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THE ISOLATION AND CHARACTERIZATION OF HELLEBRIGENIN 3-ACETATE AND HELLEBRIGENIN 3,5-DIACETATE, BUFADIENOLIDE TUMOR INHIBITORS FROM BERSAMA ABYSSINICA [a]

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In the course of a continuing search for tumor inhibitors of plant origin, an alcoholic extract of the stem bark of Bersama abyssinica Fresen. (Melianthaceae), collected in Ethiopia in March 1965, was found to show significant inhibitory activity against cells derived from human carcinoma of the nasopharynx carried in cell culture (KB).[b] We report herein the isolation and characterization of hellebrigenin 3-acetate (I) and hellebrigenin 3,5-diacetate (II), two novel naturally-occurring tumor inhibitors from B. abyssinica.[c] The two hellebrigenin acetates appear to be the first recognized naturally-occurring bufadienolide 3-esters; the 3,5-diacetate appears to be the first reported 5β-acetoxy steroid. Furthermore, although other cardenolides (1-3) and bufadienolides (3) have been found earlier to possess marked cytotoxic activity against KB tissue culture, hellebrigenin 3-acetate appears to be the first

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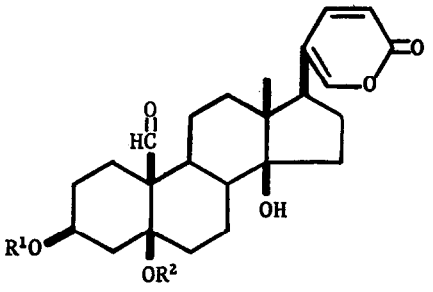
a) Tumor Inhibitors. XXVII. Part XXVI: S. M. Kupchan, M. Mokotoff, R. S. Sandhu, and L. E. Hokin, J. Med. Chem. in press.

b) Cytotoxicity and in vivo inhibitory activity were assayed under the auspices of the Cancer Chemotherapy National Service Center (C.C.N.S.C.), National Cancer Institute, National Institutes of Health, by procedures described in Cancer Chemotherapy Rept. 25, 1 (1962).

c) Hellebrigenin 3-acetate and hellebrigenin 3,5-diacetate showed significant cytotoxicity (ED_{50}) against KB cell culture at 10^{-7} and 10^{-3} μg./ml., respectively. Hellebrigenin 3-acetate showed significant inhibitory activity against intramuscular Walker carcinosarcoma 256 in rats at 8 mg/kg.

cardiotonic steroid recognized to show significant and reproducible inhibitory activity against an in vivo tumor system.



I: R^1 = Ac; R^2 = H

II: R^1 = R^2 = Ac

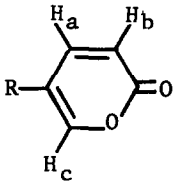
III: R^1 = R_2 = H

Fractionation of the ethanol extract of B. abyssinica, guided by assay against KB, revealed that the active principles were concentrated, successively, in the chloroform layer of a chloroform-water partition and in the aqueous methanol layer of a 10% aqueous methanol-petroleum ether partition. The 10% aqueous methanol solubles, dissolved in methanol, were treated with a saturated methanolic solution of neutral lead acetate. The active filtrate was freed from excess lead by treatment with hydrogen sulfide and the methanol evaporated. The residue was chromatographed on silicic acid to yield two discrete cytotoxic fractions (A and B) upon elution with 2% methanol in chloroform. Fraction A was rechromatographed on silicic acid and, upon elution with 1% methanol in chloroform, yielded active fraction C. Similar rechromatography of fraction B gave D and E.

Fractions D and E were crystallized from methanol to yield colorless prisms, m.p. 230-232°, $[\alpha]_D^{30}$ + 30°. The latter physical constants and infrared, ultraviolet, and nmr spectra indicated the compound to be hellebrigenin 3-acetate (I), and the identity was confirmed by direct comparison with an authentic sample (prepared by acetylation of hellebrigenin) kindly supplied by Professor T. Reichstein.

Fraction C was crystallized from methanol-ether to yield colorless prisms (II), $C_{28}H_{36}O_8$; m.p. 217-219°; $[\alpha]_D^{28}$ - 23° (c 0.51, $CHCl_3$); λ_{max} 298.5

(ϵ 5,650); $\lambda_{max}^{CHCl_3}$ 2.90, 3.38, 5.80, 6.12, 6.50, 7.95 μ; and nmr signals (in $CDCl_3$) at τ 0.00 (1H, s, -CHO), 2.20 (1H, dd, J=2 and 9.5 cps, IV, Ha), 2.76 (1H, dd, J=2 and 1 cps, IV, Hc), 3.75 (1H, dd, J=9.5 and 1 cps, IV, Hb), 4.84 (1H, m, >CH-OAc), 7.0-9.0 (22H, m), 8.01 (3H, s, -O-COCH$_3$), 8.05 (3H, s, -O-COCH$_3$), 9.32 (3H, s, C-18 methyl).



IV

The nmr spectrum of II closely resembled that of I, and the main difference was that II showed an additional acetate grouping and one less proton in the large multiplet. This suggested that II is a hellebrigenin diacetate derivative. Furthermore, the presence of only one >CH-OAc proton implied that the additional acetate is tertiary, i.e., at C-5 or C-14. To test this hypothesis, the synthesis of II from hellebrigenin (III) was undertaken.

A sample of hellebrin was hydrolyzed to hellebrigenin with Clarase enzyme preparation in a phosphate buffer. The solution was incubated with rapid stirring at 37° for 10 days. The formation of hellebrigenin was followed by tlc. When hydrolysis was complete, the solution was extracted with chloroform, the chloroform was evaporated, and the residue was crystallized from acetone-ether to yield hellebrigenin (III). This method improves on the older two-step method (4) in giving a high yield (80%) of product.

Acetylation of III was effected in chloroform and isopropenyl acetate with a catalytic amount of p-toluenesulfonic acid at 45-50° for 3-4 hours. The major product isolated by silicic acid chromatography was hellebrigenin 3-acetate (I). The minor product isolated by chromatography was found to be identical to II. Hence, the structural possibilities for II could be limited to hellebrigenin 3,5-diacetate or 3,14-diacetate alternatives.

Characterization of II as hellebrigenin 3,5-diacetate was made possible

by a study of the behaviors of I and II under conditions which favor base-catalyzed ester solvolysis. Earlier detailed studies had shown that the solvolysis of the 3-axial acetate in strophanthidin 3-acetate is a general acid-general base catalyzed reaction which is facilitated both by the hydroxyl group (at C-5) bearing a 1,3-diaxial juxtaposition to the acetate and by the C-19 aldehyde function (5). Hellebrigenin 3-acetate (I) differs from strophanthidin 3-acetate only in the lactone ring, and, in accord with expectation, treatment of I in 20% aqueous methanol with triethylamine at room temperature for 16 hours led to essentially complete solvolysis to hellebrigenin (III). In contrast, treatment of II under the same conditions effected no solvolysis, and II was recovered essentially quantitatively. The stability of II toward base-catalyzed solvolysis indicates the absence of a free 5β-hydroxyl group in II, and, therefore, strongly favors assignment of the hellebrigenin 3,5-diacetate structure.

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