For the isolation of I, the unidentified fungus was fermented in a modified Czapek-Dox medium[4] under conditions of aeration and agitation at 24 °C for 12 days. The product was extracted from the culture filtrate at pH 2.5 (300 l) into chloroform. The extract was washed with dilute sodium bicarbonate, concentrated to a residue and chromatographed on silica gel (Grace, chromatographic grade) with hexane-chloroform (3:1). Eluate fractions containing material giving a blue color with ferric chloride were combined and evaporated to dryness. The residue was crystallized from hexane-ethyl ether (4:1) to yield 5.7 g of I. Anal. C, 67.45; H, 5.85. Calcd. for $C_{10}H_{10}O_3$ (mellein): C, 67.43; H, 5.61, m.p. 51.5–52 °C; $[\alpha]_D^{25}$ + 88 (c, 1.03 in methanol), $[\alpha]_D^{25}$ + 102 (c, 1.07 in chloroform). λ_{max} (log ε) = 212 (4.30); 245 (3.86); 312 (3.62) nm in methanol. Alkali shifted the maximum at 312 nm to 344 nm, which was reversed with acid. The IR-spectrum of I was the same in all essential respects with that reported for synthetic (±)-mellein[5].

The nuclear magnetic resonance spectrum of I ($CDCl_3$, 60 MC/sec, in PPM from tetramethylsilane as internal standard) showed a total of 10 protons. These could be assigned to O–CH–C*H*$_3$ (δ = 1.53, doublet); CH-C*H*$_2$-phenyl (δ = 2.97, doublet); O–C*H*–CH$_3$ (δ = 4.75, quartet); three ortho-substituted aromatic protons (δ = 6.73, doublet; δ = 6.87, doublet; and δ = 7.45, triplet), and one phenolic proton (δ = 11.05, singlet)[6]. This interpretation is consistent with structure I for the metabolite.

Potassium hydroxide fusion at 185 °C for 45 min[7] converted I to the acid II. The product was recovered from an acidified, aqueous solution of the alkali melt by ethyl ether extraction and crystallization from hexane-ethyl ether (2:1). Yield, 60%. Anal. C, 67.54; H, 5.52. Calcd. for $C_{10}H_{10}O_3$: C, 67.43; H, 5.61, m.p. 165 °C with sublimation; λ_{max} (log ε) = 254 (4.08); 318 (3.64) nm in methanol. The IR absorption spectrum of II showed broad absorption in the region of 2500 cm^{-1} and strong peaks at 1630 and 1130 cm^{-1} expected of a chelated carboxylic acid[8]. In the spectrum of I the carbonyl absorption was at 1660 cm^{-1} and there was a strong peak at 1160 cm^{-1} attributed to an ester linkage[8], which were not present in the spectrum of II. These data indicate that II is 6-trans-propenyl-salicylic acid, the alkali-fusion product of mellein[9,11].

Zusammenfassung. Ein Stoffwechselprodukt eines nicht klassifizierten Pilzes wurde als (+)-3-methyl-3,4-dihydro-8-oxyisocumarin, der optischen Antipode von Mellein, identifiziert.

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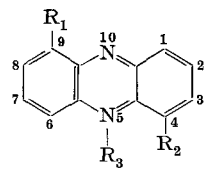
In vivo Oxidative Coupling of Anilines and Phenolic Anilines[1]

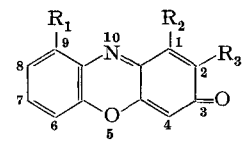
The principle of oxidative coupling in nature has received wide attention in recent years. According to a hypothesis by BARTON[2] various classes of natural products, such as alkaloids, plant pigments and microbial metabolites, contain numerous representatives which may be thought to arise biogenetically by oxidative coupling of phenolic precursors. Recent feeding experiments with labeled precursors, e.g. by BATTERSBY[3], proved the validity of the phenolic oxidative coupling principle in the biosynthesis of certain alkaloids.

Inasmuch as phenolic oxidative coupling is believed to proceed via radical pairing, the possibility that also anilines or phenolic anilines are subject to in vivo coupling seems obvious. The previously demonstrated in vitro feasibility of such coupling, either by electron transfer agents[4] or in air under catalysis of enzymes isolated from higher animals[5], suggests a similar pattern in nature. However, no in vivo coupling has heretofore been observed.

Inspection of presently known natural products shows that examples on which this hypothesis may be tested are restricted to compounds with the phenazine (I) and 3(3H)-phenoxazinone (II) skeleton.



I



II

Besides the type II ommochromes, phenazines and 3(3H)-phenoxazinones have been found exclusively as pigmented metabolites of microorganisms. Considered as a group there is a noticeable recurrence of either an acyl or hydroxyl group in positions 1, 4, 6, or 9 of I and an acyl group in positions 1 and 9 and a nitrogen function in

[1] This work was supported by Grant GB-2290 of the National Science Foundation.
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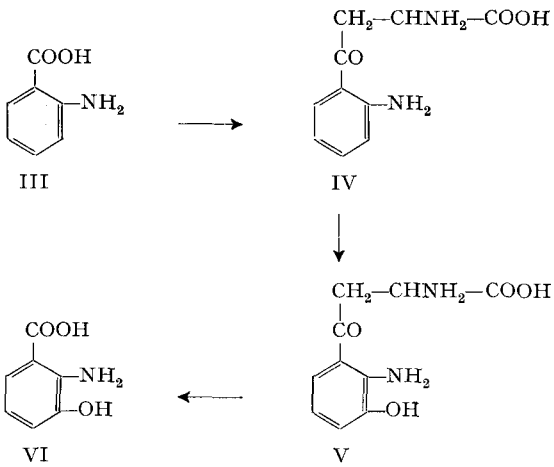
position 2 of II[6]. Therefore, the formation of the 20 known metabolites of types I and II may be envisaged from aromatic precursors substituted in adjacent positions by an acyl, amino and hydroxyl group. Such requirements are met by a sequence of compounds known to represent intermediates in the biosynthetic pathway to nicotinamide, in the order: anthranilic acid (III), kynurenine (IV), 3-hydroxykynurenine (V) and 3-hydroxyanthranilic acid (VI)[7].



A scheme was developed to synthesize III–VI labeled in the benzene ring. The reason for such site of label was twofold. Several metabolites to be investigated do not contain carbon side chains, presumably because of elimination before or after oxidative coupling. Thus measurement of incorporation of III–VI would be impossible if only the side chain is labeled. Furthermore, the loss of labeled side chain carbons may lead to their incorporation into the benzene ring at the pre-aromatic stage of the biosynthetic pathway, yielding ambiguous results[8]. It was found advantageous to prepare the suspected precursors from one common starting material. Uniformly labeled C-14 benzene (333 μc/ml) was converted via acetophenone into a mixture of 2-nitro (VII) and 3-nitroacetophenone (VIII). After separation by preparative vapor phase chromatography each of the isomers was diluted ten times with inactive compound. Isomer VII was converted into III and, using a modification of BUTENANDT's[9] procedure, into IV. From VIII a mixture of 2-nitro-3-hydroxyacetophenone (IX) and several isomers and dinitro products was obtained. Compound IX, isolated by partition chromatography, served for the synthesis of V and VI.

Results of feeding experiments

Experiment No.	C-14 precursor fed	Metabolite isolated	mμM incorporated
1	3-Hydroxykynurenine	Questiomycin A	10
		Questiomycin B	48
2	Anthranilic acid		370
3	Kynurenine sulfate	Phenazine-1-	75
4	3-Hydroxykynurenine	carboxylic acid	30
5	Anthranilic acid		644
6	Kynurenine sulfate	Pyocyanine	1100
7	3-Hydroxykynurenine		100

Ring labeled V was fed to *Streptomyces chromofuscus* (strain 31 IPRC-31)[10], producer of questiomycin A (II, $R_1 = R_2 = H$, $R_3 = NH_2$) and questiomycin B (*o*-aminophenol)[11]. Ring labeled III, IV and V was fed to *Pseudomonas aureofaciens* (ATCC 13985), producer of phenazine-1-carboxylic acid (I, $R_1 = COOH$, $R_2 = H$, R_3 absent)[12] and to *Pseudomonas aeruginosa* (ATCC 10145)[13], producer of pyocyanine (I, $R_1 = O^-$, $R_2 = H$, N_5–$R_3 = N_5^+$–CH_3). In each experiment the microorganisms were grown on a rotary shaker in 14 flasks, each containing 100 ml broth. A concentrated aqueous solution of 100 mg of the labeled compound was distributed equally over the 14 flasks at the beginning of the growth period. Shaking was continued at the specified temperature and for the optimum time indicated in the literature. The metabolites were isolated, according to known procedures, and purified by chromatographic techniques. After conversion into barium carbonate the samples were counted in a windowless flow counter.

The results, presented in the Table, show that in all experiments incorporation was found in the isolated metabolites. The level of incorporation is of the same order as that found in similar experiments[8,14]. The evidence obtained indicates that the investigated metabolites are generated by oxidative coupling of III, IV and V. In experiment 1 the side chain may have been lost before or after coupling. Isolation of questiomycin B (*o*-aminophenol), possessing a specific activity ($2.1 \cdot 10^{-2}$ μc/g) equal to that of questiomycin A, requires the first path. The side chain may have been eliminated before or after coupling in one ring (experiments 2, 3 and 4) and in both rings (experiments 5, 6 and 7). Loss of alanine and the hydroxyl group must have occurred in experiments 3 and 4 and the hydroxyl group in one ring must have been lost in experiment 7. Experiments 5, 6 and 7 further require methylation at N_5.

The formation of other metabolites of type I and II is presently under investigation.

Zusammenfassung. Im Kern markierte ^{14}C-Anthranilsäure und Derivate wurden an Mikroorganismen, die Phenazin- und Phenoxazon-Farbstoffe produzieren, verfüttert. Der erfolgte Einbau beweist die oxydative Kupplung von Anilinen und phenolischen Anilinen in vivo.

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