Phytochemistry, 1978, Vol 17, pp. 1062–1064 © Pergamon Press Ltd Printed in England 0031–9422/78/0601–1062$02.00/0

CUCURBITACIN 19-HYDROXYLASE IN *CUCURBITA MAXIMA*

JOHANNES C. SCHABORT
Department of Biochemistry, Rand Afrikaans University, P.O. Box 524, Johannesburg 2000, South Africa

(*Revised received* 7 *November* 1977)

Key Word Index—*Cucurbita maxima*; Cucurbitaceae; cucurbitacin 19-hydroxylase; enzymic hydroxylation; triterpenoids.

Abstract—The enzymatic hydroxylation of the C-19-methyl group of cucurbitacin B and D was observed in partly purified preparations obtained from the unripe fruit of *Cucurbita maxima*. Assay methods were developed and the pH optimum, cofactor requirements, and substrate specificity determined.

INTRODUCTION

The important role of cucurbitacin B Δ^{23}-reductase (NAD(P)H: cucurbitacin B Δ^{23}-oxidoreductase) [1, 2] in the breakdown of toxic bitter principles [3] in plants has been reported [1, 4]. Schabort and Teijema also postulated that several other enzymes such as a cucurbitacin 19-hydroxylase may play a role in these breakdown processes [4]. The constitutional and stereochemical formulae of the different cucurbitacins were published by Enslin, Lavie *et al.* [5–10]. The structural interrelationship of the different bitter principle are, therefore, well established. In this communication, evidence is presented for the existence of a cucurbitacin 19-hydroxylase enzyme which catalyses the chemical conversion of cucurbitacins B (**1**) to A (**2**) and D (**3**) to N (**4**). The chemical structure of cucurbitacins B, D, A and N are given in Scheme 1.

RESULTS AND DISCUSSION

Assay methods

When cucurbitacin A or N were used as substrates, no activity was observed by both the spectrophotometric and chromatographic assays. Blank determinations where cucurbitacin B (or D) or enzyme were omitted showed no activity. Studies with various metal ions (conc 1–3 mM), tested as possible cofactors, showed no increase in activity, which demonstrate that no metal cofactor is required for hydroxylating activity.

Both the spectrophotometric as well as the chromatographic assays showed no hydroxylating activity under anaerobic conditions, determined in Thunberg cuvettes in the presence of N_2 indicating the need for molecular oxygen in the C-19-hydroxylation of cucurbitacin B and D. Cucurbitacin-Δ^{23}-reductase [1, 2] was eliminated in these activity measurements by the absence of any metal ion by using dialyzed enzyme preparations for all assays.

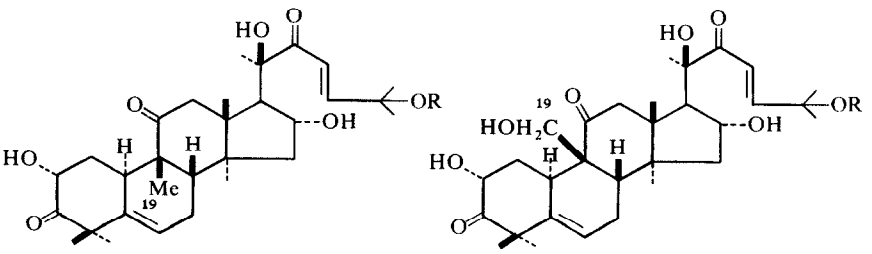
Except for the PC and TLC identifications for the formed cucurbitacin A and N containing the C-19-hydroxyl group (see Experimental), these C-19-hydroxylated bitter principles were also identified by their UV, visible and IR spectra [5–8], after extraction from reaction mixtures by chloroform.

Isolation of cucurbitacin 19-hydroxylase

A partly purified preparation of the enzyme was obtained by extraction, alcohol fractionation, heat precipitation and pH precipitation. The enrichment of hydroxylating activity obtained after the pH precipitating step was 32.6 with a yield of 61 % (Table 1). A linear relationship was found between reaction velocity and enzyme concentration. The pH optimum determined by the spectrophotometric assay at 340 nm was 6.4 with a pH range of activity of 5 to 7.5.

Substrate specificity

In comparison with NADPH which was found to be an effective electron donor, NADPH showed no activity. The relative specificity of the hydroxylase enzyme with respect to the different cucurbitacins is demonstrated by



1 Cucurbitacin B: R = Ac **2** Curcurbitacin A: R = Ac
3 Cucurbitacin D: R = H **4** Cucurbitacin N: R = H

Scheme 1. Chemical formulae of cucurbitacins A, B, D and N.

Table 1. Purification of cucurbitacin 19-hydroxylase

Isolation step	Total protein (mg)	Total enzyme activity (arbitrary units × 10^{-3})	Specific activity (arbitrary units per mg protein)	Yield (%)	Purification factor
1. Extraction	4520	176·5	39·0	100	1·0
2. Alcohol fractionation	477	134·1	281·1	76	7·2
3. Heat precipitation	286	127·1	445·1	72	11·4
4. pH precipitation	85	107·7	1272·7	61	32.6

the specific activities expressed in arbitrary units/mg protein found for 23,24-dihydrocucurbitacin B, cucurbitacin B and cucurbitacin D viz 2140, 1270 and 980 arbitrary units respectively. Enzyme obtained after the pH precipitation step was used employing the spectrophotometric assay. Other 23,24 dihydrocucurbitacins were not available for these studies. The fact that 23,24-dihydrocucurbitacin B was found to be the best substrate supports the conclusion of Schabort and Teijema that the 23,24-dihydro-derivatives of cucurbitacins A, B, D and E are better substrates for *in vitro* breakdown than their precursor cucurbitacins with unreduced side-chains, namely cucurbitacins A, B, D and E. From these results it seems likely that the cucurbitacin 19-hydroxylase (a soluble hydroxylase enzyme resembling other known soluble hydroxylases [11, 12] in many respects) may follow the cucurbitacin-Δ^{23}-reductase enzyme in the breakdown of the cucurbitacins in plants.

EXPERIMENTAL

Materials

Green Hubbard fruits of *Cucurbita maxima* were obtained from the local market. Cucurbitacins were supplied by P. R. Enslin and J. R. Bull of the National Chemical Research Laboratories, CSIR, Pretoria and NADPH and NADH were purchased from Boehringer, Mannheim, Germany. All other chemicals were of analytical grade quality.

Assay methods

Spectrophotometric assay at 340 nm. The enzymatic activity was determined by measuring the rates of decrease in *A* of a reaction mixture containing the enzyme prepn, cucurbitacin B or D and NADPH, at 340 nm employing the time drive attachment of the Perkin–Elmer model 124 double beam spectrophotometer. The μmol of NADPH oxidized and thus the μmol cucurbitacin B or D hydroxylated during the reaction were calculated from the molar *A* coefficient of NADPH at 340 nm which is given as $6{\cdot}22 \times 10^3$ M/cm in ref. [13]. Activity was expressed in μmol NADPH oxidized or cucurbitacin B or D hydroxylated/min. and sp. act. as μmol/min/mg protein. Standard reaction mixtures used for this assay contained 105 μmol of sodium maleate (pH 6.6); 0.56 μmol NADPH, 3.5 μmol cucurbitacin B or D in 8.3% MeOH (final concn) and the enzyme prepn in the sample cell with a final vol. of 3 ml. In the reference cell cucurbitacin was omitted in most cases but in some cases the enzyme was omitted and replaced by buffer. Because of their low solubility in H_2O the cucurbitacins were dissolved in MeOH. The reaction was initiated by rapid injection of the cucurbitacin soln into the sample cell by a micro-pipette. All measurements were carried out at 25°, in a temp. regulated cell holder. The amount of enzyme taken was such that the reaction was zero order with respect to both cucurbitacin and NADPH.

PC and TLC assay. Incubations of cucurbitacin substrates (1.2 mg) with enzyme prepns were performed in 0·05 M sodium maleate buffer (pH 6.6) in the presence of 2.5 μmol NADPH at 30° in a final MeOH concn of 5% and a total vol. of 5 ml for 1 to 30 hr. After incubation the cucurbitacins were extracted quantitatively from the incubation mixture with $CHCl_3$ (3 × 10 ml). The combined $CHCl_3$ extracts were evapd to dryness. The residue was dissolved in MeOH and about 400–800 μg was spotted on paper or thin-layer chromatoplates. Chromatographic methods [14, 15] already reported were used to detect cucurbitacin A and N formed from cucurbitacin B and D, respectively.

Enzyme preparation

The spectrophotometric assay method at 340 nm as described above, was used at pH 6.6. An arbitrary unit of enzyme activity was defined as the amount of enzyme which will produce a decrease of 0·001 in *A* at 340 nm in 5 min under standard assay conditions. All isolation procedures except heat pptn were carried out at 0 to 5°. Protein was determined after each step by the method of ref. [16] and total nitrogen by a micro-Kjeldahl method [17].

Extraction. The edible part of fresh, skinned, unripe Green Hubbard fruits (total weight 4.5 kg) was homogenized in the presence of 150 ml of 0.05 M sodium maleate buffer (pH 6.6) for 20 sec. The homogenate was pressed through 8 layers of cheese cloth and centrifuged at 600 *g* for 5 min to yield 1500 ml of supernatant. This supernatant was then centrifuged at 44000 *g* for 30 min in a Spinco model L4 ultracentrifuge to yield a clear supernatant of 1450 ml. The sediment was discarded. A small portion of the supernatant was prepared for assay by dialysis for *ca* 18 hr against 2 l. 0.05 M sodium maleate buffer (pH 6.6) changing the buffer twice.

Alcohol fractionation. Precooled EtOH, at −20 to −30°, was added to the above supernatant over a period of 20 min until the alcohol concn was 20% (v/v). The resulting ppt. was removed by centrifugation at 2000 *g* for 10 min at −10°. The alcohol concn of the supernatant was adjusted to 50% (v/v), and the ppt. collected as above. This ppt. was suspended in 0.05 M sodium maleate buffer (pH 6.6) and dialysed *ca* 18 hr against 2 l. of the same buffer, changing the buffer twice. After dialysis, the suspension was centrifuged at 20000 *g* for 20 min and the sediment discarded. The supernatant soln (205 ml) contained 76% of the total hydroxylating activity.

Heat precipitation. Heat pptn was performed on the supernatant at 55° for 5 min. After the heat treatment the enzyme preparation was immediately cooled in an ice-bath before centrifugation at 20000 *g* for 20 min. The sediment was discarded and the supernatant (200 ml) assayed for enzymatic activity.

pH precipitation, The pH of the soln obtained was adjusted to 4.4 by gradual addition of a 5% HOAc. The white ppt was immediately removed by centrifugation at 20000*g* for 20 min. The supernatant (218 ml) was decanted and the pH immediately adjusted to 6.6 by the addition of 5% NaOH. This soln was dialysed *ca* 18 hr against 2 l. of 0.05 M sodium maleate buffer (pH 6.6) changing the buffer twice, to yield a diffusate of 280 ml. The ppt. contained no hydroxylating activity.

Acknowledgments—The author wishes to thank the Rand Afrikaans University, Johannesburg, and the South African Council for Scientific and Industrial Research, Pretoria, for financial assistance and Drs P. R. Enslin and J. R. Bull of the National Chemical Research Laboratories, CSIR, Pretoria, for providing the cucurbitacins.

REFERENCES

1. Schabort, J. C., Potgieter, D. J. J. and De Villiers, V. (1968) *Biochim. Biophys. Acta* **151**, 33.
2. Schabort, J. C. and Potgieter, D. J. J. (1968) *Biochim. Biophys. Acta* **151**, 47.
3. Steyn, D. G. (1950) *S. African Med. J.* **24**, 713.
4. Schabort, J. C. and Teijema, H. L. (1968) *Phytochemistry* **7**, 2107.
5. De Kock, W. T., Enslin, P. R., Norton, K. B., Barton, D. H. R., Sklarz, B. and Bother-By, A. A. (1963) *J. Chem. Soc.* 3828.
6. Enslin, P. R., Hugo, J. M., Norton, K. B. and Revitt, D. E. A. (1960) *J. Chem. Soc.* 4779.
7. Enslin, P. R. and Norton, K. B. (1964) *J. Chem. Soc.* 529.
8. Van der Merwe, K. J., Enslin, P. R. and Pachler, K. (1963) *J. Chem. Soc.* 4275.
9. Lavie, D. and Glotter, E. (1971) *Progr. Chem. Organ. Natural Products* **29**, 307.
10. Lavie, D., Shvo, Y. Willner, D., Enslin, P. R., Hugo, J. M and Norton, K. B. (1959) *Chem. Ind. (Lond.)* 951.
11. Young, R. B., Bryson, M. J. and Sweat, M. L. (1965) *Arch. Biochem. Biophys.* **109**, 233.
12. Spector, T. and Massey, V. (1972) *J. Biol. Chem.* **247**, 5632.
13. Horecker, B. L and Kornberg, A. (1948) *J. Biol. Chem.* **175**, 385.
14. Schwartz, H. M., Biedron, S. I., Van Holdt, M M. and Rehm, S. (1964) *Phytochemistry* **3**, 189.
15. Schabort, J. C. and Potgieter, D J. J. (1967) *J Chromatog.* **31**, 235 and references therein.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
17. McKenzie, H. A. and Wallace, H. S. (1954) *Australian J. Chem.* **7**, 55.

Phytochemistry, 1978 Vol 17, pp. 1064–1065. © Pergamon Press Ltd Printed in England 0031–9422/78/0601–1064$02.00/0

PRUNIN-6″-*O*-*p*-COUMARATE, A NEW ACYLATED FLAVANONE GLYCOSIDE FROM *ANACARDIUM OCCIDENTALE*

WASIUR RAHMAN*, KHWAJA ISHRATULLAH*, HILDEBERT WAGNER ., OTTO SELIGMANN ., V. MOHAN CHARI* and BENGT-GÖRAN ÖSTERDAHL‡

* Department of Chemistry, Aligarh Muslim University, Aligarh, U.P., India;† Institut für pharmazeutische Arzneimittellehre der Universität München, Karlstr. 29, D-8000 München, 2, BRD, ‡ Institute of Chemistry, Organic Chemistry Department, University of Uppsala, Uppsala, Sweden

(*Received* 16 *December* 1977)

Key Word Index—*Anacardium occidentale*, Anacardiaceae; prunin-6″-*O*-*p*-coumarate, ^{13}C-NMR spectroscopy; acylated flavanone glycoside.

From the acetone extract of the defatted nut shells of *Anacardium occidentale* L. two phenolic compounds AO-1 and AO-2 could be isolated. Whereas AO-1 was shown to be identical with naringenin, the latter was a hitherto unknown glycoside mp 153–154°. Hydrolysis of AO-2 with 5% ethanolic HCl yielded naringenin, glucose and *p*-coumaric acid as hydrolysis products. Lack of characteristic shift of the UV maximum at 285 nm upon addition of NaOAc indicated the attachment of glucose to the C_7-OH group. After alkaline methanolysis naringenin-7-*O*-*β*-D-glucoside (prunin) and methyl *p*-coumarate were identified by TLC comparison with authentic samples. This showed that AO-2 was a *p*-coumarate of prunin as was also indicated by the presence of two carbonyl absorptions in the IR spectrum at 1675 and 1625 cm^{-1}. The peak at *m/e* 365 in the mass spectrum of the permethylation product of AO-2, corresponding to the tetra-*O*-methyl ether of the coumaryl-glucosyl ion, clearly demonstrated that the acyl residue was on the sugar moiety. The M^+ peak at *m/e* 678 and other fragments in the mass spectrum were in agreement with the formulation of the methylation product as prunin chalcone *p*-coumarate heptamethyl ether. A similar observation [1] has been made in the permethylation of the naturally occurring naringenin-7-*O*-(6″-*O*-galloyl)-*β*-D-glucoside. The ^{1}H-NMR spectra of AO-2 and its acetate were in agreement with the structure of naringenin-7-*O*-(coumaryl)-*β*-D-glucopyranoside.

The position of the *p*-coumaryl residue on the sugar was unequivocally determined by ^{13}C-NMR spectroscopy. The signals for the sugar carbon atoms $C_{2''}$, $C_{3''}$, $C_{4''}$, $C_{5''}$ and $C_{6''}$ of AO-2 appeared in the region 76.1 ppm to 63.3 ppm. Compared with the corresponding carbon resonances in the spectrum of prunin, the $C_{6''}$ signal in AO-2 was 2.7 ppm downfield and the $C_{5''}$ signal 3.3 ppm upfield. The assignment for $C_{6''}$ was confirmed by taking the off-resonance spectrum. Such changes in the chemical shifts of $C_{6''}$ and $C_{5''}$ can only be explained [2] if the primary hydroxyl group at $C_{6''}$ is esterified. This thus establishes the structure of AO-2 as naringenin-7-*O*-(6″-*O*-*p*-coumaryl)-*β*-D-glucoside.

EXPERIMENTAL

Mps are uncorr. The ^{1}H-NMR spectra were recorded on a Varian A-60A instrument; the ^{13}C-NMR spectra were recorded on a Jeol FX-100 NMR spectrometer. TLC was performed on Si gel plates with (A) C_6H_6–Py–HCO_2H (36:9:5); (B) Tol–EtOAc (2:1); (C) EtOAc–MeOH–H_2O (100:16.5:13.5); (D) $CHCl_3$–MeOH (3:1). Authentic naringenin-7-*O*-*β*-D-glucoside was prepared by partial hydrolysis of naringin [3].

Isolation of AO-1 and AO-2. The nut shells of *Anacardium occidentale* (1.5 kg), procured from Travancore, Kerala, India, were crushed and defatted with petrol. Subsequently they were extracted with boiling Me_2CO and the combined Me_2CO extracts concd under diminished pressure. The concentrate was successively digested with petrol, C_6H_6 and EtOAc The EtOAc soln was filtered and evapd to a brown mass (10 g). This residue gave the usual colour reactions for flavonoids and