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# 64. Mikio Yamazaki,*1 Taeko Usui,*1 and Shoji Shibata*2 : The Biogenesis of Plant Products. II.*3 The Biogenesis of Thymol.

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Although numerous works have been made on the biogenesis of steroids and triterpenoids using animal and microörganisms, only a few studies have been reported on the biogenesis of lower terpens in higher plants. Sandermann and Stockmann[1] showed the biogenesis of pulegone from 3-methylcrotonic acid in *Mentha pulegium* L., and Mitsuhashi *et al.*[2] proved the incorporation of acetate (1-$^{14}$C) into *l*-menthol of *Mentha arvensis* L.

Thymol, a main component of the essential oil of *Orthodon japonicum* Benth. et Oliv. (: *Mosla japonica* (Benth.) Maxim.) is regarded as an isoprenoid compound, though it is completely aromatic.

Therefore, it seems quite possible that thymol would be formed in the plant by the generally accepted biosynthetical pathway of terpenoids.

This has been proved by the present study which revealed the incorporation of acetate (1-$^{14}$C) and mevalonic acid (2-$^{14}$C) into thymol during the cultivation of the plant.

## Experimental

**Plant Material**——*Orthodon japonicum* Benth. et Oliv. (: *Mosla japonica* (Benth.) Maxim.) (Japanese name : Yamajiso) supplied from the Medicinal Plant Garden attached to the Pharmaceutical Faculty of Chiba University was employed as the material. The 3-months old plant was transplanted from the ground to the Wagner pot, and the administration of $^{14}$C-labelling compounds was carried out as follows: Aqueous solution of AcONa (1-$^{14}$C) (50 μc (0.1 mc./7.5 mg.)), and mevalonic lactone (2-$^{14}$C) (50 μc (0.1 mc./3.9 mg.)) in 1 cc. of water were used for the first and second experiment, respectively.

The radio-active solution was administered into the stem by the cotton twine method.[3] After 10 days, the plant was harvested and subjected to the steam distillation to obtain the volatile oil (yield: 0.5 cc. from 40 g. of half dried plant material (Exp. I); 0.7 cc. from 62 g. material (Exp. II)).

**Extraction and Isolation of Thymol**($^{14}$C)——The steam distillate containing essential oil was extracted twice with 10 cc. each of petr. ether, and the petroleum extract was shaken with the equal volume of 5% NaOH solution. After 3 times extraction with aq. NaOH solution, the combined alkaline solution was acidified with dil. HCl. Thymol was separated from the solution by seeding minute crystals of thymol and cooling in an ice-bath. M.p. 49° (from petr. ether), yield : Exp. I, 357 mg.; Exp. II, 344 mg.

The dark colored residual by steam distillation was concentrated *in vacuo*, and passed through a column of Amberlite IR-120 ion-exchange resin to separate the fractions of amino acids and sugars.

The radioactivity of each fraction was measured.

**Degradation of Thymol** ($^{14}$C)——To a solution of thymol ($^{14}$C) (300 mg.) in conc. $H_2SO_4$, a mixture of $HNO_3$ (sp. gr. 1.42) and 95% $H_2SO_4$ (3 cc. each) was added under ice-cooling. After heating for 3~4 min. on a boiling water bath, the reaction mixture was poured into water (60 cc.), and allowed to stand overnight, when crystals of trinitrocresol separated out.

Recrystallization from water containing a small amount of HCl formed colorless fine needles, m.p. 109°, which were identified as trinitrocresol by the mixed fusion and the comparison of IR spectra. $CO_2$ liberated during the nitration reaction was trapped as $BaCO_3$ whose yield was about 50% of the theoretical amount.

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*3 Part I : S. Shibata, M. Yamazaki : This Bulletin, **6**, 42 (1958).

1) W. Sandermann, H. Stockmann : Chem. Ber., **91**, 930 (1958).
2) H. Mitsuhashi, K. Kaneko, S. Eguchi, M. Otsu : Yakugaku Zasshi, **80**, 268 (1960).
3) C. L. Comer : "Radioisotopes in Biology and Agriculture," p. 155 (MeGraw Hill, N. Y (1955)).

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To a solution of trinitrocresol (30 g.) dissolved in the saturated solution of $Ca(OH)_2$ (1 cc.), a paste (10 g.) of $Ca(OH)_2$ (7 g. in 30 cc. of $H_2O$) was added at 0°, and then $Br_2$ (2.5 cc.) was dropped into the mixture under mechanical stirring. After keeping the reaction mixture at 10° for 1~1.5 hr., when the yellow color of the mixture decolorized, it was distilled to collect the distillate (10 cc.) into a centrifuging tube.

The distillate was centrifuged, and the supernatant was removed to separate from the oily precipitate, which was washed 3~4 times with water, and finally dried in a vacuum desiccator.

**Measurement of Radioactivity**——The radioactivity of each fragment of degradation reactions was measured at the stage of $BaCO_3$ derived by the Van Slyke-Folch reaction,[4] using the Aloka LBC-1 type low back ground GM. counter (Nihon Musen Electro-Medical Laboratory, Ltd., Tokyo).

The amino acids and sugar fractions were paper chromatographed using a mixture of BuOH-AcOH-$H_2O$ (4:1:5) as the developing solvent to take the radioautograms.

## Results and Discussion

The total radioactivies of each fraction are shown in Table I.

Table I.

| | Petroleum ether soluble-fraction (c.p.m.) | Residual aqueous solution (c.p.m.) Sugar fraction | Amino acid fraction |
|---|---|---|---|
| Experiment I | 2900 | $12.96\times10^5$ | |
| | | $8.36\times10^5$ | $2.66\times10^5$ |
| Experiment II | 2800 | $36.42\times10^5$ | |
| | | $34.20\times10^5$ | $4.52\times10^3$ |

When acetate (1-$^{14}$C) was administered (Exp. I), the radioactivities of amino acid ond sugar fractions were almost in the same level, whereas in the case of using mevalonate as the precursor (Exp. II) the radioactivity of residual solution was dominant in the sugar fraction.

On the paper chromatogram of amino acid fraction obtained by the Experiments I and II, two spots were observed when developed by the ninhydrine reagent, which were identified as glycine and alanine, respectively. In the sugar fraction four spots were shown by spraying with the Tollens reagent. Three spots (Rf: 0.84, 0.60, and 0.23) out of four gave blue fluorescence under ultraviolet illumination, while the remaining one (Rf. 0.18) was identified as being glucose. The radioautography of the fraction obtained by the Experiment I gave no active spot. On the radioautogram of sugar fraction obtained by the Experiment II, an active spot (Rf. 0.30) was observed, which was deduced as a product derived directly from mevalonate (2-$^{14}$C). The radioactivites of the fragments obtained by the degradation of thymol($^{14}$C) isolated from the plant were determined as shown in the Charts 1 and 2.

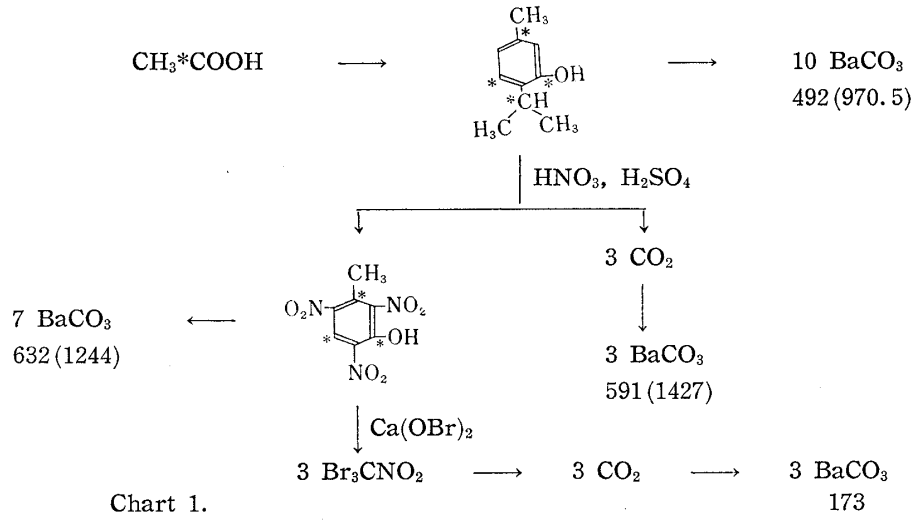


Chart 1.

4) D. D. Van Slyke, J. Plazin, J. R. Weisiger : J. Biol. Chem., **191**, 299 (1951).

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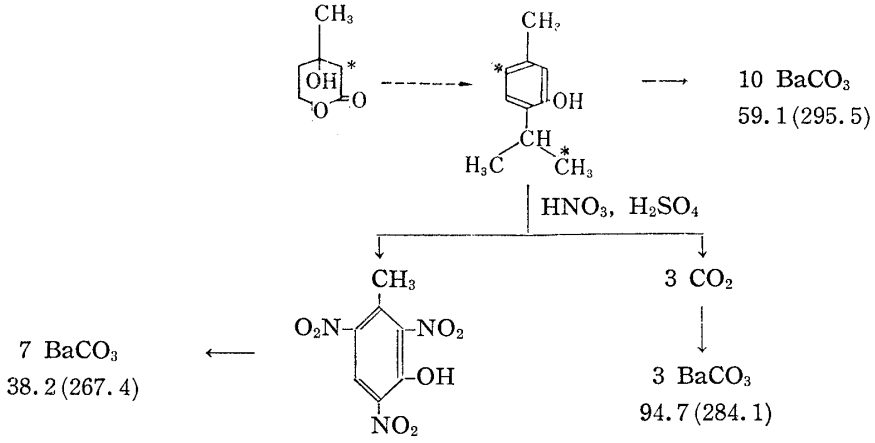


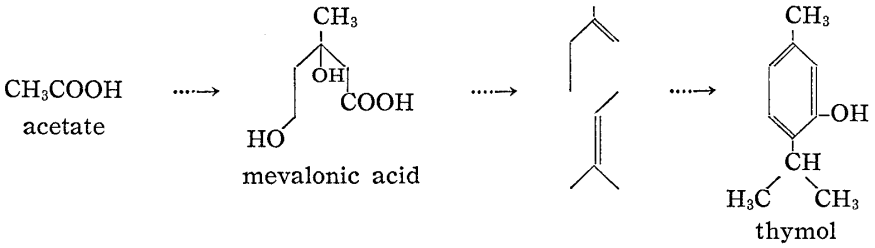

Chart 2.

In the Charts 1 and 2, the figures show the specific radioactivities c.p.m./m.mole, and the figures in parenthesis give the radioactivities of labeled carbon atoms calculated referring the randomized activities of unlabeled carbons.

The incorporation ratio of acetate-(1-$^{14}$C) and mevalonate (2-$^{14}$C) into thymol in the present experiments using higher plant such as *Orthodon japonicum* were unexpectedly low in comparison with earlier results of isoprenoid biosyntheses in microorganisms or animal organs.

The observation of seasonal variation of thymol content in *Orthodon japonicum*[5] which showed a gradual increase of thymol without a certain period of rapid formation of the principle may suggest that only a small amount of thymol can be formed during 1 or 2 weeks period of the present experiments.

The present results has indicated that thymol in *Orthodon japonicum* is biosynthesized from acetate via mevalonate by the generally accepted scheme of isoprenoid biogenesis.




The lower incorporation of mevalonate into thymol in comparison with that of acetate which has been shown in the present experiment would be resulted by the different permeability of the precursors through the cell-membrane of the plant.

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## Summary

The biogenesis of thymol in *Orthodon japonicum* Benth. et Oliv. was studied by radioisotope tracer technique.

It has been established that thymol is biosynthesized from acetate *via* mevalonate by the general pathway of isoprenoid biosynthesis.

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5) M. Yamazaki, T. Usui : Unpublished data.

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