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Biosynthesis of Natural Products. VI.[1] Biosynthesis of Usnic Acid in Lichens. (1). A General Scheme of Biosynthesis of Usnic Acid[2]

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Methylphloroacetophenone was proved to be an intermediate in usnic acid biosynthesis by the tracer experiments. The origin of methylphloroacetophenone was also shown to be derived from acetate–malonate and C_1-unit.

The biogenetical hypothesis of usnic acid (I) which is one of the most common lichen substances was first suggested by Schöpf and Ross in their report on the structural study of this compound,[4] as it would be formed in lichen by the condensation of two molecules of methylpholoroacetophenone (II). Later, Barton, *et al.* performed the synthesis of *dl*-usnic acid by the phenol oxidative coupling of methylphloroacetophenone (II).[5]

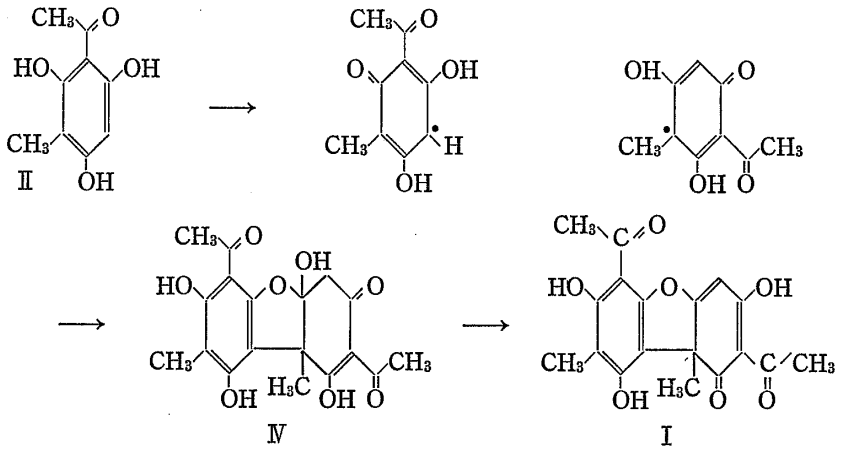
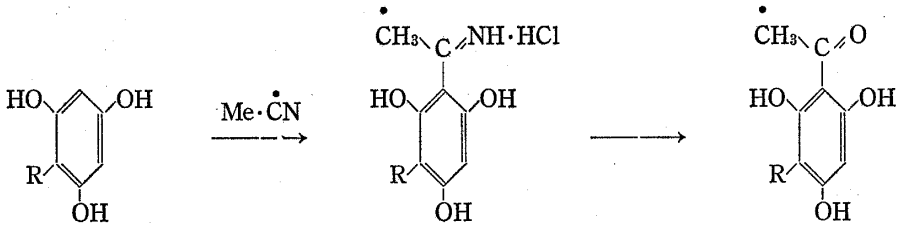


Chart 1

In the present paper the biosynthesis of usnic acid is discussed, and the intermediary steps of the scheme are elucidated. As the material to feed lichen ^{14}C-labelled phloroacetonphenone (III) and methylphloroacetophenone (II) were synthesized. Phloroglucinol or methylphloroglucinol was reacted with acetonitrile (Me-^{14}C) under the presence of zinc chloride and dry hydrogen chloride gas. Resulted ketimine was hydrolyzed into phloroacetophenone ($CO^{14}Me$) or methylphloroacetophenone ($CO^{14}Me$). Hydrated usnic acid (IV), which was expected to be an intermediate of usnic acid biosynthesis, was prepared by the Barton's method[5] and labelled with tritium. Sodium acetate (2-^{14}C), diethyl malonate (2-^{14}C) and sodium formate (^{14}C) were also used for feeding experiments.

1) Part V: U. Sankawa and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), **17**, 2025 (1969).
2) A part of this study has been reported: Tetrahedron Letters, **1966**, 521.
3) Location: *Hongo, Bunkyo-ku, Tokyo*; *a*) Present address: *Tsumura Institute, 1412, Izumi, Komae-machi, Kitatamagun, Tokyo.*
4) C. Schöpf and F. Ross, *Ann*, **546**, 1 (1941).
5) D.H.R. Barton, A.M. Deflorin and D.E. Edward, *J. Chem. Soc.*, **1965**, 530.



R=H or CH_3

Ⅱ: R=CH_3
Ⅲ: R=H

Chart 2

Experimental

Materials and Methods

Radioactive Compounds——Na Acetate (2-^{14}C), diethyl malonate (2-^{14}C), Na formate (^{14}C), $BaCO_3$ (^{14}C) and acetonitrile (Me-^{14}C) were purchased from Dai-ichi Chemical Co., Ltd.

Lichens——*Usnea longissima* Ach. was collected at Okutadeshina in Nagano-Ken; *Usnea diffracta* Wain. and *Cladonia mitis* Sandst. were collected at Lake District of Mt. Fuji; *Parmelia caperata* Ach. was collected at Sugadaira in Nagano-Ken.

Enzymes——Lacase of *Rhus succedamea* L. (optimum pH 6.5) and *Rhus vernicifera* Stokes (optimum pH 7.8) were supplied by Prof. Ogura, Faculty of Sciences, University of Tokyo. Horse radish peroxidase was prepared from horse radish by the standard method.

Feeding Experiments——Most of the feeding experiments were carried out with the soaking method. Fresh lichen thalli (5—10 g) were washed with water, fragmented and put into a flask containing 30 ml steralized Czapeck–Dox medium. Aqueous or ethanolic solution of labelled compound was added to the medium and the flask was shaken for 3 days at 25° under illumination with a white electric bulb (100V, 200W) from 1 metre distance. The dry feeding method was adopted only to *Parmelia caperata*. The fresh lichen thalli were fixed on a sheet of thick paper and a small volume of aqueous or 30% ethanolic solution of labelled compound was dropped on the surface of the thalli. They were left for a suitable period under artificial illumination. Feeding experiment with $^{14}CO_2$ was carried out in specially designed glass apparatus equipped with a dropping funnel and out let having a cock. Lichen thalli were placed in the apparatus together with small flask containing $Ba^{14}CO_3$. A small beaker filled with water was placed in the apparatus to maintain suitable humidity. Sulphuric acid was added to the small flask from the dropping funnel through a glass tubing to evolve $^{14}CO_2$. After the whole apparatus had been illuminated artificially for a suitable period, air inside the apparatus was swept out by the stream of nitrogen into $Ba(OH)_2$ to precipitate $BaCO_3$.

Measurement of Radioactivity——All the radioactive samples were measured with a liquid scintillation counter. The soluble compound such as usnic acid was measured in a toluene scintillator fluid containing 0.4% PPO and 0.01% POPOP. The other insoluble or deep coloured compounds and $BaCO_3$ were converted into CO_2 with conc. H_2SO_4 or van Slyke–Floch solution.[6] Evolved CO_2 was trapped with Hyamine 10–X (Packard). The Hyamine solution was mixed with the toluene scintillator fluid and submitted to measurement. The radioactivity of the medium recovered from feeding experiment was measured in dioxane scintillator fluid containing 10% naphthalene, 0.7% PPO and 0.05% dimethyl POPOP.

Enzymatic Oxidation of *p*-Cresol and Methylphloroglucinol

Oxidation of *p*-Cresol——To the 1/15M phosphate buffer (25 ml) solution of *p*-cresol (200 mg) the solution enzyme (2—5 ml) was added at 27°. In the case of peroxidase a small volume of 0.3% solution of hydrogen peroxide was added several times during the course of the reaction. pH of the buffer solution was adjusted to the optimum pH of enzymes. The reaction mixture was kept stirring for 10—20 hr at 27°. The ether extract of the reaction mixture was extracted with 1% NaOH to remove phenolic compounds. Ether was removed and the residue was chromatographed over silicic acid to give recemic Pummer's ketone.[5]

Oxidation of Methylphloroacetophenone——To the 1/15M phosphate buffer (150 ml) solution of methylphloroacetophenone (150 mg) the solution of the peroxidase was added. 0.3% Hydrogen peroxide was continuously added dropwise to the reaction mixture under stirring for 2 hr. The reaction was stopped by the addition of 6N H_2SO_4, and the reaction mixture was extracted with ether. Ether was removed and the residue was chromatographed over silicic acid to obtain racemic hydrated usnic acid (7 mg). On the contrary no hydrated usnic acid was observed on TLC when lacase was reacted upon methylphloroacetophenone instead of peroxidase.

Synthesis of Labelled Compounds

Methylphoroacetophenone (CO^{14}Me)——Methylphloroglucinol[6] (300 mg) was dissolved in a small volume of anhydrous ether and acetonitrile (Me-^{14}C) (70 mg, 300 μci) and $ZnCl_2$ (freshly fused) (150 mg) was added

6) A. McGookin, R. Robertson and T.H. Simpson, *J. Chem. Soc.*, **1951**, 2021.

to the solution. Dried HCl gas was babbled into the mixture for 5 hr. The reaction mixture was left overnight in a refrigerator to precipitate ketimine. The ketimine was separated from ether solution and treated with water on a hot water bath for 30 min. Precipitated crystals were recrystallized from acetone–benzene repeatedly until a constant specific redioactivity was attained. Methylphloroacetopheaone ($CO^{14}Me$) (9.2×10^8 dpm/mM) gave mp 213—214° and showed a single spot on TLC. Nonradioactive sample obtained in cold run showed following physical data.

UV $\lambda_{max}^{ethanol}$ mμ (log ε): 223 (4.02), 292 (4.20), 330 (3.44). NMR (in $CDCl_3$) δ 1.85 (Me), 2.75 (COMe), 6.02 (arom. H), 10.27, 10.51 and 13.92 (OH). *Anal.* Calcd. for $C_9H_{10}O_4$: C, 59.33; H, 5.53. Found: C, 59.46; H, 5.68.

Phloroacetophenone ($CO^{14}Me$)——To a mixture of anhydrous phloroglucinol (200 mg), acetonitrile (Me-^{14}C) and $ZnCl_2$ (freshly fused) (100 mg) in anhydrous ether (30 ml), dried HCl gas was babbled for 5 hr. Resulted precipitate was collected and treated with hot water for 30 min. After cooling resulted precipitate was collected by filtration and recrystallized repeatedly until the radioactivity was maintained at a constant level. Phloroacetophenone ($CO^{14}Me$) (3.4×10^7 dpm/mM) gave a single spot on TLC. A sample obtained in cold run showed following physical data. UV $\lambda_{max}^{ethanol}$ mμ (log ε): 227 (4.11), 286 (4.24). *Anal.* Calcd. for $C_8H_8O_4$: C, 59.33; H, 5.53. Found: C, 59.46; H, 5.68.

Hydrated Usnic Acid (^{3}H)——Hydrated usnic acid was prepared from methylphloroacetophenone by the method of Barton, *et al.*[5] It showed mp 187—190°. NMR (in $CDCl_3$) δ 1.66 (angular Me), 2.56 and 2.59 (COMe), 3.20 (CH_2), 13.35, 10.40 and 18.20 (OH). *Anal.* Calcd. for $C_{18}H_{18}O_8$: C, 59.65; H, 5.00. Found: C, 59.67; H, 4.84. Hydrated usnic acid was labelled with ^{3}H by the method of Wilzbach.[7] Its specific activity was 1.7×10^{10} dpm/mM.

Isolation and Degradation of ^{14}C-Labelled Usnic Acid

Isolation of Usnic Acid——Lichen thalli which were harvested from feeding experiment were extracted with ether in Soxhlet extractor. Solvent was removed and the residue was dissolved in a small volume of benzene. The solution was poured on the top of silicic acid column made in benzene. A yellow band on the column developed with benzene, was collected, and the solvent was removed to remain a yellow residue which was recrystallized repeatedly from benzene–ethanol to give usnic acid of mp 204—206°.

Iodoform Reaction——Wagner's solution (KI–I_2–H_2O) (30 ml) was added dropwise to the solution of usnic acid in 10% KOH. Iodoform which precipitated out from the solution was collected and recrystallized from MeOH–H_2O to give yellow plates of mp 121—122°.

Kuhn-Roth Oxidation and Schmidt Degradation——Usnic acid (120 mg) was submitted to the Khun–Roth oxidation followed by the Schmidt degradation as described in earlier paper from this laboratory.[9]

Ozonolysis of Diacetyl Usnic Acid[9]——Diacetyl usnic acid (500 mg) was dissolved in CCl_4 (50 ml). Ozonized oxygen was introduced to the solution under ice cooling until the yellow colour of usnic acid disappeared. Ozonide precipitated out was collected by filtration and treated with hot EtOH (7 ml) for 7 min. On evaporation of EtOH an oily residue was remained in the flask. It was treated further with conc. H_2SO_4, and the reaction mixture was poured into ice–water. Yellow precipitates resulted were collected and recrystallized from EtOH–H_2O. Desacetyl lactone A was obtained as needles of mp 223° (decomp.). Lactone A was obtained when the suspension of ozonide was treated directly with EtOH. It showed mp 130—132°.

Results and Discussion

The results of the feeding experiments are summerized in Table I, in which higher incorporation ratio of radioactivities is shown to be given by the dry feeding method (see experimental). This method was very successfully adopted for foliose lichens but could not be applied to fruticose lichens.

As shown in Table I, some fluctuation of incorporation ratios were observed. It is mainly due to the seasonal variation in the biosynthesis of usnic acid and this problem will be discussed in the following paper.[10] Usually over 90% of radioactive precursor was absorved from the medium. The repeated feeding experiments made it quite clear that the secondary metabolism of lichen is not so slow as it had been presumed. The ^{14}C-labelled acetate was introduced into usnic acid in lichens within 10 min.[10]

7) K.E. Wilzbach, *J. Am. Chem. Soc.*, **79**, 1013 (1957).
8) S. Shibata, U. Sankawa, H. Taguchi and K. Yamasaki, *Chem. Pharm. Bull.* (Tokyo), **14**, 474 (1966).
9) S. Shibata, J. Shoji, N. Tokutake, Y. Kaneko, H. Shimizu and Hsüch-Ching Chang, *Chem. Pharm. Bull.* (Tokyo), **10**, 477 (1962).
10) H. Taguchi, U. Sankawa and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), **17**, 2061 (1969).

Table I.

Precursor	^{14}C-Labelled compound added (μ ci)	Amount of ^{14}C absorbed (%)	Lichen	Usnic acid: Specific activity $\times 10^4$ dpm/mM	Usnic acid: Incorporation ratio (%)
Na acetate (2-^{14}C)	16.7	88	*U. longissima*	3.3	0.052
	25.0	—	*U. diffracta*	6.6	0.034
	25.0	98		220.0	0.71
	50.0	—	*P. caperata*	8.2	0.017
	20.0	98		71.0	0.21
	20.0[a)]	—		630.0	1.11
	25.0	—	*C. mitis*	18.0	0.024
	25.0	—		190.0	0.16
Diethyl malonate (2-^{14}C)	33.3	88	*U. diffracta*	9.5	0.04
	25.0	99		71.0	0.25
	33.3	94	*P. caperata*	4.4	0.005
	50.0	90		5.8	0.007
	33.3	96	*C. mitis*	310.0	0.33
	25.0	81		22.0	0.21
Na formate (^{14}C)	16.7	—	*U. loggissima*	0.65	0.006
Phloroacetophenone ($CO^{14}CH_3$)	0.19	—	*C. mitis*	0	0
Methyl phloroacetophenone ($CO^{14}CH_3$)	6.9	—	*C. mitis*	22.0	0.23
	0.5	—		36.0	7.5
	8.2[a)]	—	*P. caperata*	8.9	0.07
	2.2	—		19.0	0.28
	5.0	—		19.0	0.18
Hydrated usnic acid (^{3}H)	43.7	48	*U. diffracta*	3.0	0.02
	43.7	58	*C. mitis*	1.1	0.009

a) ^{14}C-Labelled compounds were fed with dry feeding method.

Degradation reaction was carried out on the various samples of ^{14}C-labelled usnic acid, which were obtained from the separate feeding experiments with sodium acetate (2-^{14}C), diethyl malonate (2-^{14}C) and sodium formate (^{14}C), as shown in Chart 3 and 4. The labelling pattern of usnic acid clearly indicates that two unit of C_8-polyketide are involved in the biosynthesis.

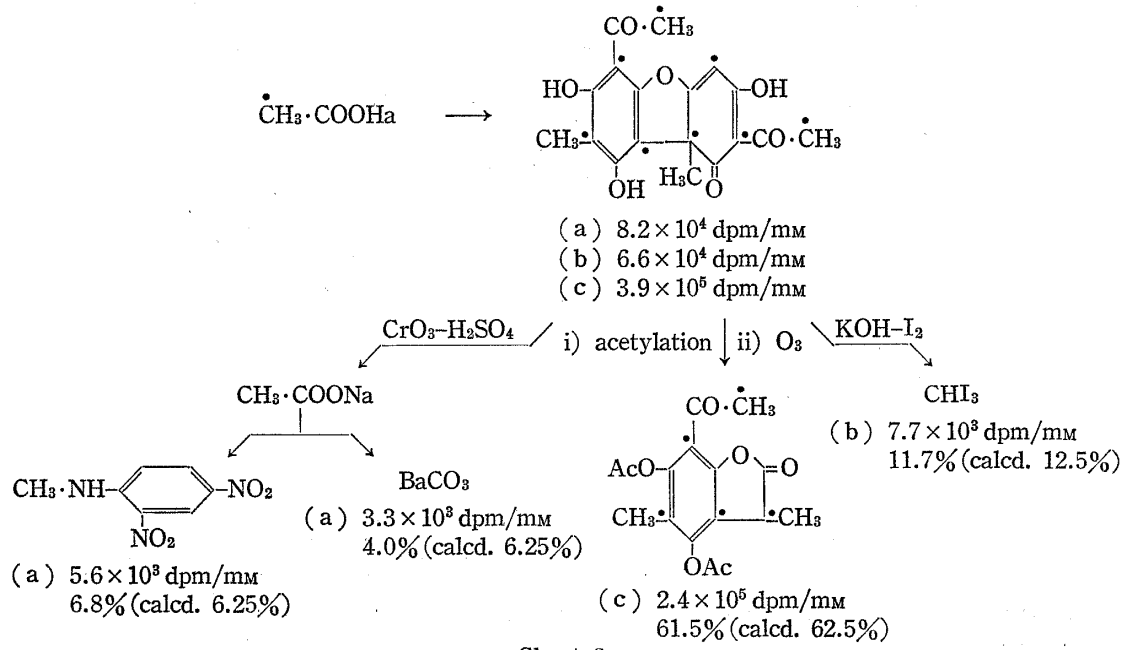


Chart 3

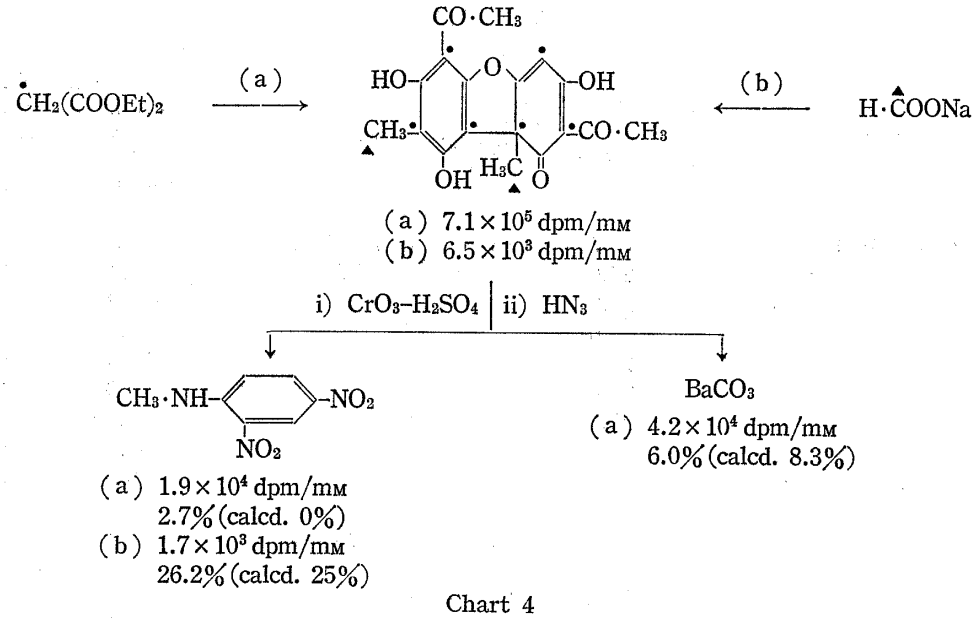


Chart 4

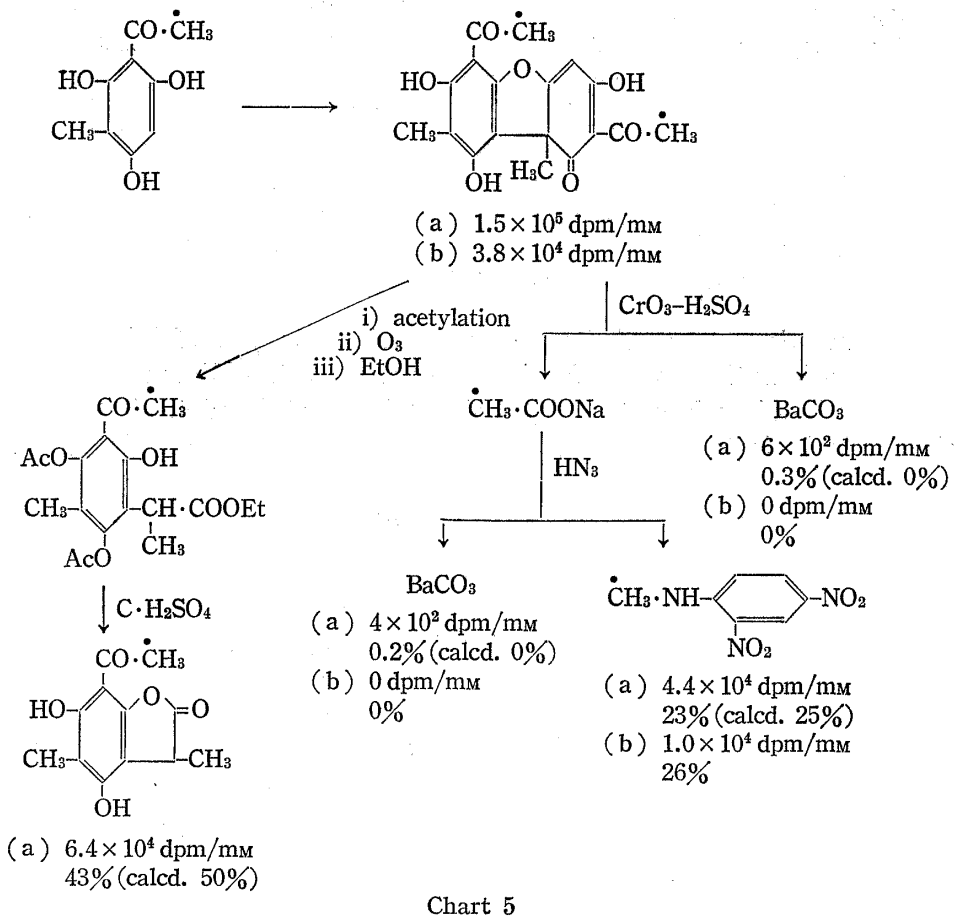


Chart 5

Two methyl groups in the usnic acid molecule, aromatic and angular ones, are confirmed to be derived from C_1-unit. Although the incorporation ratio of methylphloroacetophenone ($CO^{14}Me$) were not at a constant level, its actual rôle as an intermediate in the biosynthesis of usnic acid was confirmed and the results of the degradation of this feeding experiment is summerized in Chart 5.

On the other hand no incorporation of radioactivity was observed from phloroacetophenone ($CO^{14}Me$) into usnic acid (Table I). This result clearly indicates that the methyl group of methylphloroacetophenone is not introduced into phloroacetophenone but into C_8-polyketide prior to its cyclization. Our observation agrees well with that of Bentley, *et al.* who found that ethionine blocked the biosynthesis of stipitatic acid and cauesd an accumulation of nonaromatic C_8-polyketide.[11]

Although methylphloroacetophenone has not been isolated as yet from lichen by the ususal methods, its presence in lichen has now been confirmed by the isotopic dilution method. Nonactive methylphloroacetophenone was added to the lichen extract of *Cladonia mitis*, which had been contacted with $^{14}CO_2$ for 10 days. Recovered methylphloroacetophenone and usnic acid showed considerable radioactivity as shown in Table II.

Table II

Substance	Yield or amount added	Specific activity dpm/mM	Total activity dpm
Usnic acid	121 mg	4.8×10^7	1.7×10^7
Methyl phloroacetophenone	40.7 mg	5.8×10^4	1.3×10^4

The first product formed by the oxidative coupling of methylphloroacetophenone would be hydrated usnic acid. To investigate the enzymic oxidation of methylphloroacetophenone some experiments using horse radish peroxidase and *Rhus* laccase were carried out. When *p*-cresol was treated with laccase or peroxidase Pummer's ketone was obtained in 10—20% yield. However *dl*-hydrated usnic acid was formed only with horse radish peroxidase. Table III shows the summerized results of enzymic oxidation. During the course of this experiments, Fales, *et al.* reported almost similar result.[12]

Table III

Enzyme	pH	Hydrated usnic acid %	Pummerer's ketone %
Laccase (*Rhus succedanea*)	6.5	—	*dl*-, 10
Laccase (*Rhus vernicifera*)	7.8	—	*dl*-, 10
Peroxidase (horse radish)	6.5	*dl*-, 5	*dl*-, 20

Some attempt to prepare soluble enzyme from lichen gave no successful result. However the oxidative coupling of methylphloroacetophenone in lichen sould be stereospecific, since usnic acid occurs mainly in an optically active form.[14] Moreover it seems probable to assume, not only the position and steric factor of oxidative coupling but also the formation of ether linkage is controlled by enzymes, because the occurence of isousnic acid was found in many lichens along with usnic acid[14] and a certain species of New Zealander *Sphaerophorus* contains *d*-isousnic acid.[15]

The final stage of the biosynthesis of usnic acid is the dehydration of hydrated usnic acid. This step was also confirmed with lichen by the conversion of ^3H-labelled hydrated usnic

11) R. Bentley and P.M. Zwitzkowitz, *J. Am. Chem. Soc.*, **89**, 676; *ibid.*, 681 (1967).
12) A. Penttila and H.M. Fales, *Chem. Comm.*, **1966**, 656.
13) Y. Asahina and S. Shibata, "Chemistry of Lichen Substances," Japan Society for the Promotion of Sciences, Tokyo, **1954**, p. 172.
14) H. Taguchi and S. Shibata, *Tetrahedron Letters*, **1967**, 4867.
15) H. Taguchi and S. Shibata, Unpublished observation.

acid (Table I). From these experiments the pathway of the biosynthesis of usnic acid has been established as summerized in Chart 6.

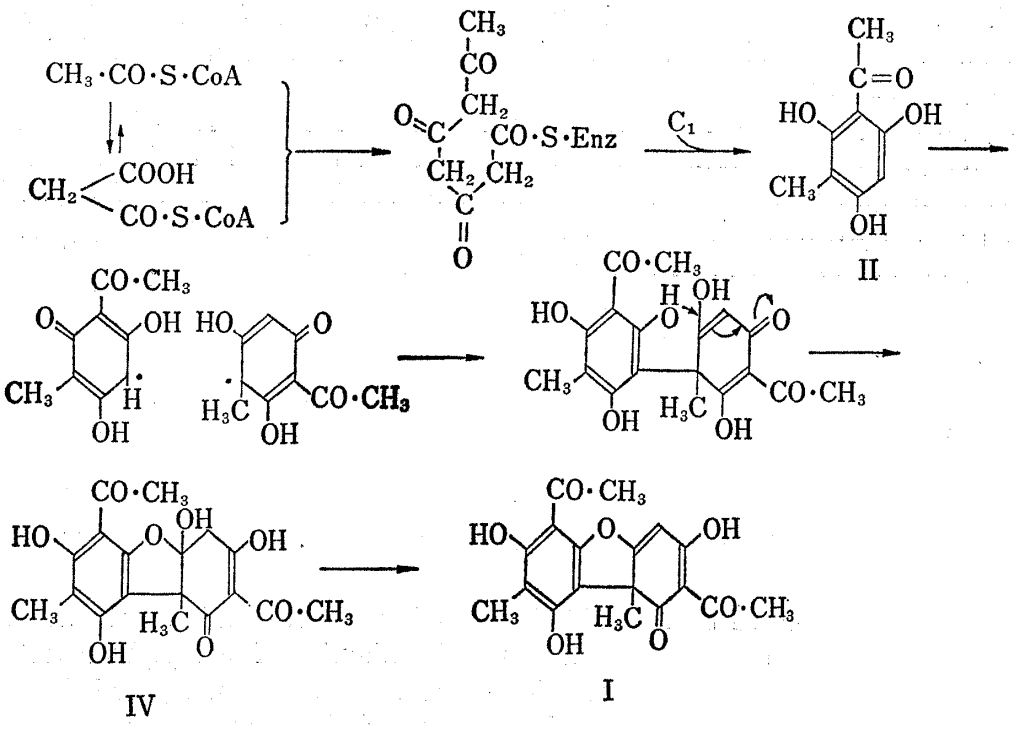


Chart 6

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