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BIOSYNTHESIS OF OCHRATOXIN A

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Ochratoxin A is a fungal toxic metabolite of Aspergillus ochraceus Wilh. and its chemical structure has been elucidated by van der Merwe et al.[1] The toxin contains 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3-methylisocoumarin linked through its carboxyl group to the amino group of L-β-phenylalanine. The biosynthesis of the isocoumarin moiety of the toxin seems very plausible to involve the condensation of the acetate and malonate units and the participation of C_1-unit and chlorine atom to the polyketide chain derived through such a way as given in Fig. 1.

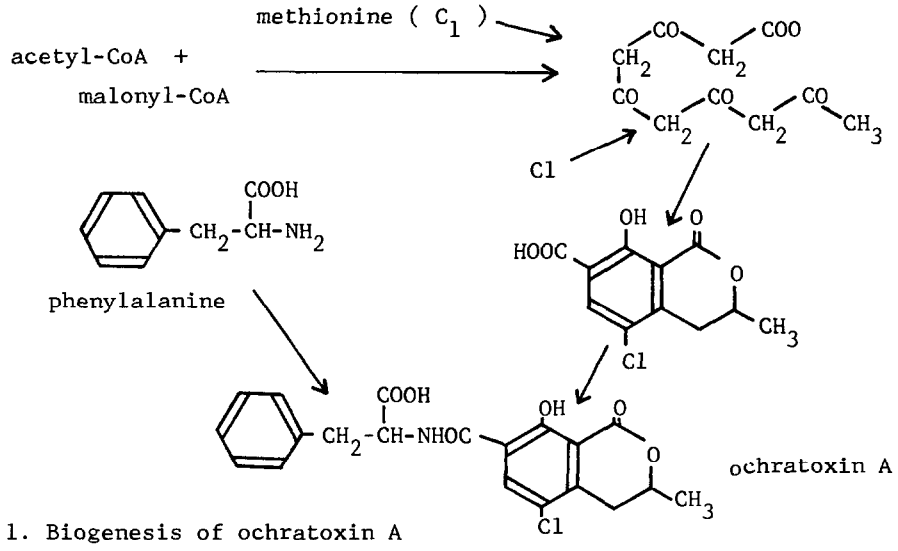

Fig. 1. Biogenesis of ochratoxin A

On some analogous fungal metabolites, e.g., citrinine in Aspergillus candidus[2] and oospolactone in Oospola astringenes[3], some biosynthetic studies had been done with the aid of the radioisotopic technique. The isocoumarin thus derived may be coupled subsequently with phenylalanine to build up the complete form of ochratoxin A.

We would like to report here some results of the experiments in which the inhibitional effect of ethionine to the participation of C_1-unit on the biosynthesis of ochratoxin A and then the direct incorporation of labeled C_1-unit to the toxin were examined. Aspergillus ochraceus Wilh. (IFM 4443) was grown in the liquid medium with a similar formula to that reported previously[4] except the further addition of 20g of yeast extract. Ethionine and the labeled precursors were added to the basal medium at the biginning of incubation in each experiment. The chloroform extract of the acidified culture filtrate was concentrated. The residue was chromatographed on silicic acid column and a fluorescent band of ochratoxin A was eluted out with $CHCl_3$-AcOEt (3 : 1). The crude toxin thus collected was purified by the recrystallization with benzene. Colorless needles, $C_{20}H_{18}O_6NCl \cdot C_6H_6$, mp 92°C. The provision of phenylalanine-U-^{14}C (0.1 mCi) and sodium malonate-2-^{14}C (0.1 mCi) to the medium gave the active ochratoxin A in several experiment. The radioactivity from phenylalanine-^{14}C and malonate-^{14}C was confirmed to be localized in the phenylalanine and the isocoumarin moieties of ochratoxin A respectively, since the radioactivity of the resulting active toxin was retained in respective part after hydrolysis of the toxin with conc. HCl.

In order to establish the actual derivation of the carboxyl group of isocoumarinic acid from C_1-unit, the fungus was incubated in a liquid medium containing a various concentration of ethionine. When ethionine was added 50mg to 1 liter of the medium, the production of ochratoxin A was entirely inhibited.

This result probably indicates that the carboxyl group of the isocoumarinic acid could be derived from the C_1-pool such as methionine. Steyn et al[5] had reported else actually a good incorporation of methionine- $S^{14}CH_3$ to the carboxyl group of ochratoxin A.

Table I.

Inhibition of ochratoxin A production by the addition of a various concentration of ethionine.

conc. of ethionine (mg/l)	final pH	dry mycelia (g*)	ochratoxin A (mg*)
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

* All values indicate the yield per liter medium.

To achieve the further confirmation of the participation of C_1-unit, sodium formate-^{13}C was provided. Sodium formate-^{13}C (65.4%, Merck, Sharp and Dohme of Canada) was added 250mg to 1 liter of the medium containing 0.5% of L-phenylalanine. The enriched ochratoxin A obtained was hydrolised to give isocoumarinic acid and methylated with diazomethane in ether containing methanol in 10% to yield methoxyisocoumarin carboxylate methyl ester. The dimethyl isocoumarin derivative is better dissolved in chloroform. The ^{13}C-nuclear magnetic resonance (NMR) spectra were measured in chloroform at 15 MHz using Hitachi spectrometer, Model R-20 equipped with a signal averaging analyzer, A-1600A and a proton wide-band decoupler, R-208 PWD. An external reference used was benzene contained in a small tube inserted in a sample tube. In Fig. 2-A and B, the ^{13}C-NMR spectra of ochratoxin A in natural abundance and ^{13}C-enriched were demonstrated. In either, the signals were observed as singlets by the applica-

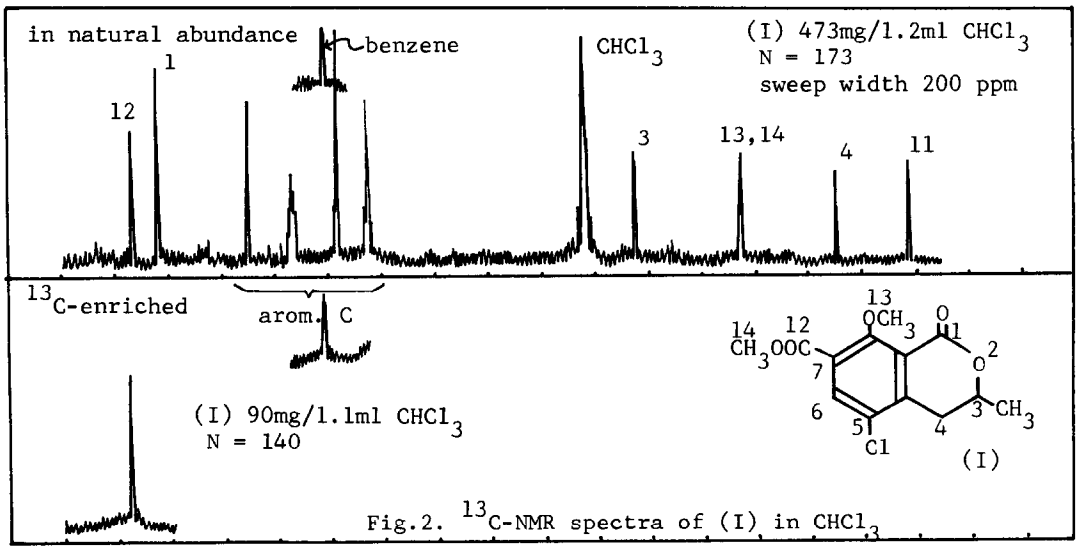

Fig.2. ^{13}C-NMR spectra of (I) in $CHCl_3$

tion of proton wide-band decoupling technique. The two signals in the most low-field (-31.0 and -36.7 ppm from benzene) were assigned as that of two carbonyl carbons (the lactonyl and carboxyl carbons) of dimethyl isocoumarin derivative.[6] It became evident from the result shown in Fig. 2 that the sole incorporation of ^{13}C into the carbon of the carboxyl of ochratoxin A from formate was carried out in the biosynthetic process of the toxin.

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