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Application of ^{13}C-NMR to the Biosynthetic Investigations. I. Biosynthesis of Ochratoxin A[1)]

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Incorporation of L-phenylalanine-U-^{14}C, Na-acetate-2-^{14}C into ochratoxin A was demonstrated in *Aspergillus ochraceus*, suggesting that the biosynthesis of ochratoxin A involves so called "acetate-malonate" and "shikimic acid" pathways. By the aid of ^{13}C-NMR spectrometric method, the participation of Na-formate-^{13}C for the formation of carboxyl group was confirmed.

The biosynthesis of ochratoxin A (**1**), a hepatic mycotoxin which have first been isolated from *Aspergillus ochraceus* by the South African research group[3)] seems to involve the condensation of acetate and malonate units to form isocoumarin skeleton through "acetate-malonate pathway" as observed in that of oospolactone,[4)] and citrinin.[5)] The participation of C_1-unit to bring the carboxyl group and also an addition of chlorine to make chloroisocoumarin carboxylic acid (**2**) may undergo subsequently although the stage when these reactions occur is not completely obvious yet.

Fig. 1. Biogenetic Route of Ochratoxin A

We have found and reported that addition of L-phenylalanine to the culture media markedly increased the production of the toxin.[6)] It is probably thought therefore that this amino acid may have a direct role as a precursor for the biosynthesis of ochratoxin. In the present

1) A part of this work was reported at the 91th Annual Meeting of the Pharmaceutical Society of Japan, Fukuoka, April, 1971 and in *Tetrahedron Letters*, **1971**, 2301 as a preliminary communication.
2) Location: *Izumicho, Narashino-shi, Chiba.*
3) K.J. van der Merwe, P.S. Steyn, L. Fourie, de.B. Scott, and J.J. Theron, *Nature*, **205**, 1112 (1965); K.J. van der Merwe, P.S. Steyn, and L. Fourie, *J. Chem. Soc.* (*C*), **1965**, 7083.
4) K. Nitta, Y. Yamamoto, T. Inoue, and T. Hyodo, *Chem. Pharm. Bull.* (Tokyo), **14**, 363 (1966).
5) A.J. Birch, P. Fitton, E. Pride, A.J. Ryan, H. Smith, and W.B. Whalley, *J. Chem. Soc.*, **1958**, 4576.
6) M. Yamazaki, Y. Maebayashi, and K. Miyaki, *Appl. Microbiol.*, **20**, 452 (1970).

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investigation, the efficient incorporation of labeled phenylalanine (**3**) into the toxin was shown in the tracer feeding experiment. By feeding L-phenylalanine-U-^{14}C to the fungus, redioactive ochratoxin A having a specific activity, 4.8×10^7 dpm/mmole was obtained. After hydrolysis, it was confirmed that the radioactivity was localized only in the phenylalanine moiety of the toxin. As same as above, ochratoxin A having a specific activity, 6.7×10^6 dpm/mmole was obtained by feeding sodium malonate-2-^{14}C, however its radioactivity was confirmed localizing only in the isocoumarin moeity in contrast to the result mentioned above (see Experimental part).

In order to elucidate the actual participation of C_1-unit for the formation of carboxyl group in isocoumarin carboxylic acid, the fungus was incubated in liquid media containing a various concentration of ethionine which is known as an effective inhibitor for the biological transmethylation. The production of ochratoxin A was detected and estimated by thin-layer chromatography-fluorodensitometry as previously reported.[4] It was evident as shown in Table I that ethionine inhibited the biosynthesis of ochratoxin A.

Table I. Inhibitory Effect of Ethionine to the Production of Ochratoxin A

Concentration of ethionine (mg/liter)	Final pH	Dry weight of mycelia (g)[a]	Ochratoxin A (mg)[a]
0.0	7.6	26.5	210
6.25	7.6	24.2	90
25.0	7.8	24.4	65
50.0	7.6	24.7	trace

a) All values indicate the yield of mycelia and ochratoxin A per liter of the medium.

Subsequently, to obtain more conclusive evidence concerning with the formation of carboxyl group in isocoumarinic acid (**2**), feeding of sodium formate-^{13}**C** to the fungus was examined. On the use of ^{13}C-NMR spectrometry in biosynthetic studies, Tanabe,[7] *et al.* have reported some results. A great advantage of this method for the application to the biosynthetic work on natural products has been recognized during this several years by many investigators but various difficulties mainly regarding to the techniques for measurement and lack of informations on ^{13}C-chemical shifts, have long disturbed its actual development.

Sodium formate-^{13}C (65.4% excess) was added to the medium containing 0.5% of L-phenylalanine and ^{13}C-enriched ochratoxin A was obtained after cultivation. Isocoumarinic acid (**2**) was given by hydrolysis, which was further methylated with diazomethane. Methyl methoxyisocoumarin carboxylate was thus obtained, which was better soluble in $CHCl_3$ and profitable for the measurement of NMR spectrometry. In this investigation, Hitachi 20B equipped with a signal averaging analyzer, A-1600A and a proton wide band decoupler, R 208 PWD, and JEOL, JNM-PFT-100, equipped with JEC-6 were used through the kindness of Hitachi Ltd. and Japan Electron Optics Laboratory Co., Ltd. Especially the use of Fourier transformation technique with PFT-100 gave us very useful informations for the assignment of signals. The schematic figure of spectra of ^{13}C-NMR of dimethylated sample (**4**) was shown in Fig. 2, and from the splitting pattern of each signals obtained by off center resonance technique as shown in Fig. 3, assignment for each carbon was determined. Two signals in the lowest field (31.56, 27.65 ppm from CS_2) were assigned as of two carbonyl carbons. The signals at 71.08 and 49.12 ppm may represent carbons of the aromatic. The signals observed in high field may be assigned as of C-11,

7) M. Tanabe, H. Seto, and L.F. Johnson, *J. Am. Chem. Soc.*, **92**, 9257 (1970); M. Tanabe, T. Hamasaki, M. Seto, and L.F. Johnson, *Chem. Commun.*, **1970**, 1539; M. Tanabe, T. Hamasaki, D. Thomas, and L.F. Johnson, *J. Am. Chem. Soc.*, **93**, 273 (1971).

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4, 13, 14, 3, of the compound (**4**) by their chemical shifts (171.95, 158.66, 139.80, 128.19 ,and 118.79 ppm from CS_2) and splitting patterns shown in Fig. 3. The result as shown in Fig. 2-a and b indicated that carbon-13 from formate fed was incorporated only into the carboxyl carbon of ochratoxin A. Because, only one signal at 27.65 ppm from CS_2 was observed as of the sample compound (**4**) obtained by feeding ^{13}C-formate. Steyn, *et al.*[8] have also reported recently that an actual incorporation of radioactivity from methionine-S-$^{14}CH_3$ into the carboxyl was observed.

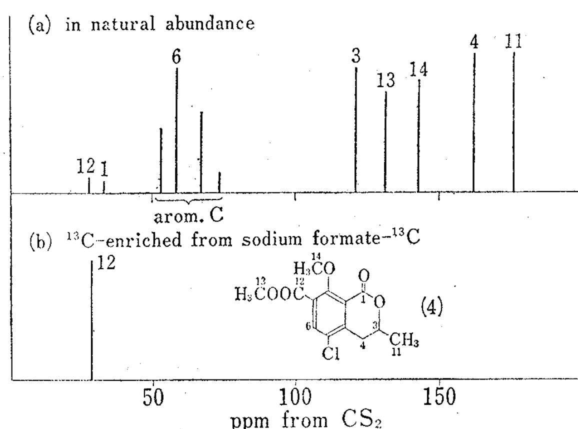

Fig. 2. ^{13}C–NMR Spectra of Compound (4)

Hitachi, R-20B-^{13}C (15 MHz) with A1600A
sample=90 mg/1.1 ml $CHCl_3$
repetition=40 sec/scan × 140 times
Ref.=C_6H_6–$CHCl_3$, lock=external

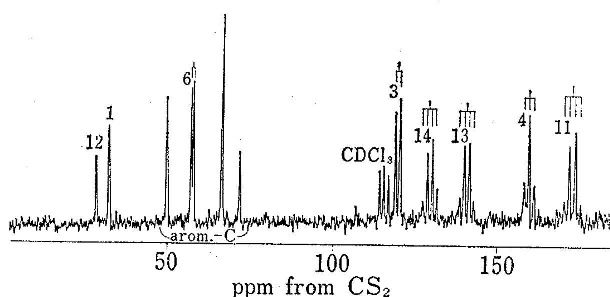

Fig. 3. ^{13}C-NMR Spectrum (off resonance) of Compound (4)

JNM-PFT-100 (25.15 MHz) with JEC6
F=6112.5 Hz sample=200 mg/1.2 ml $CDCl_3$
tw=11 μsec (45°)
repetition=4 sec × 200 times
Ref.=$CDCl_3$ from CS_2
lock=$CDCl_3$ internal

Experimental

Microorganism——Aspergillus ochraceus Wilh. (IFM 4443) was isolated from moldy rice and maintained on malt extract agar and Czapek agar containing 20% sucrose. The fungus was cultivated in liquid media as reported previously.[6] Ethionine and labeled precursors were added to the media at the beginning of incubation in each experiment. The cultures were incubated in a stationary manner at 27° for 7—12 days in dark.

Feeding of L-Phenylalanine-U-^{14}C——The fungus was cultured in Roux's flasks each containing 200 ml of sterilized medium. One ml of the solution of ^{14}C-labeled L-phenylalanine (0.1 mCi/5 ml H_2O) was added to each flask. The mycelia were separated from culture liquid by filtration, dried and extracted repeatedly with ethylacetate. The filtrate was adjusted to pH 3 with HCl and extracted with $CHCl_3$. Solvent was evaporated *in vacuo* and the residual solid was subjected to silica gel column chromatography. Crude toxin eluted with EtOAc–$CHCl_3$ (1: 3) was recrystallized from benzene to yield 20 mg of ochratoxin A having specific activity of 4.8×10^7 dpm/mmole. Radioactivity was measured with a liquid scintillation counter, Beckman Model LS 150, dissolving the sample in toluene scintillator containing PPO in 0.5%.

Feeding of Sodium Malonate-2-^{14}C——The fungus was grown in 1 liter of medium containing sodium malonate-2-^{14}C (0.1 mCi) and 5 g of L-phenylalanine. The mycelia and culture filtrates were separated and extracted with EtOAc and $CHCl_3$ respectively. Labeled ochratoxin A (151 mg) having specific activity of 6.7×10^6 dpm/mmole was obtained from 2 g of crude extract by silica gel column chromatography.

Hydrolysis of Ochratoxin A with Carboxypeptidase A——With 1 ml of carboxypeptidase A solution, radioactive ochratoxin A (10 μg, 0.58 mCi) was incubated in 0.1M NaCl–0.02M Tris buffer of pH 7.5 at 37° for 60 min. The reaction was stopped by addition of 1 ml of 10% HCl solution and immediately the mixture was extracted with $CHCl_3$. Solvent was evaporated *in vacuo* and the residue was dissolved again in 1 ml of $CHCl_3$ to be subjected to thin–layer chromatographic analysis. Aliquots of the $CHCl_3$ solution were spotted on silica gel plates and developed with benzene–AcOH (4:1). The spots of ochratoxin A and isocoumarinic hydrolyzate (2) were detected under ultraviolet light. The radioactivity in each spot was detected by autoradiography.[9]

The Hydrolysis of Labeled Ochratoxin A with Conc. HCl——The radioactive ochratoxin A (22 mg) obtained by feeding sodium malonate-2-^{14}C, was suspended in 60 ml of conc. HCl and refluxed for 26 hr under

8) P.S. Steyn, C.W. Holzapfel, and N.P. Ferreira, *Phytochem.*, 9, 1977 (1970).
9) M. Yamazaki, S. Suzuki, Y. Sakakibara, and K. Miyaki, *Japan. J. Med. Sci. Biol.*, 24, 245 (1971).

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N_2. The mixture was extracted with $CHCl_3$ after cool and by removal of the solvent, 11 mg of crude crystals were obtained. By silica gel column chromatography, pure isocoumarin carboxylic acid (2) having specific activity of 6.67×10^6 dpm/mmole was obtained.

Inhibition of Ochratoxin Production by Addition of Ethionine——Ethionine of each 50, 25, 12.5, 6.25 and 3.125 mg was added to 1 liter of the medium. Cultures were incubated stationary for 7 days at 27° in dark. The mycelia and culture filtrates were extracted with EtOAc and $CHCl_3$ separately. The extracts thus prepared were dissolved in 1 ml of $CHCl_3$ and 2 μl of the solution were quantitatively spotted on TLC-plate of silica gel (Merck). The plate was developed with benzene–AcOH (4:1). The quantity of ochratoxin A on each spot was estimated with a fluorodensitometer, Hitachi MPF-2A according to the method as previously reproted.[6]

Feeding of Formate-^{13}C——The fungus was inoculated into 1 liter of the medium containing 5 g of L-phenylalanine and 250 mg of formate-^{13}C (65.4% excess, purchased from Sharp and Dohme of Canada). The culture was incubated at 27° for 10 days in dark. The mycelium and culture filtrate were extracted together with $CHCl_3$. Solvent was evaporated in vacuo and the residue (997 mg) was subjected to silica gel column chromatography. The crude ochratoxin A was immediately dissolved in 100 ml of conc. HCl and refluxed for 23 hr under N_2. The isocoumarinic acid thus obtained was methylated with diazomethane in ether containing methanol in 10% at room temperature. After purification by silica gel column chromatography, 90 mg of methyl methoxyisocoumarin carboxylate (4) was obtained and subjected to ^{13}C-NMR spectrometry.

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