Phytochemistry, 1972, Vol. 11, pp. 819 to 822. Pergamon Press. Printed in England.

SHORT COMMUNICATION

A NEW STEROID FROM SAFFLOWER

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(*Received* 24 *March* 1971)

Abstract—A previously unreported steroid glycoside has been isolated from safflower meal. Spectral analysis of the steroid by MS, NMR, UV and IR reveals it to be a new structure of the form 15α,20β-dihydroxy-Δ^4-pregnen-3-one.

INTRODUCTION

AN INVESTIGATION of the bitter principles of safflower meal (*Carthamus tinctorus* L. Compositae) led to the isolation of a new steroid glycoside with unusual structural features for a naturally occurring compound. Structural analysis of the steroid, here presented, reveals it to be 15α,20β-dihydroxy-Δ^4-pregnen-3-one (I), present in glycosidic form in safflower seed. To the authors' knowledge, this steroid has not been reported previously.

In 1955, Wettstein,[1] discussing the microbiological conversion of steroids, regarded the C-15 position, as well as other positions, as 'unnatural' for hydroxyls. However, a year later, Djerassi *et al.*[2] presented the complete structure of digitogenin, stating that it represented 'the first naturally occurring C-15 hydroxylated steroid'. Other C-15 oxygenated steroids were found among the digitanol glycosides[3] although only one (purpnigenin) possesses a C-15 hydroxyl; the others are C-15 keto. The orientations of the hydroxyls in these two compounds differ in that digitogenin is a 15β-hydroxy and purpnigenin is a 15α-hydroxy steroid.

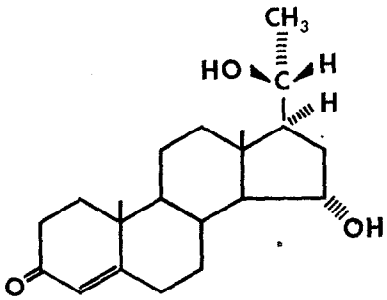
Most plant steroids have a hydroxyl at C-3 (sterols). The safflower steroid, having a Δ^4-3-keto structure, is more closely related to animal steroids and would appear to be a progesterone analogue. Progesterone has been found in a plant,[4] and although rare, there are other instances of plant steroids having the 3-keto structure. However, this type of structure as well as the C-20 hydroxyl is rare among plant steroids.

Isolation procedures for separating the steroid glycoside from safflower meal were identical to those for isolating a bitter lignan glucoside[5] except for differences in elution volumes and R_fs. Acid hydrolysis of the glycoside showed the sugar moiety to be glucose. The aglycones from both acid hydrolysis and enzymatic hydrolysis were identical.

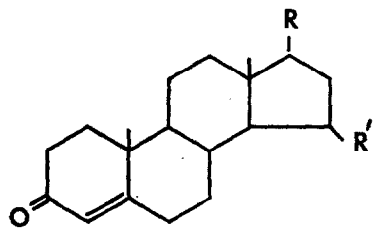
Identification of the steroid aglycone proceeded from the following evidence. High resolution mass spectrometry showed a parent peak at *m/e* 332·2330 from which an empirical

[1] A. WETTSTEIN, *Experientia* XI, 465 (1955).
[2] C. DJERASSI, T. T. GROSSNICKLE and L. B. HIGH, *J. Am. Chem. Soc.* **78**, 3166 (1956).
[3] R. TSCHESCHE, *Progress in the Chemistry of Organic Natural Products* (edited by L. ZECHMEISTER), Vol. 24, p. 99. Springer–Verlag, New York (1966).
[4] M. LEBOEUF, A. CAVÉ and R. GOUTAREL, *C.r. Acad. Sci., Paris* **259**, 3401 (1964).
[5] R. PALTER and R. E. LUNDIN, *Phytochem.* **9**, 2407 (1970).

formula of $C_{21}H_{32}O_3$ could be established. A steroid skeleton was then hypothesized. The presence of an α,β unsaturated CO group was indicated by UV absorption at 245 nm, a PMR peak at $\delta = 5{\cdot}73$ ppm (1H), and sharp IR absorption bands at 1660 and 1610 cm^{-1}. This was further substantiated by MS from an ion peak at *m/e* 124, which is characteristic of this grouping occurring in the A ring of a steroid.[6] On the basis of these data the assumption was made that it was a Δ^4-3-keto C-21 steroid. The IR spectrum showed adsorption bands for OH, as expected in the aglycone. A diacetate was formed on acetylation, as determined by PMR. The steroid skeleton therefore lacked only the positioning of the two hydroxyls and a methyl to satisfy the emprical formula. These positions were established through analysis of the PMR spectra of the aglycone (I), its diacetate (IIa), and its oxidation product (IIb).



(I)



(II) a. R = βCH(βOCOCH$_3$); R′ = αOCOCH$_3$
b. R = βCOCH$_3$; R′ = O
c. R = βCH(βOH)CH$_3$; R′ = H
d. R = βCOCH$_3$; R′ = αOH

The presence of a C-20 hydroxyl and a C-21 methyl group was strongly indicated by the shielding and splitting of the resonance assigned to the C-21 methyl protons in the aglycone and its derivatives. A sharp singlet centered at $\delta = 2{\cdot}22$ (3H) occurs in the oxidation product spectrum and can be assigned to a methyl group (C-21) adjacent to a carbonyl. This peak becomes a doublet centered respectively at $\delta = 1{\cdot}15$ ($J = 6$ Hz) and 1·14 in the spectra of the aglycone and its diacetate, indicating a methyl adjacent to a hydroxyl. As model compounds for these resonances the C-21 methyl proton singlet of 15α hydroxyprogesterone (IId) occurs at $\delta = 2{\cdot}13$, and for 20β hydroxy-Δ^4-pregnen-3-one (IIc) the corresponding doublet (6 Hz) is centered at $\delta = 1{\cdot}14$.

It is now generally recognized that the positions of the resonances from the angular methyl protons accurately reflect in an additive fashion the locations and types of steroid substituents.[7] By use of the substituent shifts tabulated by Bhacca and Williams[8] and

[6] H. BUDZIKIEWICZ, C. DJERASSI and D. H. WILLIAMS, *Structural Elucidation of Natural Products by Mass Spectrometry*, Vol. II, p. 87. Holden-Day, San Francisco (1964).
[7] R. F. ZÜRCHER, *Helv. Chim. Acta* **46**, 2054 (1963).
[8] N. S. BHACCA and D. H. WILLIAMS, *Applications of NMR Spectroscopy in Organic Chemistry. Illustrations from the Steroid Field*, p. 13. Holden-Day, San Francisco (1964).

Zürcher,[9] the chemical shifts of the C-18 and C-19 methyl protons were calculated for the aglycone, its diacetate, and its oxidation product for various substituent locations consistent with all the available spectral and chemical information. The only structure, for which sufficient substituent shift data was available and gave agreement within 0·02 ppm between observed and calculated shifts for all three compounds, was for a 17β configuration for the CH(β OH)CH_3 group and for the skeleton hydroxyl to be at the 15α position. The excellent agreement between observed and calculated values for the aglycone, its two derivatives and two closely related steroids of known structure is shown in Table 1.

TABLE 1. CALCULATED* AND OBSERVED SHIELDINGS OF ANGULAR METHYL RESONANCES FOR SEVERAL SUBSTITUTED PREGNENES

	Methyl proton shielding value (δ)			
	C-18		C-19	
Compound†	Calc.	Obs.	Calc.	Obs.
I	$0{\cdot}84_2$	0·83	$1{\cdot}20_9$	1·21
IIa	$0{\cdot}75_1$	0·76	$1{\cdot}20_1$	1·19
IIb	$0{\cdot}75_9$	0·74	$1{\cdot}20_9$	1·20
IIc	$0{\cdot}80_9$	0·80	$1{\cdot}20_1$	1·18
IId	$0{\cdot}71_7$	$0{\cdot}69_5$	$1{\cdot}20_9$	1·20

* From values given in Ref. 8.
† In $CDCl_3$.

Introduction of a 15α-hydroxyl has been successfully achieved microbiologically in progesterone, cortexone and testosterone.[10] To verify the proposed structure of the steroid aglycone, we started with 20β-hydroxy-Δ^4-pregnen-3-one (IIc) and followed the procedure of Fried *et al.*,[11] using an inoculum of *Penicillium* species ATCC 11598. The isolated product was compared by mass spectral and NMR analysis to the steroid aglycone (I) from safflower and found to be identical.

For further verification, we obtained an authentic sample of 15α-hydroxyprogesterone (IId) which we oxidized to 15-ketoprogesterone (IIb) for comparison with the oxidation product of the safflower steroid aglycone. Again the mass spectra and NMR spectra were identical.

Thus the structure of the steroid aglycone from safflower has been established. The composition and stereochemistry of the glucose moieties have not yet been determined.

EXPERIMENTAL

PMR spectra, with $CDCl_3$ as solvent, were obtained with an internally locked Varian HR 100 Spectrometer with TMS as the internal standard. Relative peak areas were consistent with their assignments. Mass analyses were performed on a Consolidated Electrodynamics 21–100 Mass Spectrometer. IR spectra were

[9] R. F. ZÜRCHER, in *Progress in Nuclear Magnetic Resonance Spectroscopy* (edited by J. W. EMSLEY), Vol. 2, 230. Pergamon Press, Oxford (1967).
[10] A. GUBLER and C. TAMM, *Helv. Chim. Acta* **41**, 301 (1958).
[11] J. FRIED, R. W. THOMA, D. PERLMAN and J. R. GERKE (to Olin Mathieson Chemical Corp.) U.S. Patent 2,753,290 (3 July, 1956); *Chem. Abstr.* **51**, 2071 (1957).

determined on a Perkin–Elmer 257 and UV spectra on a Cary Model 15. Optical rotations were taken on a Cary Model 60 Spectropolarimeter. Analytical and preparative layer silica gel plates were purchased from Brinkmann Instruments.

Extraction and isolation procedures. 15α,20β-Dihydroxy-Δ^4-pregnen-3-one glycoside was isolated concurrently with matairesinol monoglucoside[5] from oil-free safflower meal by MeOH and EtOAc extraction procedures. Dialysis, ion-exchange (Dowex-50(H^+)) and gel filtration (Sephadex LH-20) chromatography, and PLC completed the isolation. The ion-exchange column elution volume for the steroid glycoside was 15·8–20·4 l. Effluent volume from the gel filtration column was 1833–2045 ml. The steroid glycoside is a hygroscopic white powder, dec. 175–178°, $[\alpha]_D^{27}$ + 72 in EtOH.

Since the last step in the preparation of the steroid aglycone and its derivatives was purification on silica gel plates, appreciable amounts of contaminants derived from the plates were apparent in almost all PMR spectra. The nature of these impurities is unknown.[12] Although their spectra are quite unlike steroid spectra, their presence made total integrations impossible and prevented a detailed spectral analysis particularly in the region 3·5–3·9 ppm. A 'blank' silica gel extraction preparation qualitatively accounted for the extraneous peaks seen in all spectra.

Glycoside hydrolysis. A 56·2-mg sample of the purified glycoside was hydrolyzed with 5 ml 0·5 N HCl for 4 hr at 100° in an evacuated sealed tube. The aglycone was extracted with $CHCl_3$ and purified by the same PLC technique described previously.[5] The sugar was similarly isolated and identified as glucose.

A 20-mg sample of glycoside was hydrolyzed enzymatically by reaction with 2 ml of a filtered Takamine cellulase solution (33 mg/ml 0·1 M sodium acetate buffer, pH 5·0) for 48 hr at 40°. The resulting precipitate, after being washed with water, was dissolved in $CHCl_3$ and applied to a silica gel semi-preparative plate for development with EtOAc–$CHCl_3$ (9:1). The aglycone band was located under UV light, extracted from the silica gel with $CHCl_3$, and dried. The measured mass of the aglycone was 332·2330; calc. mass for $C_{21}H_{32}O_3$ is 332·2351. The major fragmentation peaks of the steroid aglycone occurred at *m/e* 55, relative abundance 34%; *m/e* 45, 24%; *m/e* 91, 23%; *m/e* 333, 21%; *m/e* 105, 17%; *m/e* 107, 16%; *m/e* 124, 16%; *m/e* 147, 15%. The steroid aglycone has a m.p. of 222° and $[\alpha]_D^{27}$ + 110 in $CHCl_3$.

Derivatives. Steroid aglycone (I) (3 mg) was acetylated with 0·1 ml pyridine and 0·1 ml Ac_2O at 37° for 17 hr. The excess reagents were removed under an N_2 stream. Analytical TLC showed the reaction to be quantitative. The measured mass was 416·2583; calc. mass for $C_{25}H_{36}O_5$ is 416·2563. A new peak in the PMR spectrum at δ = 2·00 (6H) showed that a diacetate had been formed. Only the residual impurity band centered at δ = 3·75 remained in the vicinity of δ = 4·0, and a new broad band centered at δ = 4·80 showed that both hydroxyl-bearing carbons had been acetylated. Strong irradiation of this band caused the C-21 proton doublet centered at δ = 1·14 (3H) to collapse to a singlet, showing that the hydroxyl on the adjacent carbon had been acetylated.

The safflower steroid aglycone was oxidized by use of the Jones reagent in acetone.[13] Aglycone (6 mg) in 3 ml of acetone was allowed to react with two drops of Jones reagent at 20° for 2 hr. The reaction mixture was applied to a preparative silica gel plate and developed with $CHCl_3$–acetone (9:1). The oxidized aglycone (IIb) band was located under UV light, extracted with $CHCl_3$, and dried. The measured mass was 328·2063; calc. mass for $C_{21}H_{28}O_3$ is 328·2038. As expected, the C-21 methyl protons appear in the PMR spectrum at δ 2·22 (3H), and the doublet centered at δ = 1·15 in the aglycone spectrum ascribed to this group was absent. No peaks were present below δ = 2·6 except for the C-4 proton singlet.

The authentic sample of 15α-hydroxy progesterone (IId) was oxidized to 15-keto progesterone (IIb) in the identical manner. The PMR spectrum of IIb was virtually identical to that of the oxidized aglycone.

Microbiological synthesis of 15α,20β-dihydroxy-Δ_4-pregnen-3-one. 20β-Hydroxy-Δ_4-pregnen-3-one (IIc) (500 mg) (Mann Research Laboratories) was treated with an inoculum of *Penicillium* species ATCC 11598 according to the method of Fried *et al.*[9] After pH adjustment and filtration the desired product was isolated from the growth medium by extraction with $CHCl_3$. The $CHCl_3$ extract, dried *in vacuo*, was applied to silica gel preparative plates and developed with EtOAc–$CHCl_3$ (9:1). The pertinent band was extracted with $CHCl_3$ and dried. Complete purification was achieved by a second PLC development with $CHCl_3$–MeOH (9:1). The measured mass was 332·2369; calc. mass for $C_{21}H_{32}O_3$ is 332·2351.

Acknowledgements—The authors wish to thank Dr. William F. Haddon for the mass spectra analyses and Dr. James C. Lewis for his assistance with the microbiological aspects of this work. The gift of 15α hydroxyprogestrone from Dr. P. A. Diassi of the Squibb Institute for Medical Research is greatly appreciated.

[12] H. SPITZ, *J. Chromatog.* **42**, 384 (1969).
[13] L. F. FIESER and M. FIESER, *Reagents for Organic Synthesis*, p. 142. Wiley, New York (1967).

Key Word Index—Carthamus tinctorus; Compositae; steroid; 15α,20β,-dihydroxypreg-4-ene-3-one.