Tumor Inhibitors. XXIII.[1] The Cytotoxic Principles of *Marah oreganus* H.[2]

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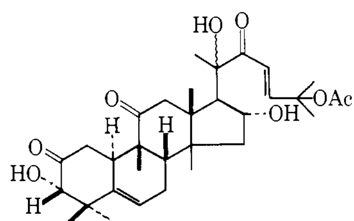
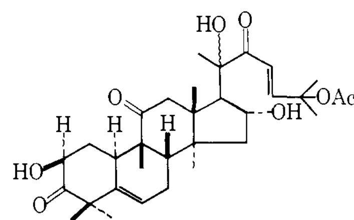
An alcoholic extract of the root of *Marah oreganus* H. was found to show significant inhibitory activity against human carcinoma of the nasopharynx carried in cell culture (KB). Systematic fractionation of this extract has led to the isolation and characterization of the cytotoxic tetracyclic triterpenes, isocucurbitacin B, cucurbitacin B, dihydrocucurbitacin B, and cucurbitacin E.

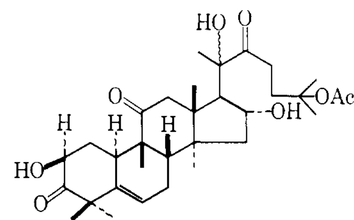
In the course of a continuing search for tumor inhibitors of plant origin, an alcoholic extract of the roots of *Marah oreganus* Howell was found to show significant inhibitory activity against human carcinoma of the nasopharynx carried in cell culture (KB).[3,4] We report herein the systematic fractionation of the active extract of *Marah oreganus* H. and the isolation and characterization of the cytotoxic principles: isocucurbitacin B (I), cucurbitacin B (II), dihydrocucurbitacin B (III), and cucurbitacin E (IVa).



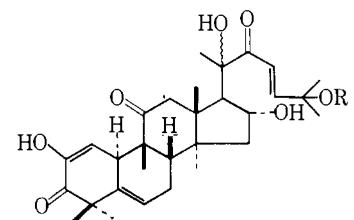
I



II



III



IVa, R = Ac
b, R = H

Although no prior chemical investigations appear to have been carried out on any *Marah* species, extracts from several other plants belonging to the *Cucurbitaceae* family have been reported to possess tumor-inhibitory properties.[5] Various plants in this family elaborate cucurbitacins, a series of highly oxygenated tetracyclic triterpenes.[6] The ability of certain cucurbitacins to inhibit tumor growth has been reported earlier.[7,8]

The dried ground root of *M. oreganus* was extracted continuously with ether followed by methanol for several hours (Scheme I). Since the activity (Table I) was only partially extracted by ether, in all subsequent fractionations the ether extraction was omitted and the root was extracted directly with methanol. Partition of a portion of the concentrated methanolic extract (C) between water and chloroform resulted in a concentration of the activity in the chloroform phase (F). The brown residue from the chloroform layer was defatted by partitioning between 10% aqueous methanol and petroleum ether (Skellysolve B), whereupon the activity was concentrated in the aqueous methanol layer (G). The material recovered from the aqueous methanol layer was dissolved in methanol and treated with a saturated methanol solution of neutral lead acetate. Removal of the precipitate by centrifugation and of the excess lead with hydrogen sulfide gave the active extract (I). Subsequent fractionations of the root gave extracts corresponding to I which had comparable cytotoxic activity.

Further fractionation of fraction I was effected by adsorption chromatography on a silicic acid column, whereby the activity was concentrated in the fractions eluted with 1% methanol in chloroform. These active fractions were combined on the basis of analysis by thin layer chromatography and, upon treatment with ether, two crystalline materials were obtained (J, K).

Fraction J, consisting mainly of the higher R_f com-

(1) Part XXII in the series: S. M. Kupchan and M. I. Suffness, *J. Pharm. Sci.*, in press.

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(3) The roots were collected in California in April and Sept 1964. We acknowledge the receipt of the dried plant material from Dr. Robert E. Perdue, Jr., U. S. Department of Agriculture, Beltsville, Md., in accordance with the program developed with the U. S. Department of Agriculture by the Cancer Chemotherapy National Service Center.

(4) The evaluation of the KB assay results by the Cancer Chemotherapy National Service Center in sequential testing is such that a purified compound is considered active if the average ED_{50} of two tests $\leqq 4$ μg/ml and if this result is reproducible by a second screener. In the event that a compound has an $ED_{50} < 1$ μg/ml in the first test, the second sequential test is omitted and it is submitted to a second screener for confirmation. The procedures were those described in *Cancer Chemotherapy Rept.*, **25**, 1 (1962).

(5) N. R. Farnsworth, *J. Pharm. Sci.*, **55**, 225 (1966).

(6) G. Ourisson, P. Crabbé, and O. Rodig in "Tetracyclic Triterpenes," E. Lederer, Ed., Holden-Day, Inc., San Francisco, Calif., 1964, p 173.

(7) S. Gitter, R. Gallily, B. Shohat, and D. Lavie, *Cancer Res.*, **21**, 516 (1961).

(8) R. Gaillily, B. Shohat, J. Kalish, S. Gitter, and D. Lavie, *ibid.*, **22**, 1038 (1962).

Scheme I
Fractionation of the Cytotoxic Principles of *Marah oreganus* H.

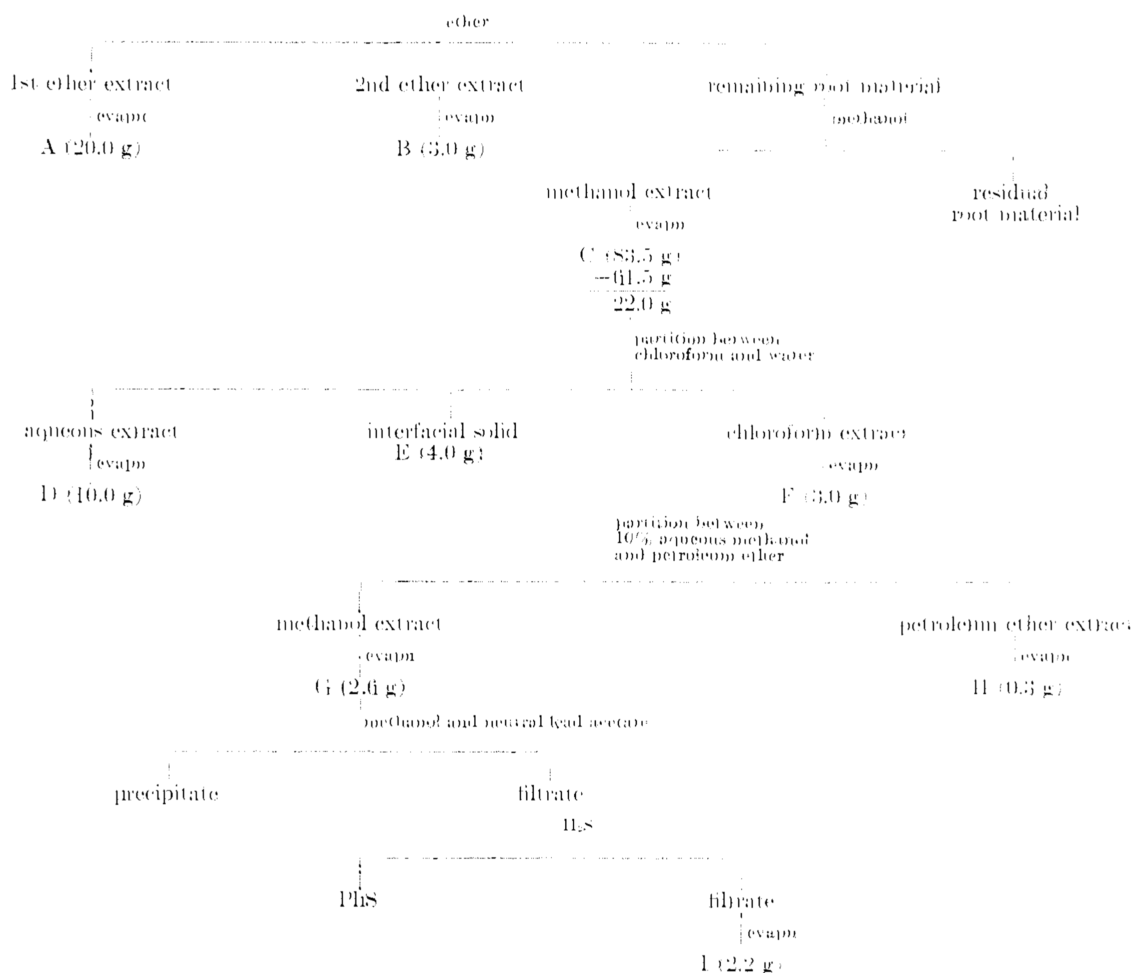

Table I
Cytotoxicity of Fractions from *Marah oreganus* H.

Fraction	ED_{50}, μg/ml
A	0.23
B	1.8
C	0.50
D	23.0
E	2.3
F	0.12
G	0.0024
H	6.5
I	0.00015, 0.014, 0.065

ponent, was further purified by repeated recrystallization, to yield a compound characterized as "2-epicucurbitacin B" (I) by comparison of its physical properties with reported values[9] and with an authentic sample.[10a] This compound was initially thought[9] to have a 3-oxo-2-(axial)-hydroxyl structure and was named "2-epicucurbitacin B" on the basis that it was a C-2 epimer of cucurbitacin B (II). Subsequent work,[10b,c] however, has led to proposal of the 2-oxo-3α-(equatorial)-hydroxyl structure (I). Since I is not epimeric at C-2 but is actually an isomer of cucurbitacin B, we propose that the name isocucurbitacin B be adopted for this compound.

The crystalline fraction K appeared by thin layer chromatography to consist mainly of two components of lower R_f in addition to small amounts of isocucurbitacin B. The low R_f material was separated by preparative thin layer chromatography to give two crystalline compounds. The compound of lowest R_f was identified as cucurbitacin B (II) by comparison of its physical properties with reported values[11] and by conversion to the known[12,13] cucurbitacin I (IVb) by bismuth oxide oxidation.[14]

Bismuth oxide oxidation of isocucurbitacin B and cucurbitacin B, using a modification of the procedure described by Lavie and Shvo[13] for the oxidation of cucurbitacin D to cucurbitacin I, yielded the deacetylated diosphenol cucurbitacin I (IVb). Hydrolysis of the allylic acetate apparently occurred during work-up of the reaction mixture, because, when a procedure described by Enslin[15] involving milder isolation condi-

(9) D. Lavie, Y. Shvo, O. R. Gottlieb, R. B. Desai, and M. L. Khorana, *J. Chem. Soc.*, 3259 (1962).

(10) (a) We thank Professor David Lavie of the Weizmann Institute of Science, Rehovoth, Israel, for an authentic sample of "2-epicucurbitacin B." (b) In a private communication, Professor Lavie has proposed the 2-oxo-3α-(equatorial)-hydroxyl structure for I. (c) D. Lavie and B. S. Benjaminov, *J. Org. Chem.*, **30**, 607 (1965).

(11) W. O. Eisenhut and C. R. Noller, *ibid.*, **23**, 1984 (1958).

(12) D. Lavie and D. Willner, *J. Am. Chem. Soc.*, **80**, 710 (1958).

(13) D. Lavie and Y. Shvo, *ibid.*, **82**, 966 (1960).

(14) W. Rigby, *J. Chem. Soc.*, 793 (1951).

(15) We thank Dr. P. R. Enslin (National Chemical Research Laboratory, South African Council for Scientific and Industrial Research, Pretoria, South Africa) for furnishing his procedure for the oxidation of cucurbitacin B to cucurbitacin E and for a generous sample of cucurbitacin B.

tions was employed, cucurbitacin E (IVa) was obtained.

The compound with R_f slightly higher than cucurbitacin B was characterized as dihydrocucurbitacin B (III) by comparison of its physical properties with reported values[16] and by direct comparison with a sample prepared by hydrogenation of cucurbitacin B. Although dihydrocucurbitacin B has been prepared previously from cucurbitacin B, the isolation from *M. oreganus* appears to represent the first evidence of its occurrence in nature.

During the course of only one of several chromatographic separations of fraction I, a material was eluted from the column with 1% methanol in chloroform (prior to isocucurbitacin B) which crystallized from ether. Recrystallization of the latter material gave cucurbitacin E (IVa), characterized by comparison of its physical properties with reported values[17] and by direct comparison with a sample obtained by bismuth oxide oxidation[15] of cucurbitacin B. Since this compound was not obtained in subsequent chromatographic separations of fraction I, it appears likely that the diosphenol (IVa) was an artifact which arose by oxidation of I or II.

The *in vitro* cytotoxicity (Table II) of the cucurbita-

TABLE II

CYTOTOXICITY OF COMPOUNDS FROM *Marah oreganus* H.

Compound	ED_{50}, μg/ml
I	4.0×10^{-1}
II	2.5×10^{-6}, 5.3×10^{-10}
III	1.7×10^{-3}, 2.6×10^{-2}
IVa	4.5×10^{-7}, 5.8×10^{-9}
IVb[a]	3.1×10^{-4}

[a] Prepared by synthesis; not isolated from this plant.

cins isolated from *M. oreganus* was determined by assay against cells derived from human carcinoma of the nasopharynx (KB).[4] Compounds II and IVa (cucurbitacins B and E, respectively) clearly possess a very high order of cytotoxicity, which has been confirmed by repeated assays. In view of the high level of cytotoxicity exhibited by II and IVa, these compounds were evaluated for inhibitory activity against two *in vivo* tumor systems (Table III).[18] Although cucurbitacin B (II) gave acceptable T/C values in two *in vivo* tumor systems, the low margins between active and toxic doses render the material unpromising as a therapeutic agent.

TABLE III

ACTIVITY OF CUCURBITACINS B AND E AGAINST *in Vivo* TUMOR SYSTEMS

Compd	Tumor system	Dose, mg/kg	Survivors	Animal wt chg dif (T − C)	Tumor wt, mg (T/C)	T/C × 100
II	WM[a]	3.2	0/4	...	...	Toxic
		1.6	3/4	−7.0	1400/4600	30
		0.8	4/4	−5.0	4600/4600	100
	LL[b]	1.6	0/4	...	...	Toxic
		0.8	4/4	−2.0	514/1216	42
		0.4	4/4	+0.5	593/1216	48
IVa	WM	10.0	1/4	+2.0	2300/7400	Toxic
		5.0	4/4	−4.0	6400/7400	86
		2.5	4/4	0.	8300/7400	112

[a] Intramuscular Walker carcinosarcoma 256 in rats. [b] Lewis lung carcinoma in mice.

Experimental Section[19]

Extraction and Preliminary Fractionation.—The dried ground root (420 g) was extracted continuously with ether in a Soxhlet extractor for 15 hr. Evaporation of the ether solution under reduced pressure gave 20.0 g of brown oil (A). Fresh ether was added to the root material which was extracted for an additional 24 hr. The second ether extract was evaporated to yield an additional 3.0 g of brown oil (B). The remaining root material was then continuously extracted with MeOH for 20 hr and the MeOH extract was evaporated under reduced pressure to yield 83.5 g of a thick brown foam (C). A portion of fraction C (22.0 g) was partitioned between water (250 ml) and three 250-ml portions of $CHCl_3$. The resulting $CHCl_3$ layer was washed with water, dried (Na_2SO_4), and evaporated under reduced pressure to yield 3.0 g of brown foam (F). Evaporation of the combined aqueous layer and washings under reduced pressure yielded 10.0 g of residue (D). The interfacial solids after drying amounted to 4.0 g (E).

The chloroform soluble fraction (F) was partitioned between 10% aqueous MeOH (200 ml) and four 250-ml portions of petroleum ether (bp 60–68°). Evaporation of the 10% aqueous MeOH layer yielded 2.6 g of brown foam (G). The combined petroleum ether extracts were washed with water, dried (Na_2SO_4), and evaporated under reduced pressure to yield 0.3 g of residue (H). The aqueous methanol-soluble fraction (G) was dissolved in MeOH (100 ml) and treated with a saturated MeOH solution of $Pb(OAc)_2$. The precipitate was removed by centrifugation and washed twice with MeOH. The combined supernatant methanol solution was freed from excess lead by treatment with H_2S. The PbS was removed by filtration, and the filtrate was evaporated under reduced pressure to yield 2.2 g of brown foam (I). In all subsequent fractionations of this plant, the extraction with ether was omitted and the ground root was subjected directly to continuous MeOH extraction. When this fractionation was carried out on a large scale, 4.1 kg of the dried ground root afforded 138 g of material comparable to fraction I.

Isolation of Isocucurbitacin B (I).—Fraction I was further fractionated by adsorption chromatography on a silicic acid (Mallinckrodt, 3 kg) column, 90 × 9.5 cm. Fraction I (100 g) was dissolved in $CHCl_3$ (200 ml) and applied to the column. The column was eluted with 0.5% MeOH in $CHCl_3$ (14 l.) and then 1% MeOH in $CHCl_3$ (2.2 l.) to give 21 g of brown oil. Continued elution with 1% MeOH in $CHCl_3$ (6 l.) afforded a fraction (3.28 g) which crystallized from ether to give a white powder (J, 0.98 g). Fraction J was recrystallized several times from EtOH (95%) followed by MeOH to give isocucurbitacin B (I, 0.40 g): R_f on tlc 0.33 (ether); mp 223–223.5° dec; $[\alpha]^{26}D$ +43° (*c* 1.61, $CHCl_3$); λ_{max}^{KBr} 2.80, 5.74, 5.81, 5.90, 6.15, 8.08, and 9.08 μ; λ_{max}^{EtOH} 230 mμ (ε 11,200) (lit.[9] mp 229–231° dec; $[\alpha]D$ +41° (*c* 1.1, $CHCl_3$); λ_{max}^{KBr} 2.82, 5.78, 5.90, 6.14, 7.94, and 9.08 μ; λ_{max}^{EtOH} 230 mμ (ε 11,000)). An authentic sample of isocucurbitacin B was obtained[10a] and recrystallized from MeOH to yield product with mp 218–221°. The melting point of our compound was not depressed by admixture with the authentic isocucurbitacin B, and the infrared spectra (KBr) of the respective samples were identical. The two samples showed identical R_f on tlc (3% MeOH in $CHCl_3$).

Isolation of Cucurbitacin B (II) and Dihydrocucurbitacin B (III).—In a typical experiment, a solution of fraction I (100 g) in $CHCl_3$ (200 ml) was chromatographed on a silicic acid (3 kg) column. The column was eluted with 1% MeOH in $CHCl_3$ and the course of the chromatography was followed by tlc using 80% ether in benzene as the developing solvent. After most of the material corresponding to isocucurbitacin B had been eluted, two components of lower R_f were eluted simultaneously from

(16) W. Schlegel, A. Melera, and C. R. Noller, *J. Org. Chem.*, **26**, 1206 (1961).

(17) D. Lavie and S. Szinai, *J. Am. Chem. Soc.*, **80**, 707 (1958).

(18) The evaluation of *in vivo* assay results (in the systems cited in Table III) by the Cancer Chemotherapy National Service Center on a statistical basis in sequential testing is such that a material is considered active if it causes reduction of tumor weight to 42% or less. For further details compare protocols described in ref 4.

(19) Melting points were determined on a Fisher-Johns melting point apparatus and are corrected. Infrared spectra were determined on Perkin-Elmer 421 and Beckman IR-5A infrared spectrophotometers. Ultraviolet spectra were measured on a Beckman DK-2A recording spectrophotometer. Thin layer chromatography was carried out on silica gel G (E. Merck) plates, and the chromatograms were sprayed with a $Ce(SO_4)_2$–H_2SO_4 solution followed by heating until brown spots appeared.

the column. Crystallization of this fraction from ether gave a white powder (K, 3.0 g) which appeared (tlc) to consist mainly of the two lower R_f components in addition to a small amount of material corresponding to isocucurbitacin B.

Fraction K was separated into its main components by preparative tlc. A solution of K (1.7 g) in chloroform–benzene was applied to sixteen 20 × 38 cm glass plates coated with silica gel HF 1 mm in thickness. The plates were developed in ether and viewed under ultraviolet light, whereupon three blue fluorescent bands were observed. The two lower R_f areas were removed from the plates and the silica gel was extracted with four 50-ml portions of warm MeOH. Evaporation of the MeOH under reduced pressure gave two residues which were taken up in warm benzene and filtered through sintered-glass funnels. Evaporation of the benzene under reduced pressure afforded the two low R_f fractions (L and M).

Fraction L (0.580 g), obtained from the lowest R_f material, crystallized from ether to give a white powder (N, 0.343 g). Recrystallization of N from acetone–Skellysolve B gave cucurbitacin B (II, 0.295 g): R_f on tlc 0.22 (ether); mp 181–183°; $[\alpha]^{27}$D +87° (c 0.96, absolute EtOH); λ_{max}^{KBr} 2.92, 5.84, 5.94, 6.17, and 8.01 μ; λ_{max}^{EtOH} 229 mμ (ϵ 13,800) (lit.[11] mp 178–179°; $[\alpha]^{25}$D +87° (c 0.89, absolute EtOH); λ_{max}^{KBr} 2.92, 5.82, 5.92, and 6.17 μ; λ_{max}^{EtOH} 228 mμ (ϵ 10,500)).

Fraction M (0.297 g), which is the material of slightly higher R_f than fraction L, crystallized from ether to give a white powder (O, 0.190 g). Recrystallization of O from benzene–Skellysolve B gave dihydrocucurbitacin B (III, 0.122 g) as needles; R_f on tlc 0.27 (ether); mp 163.5–164.5°; $[\alpha]^{25}$D +53° (c 0.95, $CHCl_3$); λ_{max}^{KBr} 2.79, 2.89, 5.79, 5.82, 5.91, and 7.96 μ; λ_{max}^{EtOH} 278 mμ (ϵ 350) (lit.[16] mp 160–163°; $[\alpha]^{25}$D +57° (c 0.91, $CHCl_3$); $\lambda_{max}^{CHCl_3}$ 2.92, 5.79, 5.85, 5.89, and 8.10 μ; λ_{max}^{EtOH} 282 mμ (ϵ 210)). The melting point was not depressed by admixture with a sample of dihydrocucurbitacin B obtained by hydrogenation of cucurbitacin B. The infrared spectra of the two samples ($CHCl_3$) were identical and both showed identical R_f on tlc (ether).

Bismuth Oxide Oxidation of Isocucurbitacin B (I).—A modification of the procedure described by Lavie and Shvo[13] for the oxidation of cucurbitacin D to cucurbitacin I ("elatericin A" to "elatericin B") was employed. A mixture of isocucurbitacin B (90 mg) and Bi_2O_3 (90 mg) in glacial acetic acid (5 ml) was stirred and heated under reflux for 1.5 hr. The reaction mixture was worked up according to the literature method to give the crude diosphenol (37 mg) as a pale yellow oil. A solution of the oil in $CHCl_3$ was chromatographed on silicic acid–Celite 545 (Johns-Manville) (1:1, 10 g). The column was eluted with 1% MeOH in $CHCl_3$ and the resulting material crystallized from ethyl acetate–benzene–Skellysolve B to give cucurbitacin I (IVb, 10 mg), mp 125–130°, homogeneous upon tlc (3% MeOH in $CHCl_3$). After several recrystallizations from ethyl acetate–benzene, the melting point was raised to 148–150°; R_f on tlc 0.15 (ether); $[\alpha]^{29}$D −50° (c 1.20, $CHCl_3$); λ_{max}^{KBr} 2.91, 5.94, 6.01, 6.14, 7.10, 9.21, and 9.98 μ; λ_{max}^{EtOH} 233 mμ (ϵ 13,600), 268 mμ (ϵ 8600, shoulder) (lit.[12,13] mp 149–151°; $[\alpha]$D −51° (c 0.80, $CHCl_3$); $\lambda_{max}^{CHCl_3}$ 2.93, 5.94, 6.02, 6.14, 6.22, 7.08, 9.17, and 9.95 μ; λ_{max}^{EtOH} 234 mμ (ϵ 11,000), 266 mμ (ϵ 6850, shoulder)).

Bismuth Oxide Oxidation of Cucurbitacin B (II). **Method A.**—Utilizing the procedure[13] described above, a mixture of cucurbitacin B (137 mg) and Bi_2O_3 (137 mg) in glacial acetic acid (5 ml) was stirred and heated under reflux for 1.5 hr. After work-up of the reaction mixture, the crude diosphenol (50 mg) was chromatographed on silicic acid–Celite 545 (1:1, 10 g). The diosphenol was eluted from the column with 1% MeOH in $CHCl_3$ and crystallized from ethyl acetate–benzene to yield 15 mg of solid, mp 128–133°. Recrystallization from ethyl acetate–benzene raised the melting point to 140–145°, not depressed by admixture with a sample of cucurbitacin I obtained from isocucurbitacin B; the infrared spectra (KBr) of the two samples were identical. Both samples showed identical R_f on tlc (3% MeOH in $CHCl_3$).

Method B.—The procedure of Enslin[15] was employed. Cucurbitacin B (100 mg) was dissolved in glacial acetic acid (1 ml) and heated with stirring at 100° with Bi_2O_3 (74 mg, freshly prepared by heating bismuth subcarbonate) for 30 min. The reaction mixture was then cooled, diluted with water (2 ml), and extracted ($CHCl_3$). The extract was washed several times with water and dried (Na_2SO_4). After evaporation of the $CHCl_3$, the product was crystallized from MeOH to give cucurbitacin E (49 mg), mp 233–235°.

Hydrogenation of Cucurbitacin B.—A modification of the procedure described by Noller and co-workers[16] was employed. A solution of cucurbitacin B (67 mg, 0.12 mmole) in EtOH–EtOAc (1:1, 2 ml) was added to 10% Pd–C (60 mg) saturated with H_2 in EtOH–EtOAc (1:1, 2 ml). The suspension was stirred for 5 min in a hydrogen atmosphere until the consumption of H_2 ceased (3.4 ml = 1.26 molar equiv). The catalyst was removed by filtration and the solvents were removed under reduced pressure. The residue (70 mg) was dissolved in $CHCl_3$ and applied to a 20 × 20 cm glass plate coated with silica gel HF 1 mm in thickness. The plate was developed in ether and viewed under ultraviolet light. The lowest R_f blue fluorescent band was removed and the silica gel was extracted (MeOH). The residue obtained after evaporation of the MeOH was taken up in benzene and filtered through a sintered-glass funnel. After evaporation of the benzene solution, the product was crystallized from benzene–Skellysolve B to give dihydrocucurbitacin B (12 mg), mp 163–164°.

Isolation of Cucurbitacin E (IVa).—A solution of fraction I (100 g) in $CHCl_3$ (200 ml) was applied to a column of silicic acid (3 kg) and the column was eluted with $CHCl_3$ until the first yellow band reached the bottom of the column. Elution with 0.5% MeOH in $CHCl_3$ (11 l.) and then 1% MeOH in $CHCl_3$ (4 l.) gave a brown oil (12.9 g). Continued elution with 1% MeOH in $CHCl_3$ (8 l.) gave a foam (4.2 g) which crystallized from ether to give a white powder (0.566 g). Recrystallization from MeOH gave IVa (0.328 g) as colorless hexagonal plates: R_f on tlc 0.40 (ether); mp 233–235° dec; $[\alpha]^{26}$D −58° (c 1.0, $CHCl_3$); λ_{max}^{KBr} 2.97, 5.84, 5.94, 5.98, 6.03, 6.16, 7.04, 7.35, 8.93, 9.17, and 10.14 μ; λ_{max}^{EtOH} 233 mμ (ϵ 11,700), 267 mμ (ϵ 8400, shoulder) (lit.[27] mp 232–233° dec; $[\alpha]$D −59° (c 0.7, $CHCl_3$); λ_{max}^{KBr} 2.90, 5.80, 5.94, 6.02, 6.15, 7.08, 7.30, 8.85, 9.17, and 10.11 μ; λ_{max}^{EtOH} 234 mμ (ϵ 11,700), 267 mμ (ϵ 8350 shoulder)). The melting point was not depressed by admixture with a sample of cucurbitacin E obtained by Bi_2O_3 oxidation of cucurbitacin B, and the infrared spectra (KBr) of the two samples were identical. Both samples showed identical R_f on tlc (3% MeOH in $CHCl_3$).