

# The Utilization of Sodium Acetate-Carboxyl-C<sup>14</sup> by *Swertia japonica* Mak.

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Sodium acetate-carboxyl-C<sup>14</sup> was fed to *Swertia japonica* plants in culture solution and the distribution of carbon-14 in various tissues of plants, residual culture solution and respired carbon dioxide was measured. Carbon-14 labeled oleanolic acid, swertiamarin and sugars were separated from the plants and their specific activity was determined. The oleanolic acid was subjected to the Schmidt reaction and the evolved carbon dioxide had no radioactivity. It was thereby found that C<sub>28</sub> of oleanolic acid was not derived from the carboxyl carbon of acetate, but probably derived from methyl carbon of acetate.

## INTRODUCTION

The herb of *Swertia japonica* Makino is one of the most wide-distributed medicinal plant with its characteristic bitter taste and the dried plant material is commonly used for the purpose of stomachic as well as European *Gentiana* root. Several components of *Swertia japonica* have already been identified in the paper of Kariyone and Matsushima<sup>1)</sup>, in which two components, namely, swertiamarin, a bitter glucoside and "Toyakuic acid" were reported whereas the latter acid was proved to be identical to oleanolic acid by Kuwada and Matsukawa.<sup>2)</sup>

Recently it has been elucidated that a two-carbon unit of acetic acid is an extremely important intermediate in the metabolic process of the organism. However, the specific role of acetic acid in higher plant product, oleanolic acid has not been studied. The purpose of the present work is to investigate the utilization of acetate by higher plant and further to determine the incorporation of acetate into oleanolic acid skeleton. The authors have previously studied that *Swertia japonica* grew well by hydroponic culture and 30 mg. of sodium acetate in 1 l of the solution was found not to be toxic to the plants growth. The plant materials obtained showed fairly high content of oleanolic acid. Thus, *Swertia japonica* was regarded to be suitable material in order to biosynthesize the labeled oleanolic acid by using the culture solution of sodium acetate-carboxyl-C<sup>14</sup>.

## EXPERIMENTAL

### Material and Method

*Swertia japonica* plants about 15~20 cm. in height which had been grown

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at Yamazaki in Kyoto Prefecture were lifted from the ground on September 28, 1957. The plants were transplanted into the Knopp's culture solution. The culture solution was changed frequently every two days. After one week in the stage of flower bud formation, the plants were arranged for feeding with sodium acetate-carboxyl- $C^{14}$ . The plants were divided into three groups, each group consisting of 160 plants (120 g) and were fed 1 l of hydroponic solution. The components of the solution employed for the cultivation are given in Table 1.

Table 1. Component of hydroponic solution.

$Ca(NO_3)_2 \cdot 4H_2O$	1g/l
$KNO_3$	0.25
$KH_2PO_4$	0.25
$MgSO_4 \cdot 7H_2O$	0.25
$FeCl_3$	0.003
Minor elements*	Trace
$CH_3C^{14}OONa$	0.03(150 $\mu$ c)
pH of the solution was adjusted to 6.0	

\* Arnon A<sub>5</sub> (B, Mn, Zn, Cu, Mo) and Arnon B<sub>6</sub> (V, Cr, Ni, Co, W, Ti) were added.

Group A was cultivated in a closed system, while groups B and C were cultivated in the open air. Previous experiments had shown that the acetic acid was consumed in the culture solution due to the bacterial action of roots when cultivation period was long. In order to avoid this prospect, a fairly large amount of plants was employed, so that the cultivation period could be shortened. All feeding were made at the same period and the temperature range recorded during the cultivation was 18~23°C.

Group A was cultivated as follows. The plants were dipped in the medium and the pot was sealed in a glass cylinder with a tight glass lid, through which inlet and outlet tubing were fitted. The inlet tube was connected with an absorption tube containing sodalime and the outlet tube was connected to 8 trap bottles containing 0.2 N-Ba(OH)<sub>2</sub> to hold CO<sub>2</sub> generated from the plants. The last trap bottle was connected to suction. The experiment was made in a hood under illumination by a fluorescent lamp. Groups B and C were cultivated in the open air, avoiding direct sunlight.

After 24 hours, the plants were removed and the roots were washed with water. The washing water was added to the culture solution and the total volume made up to 1.5 l. Five ml. of the sample was introduced into a combustion flask. After diluting with nonradioactive sodium acetate and neutralizing with sodium hydroxide, the sample was evaporated to dryness under reduced pressure. The plants were rapidly dried at 80~90° and each part of the plants were separated. The plant materials were weighed and pulverized. For the determination of radioactivity, all samples were oxidized by a wet combustion method and CO<sub>2</sub> was absorbed in 0.2 N-Ba(OH)<sub>2</sub> solution. To calculate the total amount of BaCO<sub>3</sub> the excess of Ba(OH)<sub>2</sub> was titrated with 0.2 N-HCl. The activity was counted at infinite thickness with a G-M counter tube and

compared with the count of a standard  $BaCO_3$ . Since  $BaCO_3$ , released from plants respiration, showed very high activity, a small amount of sample was treated with perchloric acid in the wet combustion flask and  $CO_2$  was absorbed in 1 N-NaOH solution. A calculated amount of  $Na_2CO_3$  solution was added as carrier to the 1 N-NaOH solution, derived to  $BaCO_3$  by the usual manner and plated at infinite thickness. The results of the radioactivity measurements made are shown in Tables 2 and 3. The radioautogram of a plant (group B) is shown in Fig. 1.

Table 2. Localization of carbon-14 in leaves, stems and roots.

	Group	Total, dried, g.	Total activity $\mu c$	Recovery %	Specific activity $\mu c/g.$
Leaves*	A	10.7	23.95	15.95	2.24
	B	11.3	4.69	3.12	0.415
	C	11.4	6.71	4.47	0.588
Stems	A	10.6	15.00	10.00	1.42
	B	12.7	7.21	4.81	0.568
	C	11.9	7.01	4.67	0.589
Roots	A	5.01	25.40	16.91	5.07
	B	5.70	36.32	24.2	6.37
	C	5.00	39.90	26.6	7.98

\* Mixed some flower buds.

Table 3. Distribution of carbon-14 in residual culture solution, respired  $CO_2$  and plant material.

	Activity	A	B	C
Residual culture solution	Total activity, $\mu c$	10.51	6.17	7.70
	Recovery, %	7.01	4.12	5.14
Respired $CO_2$	Yield of $BaCO_3$ , g.	2.977	—	—
	Total activity, $\mu c$	81.70	—	—
	Recovery, %	54.50	—	—
Plant material	Total dried, g.	26.31	29.7	28.3
	Total activity, $\mu c$	64.33	48.2	53.6
	Recovery, %	42.9	32.1	35.8
Total	Activity, $\mu c$	156.561	—	—
	Recovery, %	104.00	—	—

### Isolation of Principles from Plant

Powdered materials of groups B and C were mixed. The combined mixture of B and C and powdered material of group A were extracted separately as follows. The plant materials were extracted in a Soxhlet extractor first with ethyl acetate for 9 hours and then with 90 % methanol for 7 hours. The yield and radioactivity of extracts are shown in Table 4. The method of isolation of principles from extract is illustrated in Table 5.

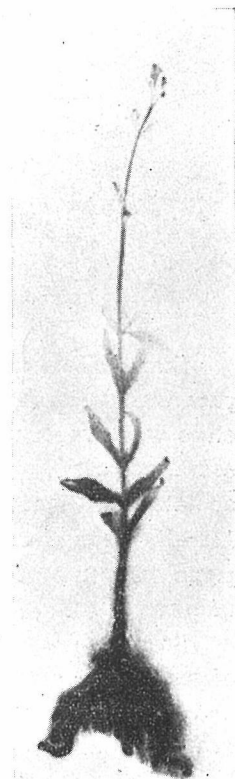
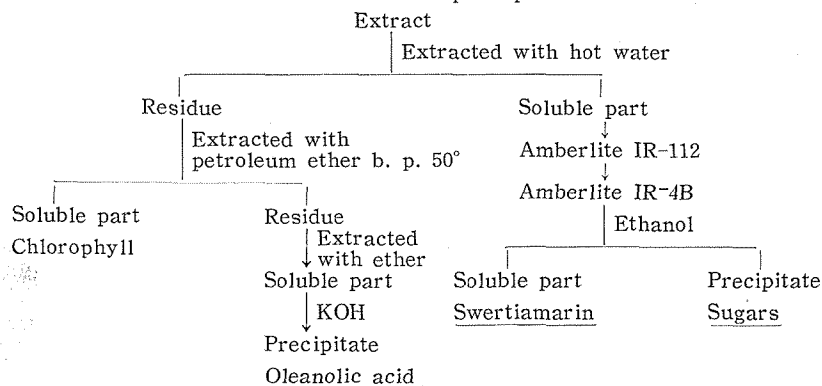
Fig. 1. Radioautogram of *Swertia japonica*

Table 4. Yield and radioactivity of extract.

Group	Total, dried g.	Total activity $\mu\text{C}$	Recovery %	Specific activity $\mu\text{C/g.}$
A	7.90	20.77	13.82	2.26
B + C	20.12	15.34	5.12	0.76

Table 5. Method of isolation of principles from extract.



**Swertiamarin (I).** The extract was treated with hot water and soluble part was purified by passing through both Amberlite IR-112 and IR-4B. The elute was evaporated to dryness under reduced pressure and the residue was extracted with ethanol. The soluble part was recrystallized from hot ethyl acetate and then ethanol-ether mixture. Colorless amorphous powder of m. p. 112~114° was obtained. The nature of this compound was identical in all respects with that of authentic sample of swertiamarin separated by the method of Rariyone and Matsushima.<sup>13</sup>

**Sugars (II).** The ethanol insoluble residue was dissolved in a small amount of water and precipitated by ethanol. Thus, the reprecipitation was repeated and colorless, hygroscopic powder resulted was examined by paper chromatography using the developing solvent of butanol, water and acetic acid (5 : 4 : 1, Vol.) and the conventional solution of aniline hydrogen phthalate as a color reagent. Rf values of spots on papergram were equivalent to those of glucose, fructose and sucrose respectively. Close by the start line on paper a small spot was located to which no further examination was performed. Amongst those sugars the glucose was dominant.

**Oleanolic acid (III).** The residual part from the hot water extraction of (I) was treated in a Soxhlet extractor by petroleum ether (b. p. 35~50°) for 2 hours to remove colored materials. The petroleum ether insoluble part was extracted by ether for 3 hours in a same apparatus. The ether solution was concentrated to a half volume and 10 % KOH aqueous solution was added, then well agitated. After standing overnight insoluble potassium oleanolate was separated which was converted to oleanolic acid by acidifying with the mixture of 10 % hydrochloric acid and ether. The supernatant ether layer was evapo-

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rated and the residue was recrystallized thrice from ethanol. The final pure product formed colorless needles, m. p. 304°. This was found to be identical with authentic sample of oleanolic acid separated by the method of Kariyone and Matsushima<sup>1)</sup> or Kuwada and Matsukawa.<sup>2)</sup>

The yield and radioactivity of principles are shown in Table 6.

Table 6. Yield and radioactivity of principles.

Principle	Group	Yield g.,	Total activity $\mu$ c	Recovery %	Specific activity $\mu$ c/m. mole
Swertiamarin	A	0.51	3.63	2.42	2.770
	B+C	1.49	0.17	0.057	0.044
Sugars	A	0.30	1.20	0.80	0.723*
	B+C	0.84	0.22	0.073	0.047
Oleanolic acid	A	0.19	0.06	0.04	0.149
	B+C	0.42	0.11	0.03	0.119

\* Calculated from the formula C<sub>30</sub>H<sub>48</sub>O<sub>6</sub>.

**Schmidt reaction of oleanolic acid.** A mixture of oleanolic acid (390 mg, 0.86 m. mole) separated from groups B and C, chloroform (20 ml.) and sodium azide (195 mg, 3 m. mole) was cooled in an ice-bath and conc. sulfuric acid (2 ml.) was added. The reaction mixture was stirred magnetically and the flask swept with a stream of CO<sub>2</sub> free N<sub>2</sub>. The effluent gases were first passed through a solution of 5% KMnO<sub>4</sub> in 1 N sulfuric acid and then through CO<sub>2</sub> free 1 N NaOH. The reaction was maintained at 0° for 30 minutes and then warmed to 50~60° for 3 hours. The BaCO<sub>3</sub> was precipitated in the usual manner, centrifuged, washed with water and ethanol. The precipitate was then homogenized and plated on a stainless steel disk. The yield of BaCO<sub>3</sub> was 67.5 mg (40%) and this sample contained an activity indistinguishable from the background.

#### DISCUSSION

It is observed on Tables 2 and 3 that about a half of carbon-14 was respired and a greater amount of carbon-14 accumulated in the roots than in leaves and stems. It should be noted, however, that the specific activity of leaves and stems in group A was much higher than that of B and C. This indicates that some reabsorption of C<sup>14</sup>O<sub>2</sub> took place in plants treated in the closed system. This interpretation is supported by the following observations. The specific activity of extract, sugars and swertiamarin, separated from group A, is much higher than that of B and C. It is considered that the large amounts of the reabsorbed C<sup>14</sup>O<sub>2</sub> was incorporated into sugar fraction rather than the oleanolic acid.

The recovery of carbon-14 of isolated principles is lower than that of plant materials and extracts. It can be certified that the acetate was utilized to build up oleanolic acid in *Swertia japonica*, although its specific activity was compa-

ratively low. Somewhat similar results are reported by Djao and Youngken<sup>4)</sup> in which they obtained fairly low specific activity of steroidal glucosides of *Digitalis purpurea* by 10 day's hydroponic cultivation of plants using labeled acetate solution.

Consequently, a large amount of acetate-carboxyl-C<sup>14</sup> was respired by plants and the remaining carbon-14 was incorporated into cellulose, lignin or related substances. It is also assumed that labeled acetate is converted into water soluble acids such as citric or malic acids which are regarded to be respiratory intermediate product though these compounds were not identified by strictly purifying procedures. Anyhow, it is concluded that the experimental results obtained in this study was in accord with those of Djao and Youngken<sup>4)</sup> so far as the pathway of acetate-carboxyl carbon is concernd.

A nuber of recent reports has been pertaining to the biosynthetic pathway of isoprenoids or triterpenoids since the speculative proposition by Ruzicka and his associates.<sup>5)</sup> Meanwhile, a tetracyclic triterpenoid, eburicoic acid was successfully biosynthesized by Dauben and his associates<sup>6)</sup> in which *Polyporus sulfureus* was inoculated on a medium containing carboxyl-labeled acetate. Thus, labeled eburioic acid has been obtained and the degradation of its molecule was performed by which the fact was verified that the acetate was utilized as a two carbon unit and some carbon atoms of the molecule were labeled while

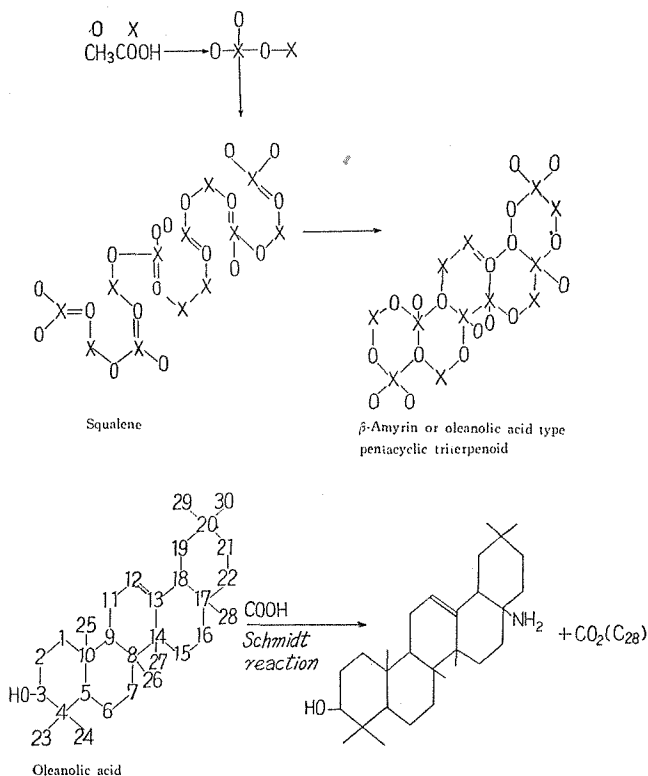


Fig. 2

several others were not labeled. The location of the labeled atoms was proved to be identical to the squalene hypothesis concerning the mechanism of biosynthesis for triterpenes or steroids. None was performed hitherto concerning the biosynthesis for pentacyclic triterpenes which are labeled with isotopes, although numerous works have been appeared concerning the studies on the labeled steroids<sup>6)</sup> or tetracyclic triterpenes.<sup>6)</sup> The authors have tried, therefore, in this study to know whether or not a pentacyclic triterpene, oleanolic acid would be biosynthesized by incorporating acetate as a two carbon unit in accordance with the results obtained in the study on eburicoic acid by Dauben.<sup>6)</sup> The carboxyl carbon (C<sub>28</sub>) of oleanolic acid should be derived from the methyl carbon of acetate, if the acetate were incorporated according to the rule mentioned above. Namely, the distribution of the individual carbon atom of acetate in the oleanolic acid molecule is expected to be as in the following formula (Fig. 2).

The labeled oleanolic acid obtained from *Swertia japonica* by the authors was decarboxylated *via* the Schmidt reaction to evolve carbon dioxide, which had no radioactivity. Thus, C<sub>28</sub> of oleanolic acid does not arise from the carboxyl of acetate and therefore, it is most likely that it has its origin in the methyl carbon of acetate.

Oleanolic acid, isolated from group A, was not subjected to the Schmidt reaction because it was suspected that some randomization had occurred. Specific activity of oleanolic acid, isolated from groups B and C, was very low, therefore, a total yield of sample was subjected to the Schmidt reaction. In this experiment, stepwise degradation of oleanolic acid could not be undertaken by the above reason. Further experiment of degradation of oleanolic acid will be performed subsequently.

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#### REFERENCES

- (1) K. Kariyone and G. Matsushima, *J. Pharm. Soc. Jap.* **47**, 133 (1927).
- (2) S. Kuwada and T. Matsukawa, *ibid.* **53**, 680 (1933).
- (3) D. I. Arnon, *Am. J. Bot.*, **25**, 332 (1938).
- (4) E. H. Djaou and H. W. Youngken, Jr., *J. Am. Pharm. Assoc. Scientific Ed.*, **43**, 425 (1954); **44**, (1955).
- (5) L. Ruzicka, *Experientia*, **9**, 357 (1953); L. Ruzicka, Eshenmoser and O. Jeger, *Helv. Chim. Acta*, **38**, 1890 (1955).
- (6) W. G. Dauben and J. H. Richards, *J. Am. Chem. Soc.*, **78**, 5329 (1956); W. G. Dauben, Y. Ban and J. H. Richards, *ibid.*, **79**, 968 (1957).