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CONSTITUENTS OF *CITRULLUS COLOCYNTHIS* (L.) SCHRAD.*

D. LAVIE, D. WILLNER† and Z. MERENLENDER‡

The Daniel Sieff Research Institute, the Weizmann Institute of Science, Rehovoth, Israel

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Abstract—The chemical constituents of *Citrullus colocynthis* (L.) Schrad. have been found to consist mainly of glycosides which upon enzymatic hydrolysis yield elaterin (cucurbitacin E), elatericin B (cucurbitacin I) and dihydroelatericin B (cucurbitacin L).

DURING recent years, we have been studying the substances occurring in certain species of the botanical family *Cucurbitaceae.*[1] *Citrullus colocynthis* (L.) Schrad. is a well-known member of this group which has been the object of innumerable investigations.

Citrullus colocynthis has been used for its medicinal properties since ancient times. Mention is to be found in old herbals and even in the known Papyrus Ebers. However, the oldest record is supposed to be found in connection with prophet Elisha's miracle.[2] During the second half of the nineteenth century, Walz[3] isolated a bitter glycoside named colocynthin which, upon acid hydrolysis, yielded an amorphous substance, colocynthein. A tasteless crystalline substance colocynthetin was also isolated. While Henke[4] could not establish the glycosidic character of colocynthin, Johannson[5] described its hydrolysis products and reported the isolation of colocynthein and α-elaterin together with other substances. Later Naylor and Chappel[6] obtained colocynthin in a crystalline form. Power and Moore,[7] who doubted the purity of the substances obtained by their predecessors, reinvestigated the plant and isolated citrullol, α-elaterin, an alkaloid and various other components. While Hamilton and Kermack[8] could not repeat the previous work and did not isolate α-elaterin, Siddiqui *et al.*[9] obtained a series of crystalline compounds including α-elaterin. A recent publication by El Khadem and Abdel Rahman[10] reported the isolation of a glycoside following our procedure.[11]

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[1] (a) D. LAVIE, Y. SHVO, O. R. GOTTLIEB and E. GLOTTER, *J. Org. Chem.* **27**, 4546 (1962); (b) *ibid.* **28**, 1790 (1963), and references cited therein.

[2] 2 Kings iv., 39–41.

[3] WALZ, *Jahrb. Pharm.* **9**, 16, 225 (1858); **16**, 10 (1861).

[4] HENKE, *Arch. Pharm.* **221**, 200 (1883).

[5] JOHANNSON, *Zeit. Anal. Chem.* **24**, 154 (1885).

[6] W. R. H. NAYLOR and F. S. CHAPPEL, *Pharm. J.* **25**, 117 (1907).

[7] F. B. POWER and C. W. MOORE, *J. Chem. Soc.* **97**, 99 (1910).

[8] B. HAMILTON and W. O. KERMACK, *J. Chem. Soc.* 5051 (1952).

[9] R. H. SIDDIQUI, I. R. SIDDIQUI and S. MUHAMMAD, *J. Indian Chem. Soc.* **32**, 669 (1955).

[10] H. EL KHADEM and M. M. A. ABDEL RAHMAN, *Tetrahedron Letters*, 1137 (1962). In this publication a phytosterol m.p. 162–164° is reported which has the same m.p. as spinasterol previously identified in the same plant.[8]

[11] D. LAVIE, D. WILLNER, M. BELKIN and W. G. HARDY, *ACTA Unio Int. Contra Cancrum* **15** *bis*, 177 (1959).

The distribution of cucurbitacins among the various species of the *Cucurbitaceae* has been studied extensively by Enslin and a group of South African workers.[12] They have found that these substances occur in nature as glycosides or as aglycones according to the presence in the plant of an enzyme named elaterase,[13] a glycoside hydrolase of undetermined specificity which is capable of rapidly hydrolysing the glycosides. A comparative study of several species showed that elaterase is present in high concentration in genera such as *Cucumis* and *Lagenaria*, whereas it is absent in *Citrullus* and *Cucurbita*. The complicated structure of the cucurbitacins and their high sensitivity to hydrolytic agents accounts for the difficulties encountered by several authors in the identification and the study of the nature of the aglycones and their glycosides. This identification could best be done when they were extracted and isolated from plants in which the enzyme was present, as for instance in *Ecballium elaterium* or other species.[12]

RESULTS

In our studies[1] on the structure elucidation of the cucurbitacins we have been looking for sources of starting material and have used elaterase which was obtained from fresh juice of *Ecballium*. The enzyme was used on fresh fruit of *Citrullus* collected locally,[14] on various samples of commercially available colocynth extracts, as well as on the glycoside elaterinide. In order to prepare the glycoside, the crude commercial alcoholic extract was extracted with chloroform and the product of this extraction treated with petroleum ether and ether. A yellow substance crystallized out of the ethereal solution and was identified as the glycoside elaterinide.[11] It showed one single spot on paper chromatogram and yielded pure elaterin (I)[15] and glucose upon enzymatic hydrolysis with elaterase.

In order to determine whether other aglycones are present in *Citrullus colocynthis*, experiments were performed with fresh fruit percolates as well as with the commercial extracts. The pH of the solutions was adjusted to 5·2–5·4 and the enzyme was added. After 24 hr a sediment was formed and found to consist mainly of elaterin. The supernatant solution was filtered, exhaustively extracted with ether, and the residue, after removal of the ether, was dissolved in ethyl acetate and on adding benzene gave a white crystalline substance. This was readily identified as elatericin B (II) (cucurbitacin I) by comparison with an authentic sample.[16] The mother liquor from this crystallization still contained a mixture of substances which were separated, using cold aqueous alkali, into two main fractions according to a procedure previously described.[16] The fraction insoluble in alkali gave a negative reaction with a ferric chloride solution, and with triphenyl tetrazolium chloride formed a red precipitate of formazan indicating the presence of cucurbitacins possessing an α-hydroxy ketone in ring A;[1b] this fraction awaits further purification for complete identification. The substance soluble in aqueous alkali was collected after acidification and chromatographed twice, leading to a crystallizable fraction which gave a single spot on chromatoplate and had the same spectroscopic characteristics and R_f value as a synthetic sample of dihydroelatericin B (III).

[12] S. REHM, P. R. ENSLIN, A. D. S. MEEUSE and J. H. WESSELS, *J. Sci. Food Agr.* **8**, 679 (1957).
[13] A. BERG, *Bull. Soc. chim. France* **17**, 85 (1897); *C.R. Acad. Sci. Paris* **154**, 370 (1912).
[14] It is noteworthy that with fruit collected and dried in our laboratory the results of our experiments were rather consistent, while the samples obtained commercially gave varied results, probably due to the different methods of processing.
[15] D. LAVIE and S. SZINAI, *J. Amer. Chem. Soc.* **80**, 707 (1958).
[16] D. LAVIE and D. WILLNER, *ibid.* **80**, 710 (1958).

This substance together with synthetic dihydroelatericin B, were compared with a sample of cucurbitacin L (m.p. 122–127°, $[\alpha]_D - 41°$)[17] kindly supplied by Dr. P. R. Enslin. All three substances were found similar in all respects; producing identical u.v. and i.r. spectra, no depression of mixed m.p., and the same R_f value on the chromatoplate. In order to confirm the identity of the naturally occurring substance as dihydroelatericin B (III), it was hydrogenated over palladium on carbon. The product obtained proved to be tetrahydroelatericin B (IV) identical with a synthetic sample.[1b]

It is thus apparent that the crystalline glycoside colocynthin was most probably either elaterinide, or a mixture of glycosides of the various aglycones described above. Colocynthein we believe to be an unidentified mixture obtained from the acid treatment of elaterinide. Indeed when we treated either this glycoside or elaterin (I) itself with acid, a crude amorphous mixture was obtained which could be resolved on a chromatoplate through the crystalline

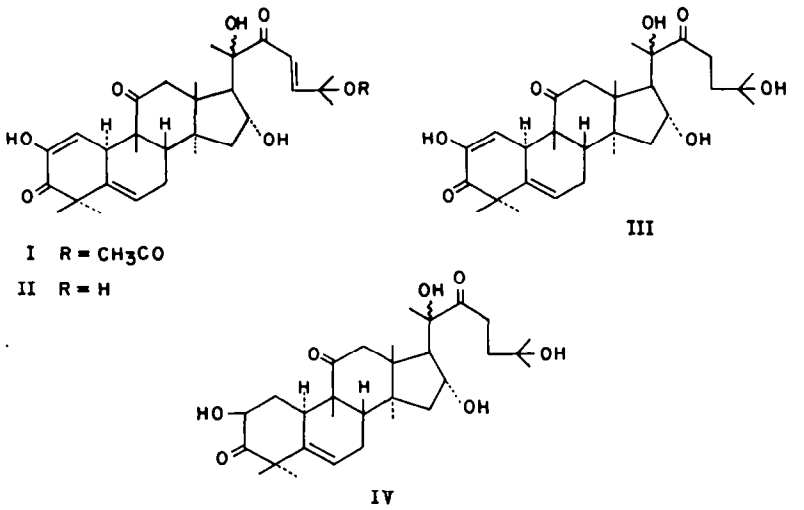

FIG. 1.

acetate into elaterin diacetate[15,18] and elatericin B diacetate,[16] which were identical with authentic samples. Colocynthein may therefore have been a similar mixture of elaterin (I) and elatericin B (II), the latter being formed by the hydrolysis of the tertiary acetoxy group of the side chain.

EXPERIMENTAL

Melting points were taken on a Kofler hot stage microscope and are corrected. Infrared spectra were determined in chloroform solution in 5–10% concentration.

Isolation of Elaterase

The enzyme was prepared from the fruit juice of *Ecballium elaterium*.[19] The fruit was minced and the juice drained and filtered. A portion of 5 l. of juice was cooled to about 5°

[17] P. R. ENSLIN, R. REHM and D. E. A. RIVETT, *J. Sci. Food Agric.* **8**, 673 (1957).
[18] J. N. T. GILBERT and D. W. MATHIESON, *Tetrahedron* **4**, 302 (1958).
[19] Collected in the vicinity of Jerusalem, Israel; cf. P. R. ENSLIN, F. J. JOUBERT and S. REHM, *J. Sci. Food Agric.* **7**, 646 (1956).

and while stirring an equal volume of cold ethanol was added, care being taken to keep the same temperature. The precipitate which was formed was centrifuged, collected, washed with cold alcohol, ether and dried *in vacuo*; 15 g crude enzyme were thereby obtained which were used without further purification.

Isolation of Elaterinide

Commercial *Citrullus colocynthis* extract (E. Merck, Darmstadt) (150 g) was thoroughly mixed with Celite (100 g) to avoid caking, and extracted in a soxhlet with chloroform for 48 hr. Evaporation of the solvent left a brown hygroscopic residue (51 g), which was mixed with Celite and extracted in a soxhlet with petroleum ether and then with ether for 24 hr each. During the process of extraction a yellow microcrystalline powder separated from the ethereal extract which was collected (1·8 g) m.p. 156°. The substance showed one spot on paper chromatogram (impregnated with formamide); its infrared spectrum displayed a strong absorption in the hydroxyl region ν_{max} 3400 cm^{-1} for the sugar moiety of the glycoside.

Enzymatic Hydrolysis of the Glycoside Elaterinide

The glycoside (4·5 g) was dissolved in ethanol (25 ml) and water (600 ml) was added. To the slightly cloudy solution, an aqueous solution of crude elaterase (100 mg) was introduced and the mixture incubated at 37° for 24 hr. The sediment which formed was collected (2·5 g) and after several crystallizations from methanol, using charcoal, pure crystalline elaterin was obtained: m.p. 233–234 dec., $[\alpha]_D - 58°$ in chloroform (c, 0·8). It was identified by comparison with an authentic sample; no depression in mixture m.p., identical infrared spectra throughout the entire range.

Extraction and Enzymatic Hydrolysis of Citrullus colocynthis *Fruit*

1. Fresh full-grown fruit of *Citrullus colocynthis* (50 kg) was minced, allowed to macerate in water (27 l.) for 6 hr, and the juice strained through a sieve. The remaining pulp was transferred to a screw press. The dried cake (5 kg) was discarded, the juice (50 kg), containing some fine pulp in suspension, was inoculated with powdered elaterase (25 g) and the mixture was incubated at 37° for a week. The supernatant (A) was then carefully decanted and the sediment centrifuged to a thick slimy mass which was air dried. The cake was pulverized and extracted in a soxhlet consecutively with hexane (the extract discarded) and with chloroform. The chloroform solution was washed with aqueous sodium bicarbonate and water, then evaporated to dryness under reduced pressure. The heavy residue was triturated with methanol yielding crystalline material (50 g) which was collected and recrystallized several times from methanol yielding pure elaterin.

The combined supernatant (A) and centrifuged liquid were continuously extracted with ether. The ethereal extract was washed as above and evaporated. The residue was dissolved in ethyl acetate and addition of an equal volume of benzene induced crystallization yielding pure elatericin B (15 g): m.p. and mixed m.p. 149–151°, $[\alpha]_D - 53°$ in chloroform (c, 0·95); identical u.v. and i.r. spectra with authentic specimen.

The mother liquor from the above was evaporated to dryness; the residue (10 g) was dissolved in a small quantity of methanol and diluted with ether (1:1). The solution was shaken with cold 4% aqueous sodium hydroxide solution (4 × 250 ml) and the combined aqueous layers shaken twice with fresh ether. The combined ether fractions were then washed

with water, dried and evaporated to dryness yielding a foam (2·28 g). This foam gave a negative $FeCl_3$ test, while with triphenyl tetrazolium chloride (TTC) a red precipitate of formazan was formed; these tests indicate the occurrence of cucurbitacins having an α-hydroxy ketone in ring A.[1b] This fraction could not be induced to crystallize. The cold aqueous layers were *immediately* acidified with cold dilute hydrochloric acid and extracted with ether. The organic layer, after being washed and dried, was distilled leaving a foam (6·25 g). This foam, which gave a positive test with a ferric chloride solution, and was negative with TTC, was chromatographed on acid-washed alumina (20 g Merck). The column was eluted with ether and with ether–methanol (95:5). The latter fractions were combined (3·5g) and rechromatographed on a mixture consisting of silicic acid (300 g, 100 mesh) and Celite (100 g). The eluent was chloroform saturated with formamide. The middle fractions of the rechromatography were combined and induced to crystallize from methanol–water, m.p. 149–152° dec., $[\alpha]_D - 38°$ in chloroform (c, 1·0); R_f 0·44 on chromatoplate (silica gel G Merck) with ethyl acetate in benzene 60 per cent. A sample of dihydroelatericin B (III) prepared by the hydrogenation of pure elatericin B (II) had the following characteristics: m.p. 158–160° dec., $[\alpha]_D - 44°$ in chloroform (c, 0·91); chromatoplate (same conditions as above): R_f 0·44. A mixture m.p. of both samples was not depressed; the u.v. and i.r. spectra of both specimens were identical. A sample of cucurbitacin L[20] was compared with the above two samples and had identical infrared spectrum throughout the entire range, and the same R_f on chromatoplate.

2. Air-dried minced fruit (1 kg) from *Citrullus colocynthis* fruit (7·5 kg), was left to macerate for a week in 5 l. 50 per cent water–ethanol mixture at a temperature of 37°. The solution was decanted and the wet fruit washed with the same solvent mixture. The combined extracts were filtered by suction through a thin layer of Celite, and concentrated to half the original volume by flash distillation in order to avoid frothing. The resulting concentrated solution was brought to pH 5·2–5·4 and, after the addition of finely ground elaterase (1·7 g) in water (25 ml), was incubated at 37° for 48 hr. The sediment and suspended material which were formed in the solution, were collected by filtration with suction through a thin layer of Celite, the latter being extracted in a soxhlet apparatus with chloroform for 3 days. The solvent was evaporated to a small volume and diluted with methanol, whereupon elaterin crystallized. The filtrate was extracted exhaustively with ether; the organic layer was washed with water and dried (Na_2SO_4). The extract was then concentrated to a foam which was dissolved in a small volume of ethyl acetate, and on dilution with an equal volume of benzene yielded crystalline elatericin B.

Tetrahydroelatericin B (IV) from Natural Dihydroelatericin B (III) (Cucurbitacin L)

Dihydroelatericin B (55 mg), isolated as described above, in ethanol solution (10 ml) was hydrogenated over palladium on charcoal (10%, 25 mg) for 24 hr, when about 1 mole of hydrogen was absorbed. The catalyst was filtered, the solvent evaporated, and the residue was dissolved in benzene and chromatographed on acid-washed alumina (Merck). The product was displaced with methanol–ether (7:93), crystallized from methanol–ether, and had m.p. 175–177°, $[\alpha]_D + 60°$ in chloroform (c, 0·53). A mixed m.p. with an authentic sample[1b] was not depressed, and the i.r. spectra were identical.

[20] The physical constants reported for cucurbitacin L, ref. 17 are: a lower melting point, 122–127° (described as unsatisfactory because of sintering), and a similar optical rotation, $[\alpha]_D - 41°$, in ethanol. It is probable that this sample contained some elatericin B as impurity.

Enzymatic Hydrolysis of Commercial Colocynth Extract

Citrullus colocynthis extract (E. Merck, Darmstadt) (100 g), was powdered and worked up in a mortar with ethanol (900 ml) into solution and diluted with water (3400 ml). The dark brown solution was filtered with suction over a thin layer of Celite, adjusted to pH 5·2–5·4, and a suspension of crude elaterase (700 mg) in water (15 ml) was introduced. The mixture was incubated 48 hr at 37°, and the crystalline sediment which was formed was collected, washed with water and repeatedly crystallized from methanol to yield elaterin (5·6 g).

The filtrate was extracted continuously with ether, and the extract washed and dried. Evaporation of the solvent left a residue which was dissolved in a small quantity of ethyl acetate and an equal volume of benzene was added. Elatericin B crystallized out (1·5 g) after seeding.

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