[Chem. Pharm. Bull. 14(4) 363~369 (1966)] UDC 581.13 : 582.288 : 547.587.51

50. Keiichi Nitta, Yuzuru Yamamoto, Toshimi Inoue, and Toshiko Hyōdo : Studies on the Metabolic Products of *Oospora astringenes*. Ⅶ.*1 Biogenesis of Oospolactone and Oosponol.

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About one hundred fungi were collected from the asthmatic patients' rooms with a view to examine the relationship between asthma and fungi or their metabolic products. By the preliminary pharmacological test, some of the fungi have been found to have contractive activity to the tracheal muscle. One of them, *Oospora astringenes* was studied on the metabolites by Y. Yamamoto, *et al.*, and oospolactone,[1,2] oosponol,[3,4] oospoglycol,[5,6] and eburicoic acid were isolated and their chemical structures were determined. Pharmacological tests of the metabolites were also studied by Kobayashi, *et al.*[7] to find that oosponol have a strong contractive activity to the tracheal muscle. Oospoglycol was discovered to have antagonistic action against oosponol. Otherwise, oospolactone has not any effect to the tracheal muscle.

About twenty isocoumarin derivatives have been found in plants and fungi, but 4-substituted isocoumarins such as oospolactone, oosponol, oospoglycol occur rarely in the nature, and particularly last two compounds are exceptional group in the point of no substituted group at 3-position.

These three isocoumarins isolated from *Oospora astringenes* have very similar structures and it is supposed that their biosynthesis are progressed in close relation among them. Previously, the authors[6] reported that oospoglycol is produced from oosponol by the reducing activity of the resting cell of *Oospora astringenes*.

In this paper the biogenesis of oosponol and oospolactone are discussed by using ^{14}C-labelled compounds.

Experimental

Cultivation of the Fungus——The culture medium had the following composition : malt extract, 20 g.; anhydrous glucose, 20 g.; peptone, 1 g.; and tap water 1 L.

In the radioisotope experiments, the culture medium was poured in 800 ml. portions into three 3 L.-Fernbach flasks, sterilized and inoculated with spore or fragment of mycelium from malt extract-glucose-peptone agar slant. The labelled compound was dissolved in water, sterilized and pipetted into the culture flasks by equal volume at proper period. Immediately after the addition of labelled compound, the cotton plug was changed to rubber stopper carrying two cotton-plugged glass tubes. The gentle stream of CO_2-free air was introduced through the tube, and respiratory CO_2 was trapped in NaOH solution and subsequently collected as $BaCO_3$.

The cultures were incubated at 27° for 30~40 days, with the checking of the UV-absorption of the diluted culture medium (1:50). When the UV-absorption at 335 mμ reached about 0.4~0.5, cultivation was stopped. The nonlabelled metabolites were obtained by the cultivation in Roux flasks.

Isolation of Oospolactone (I) and Oosponol (II)——The mycelium was harvested by filtration through gauze, washed with water three times, and washings were combined to the filtrate.

Previously, oosponol (II) was obtained by active carbon adsorption method, but in this time the method was modified as follows. The culture filtrate was concentrated in a rotary evaporator to some

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*1 Part Ⅵ. Agr. Biol. Chem., **27**, 822 (1963).

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1) I. Yamamoto, K. Nitta, Y. Yamamoto : Agr. Biol. Chem., **25**, 405 (1961).
2) K. Nitta, C. Takura, I. Yamamoto, Y. Yamamoto : *Ibid.*, **27**, 813 (1963).
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7) Y. Kobayashi, S. Ōhashi : Proc. Japan Acad., **38**, 766 (1962).

1/5 volume. After acidifying with HCl it was exhaustively extracted with benzene. Yellow benzene solution was evaporated under reduced pressure. Recrystallization from benzene and/or ethanol gave oosponol, m.p. 173~176°(yield, 50~150 mg. from 1 L. culture). This benzene-extracting method had an advantage to obtain oosponol easily in good yield without contamination of other metabolites.

Oospolactone (I) was obtained from the dried mycelium by extraction with petr. benzin in Soxhlet apparatus for about 24 hr. After removing separated eburicoic acid the benzin solution was evaporated to small volume. Crystalline oospolactone was collected and recrystallized from petr. benzin or ethanol, m.p. 128~129°(yield, 50~100 mg. from 1 L. culture).

Degradation of Oospolactone (I)——I (600 mg.) was fused with KOH according to the previous paper.[1] The reaction mixture was dissolved in water and acidified with H_3PO_4. Precipitated 6-ethylsalicylic acid (III) was collected by filtration and recrystallized from benzene (450 mg.). The filtrate was steam-distilled. The distillate was neutralized with NaOH solution to pH 7~8 and evaporated to dryness. Thus obtained NaOAc (230 mg.) was purified by redistillation, and was in part introduced to S-benzylthiuronium salt (m.p. 139°). The NaOAc (120 mg.) was decomposed into CO_2 (carbon 9) and methylamine (carbon 10) by Schmidt reaction. The reaction was carried out in Lindenbaum apparatus under vacuum, and CO_2 was trapped in $Ba(OH)_2$ solution. Methylamine was distilled with water from alkaline solution and collected as HCl salt. The HCl salt was introduced to the picrate (m.p. 211°) for measurement of radioactivity.

6-Ethylsalicylic acid (III) was decarboxylated by boiling with 85% H_3PO_4 under gentle stream of CO_2-free N_2. The evolved CO_2 (carbon 1) was trapped in $Ba(OH)_2$ solution. The resulted *m*-ethylphenol (IV) was extracted with ether and methylated with $(CH_3)_2SO_4$ without purifications, and the afforded methyl ether (V) was purified by steam-distillation from alkaline solution, then reextracted with ether from the distillate. The syrupy *m*-ethylanisol (V) was oxidized to *m*-methoxybenzoic acid (VI) by following condition: After 1 ml. of 10% NaOH was added to V prepared from 300 mg. of III, the mixture was warmed on a boiling water bath and 6 ml. of $KMnO_4$ solution (3%) was added dropwise during 10 hr. with vigorous shaking. After the color of $KMnO_4$ was faded out, MnO_2 was treated with $NaHSO_3$ and H_2SO_4. The resulted *m*-methoxybenzoic acid (VI) was extracted with ether and recrystallized from water (yield, 32 mg.), m.p. 106~107°. It was decomposed into CO_2 (carbon 8) and amine (*m*-methoxyaniline) by Schmidt reaction, and CO_2 was collected as $BaCO_3$ (39 mg.). The amine was not obtained as crystalline form.

m-Ethylphenol (IV) (prepared from 200 mg. of III) was mixed with 0.1 ml. of conc. H_2SO_4, and 0.3 ml. of 70% HNO_3 was added in small portions under ice cooling. Then the mixture was heated on a boiling water bath for 1.5 hr. After cooling, 1 ml. of fuming HNO_3 (d=1.52) was added and heated for further 5 hr. The trinitro-compound (VII) was obtained by pouring the reaction mixture into 5 ml. of water, and recrystallized from diluted H_2SO_4 (yield, 95 mg.), m.p. 82~84°. *Anal.* Calcd. for $C_8H_7O_7N_3$: C, 37.36; H, 2.74. Found: C, 37.57; H, 2.69.

It was decomposed by barium hypobromide method, and $BaCO_3$ (carbons 3 and 5) and bromopicrin (carbons 2, 4, 6) were obtained. $BaCO_3$ was purified by recycling the CO_2 into $Ba(OH)_2$ solution. Bromopicrin was oxidized to CO_2 by Van Slyke-Folch-oxidation and introduced to $BaCO_3$ after purification.

Degradation of Oosponol (II)——Oosponol (II) was treated with HIO_4 according to the method previously reported.[3] Resulted formaldehyde (carbon 11) was solidified as 2,4-dinitrophenylhydrazone (m.p. 164°). Oospoic acid (VIII) (25 mg.) was boiled with 85% H_3PO_4 for 40 min. and liberated CO_2 (carbon 10) was collected in $Ba(OH)_2$ trap with the stream of N_2 (yield, 19 mg. as $BaCO_3$). The reaction mixture was steam-distilled. Extraction of distillate with ether, evaporation, and recrystallization from petr. benzin gave 8-hydroxyisocoumarin (IX) (5 mg.), m.p. 122°. *Anal.* Calcd. for $C_9H_6O_3$: C, 66.67; H, 3.73. Found: C, 66.31, 66.95; H, 3.72, 3.71.

Oospoic acid (VIII) was treated with O_3 in chloroform by the method previously reported.[3] When the ozonide (from 650 mg. of VIII) was decomposed with hot water, the evolved CO_2 (carbon 10) was repelled with the stream of N_2 and trapped in $Ba(OH)_2$ (yield, 500 mg. as $BaCO_3$). After resulted 3-hydroxyphthalic acid (X) was extracted with ether the aqueous layer was steam-distilled. The distillate was neutralized with NaOH and evaporated, and the sodium formate (carbon 9) was introduced to S-benzylthiuronium salt (m.p. 158°).

3-Hydroxyphthalic acid (X) (carbons 1~8) was decomposed by the method reported by Gatenbeck[8] with some modifications.

The degradation methods of oospolactone and oosponol are summarized in Charts 1 and 2.

Radioisotope Experiments——Labelled compounds used in the experiments were glucose-[1-^{14}C], glucose-[6-^{14}C], ethyl malonate-[2-^{14}C], sodium formate-[^{14}C], and glucose-[^{14}C] (uniformly labelled), which were purchased from the Radioisotope Center, England. About 100 μCi of each labelled compound was administered in equal amount to three flasks which are mentioned above.

8) S. Gatenbeck: Acta Chem. Scand., **12**, 1985 (1958).

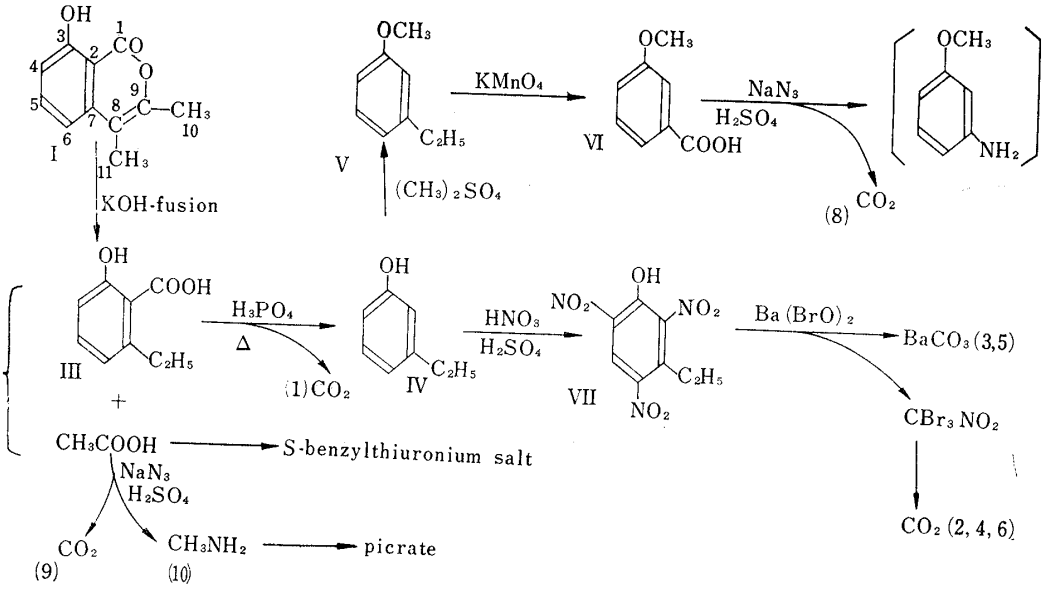

Chart 1. Degradation of Oospolactone

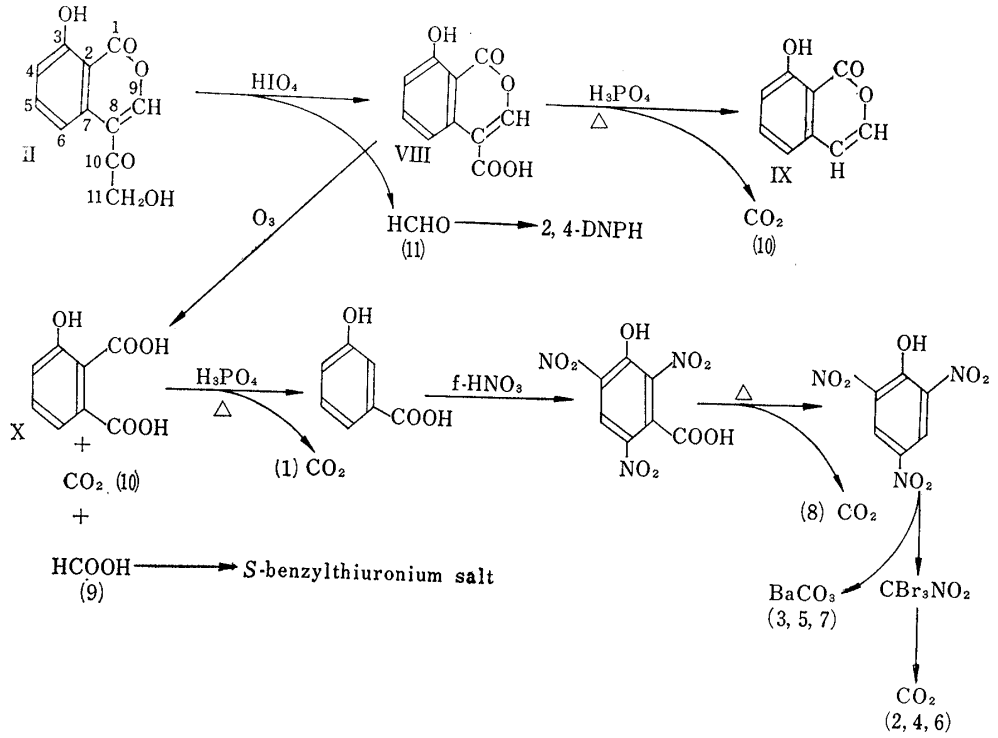

Chart 2. Degradation of Oosponol

The radioactivity of the mycelium was measured as $BaCO_3$ which was prepared by Van Slyke-Folch-oxidation. The broth was measured its radioactivity by evaporation to dryness under IR-light and combustion of evaporating residue to CO_2. To calculate the specific activity per mg. carbon, the amount of carbon of mycelium and broth were determined by the method of elementary analysis.

The extracted metabolites were properly diluted with non-labelled compounds prior to the degradation experiments. Each carbon atom of the metabolites was separated as C_1-compound such as CO_2, CH_3NH_2 or formic acid etc. which were induced to solid forms.

Solid samples (2~20 mg.) were placed in stainless-steel planchets (2.5 cm. in diameter) as even as possible with a few drops of water with a glass piece, dried, counted with a 2π gas-flow counter (Kobe Kogyo Ltd., ATS-100-series, or PR-123-series) and self-absorptions were corrected. The counting error was made within 3%.

Results and Discussion

The progress of cultivation was studied by measuring the dry weight of mycelium, pH and absorbancy of the broth, and the yield of oospolactone and oosponol. The results are shown in Fig. 1. The absorbancy of the culture medium at 335 mμ increased rapidly after the middle period (about the 20th day) of cultivation when the amount of mycelium and pH reached almost constant values. The yield of the two metabolites were paralleled with the absorbancy of the broth. It shows these were synthesized in the late stage of cultivation when the multiplication of organism was almost ceased.

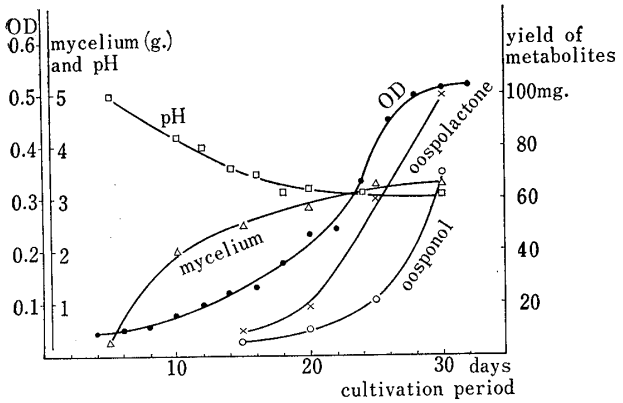

Fig. 1. The Progress of Cultivation

OD was measured by diluting the culture medium to 1:50.
The yield of metabolites was measured by extraction from 1 L. culture (5 Roux flasks).

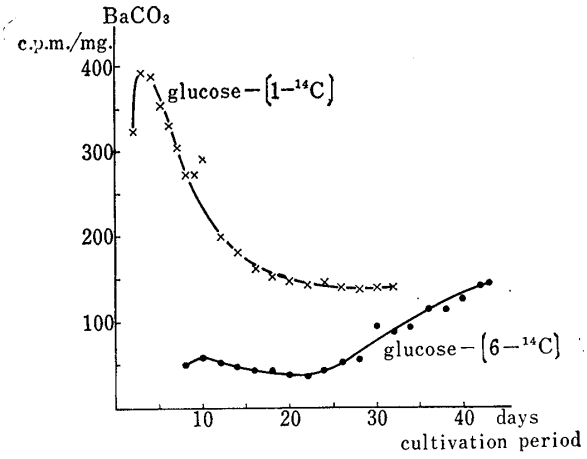

Fig. 2. Specific Activity of Respiratory CO_2 in the Experiments with Glucose-[1-^{14}C] and Glucose-[6-^{14}C]

With glucose-[1-^{14}C], which was added on the first day of cultivation, the specific radioactivity of respiratory carbon dioxide was the highest on the third day of growth, and thereafter declined. On the other hand, with glucose-[6-^{14}C], which was added on the eighth day, the specific radioactivity was low in early stage and after 22nd day increased steadily to the value of glucose-[1-^{14}C]-experiment as shown in Fig. 2.

Total radioactivities of carbon dioxide liberated were 35.3% and 21.5% with glucose-[1-^{14}C] and glucose-[6-^{14}C], respectively. These observations suggest that at least initially, there is preferential removal of C-1 of glucose, perhaps by the pentose phosphate pathway.

Malonate-[2-^{14}C] and sodium formate-[^{14}C] were added on the 18th day of cultivation, at that time oospolactone and oosponol begin to be synthesized. On the next day of addition, in both cases, the specific radioactivity of respiratory carbon dioxide reached the highest value, and thereafter decreased rapidly.

Table I shows the distribution and recovery of radioactivity. The recoveries of radioactivity of five experiments were between 55.3~88.7%. Specific radioactivities(c.p.m./mg. carbon) of oospolactone (I) and oosponol (II) resembled to those of the mycelium and culture medium, respectively. But in the experiment with sodium formate-[^{14}C], the metabolites had far higher specific activity than mycelium and culture medium. This suggests formate had a special behavior for the formation of the metabolites.

Table II shows the distribution of radioactivity in the molecule of the two metabolites.

In the experiment with formate-[^{14}C], almost all the radioactivity was distributed on each one carbon : in oospolactone (I) on the carbon 11 and in oosponol (II) on the

Table I. Distribution of Radioactivity in the Culture and the Metabolites

Experiment with		Amounts	Total activity c.p.m. $\times 10^6$	Incorporation ratio (%)	Specific activity c.p.m./mg. carbon $\times 10^3$
Glucose-[1-^{14}C]	^{14}C used		94.3		
day of addition : 1st day	mycelium	4.9 g.	6.1	6.5	2.6
	culture medium	2.4 L.	29.8	31.6	4.8
day of harvest : 30th day	respiratory CO_2	45 g.	33.3	35.3	
	total		69.2	73.4	
	oospolactone	102 mg.	0.16	0.16	2.2
	oosponol		0.02	0.02	
Glucose-[6-^{14}C]	^{14}C used		94.5		
addition : 8th day	mycelium	7.6 g.	8.3	8.8	2.3
	culture medium	2.4 L.	23.6	25.0	3.6
harvest : 36th day	respiratory CO_2		20.3	21.5	
	total		52.2	55.3	
	oospolactone	162 mg.	0.36	0.38	3.2
	oosponol	188 mg.	0.45	0.48	4.0
Malonate-[2-^{14}C]	^{14}C used		108		
addition : 18th day	mycelium	7.3 g.	14.8	13.0	4.6
	culture medium	2.4 L.	52.6	48.7	8.1
harvest : 33rd day	respiratory CO_2		29.2	27.0	
	total		96.6	88.7	
	oospolactone	340 mg.	1.06	0.98	4.5
	oosponol	216 mg.	1.18	1.09	9.1
H^{14}COONa	^{14}C used		100		
addition : 18th day	mycelium	6.4 g.	8.3	8.3	2.7
	culture medium	2.4 L.	20.9	20.9	4.0
harvest : 34th day	respiratory CO_2		57.1	57.1	
	total		86.3	86.3	
	oospolactone	146 mg.	1.36	1.36	13.4
	oosponol	247 mg.	4.27	4.27	28.8
Glucose-[^{14}C](U)	^{14}C used		110		
addition : 9th day	mycelium	6.1 g.	7.1	6.5	2.5
	culture medium	2.4 L.	30.8	28.0	4.9
harvest : 35th day	respiratory CO_2		34.8	31.6	
	total		72.7	66.1	
	oospolactone	373 mg.	0.75	0.68	2.9
	oosponol	123 mg.	0.41	0.37	5.5

carbon 9. This shows the origin of the methyl-C (carbon 11) of oospolactone, and isocoumarin ring carbon 9 of oosponol were C_1-unit such as formate. It is interesting that these two isocoumarin compounds from the same culture have the different biosynthetic origin in lactone formation.

Experiments with glucose-[1-^{14}C], glucose-[6-^{14}C], and malonate-[2-^{14}C] gave similar results : in oospolactone (I) carbons 2, 4, 6, 8, and 10 had strong and carbons 1, 3, 5, 7, and 9 had weak radioactivity. In oosponol (II) carbons 2, 4, 6, 8, and 11 had strong and carbons 1, 3, 5, 7, and 10 had weak radioactivity. This alternated arrangement of radioactivity suggests roughly the major role of acetate units in the biosynthesis of the two metabolites. Thus, the main skeletons were synthesized by "acetate-malonate condensation".

Table II. Percent Distribution of Radioactivity in Oospolactone and Oosponol

	Carbon No.	Glucose-[1-^{14}C] (%)	Glucose-[6-^{14}C] (%)	Malonate-[2-^{14}C] (%)	H^{14}COONa (%)	Glucose-[^{14}C](U) (%)
Oospolactone (I)	1	1.5	0.8	1.8	0.0	8.8
	2, 4, 6	75.2[c]	15.3[d]	17.0[d]	1.0[c]	9.2[d]
	3, 5, 7		5.6[e]	3.3[e]		9.4[e]
	8		15.4	15.6		9.4
	9	6.0	0.9	2.0	0.5[c]	8.6
	10	15.5	15.2	14.9		8.7
	11[a]	1.4	5.0	4.7	98	8.6
Oosponol (II)	1	1.2	0.8	1.1	1.3[c]	9.3
	2, 4, 6	80[c]	14.9[d]	17.3[d]		9.1[d]
	3, 5, 7		6.4[d]	2.2[d]		8.9[d]
	8		13.9	15.6		9.1
	9[b]	1.1	1.3	5.6	98	9.0
	10	2.3	0.8	0.7	0.1	9.2
	11	14.8	19.5	18.4	0.3	9.3

a) The activities were obtained by difference.
b) The activities were obtained directly by isolation of HCOOH (cf. the text) or indirectly by difference.
c) The total activities of the groups of the carbons.
d) The average activities of the three carbons.
e) The average activities of the carbons 3 and 5, carbon 7 is also presumed to have the similar activity.

In conclusion, oospolactone was formed by cyclization within polyketomethylene chain (from one acetate plus 4 malonate) and the methyl group (carbon 11) derived from C_1-unit. On the other hand, oosponol was formed by cyclization between the carbon derived from C_1-unit and terminal carboxyl group, and a part of the polyketomethylene chain remained as carbons 10 and 11, and finally terminal methyl group was oxidized to oxymethyl group.

"Acetate-malonate theory" is very probable as mentioned above, but one doubtful point remained in the experiments with glucose-[6-^{14}C] and malonate-[2-^{14}C], where there were some differences between the labelling patterns of the two metabolites: oosponol had higher radioactivity in the terminal carbon (carbon 11) than any other carbons, on the other hand, the terminal carbon of oospolactone (carbon 10) had almost the same radioactivity as other strongly labelled carbons. Even if the interconversion of malonate $\rightleftarrows$ acetate is progressed intensively, high radioactivity of the terminal carbon (carbon 11) of oosponol cannot be fully explained. Ibrahim and Towers[9] reported that hydrangenol (3-*p*-hydroxyphenyl-3,4-dihydroisocoumarin) is synthesized from three acetate units and phenylalanine. As well as the case, there remained the possibility that oosponol is biosynthesized from polyacetate chain and C_1-unit plus something else. To resolve the question, as one possible method, uniformly labelled glucose-[^{14}C] was administered to the culture on the ninth day of cultivation, but the present result did not show the participation of other mechanisms.

The authors are indebted to Prof. T. Suzuki of the Institute for Protein Research, Osaka University for his kind discussions. The elementary analyses were carried out by Mr. Y. Itatani of this Faculty to whom the authors express their thanks.

9) R. K. Ibrahim, G. H. N. Towers: Canad. J. Biochem. Physiol., **38**, 627 (1960); **40**, 449 (1962).

Summary

Biogenesis of oospolactone (I) and oosponol (II) from a fungus, *Oospora astringenes* were studied by using glucose-[1-^{14}C], glucose-[6-^{14}C], malonate-[2-^{14}C], formate-[^{14}C], and uniformly labelled glucose-[^{14}C]. Degradation methods of the two metabolites were established and the progress of cultivation was also studied.

The isotope experiments suggest that the skeletons of the two metabolites are synthesized from five C_2-units according to the "acetate-malonate condensation" and one C_1-unit. C_1-unit is incorporated as methyl-C (carbon 11) in I, on the other hand, as =CH- (carbon 9) which participates to form the lactone ring in II. It is interesting that these two isocoumarins from the same culture have the different origin of lactone ring.

(Received September 3, 1965)

[Chem. Pharm. Bull. 14(4) 369~375 (1966)] UDC 547.298.61.07 : 547.298.1.07 : 547.582.4.07

51. Tetsuji Kametani and Osamu Umezawa : A Novel Dehydrazination Reaction. V.[*1] The Formation of Various Amides from Aliphatic and Aromatic Carboxylic Acid Hydrazides in the Presence of Chloral.

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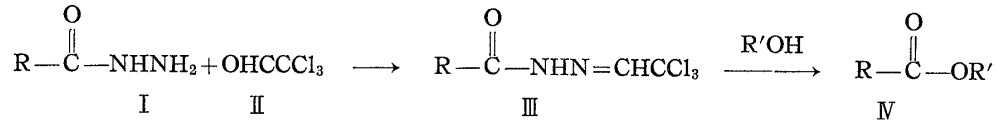
It has been previously observed that ethyl 2-bromo-4,5-dimethoxyphenylacetate was produced by the reaction between 2-bromo-4,5-dimethoxyphenylacetic acid hydrazide and either chloral or bromal in ethanol.[1a] Accordingly, the reactions between aromatic,[1b,2] aliphatic[3] and heterocyclic[*1] carboxylic acid hydrazides and either chloral or bromal in various alcohols were attempted and respective esters were obtained.



The purpose of the present investigation was to study the reaction of various acid hydrazides with chloral in the presence of various amines instead of alcohols previously reported by the authors,[*1,1a,1b,2,3] leading eventually to reveal that the formation of corresponding acid amides was recognized by the reactions between aliphatic and aromatic acid hydrazide and chloral in various amines.

After a reddish-brown mixture of 1-(benzoyl)-2-(2,2,2-trichloroethylidene)hydrazine[2] (III : R=C_6H_5-) and an excess of butylamine had been refluxed for 5 hr., removal of an excess of amine and alumina-chromatography of the residue gave the anticipated amide

*1 Part IV. T. Kametani, O. Umezawa, H. Yagi, S. Asagi : Yakugaku Zasshi, **85**, 518 (1965).
*2 Kita-4-bancho, Sendai (亀谷哲治, 梅沢 修).
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2) *Idem* : Yakugaku Zasshi, **85**, 181 (1965).
3) *Idem* : *Ibid.*, **85**, 514 (1965).