# 2,4-DIHYDROXY-4′-METHOXYBENZIL, A PROBABLE ARTEFACT FROM FORMONONETIN IN SUBTERRANEAN CLOVER EXTRACTS*

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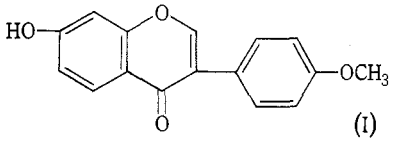
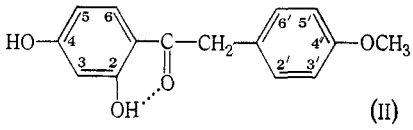
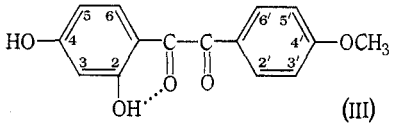
To investigate a claim that ethanolic alkali extracts of chloroplast fractions from subterranean clover contain phenolic substances having greater oestrogenic activity than the known isoflavonoid constituents, the chloroplast fraction from a large (5-ton) batch of subterranean clover was extracted by the method of Beck and Braden.[1] Biochanin A and genistein were readily separated, but as isoflavones lacking a 5-position hydroxyl group readily undergo alkaline hydrolysis, relatively little formononetin (I) was isolated. Instead there was obtained large amounts of ononetin (II), an hydrolysis product of formononetin, and in very low yield a yellow crystalline compound which has been identified as 2,4-dihydroxy-4′-methoxybenzil (III).




(I)




(II)




(III)

The benzil (III) was characterized by its spectroscopic properties and the substitution pattern followed from the resemblance of the n.m.r. spectrum to that of ononetin (II). The signals from the protons at C2′ and C6′, which are part of an $A_2B_2$ system, show a downfield shift (of 0·43 p.p.m.) relative to the signals from the corresponding protons in the spectrum of ononetin, and this shift is ascribed to the deshielding effect of the adjacent carbonyl group.

The signals from the C2′ and C6′ protons are also slightly downfield (0·33 p.p.m.) with respect to the signal from the C6 proton, possibly because of the reduced deshielding effect of the hydrogen bonded carbonyl group. The methoxyl group can be placed at C4′, and an hydroxyl at C4 as in ononetin, because the mass spectrum of the benzil showed a weak molecular ion peak at *m/e* 272, with two major fragmentation peaks at *m/e* 135 and *m/e* 137, and indicated clearly to which ring the methoxyl

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[1] Beck, A. B., and Braden, A. W., *Aust. J. exp. Biol. med. Sci.*, 1951, **29**, 273.

and hydroxyl groups were attached. Chemical proof of the structure of the benzil was obtained by ethylation and oxidation of the product to 2,4-diethoxybenzoic acid, 4-ethoxy-2-hydroxybenzoic acid, and anisic acid.

As the benzil (III) has not been detected by thin-layer chromatography as a constituent of subterranean clover extracts, its close structure relationship to ononetin suggests that like ononetin, it may be an artefact derived from formononetin. The formation of (III) under the extraction conditions used might have been expected because it has been shown[2] that deoxybenzoin in hot t-butanol containing potassium t-butoxide is rapidly converted into benzilic acid.

Neither ononetin nor the benzil (III) showed significant oestrogenic activity. Although fractions were prepared which showed strong oestrogenic activity in mice at doses of 10 $\mu$g per mouse, the compounds responsible were present in amounts too small for purification.

*Experimental*

The method used for processing the chloroplast fractions was an empirical one, based on an unexplained observation[1] that, when successive extracts were made with ethanolic sodium hydroxide, the phenols from the second and third extracts but not the first exhibited an enhanced oestrogenic activity in the mice. Yields are therefore not quantitative. Dry chloroplast (13·2 kg) prepared from the juice of about 5 tons of the Dwalganup strain of subterranean clover by a previously described method[3] was extracted in 800-g batches with boiling 1N alcoholic sodium hydroxide solution (8 l. for 10 min). The solution was cooled rapidly and filtered, and the filtrate from the first extraction was discarded. The solid recovered from filtration was extracted a second time with 0·5N ethanolic sodium hydroxide solution and after filtration a third extraction was made with 0·2N ethanolic sodium hydroxide. The second and third extracts were neutralized with 20% ethanolic sulphuric acid and the "crude phenols" and "purified phenols" were recovered from the neutral solution by the method of Beck and Braden.[1] The total amount of dry chloroplasts (13·2 kg) gave 89 g "purified phenols".

The "purified phenols" were chromatographed on silica gel with light petroleum as the eluting solvent, followed by mixtures of light petroleum and ether in which the proportion of ether was gradually increased. The following fractions were collected in sequence (yields are indicated in brackets, and all compounds named were identified by purification, determination of melting points, spectroscopic examination, and comparison with known reference compounds):

(i) a mixture of ononetin (major constituent) and ethyl *p*-hydroxybenzoate (30%);

(ii) crude biochanin A (5%);

(iii) a yellow oily fraction which contained the highly oestrogenic compounds (20%);

(iv) genistein (40%).

The isolation of benzil (III) was achieved in two ways:

(1) Part of the yellow oily fraction was submitted to countercurrent distribution (23 transfers) between benzene and aqueous phosphate buffer (pH 8·6). The first fractions (32%) were not investigated, the last fractions (12%) consisted of genistein, and fractional crystallization of the middle fractions yielded the benzil (III).

(2) The remainder of the yellow oily fraction was chromatographed on silica gel with a mixture of acetone and methylene dichloride, using gradient elution, and increasing the proportion of acetone in the mixture. Benzil (III) was isolated from the eluate.

The benzil (III) crystallized from methylene dichloride/light petroleum in yellow needles, m.p. 172° (Found: C, 66·5; H, 4·7; $CH_3O$, 11·0. Calc. for $C_{15}H_{12}O_5$: C, 66·2; H, 4·4;

[2] Doering, W. von E., and Haynes, R. M., *J. Am. chem. Soc.*, 1954, **76**, 484.

[3] Legg, S. P., Curnow, D. H., and Simpson, S. A., *Biochem. J.*, 1950, **46**, 19.

$CH_3O$, 11·4%), and yielded on acetylation 2,4-acetoxy-4′-methoxybenzil as pale prisms, m.p. 69° (Found: C, 63·9; H, 4·6. Calc. for $C_{19}H_{16}O_7$: C, 64·0; H, 4·5%). The benzil (III) had $\lambda_{min}$ 247 mμ, ε 6100, $\lambda_{max}$ 285 mμ, ε 19300; $\lambda_{infl}$ 310 mμ, ε 14500, and $\lambda_{max}$ 320 mμ, ε 14600, and i.r. bands at 3360 $cm^{-1}$ (free OH), 3200 $cm^{-1}$ (bonded OH), and 1670 $cm^{-1}$ (carbonyl). The 60-Mc/s n.m.r. spectrum, measured in a solution of deuterated dimethyl sulphoxide/deuterochloroform (1 : 1) with chemical shifts relative to tetramethylsilane (δ 0·00), showed a three-proton signal at δ 2·75 (methoxyl), and a typical $A_2B_2$ system of peaks centred at δ 6·75 and δ 7·63 (1,4-substituted phenyl ring). Signals from three other aromatic protons at δ 6·34, 6·40, and 7·30 showed chemical shifts and coupling constants consistent with the structure of (III), and a broad two-proton signal at δ 8·0–10·0 was assigned to the two hydroxyl groups.

Ethylation by heating in acetone solution with diethyl sulphate and potassium carbonate, and oxidation of the product with alkaline hydrogen peroxide in acetone, yielded 2,4-diethoxybenzoic acid, 2-hydroxy-4-ethoxybenzoic acid, and anisic acid, which were identified by gas chromatographic examination of their methyl esters and by thin-layer chromatography.