium 2-oxoglutarate-5-$^{14}C$ | | 5.8±0.2 | | | 94.2±2.7 |
| 7 | Sodium 2-oxoglutarate-5-$^{14}C$ | 18.9±0.9 | 2.6±0.1 | 2.2±0.1 | | 76.3±2.8 |
| 9 | Sodium glutamate-5-$^{14}C$ | 19.2±0.9 | 6.0±0.3 | 5.4±0.3 | | 69.4±3.0 |

a) "Exptl No." correspond to the number in Table 1.

other hand, the oxidative degradation of the radioactive pentanoic acid by the Kuhn-Roth oxidation[12] gave acetic acid involving the C-4 and C-5 carbon atoms of the pentanoic acid. The acetic acid on Schmidt's reaction gave carbon dioxide and methylamine, which was further converted into carbon dioxide by the Van Slyke-Folch oxidation.[13] Carbon dioxide obtained by the degradation as above was converted into barium carbonate for the determination of its radioactivity. The distribution of radioactivity on each carbon atom of **1** is shown in Table 2.

In the administration of sodium acetate-1-$^{14}C$, about 90 percent of the total radioactivity in protoanemonin (**1**) was located on its C-5 carbon atom.[14] The uptake of sodium malonate-2-$^{14}C$ resulted in the location of about 60 percent of the radioactivity on C-4 of **1**. The malonate-2-$^{14}C$ would biologically yield acetate-2-$^{14}C$ by the action of acetyl-CoA carboxylase. The incorporation of the acetate-2-$^{14}C$ into **1** results in the location of radioactivity on its C-4 carbon atom. Accordingly, the above-described labeling pattern indicates that the C-4 and C-5 carbon atoms of **1** came from the $C_2$-unit of acetate. However, the incorporation of these precursors resulted in the random distribution of the low radioactivity on the C-1—C-3 carbon atoms of **1**. This random labeling seems to indicate that the C-1—C-3 carbon atoms of **1** arise from a source differing from the acetate. In order to clarify the source, the feeding experiment of sodium succinate-2,3-$^{14}C_2$ was carried out, and 64 percent of the radioactivity was found to be located on the C-2 and C-3 carbon atoms of **1**. This demonstrated clearly that the C-1—C-3 carbon atoms of **1** came from succinic acid. From the results obtained in the uptake of acetate, malonate, and succinate, it was found that these precursors participate in the biosynthesis of protoanemonin (**1**) through the tricarboxylic acid cycle. This finding suggests a possibility that a pivotal intermediate for the biosynthesis of **1** might be 2-oxoglutarate.

This possibility was proved by the preferential location of almost all the radioactivity on the C-5 carbon atom in **1** biosynthesized by incorporation of 2-oxoglutarate-5-$^{14}C$ for a 1-d period. In the incorporation of this precursor for a 2-d period, the location of radioactivity on C-5 resulted in 76 percent, and 19 percent of the radioactivity was newly located on C-1 of **1**; this suggests occurrence of the randomization of the $^{14}C$-label from C-5 of 2-oxoglutarate to its C-1 by turn around of the tricaboxylic acid cycle. These facts indicate that the pivotal intermeidate for the biosynthesis of **1** is 2-oxoglutarate (**3a**). This was supported by the fact that the distribution of radioactivity in **1** biosynthesized from glutamate-5-$^{14}C$ is similar to that observed in the uptake of 2-oxoglutarate-5-$^{14}C$ during a 2-d period, since the interconversion of the glutamate and 2-oxoglutarate is of wide occurrence in a biological system. Thus, it was established that the pivotal intermediate for the biosynthesis of protoanemonin (**1**) is 2-oxoglutarate (**3a**). We will here propose that the biological formation of protoanemonin (**1**) from the 2-oxoglutarate (**3a**) may involve the lactonization between the 5-carboxyl and the 2-carbonyl groups of **3a** as well as the hydrogen elimination from **3a** for the formation of the C–C double bond.

*Stereospecific Hydrogen-loss of 2-Oxoglutarate in the Biosynthesis of Protoanemonin (1).* The hydrogen elimination in the formation of double bond at the 3-position of protoanemonin (**1**) was, therefore, investigated by examining the $^3H/^{14}C$ ratio in **1** biosynthesized from the $^3H/^{14}C$-labeled 2-oxoglutarate. 2-Oxoglutaric-3-$^3H_2$ acid (**3b**) was prepared by exchanging the hydrogen atoms at the 3-position of sodium 2-oxoglutarate by treatment with tritiated water.[15] (3*R*)-2-Oxoglutaric-3-$^3H_1$ acid (**3c**) was prepared by incubation of the tritiated 2-oxoglutaric acid (**3b**) with isocitrate dehydrogenase.[16] 2-Oxoglutaric-3,4-$^{14}C_2$ acid (**3d**) was prepared by condensation of dimethyl succinate-2,3-$^{14}C_2$ with diethyl oxalate.[17] The (3*R*)-2-oxoglutaric-3-$^3H_1$-3,4-$^{14}C_2$ and 2-oxoglutaric-3-$^3H_2$-3,4-$^{14}C_2$ acids, (**3e** and **3f**), were prepared by mixing the above-described singly labeled 2-oxoglutaric acids.

Table 3 shows the incorporations of the doubly labeled precursors into protoanemonin (**1**). The $^3H/^{14}C$ ratios in protoanemonin (**1**) thus biosynthesized are shown in Table 4. The $^3H/^{14}C$ ratio in **1** biosynthesized from 2-oxoglutaric-3-$^3H_2$-3,4-$^{14}C_2$ acid (**3f**) resulted in a half of the $^3H/^{14}C$ ratio in **3f**, whereas the $^3H/^{14}C$ ratio in **1** was retained in the feeding of

Table 3. The incorporation of (3*R*)-2-oxoglutaric-3,4-$^{14}C_2$ acid (**3e**) and 2-oxoglutaric-3-$^{3}H_2$-3,4-$^{14}C_2$ acid (**3f**) into protoanemonin (**1**) by *R. glaber*

| Exptl No. | Precursors | Radioactivities of precursors used[b] μCi | Feeding time[c] h | Sp. act. of **1**[a,b] (dpm/mmol)×$10^{-2}$ | Incorp.[d] % |
|---|---|---|---|---|---|
| 10 | **3e** | 0.249 | 12 | 7.40±0.22 | 0.61 |
| 11 | **3e** | 0.015 | 12 | 1.83±0.12 | 0.47 |
| 12 | **3f** | 0.066 | 12 | 2.32±0.18 | 0.52 |
| 13 | **3f** | 0.059 | 12 | 2.76±0.18 | 0.73 |

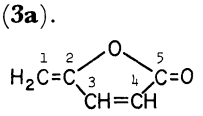
a) The specific radioactivity of **1** was determined on the basis of the radioactivity of pentanoic acid obtained from **1** by its hydrogenation. b) The radioactivity of the doubly labeled compounds refers to only $^{14}C$. c) Metabolic period after uptake of the precursors. d) The incorporation is calculated with respect to only $^{14}C$.

Table 4. The $^{3}H/^{14}C$ ratios in protoanemonin (**1**) after uptake of (3*R*)-2-oxoglutaric-3-$^{3}H_1$-3,4-$^{14}C_2$ acid (**3e**) and 2-oxoglutaric-3-$^{3}H_2$-3,4-$^{14}C_2$ acid (**3f**)

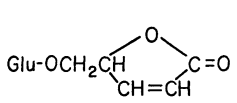
| Exptl No.[a] | Precursors | | | Protoanemonin (**1**) | |
|---|---|---|---|---|---|
| | The acids | $^{3}H/^{14}C$ Ratio | $^{3}H$:$^{14}C$ (Normalized)[b] | $^{3}H/^{14}C$ Ratio | $^{3}H$:$^{14}C$ (Normalized)[c] |
| 10 | **3e** | 1.96±0.01 | 1:1 | 1.89±0.07 | 0.96:1 |
| 11 | **3e** | 6.21±0.31 | 1:1 | 6.01±0.66 | 0.97:1 |
| 12 | **3f** | 13.9 ±0.22 | 2:1 | 6.18±0.51 | 0.89:1 |
| 13 | **3f** | 12.8 ±0.98 | 2:1 | 6.56±0.45 | 1.03:1 |

a) "Exptl No." corresponds to the numbers in Table 3. b) This is the normalized ratio as related to the number of $^{3}H$-label at C-3 of 2-oxoglutaric acid. c) The normalized $^{3}H$:$^{14}C$ ratio was obtained by dividing the $^{3}H/^{14}C$ ratio of **1** by the $^{3}H/^{14}C$ ratio of 2-oxoglutaric acid and multiplying the answer by the number of $^{3}H$-label at C-3 of the acid.

(3*R*)-2-oxoglutaric-3-$^{3}H_1$-3,4-$^{14}C_2$ acid (**3e**). These results demonstrate clearly that (i) the formation of double bond at the 3-position of protoanemonin (**1**) results from the elimination of the hydrogen atom at the 3-position of 2-oxoglutaric acid and (ii) the hydrogen elimination involves the stereospecific loss of the *pro*-3*S* hydrogen atom ($R_S$) and the retention of the *pro*-3*R* hydrogen atom ($R_R$) of 2-oxoglutaric acid (**3a**).



1



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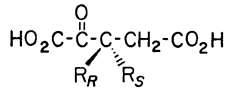
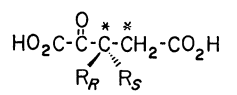


3a: $R_R$=$R_S$=H
3b: $R_R$=$R_S$=$^{3}$H
3c: $R_R$=$^{3}$H, $R_S$=H



3d: $R_R$=$R_S$=H
3e: $R_R$=$^{3}$H, $R_S$=H
3f: $R_R$=$R_S$=$^{3}$H

*=$^{14}$C

## Experimental

All the radioisotopically labeled compounds derived from radioactive protoanemonin (**1**) were purified to a constant specific radioactivity by a combination of preparative TLC with silica-gel plates (Kieselgel $GF_{254}$; 0.75 mm thick) and/or repeated recrystallization. All the compounds showed $^{1}H$ NMR and IR spectra identical with those of authentic samples. The radioactivity was counted on a liquid-scintillation spectrometer using Bray's scintillation solvent[18] and/or a 2π-gas flow low-back counter on $BaCO_3$ planchets at an infinite thickness.

*Plant Material and Labeled Precursors.* The sprigs of *Ranunculus glaber* Makino were collected in May at the damp ground in the suburbs of Hiroshima City. Each sprig used for the feeding experiments was about 20 cm in length and about 15 g in fresh wt. The singly labeled precursors, such as sodium 2-oxoglutarate-5-$^{14}C$ (13 mCi/mmol), sodium acetate-1-$^{14}C$ (23 mCi/mmol), sodium malonate-2-$^{14}C$ (15 mCi/mmol), sodium succinate-2,3-$^{14}C_2$ (33 mCi/mmol), and sodium glutamate-5-$^{14}C$ (68 mCi/mmol), were the products of the Daiichi Pure Chemicals Co., Ltd, Tokyo and used for the feeding experiments without purification. The doubly labeled precursors were prepard as described below.

*Feeding of the Labeled Precursors to* R. glaber. The labeled precursor (Tables 1 and 3) dissolved in a phosphate-buffered soln (2.0 $cm^3$; pH 7.0—7.4) was fed to the fresh-collected sprigs (*ca.* 70 g in total wt) through their cut-stem in a glass tube, and then the phosphate-buffered soln was soaked into the sprigs several times to force the uptake of the labeled precursor to completion. At the end of the metabolic periods shown in Tables 1 and 3, the sprigs were subjected to the following procedure to isolate the radioisotopically labeled protoanemonin (**1**).

*Isolation of the Radioisotopically Labeled Protoanemonin (**1**).* The sprigs (*ca.* 70 g) were steam-distilled. The distillate was extracted with five 50-$cm^3$ portions of ether. The removal of the solvent from the ether soln at temp below 25 °C under a reduced pressure in a current of nitrogen gave protoanemonin (**1**) (*ca.* 100 mg), which was then, without purification, converted into pentanoic acid [95 mg; IR (liq) 3300—2500 and 1710 $cm^{-1}$ (COOH)] by hydrogenation in MeOH (10 $cm^3$) in the presence of Adam's

platinum oxide (5 mg).[3] A portion (20 mg) of the sample of pentanoic acid was treated with *p*-bromophenacyl bromide to give the *p*-bromophenacyl derivative (45 mg): mp 65—67 °C; IR (KBr) 1740 $cm^{-1}$ (ester); $^1H$ NMR (60 MHz, $CCl_4$) $\delta$=1.03 (3H, t, Me), 1.59 (4H, bs, $-CH_2-CH_2-$), 2.52 (2H, t, $-CO-CH_2-$), 5.33 (2H, s, $-CH_2-O-$), and 7.80 (4H, m, arom. H); MS (70 eV), *m/z* (rel intensity), 300 and 298 (6%, $M^+$), 185 and 183 (100), 85 (42), 57 (36), and 28 (51). Found: C, 51.98; H, 4.89%. Calcd for $C_{13}H_{15}O_3Br$: C, 52.19; H, 5.05%. The specific radioactivities of **1** were determined by measuring the specific radioactivities of the *p*-bromophenacyl derivatives; they are shown in Tables 1 and 3.

*Labeling Pattern of Radioactive Pentanoic Acid.* After radioactive pentanoic acid (50 mg) resulted from **1** as described above was diluted 10 times with the carrier, the pentanoic acid was converted into its silver salt (800 mg) by adding a 5% $AgNO_3$ soln to it. The silver pentanoate (200 mg) on the Schmidt reaction[11] yielded carbon dioxide and butylamine (62 mg). Carbon dioxide was absorbed in a $Ba(OH)_2$ soln and converted into $BaCO_3$ (88 mg). Butylamine (60 mg) was oxidized with a 5% $KMnO_4$ soln for 30 min at 100 °C, and the reaction mixture, after the decomposition of the remaining oxidant with sodium hydrogensulfite, was extracted with $Et_2O$ by a continuous liquid-liquid extractor. The removal of the solvent from the ethereal soln gave butyric acid, which was then converted to the silver salt (92 mg). The oxidative degradation of this silver butyrate (80 mg) by the Schmidt reaction was repeated as described above, giving $BaCO_3$ (86 mg) and propionic acid (57 mg as the silver salt). Silver propionate (50 mg) was again subjected to the degradation in the same manner as above to give $BaCO_3$ (36 mg) and silver acetate (11 mg). On the other hand, the radioactive silver pentanoate (100 mg) on the Kuhn-Roth oxidation[12] afforded sodium acetate (17 mg). This acetate was then subjected to the Schmidt reaction[11] to give carbon dioxide and methylamine, which were then converted into $BaCO_3$ (21 mg) and methylamine hydrochloride (4 mg), respectively. This methylamine hydrochloride was then further converted into $BaCO_3$ (12 mg) by the Van Slyke-Folch oxidation.[13]

The distribution of radioactivity in each carbon atom originating from **1** was determined on the basis of the radioactivities of $BaCO_3$ obtained by the Schmidt reaction and the Kuhn-Roth oxidation; they are shown in Table 2.

*Preparation of 2-Oxoglutaric-3-*$^3H_2$ *Acid (**3b**).* On reference to the method described in the literature,[15] sodium 2-oxoglutarate (50 mg) dissolved in tritiated water (0.16 $cm^3$; 160 μCi) was heated at 120 °C for 30 min in a sealed glass tube. The reaction mixture was put on an Amberlite IR-45 column. After the column was washed thoroughly with $H_2O$ to remove the tritiated water, elution of the column with 0.3% HCl (50 $cm^3$) gave 2-oxogutaric-3-$^3H_2$ acid (**3b**) (31 mg; 12.5 μCi) as an eluate.

*Preparation of (3R)-2-Oxoglutaric-3-*$^3H_1$ *Acid (**3c**).* Following the method described in the literature,[15,16] the tritiated 2-oxoglutaric acid (**3b**) (7.5 mg; 3.0 μCi) was incubated with isocitrate dehydrogense (50 units; Sigma Chemical Company) in the presence of NADPH (25 mg) and $MgCl_2$ (3.6 mg) in a triethanolamine hydrochloride buffered soln (50 $cm^3$; pH 7.1) at 30 °C for 6 h. The reaction mixture was subjected to column chromatography using Amberlite IR-45 as an ion exchange resin and 0.3% HCl as an eluent to afford (3*R*)-2-oxoglutaric-3-$^3H_1$ acid (**3c**) (5.9 mg; 1.1 μCi).

*Preparation of 2-Oxoglutaric-3,4-*$^{14}C$ *Acid (**3d**).* On reference to the method described in the literature,[17] dimethyl succinate-2,3-$^{14}C_2$ (0.26 mg; 50 μCi) was added to a suspension of diethyl oxalate (2.0 mg) and potassium ethoxide [potassium (10 mg) in EtOH (0.1 $cm^3$)] in $Et_2O$ (1.0 $cm^3$) with stirring at room temp. After 10 min, a precipitated yellow mass (5.1 mg) was filtered and dissolved in concd HCl. The soln obtained was extracted with $Et_2O$ to give an oily product (2.3 mg), which was then refluxed with concd HCl (1 $cm^3$) for 1 h. The mixture was left to stand at room temp for 12 h and then extracted with $Et_2O$ to give a crude sample of 2-oxoglutaric-3,4-$^{14}C_2$ acid (**3d**). Purification of this crude sample by preparative TLC (cellulose; 0.5 mm thick) with *n*-BuOH–$CHCl_3$ (1:19, v/v) and then *n*-BuOH–AcOH–$H_2O$ (12:3:5, v/v/v) afforded the pure sample of 2-oxoglutaric-3,4-$^{14}C_2$ acid (**3d**) (3.0 μCi).

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