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MICROBIAL TRANSFORMATION OF CUCURBITACIN E 2-*O*-β-D-GLUCOPYRANOSIDE

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ABSTRACT.—Cucurbitacin E 2-*O*-β-D-glucopyranoside [**1**] was transformed by *Curvularia lunata* NRRL 2178 into cucurbitacin E [**2**] and three new metabolites. The novel metabolites were identified by one- and two-dimensional ^{1}H- and ^{13}C-nmr spectroscopic techniques and high-resolution mass spectrometry as (24*R*)-hydroxy-23,24-dihydrocucurbitacin E and (24*S*)-hydroxy-23,24-dihydrocucurbitacin E [**4** and **5**], and the 3-acetyloxy-3-methylbutyl ester of (23-27)-penta-norcucurbitacin I 22-oic acid [**6**].

Cucurbitacin E 2-*O*-β-D-glucopyranoside [**1**] is an abundant tetracyclic triterpenoid glucoside, isolated from the $CHCl_3$ extracts of *Citrullus colocynthis* L. Schrad (1) as well as other plants in the Cucurbitaceae (2,3). Numerous cucurbitacin derivatives have been isolated and identified and examined for many types of biological activity. Within the cucurbitacin class, compounds possess purgative (4), cathartic (5), diuretic, antidiabetic, and diaphoretic actions (6). Their antineoplastic (7), abortifacient (8), and cytotoxic activities (9) have also been described. While its glycoside itself is inactive as a cytotoxic agent, the aglycone, cucurbitacin E [**2**], possesses moderate cytotoxic activity against several in vitro test systems (10–12).

Relatively large quantities of cucurbitacin E 2-*O*-β-D-glucopyranoside [**1**] became available through efforts intended to identify the active principles of an Egyptian-grown sample of *Citrullus colocynthis* Shrad. Because **1** was an abundant cucurbitacin precursor, we investigated microbiological transformations as a means of preparing rare or novel cucurbitacins. Steroids are among the most well-studied substrates for microbiological transformations (13–21), and numerous steroid hormone products have been prepared in this manner on an industrial scale. Since the ring skeleton of the cucurbitacins embodies an oxygenated cyclopentanoperhydrophenanthrene system, it was logical to examine cultures well known to metabolize steroids. In our hands, screening experiments revealed that *Curvularia lunata* NRRL 2178 was capable of transforming cucurbitacin E 2-*O*-β-D-glucopyranoside [**1**] into several new metabolites. This report describes the production, isolation, and characterization of cucurbitacin metabolites produced by this microorganism.

RESULTS AND DISCUSSION

Curvularia lunata efficiently converted cucurbitacin E 2-*O*-β-D-glucopyranoside [**1**] into four major metabolites during a 14-day incubation period. The major metabolite, **2**, gave a mass spectrum with *m/z* 574 as the apparent molecular ion. This ion is consistent with a metabolite obtained by deglucosylation of the substrate to form cucurbitacin E [**2**]. Both the ^{1}H- and ^{13}C-nmr spectral data supported the absence of the glucose moiety from **1**, and no other changes in the aglycone structure were evident. All of the physical properties of the metabolite were consistent with published values (1,22,23) for cucurbitacin E [**2**].

The melting point of **4** was 148–149°, and its hrms indicated an empirical formula

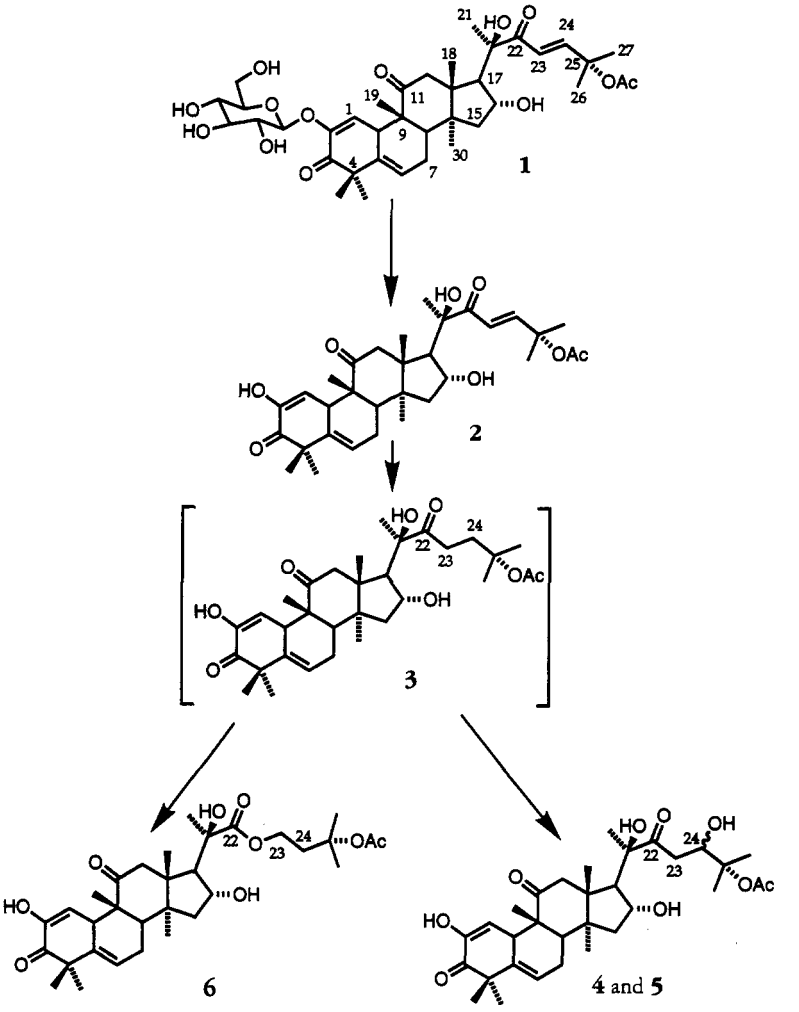

FIGURE 1. Proposed scheme for the biotransformation of cucurbitacin E 2-*O*-β-D-glucopyranoside [**1**] by *Curvularia lunata* NRRL 2178.

of $C_{32}H_{44}O_8$, consistent with the loss of glucose, and addition of H_2O to the substrate structure when compared with **1**. The ^1H- and ^{13}C-nmr spectra (Tables 1 and 2) were essentially identical to those of **1** except for the absence of signals for the glucose moiety between 60–102 ppm, and in signal differences for the C-23–C-24 double bond. The three carbonyl signals at 198.6, 213.1, and 212.9 ppm were assigned to C-3, C-11, and C-22, respectively. The downfield shift of 10.1 ppm for the C-22 signal indicated that the C-23–C-24 double bond was saturated (1,22,24). In support of this finding was the 9-ppm shielding of the C-25 carbon signal, and the absence of side-chain olefinic carbon signals at 120 and 150 ppm, respectively. A new signal at 86.1 ppm (C-24) suggested a hydroxylated aliphatic carbon (25). The ^1H-nmr spectrum of **4** lacked signals for the sugar of **1**, and proton signals between 6.8–7.1 ppm for the side-chain olefin at H-23–H-24. The methyl signal at 1.9 ppm confirmed the presence of the 25-acetate functional group, and a new signal at 4.16 ppm was assigned to a carbinol methine proton at C-24.

Metabolite **4** was subjected to HMBC nmr spectral analysis to confirm assignments shown in Table 2, and to identify the position of the new alcohol functional group.

TABLE 1. ^{1}H-Nmr (360 MHz, $CDCl_3$) Spectral Properties of Cucurbitacin Metabolites.[a]

Proton	Compound				
	1	**2**	**4**	**5**	**6**
1	6.10, d (2)	5.93, d (2)	5.93, d (2)	5.93, d (2)	5.91, d (2)
6	5.83, br s	6.75, br s	5.76, br s	5.77, br s	5.76, br s
7α	2.41, m	2.43, m	2.42, m	2.37, m	2.36, m
7β	2.13[c]	2.10[c]	2.18[c]	2.20[c]	2.17[c]
8	2.09, d (8)	2.05, d (8)	2.05, d (8)	2.04, d (8)	1.99, d (8)
10	3.69, br s	3.51, br s	3.50, br s	3.51, br s	3.51, br s
12α	3.40, d (14)	3.21, d (14)	3.26, d (14)	3.21, d (14)	3.23, d (14)
12β	2.57, d (14)	2.70, d (14)	2.69, d (14)	2.69, d (14)	2.70, d (14)
15α	1.88[c]	1.95[c]	1.90[c]	1.88[c]	1.91[c]
15β	1.46[c]	1.70[c]	1.52[c]	1.51[c]	1.59[c]
16	4.54, t (7)	4.39, t (7)	4.39, t (7)	4.46, t (7)	4.51, br t
17	2.62, d (7)	2.95, d (7)	3.04, d (7)	3.08, d (7)	3.06, d (7)
18	0.89	0.98	1.00	0.99	0.97
19	1.41	1.41	1.03	1.02	1.01
21	1.41	1.34	1.43	1.41	1.20
23	6.82, d (15)	6.46, d (15)	2.58[c] br	2.62[c] br	3.46[c] br
24	6.98, d (15)	7.04, d (15)	4.16, br t	4.06, br t	ND
26[b]	1.57	1.55	1.25	1.25	1.24
27[b]	1.54	1.56	1.47	1.45	1.30
28	1.30	1.38	1.35	1.35	1.33
29	1.27	1.26	1.24	1.26	1.35
30	1.01	1.02	1.41	1.40	1.39
OAc	2.01	2.00	1.90	1.91	1.95
1′	4.69, d (7)	—	—	—	—

[a]Chemical shifts (δ) are expressed in ppm, and coupling constants *J* in parentheses, in Hz; ND=not detected.

[b]Assignments are interchangeable.

[c]Signal was obscured.

Irradiation of the carbinol methine proton signal at 4.16 ppm enhanced carbon signals at 212.9 (C-22), 27.9 (C-26), and 28.2 (C-27) ppm, consistent with three-bond correlations between the proton and each of these carbon signals. The remainder of HMBC nmr proton-carbon correlations confirmed the structure of this metabolite as **4**. The stereochemistry of the hydroxyl group at position 24 was not assigned.

Metabolite **5** differed from metabolite **4** in mp, 162–164°, and in R_f value by tlc. Nevertheless, **5** possessed essentially identical spectral properties to those collected for metabolite **4**. These included eims and ^{1}H- and ^{13}C-nmr (Tables 1 and 2) results, and in the HMBC ^{1}H-^{13}C correlation nmr spectrum. We concluded that the metabolite is 24-hydroxy-23,24-dihydrocucurbitacin E [**5**] with the opposite stereochemistry of the hydroxyl group at position 24.

Metabolite **6** was identified as the 3-acetyloxy-3-methylbutyl ester of (23-27)-pentanorcucurbitacin I 22-oic acid. Hrfabms of **6** indicated an empirical formula of $C_{32}H_{46}O_9$ suggesting the likely loss of the glucose molecule, and the addition of H_2O to the substrate structure. Signals for the tetracyclic ring system were essentially identical to those of **1** and the other metabolites obtained. However, the ^{13}C-nmr spectrum confirmed the absence of sugar carbon signals between 60–102 ppm, and the saturation of the C-23–C-24 double bond by the absence of olefinic carbon signals at 121 and 151 ppm. Shielding of the signal for C-25 by 8.4 ppm also supported the saturation of the side-chain double bond (1,22,24). An upfield shifted carbonyl signal at 171.5 ppm was assigned to an ester carbonyl functional group (25) at the C-22 position, together with

TABLE 2. ^{13}C-Nmr (90.56 MHz, $CDCl_3$) Spectral Properties of Cucurbitacin Metabolites.

Carbon	Compound				
	1[a]	2	4	5	6
1	122.3	115.2	114.8	114.9	115.0
2	146.8	144.7	144.6	144.6	144.6
3	199.4	198.8	198.6	198.6	198.7
4	48.8	47.7	47.5	48.0	47.0
5	137.1	136.5	136.2	136.7	136.8
6	123.6	120.5	120.7	120.6	120.7
7	24.6	23.5	23.5	23.6	24.1
8	42.8	41.5	45.6	41.6	41.6
9	49.9	48.8	48.2	48.8	48.3
10	36.2	34.6	34.7	34.7	34.7
11	216.2	213.0	213.1[b]	213.6[b]	213.2
12	48.9	48.6	48.9	48.8	48.3
13	50.0	48.0	48.2	48.6	47.0
14	50.3	50.5	50.9	50.7	48.9
15	46.6	45.2	45.5	45.5	45.7
16	70.5	70.7	71.0	70.3	72.1
17	59.7	57.9	57.5	57.5	57.5
18	20.7	19.8	18.2	18.2	19.8
19	18.6	17.5	14.3	14.4	18.4
20	80.0	78.6	78.2	79.7	79.5
21	25.2	23.7	24.4	24.8	27.1
22	204.8	202.4	212.9[b]	213.1[b]	171.5
23	122.4	120.7	38.7	38.0	80.2
24	151.5	151.7	86.1	86.2	36.4
25	80.8	79.3	69.9	70.6	70.9
26	26.4[b]	26.0[b]	27.9[b]	27.9[b]	27.3[b]
27	26.8[b]	26.1[b]	28.2[b]	28.1[b]	27.9[b]
28	20.7[b]	19.8	19.7	19.7	20.2
29	28.2	27.7	30.9	30.9	30.8
30	20.7[b]	20.0	20.2	20.2	23.3
25-OAc	21.7	21.8	23.5	22.2	23.6
	171.6	170.1	164.8	164.8	169.5

[a]Signals for sugar carbons for the substrate [**1**] were 100.8 (1′), 74.0 (2′), 77.7 (3′), 71.7 (4′), 77.2 (5′), and 61.9 (6′).

[b]Assignments are interchangeable.

a hydroxylated carbon signal at 80.2 ppm at the C-23 position (26) (Table 1). The ^{1}H-nmr spectrum confirmed the presence of eight skeletal methyl groups, and the C-25 acetate methyl group signal as well as the absence of the side-chain double bond. The signal at 3.46 ppm was assigned to the C-23 oxygenated-methylene protons. When examined in an HMBC experiment, these protons were correlated with carbon signals at 171.5 (C-22), and 70.9 (C-25) ppm. The HMBC spectrum confirmed all other proton and carbon assignments.

Therefore, the biotransformation of cucurbitacin E 2-*O*-β-D-glucopyranoside [**1**] by *Curvularia lunata* NRRL 2178 gave four metabolites, cucurbitacin E [**2**], the isomeric (24*R*)- and (24*S*)-hydroxy-23,24-dihydrocucurbitacin E [**4** and **5**], and the 3-acetyloxy-3-methylbutyl ester of (23-27)-pentanorcucurbitacin I 22-oic acid [**6**]. Compounds **4**–**6** are all previously unidentified cucurbitacin derivatives. All of the metabolites lacked the glucose moiety, suggesting that the first step in the biotransformation sequence was glucolysis followed by secondary biotransformations. *C. lunata* is a well-known biocatalyst that commonly hydroxylates many types of steroids (13, 15–18) at different positions.

However, with cucurbitacin E 2-*O*-β-D-glucopyranoside [**1**], this organism catalyzed interesting biotransformations only on the side-chain and not on the main cyclic structure of the cucurbitacin. The likely biotransformation sequence based on the identification of these metabolites is shown in Figure 1. Following glucose cleavage to cucurbitacin E [**2**], the major metabolite, hydration of the double bond could form an isomeric mixture of **4** and **5**. Alternately, reduction of the conjugated 23,24-double bond would form a saturated side-chain intermediate **3** that could undergo hydroxylation via a monooxygenase-like enzyme, or Baeyer-Villiger oxidation to form the ester product **6**. To distinguish between hydration and oxygenation pathways, reactions will have to be conducted under an $^{18}O_2$ atmosphere. Reactions such as those observed with **1** have been well-documented in the steroid biotransformation field.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mps are uncorrected and were determined with a Thomas-Hoover melting point apparatus. ^{1}H- and ^{13}C-nmr spectra were obtained with a Bruker WM360 spectrometer operating at 360.13 and 90.56 MHz, respectively. All spectra were obtained in $CDCl_3$ using TMS as internal standard, with the chemical shifts expressed in δ (ppm) and the coupling constants (*J*) in Hz. Heteronuclear multiple-bond correlation (HMBC) nmr (27) experiments were obtained on a Bruker AMX-600 spectrometer. Low-resolution chemical ionization-mass spectra (cims) were recorded on a Nermag R10-10C (France) mass spectrometer using CH_4 as carrier gas. Fabms were obtained on a Kratos MS-50 triple analyzer mass spectrometer using Xe as the carrier gas and 3-nitrobenzyl alcohol (NBA) as the sample matrix. Hrfabms were obtained on a ZAB-250HF mass spectrometer at 70 eV through the mass spectral services of the Department of Chemistry, University of Nebraska at Lincoln (Midwest Center for Mass Spectrometry).

SUBSTRATE MATERIAL.—Cucurbitacin E 2-*O*-β-D-glucopyranoside [**1**] was isolated from the ripe sliced fruits of *Citrullus colocynthis*, and exhibited: mp 157–158° [lit., 157–159° (1)]; fabms, *m/z* $[M+Na]^+$ 741 for $C_{38}H_{54}O_{13}$; ^{1}H- and ^{13}C-nmr data, see Tables 1 and 2.

FERMENTATION METHODS.—*Curvularia lunata* NRRL 2178 cultures were grown according to a standard two-stage fermentation protocol (28). Screening experiments were conducted in 125 ml DeLong flasks, while preparative-scale experiments were conducted using 1-liter flasks. DeLong culture flasks held one-fifth of their volumes of the following medium: 1% glucose, 0.5% soybean meal, 0.5% yeast extract, 0.5% NaCl, and 0.5% K_2HPO_4. The pH of the medium was adjusted to 7 with 6 N HCl prior to autoclaving for 20 min at 121° and 18 psi. After being inoculated with fresh *C. lunata* slants, Stage I cultures were incubated at 30° and 250 rpm for 72 h before being used to inoculate Stage II culture flasks (10% inoculum volumes). For screening-scale experiments, 20 mg of the substrate cucurbitacin E 2-*O*-β-D-glucopyranoside [**1**] in 0.1 ml dimethylformamide (DMF) (800 μg substrate per ml of culture medium) was added to 24-h-old Stage II culture flasks which were incubated again, and sampled periodically for analysis.

SAMPLING AND CHROMATOGRAPHY.—Culture samples of 1 ml each were taken at 12, 24, 36, and 48 h, and every other day for three weeks following substrate addition. Samples were shaken for one min with 0.5 ml of EtOAc and spun at 3,000×g for 1 min in an IEC HN-SII desk-top centrifuge. Samples of 10 μl each of the extracts were spotted on Si gel GF_{254} tlc plates, developed in $CHCl_3$-CH_3OH (95:5, system A), (85:15, system B), and spots were made visible by spraying with 0.5% vanillin/H_2SO_4 reagent followed by heating at 100° for 5 min. All isolated cucurbitacin derivatives gave reddish-brown colors with this reagent, and displayed the following R_f values in systems A and (B): 0.11 (0.44), 0.68 (0.86), 0.43 (0.73), 0.25 (0.63), and 0.19 (0.53) for compounds **1**, **2**, **4**, **5**, and **6**, respectively.

Semi-quantitative tlc estimations of metabolite yields were obtained by comparing spot sizes and intensities of components of fermentation extracts with known quantities of **1**, **2**, and **4–6**, ranging from 0.05–100 μg spotted on tlc plates along with standards. Color intensities and spot sizes were compared to standards 1/2 h after visualization when maximum colors were observed.

PREPARATIVE-SCALE CONVERSION OF CUCURBITACIN E 2-*O*-β-D-GLUCOPYRANOSIDE.—Ten 1-liter Stage II cultures received a total of 2.0 g of cucurbitacin E 2-*O*-β-D-glucopyranoside [**1**] in 20 ml of DMF (1.0 mg substrate per ml of culture medium). After incubating for 14 days under the usual conditions, cultures were combined and exhaustively extracted with 3×1 liter of 10% CH_3OH in EtOAc. The solvent was dried over anhydrous Na_2SO_4 and evaporated under vacuum to yield a crude dark brown extract of 3.2 g.

The crude extract was loaded onto 120 g of Si gel in a flash column (3.5×60 cm) and eluted with mixtures of $CH_3OH/CHCl_3$ by linear gradient elution with increasing polarity up to 30% CH_3OH. The

collected fractions (10 ml) were spotted on Si gel GF_{254} tlc plates, developed in system A, sprayed with vanillin/H_2SO_4 and heated at 100° for 5 min. Similar fractions (same R_f) were pooled, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to provide 425 mg of **2**, 58 mg of **4**, and 210 mg of **5** and **6**. A total of 110 mg of unreacted **1** was recovered.

Analytical samples of **2** and **4–6** were obtained as pale-yellow amorphous powders from 1-mm thick Si gel GF_{254} tlc plates using solvent system A to provide 42 mg **1**, 80 mg **2**, 20 mg **4**, 20 mg **5**, and 50 mg **6**. On reversed-phase C_{18} Si gel using CH_3OH-CH_3CN-H_2O (40:20:40) as solvent, these compounds displayed the following R_f values: **1** (0.57), **2** (0.17), **4** (0.28), **5** (0.33), and **6** (0.46).

The major metabolite **2**, 60% yield, was obtained as colorless prisms (CH_3OH-$CHCl_3$) mp 232°; cims (CH_4) *m/z* $[M^+-60]^+$ 498 (2.5), $[C_6H_8O]^+$ 96 (100), $[C_7H_{11}O]^+$ 111 (8); ^{1}H- and ^{13}C-nmr data, see Tables 1 and 2.

Metabolite **4** was obtained as a pale-yellow amorphous powder, 2.5% yield: mp, 148–149°; cims (CH_4) *m/z* $[M^+-H_2O]^+$ 556 (3), $[M^++1-H_2O]^+$ 557 (2), 164 (11), 111 (17), 96 (6); fabms *m/z* $[M-H_2O]^+$ 556; hrfabms *m/z* 556.3251 for $C_{32}H_{44}O_8$ (calcd 556.3112); ^{1}H- and ^{13}C-nmr data, see Tables 1 and 2.

Metabolite **5**, 15% yield: mp 162–164°; cims *m/z* $[M^+-H_2O]^+$ 556 (2), $[M^++1,-H_2O]^+$ 557 (2), 164 (14), 111 (16), 96 (4); fabms (3-NBA) *m/z* $[M^+-H_2O]^+$ 556; hrfabms *m/z* 556.3241 for $C_{32}H_{44}O_8$ (calcd 556.3112); ^{1}H- and ^{13}C-nmr data, see Tables 1 and 2.

Metabolite **6**, 10% yield, was obtained as a pale-yellow amorphous powder: mp, 144–145°; cims *m/z* $[M^+-1]^+$ 573 (0.3), $[M^++1-H_2O]^+$ 557, $[M^+-H_2O]^+$ 556 (8), 401 (0.6), 164 (16), 111 (25), 101 (13), 96 (18); fabms *m/z* 574; hrfabms *m/z* 574.3416 for $C_{32}H_{46}O_9$ (calcd 574.3598); ^{1}H- and ^{13}C-nmr data, see Tables 1 and 2.

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