Phytochemistry, 1979. Vol. 18, pp. 345–346. © Pergamon Press Ltd. Printed in England. 0031-9422/79/0201-0345 $02.00/0

SEX HORMONES AND CORTICOSTEROIDS IN POLLEN OF *PINUS NIGRA**

MELITA ŠADEN-KREHULA, MELITA TAJIĆ and DRAGUTIN KOLBAH
Department of Chemistry, Faculty of Pharmacy and Biochemistry, University of Zagreb and Division of Endocrinology, University Hospital Rebro, Zagreb, Yugoslavia

(*Revised received* 28 *July* 1978)

Key Word Index—*Pinus nigra*; Pinaceae; steroid hormones; pollen.

Abstract—In continuation of earlier investigations on steroid hormones in the pollen of pine, we have isolated and quantitatively determined the following steroids by radioimmunoassay and fluorimetric methods: testosterone, testosterone together with epitestosterone, respectively, and androstenedione; progesterone was determined by radioimmunoassay alone. In addition, we have tried to isolate cortisol, cortisone, 11-deoxycortisol, corticosterone and 11-deoxycorticosterone and to characterize these compounds by specific reactions. The intensity of these reactions were used to estimate the amounts of corticosteroids in pollen.

INTRODUCTION

Members of all steroid groups, except the bile acids, have been found in plants, especially three of the main sex hormones—oestrone, progesterone, testosterone—as well as cholesterol. Obviously, these compounds are not limited to the animal kingdom [1]. Observations made on the metabolism of progesterone in *Digitalis lanata* indicated that the plant had the capacity to introduce a hydroxyl group at C-21 of this hormone and, also, to convert deoxycorticosterone into cardenolides [2]. Evidence was put forward that another plant, *Mallotus paniculatus* (Euphorbiaceae), can oxidize exogenously supplied cortisol to cortisone [3]. Corticosteroids were shown by Geuns [4] to have a hormonal function in plants and should be considered as a new group of plant steroid hormones. Geuns studied the structural requirements of corticosteroids for plant hormonal activity in etiolated mung bean seedlings.

RESULTS AND DISCUSSION

In previous work we have detected testosterone, androstenedione and progesterone in the pollen of pine. Now we report the results of quantitative determination of these steroids by radioimmunological and fluorimetric methods. The reactivity of isolated substances, when determined by these two specific procedures, was taken as additional evidence for the presence of these steroids in pine pollen. The amounts found are given in Table 1 (means of 2 or 3 determinations) and varied from sample to sample, since the extraction of pollen (from different trees *P. nigra* Ar.) was not always equally efficient. In our search for corticosteroids in extracts from the same kind of pollen, we used various semiquantitative methods of evaluation (UV spectra and tetrazolium salt reactions) and methods for quantitative determination (Porter-Silber reaction and two types of fluorescence measurement). The amounts of corticosteroids in pine pollen are given in Table 2. The results with corticosteroids, like those with sex hormones, depended on the extraction efficiency and on the reaction used in quantitative determination. The final aim of our investigations however, was not to find how much corticosteroids are contained in pollen, but to isolate these compounds by chromatographic procedures, then to identify and characterize the fractions by well known reactions for distinct functional groups within the steroid molecule.

Table 1. Amounts of steroid hormones found in pine pollen by radioimmunoassay (RIA) and by fluorimetry

	μg/10 g pollen						
	F	T	T + ET	T + ET Acetate	ΔA	ΔA Acetate	P
RIA	0.9	0.7	—	—	0.8	—	0.8
Fluorimetry	0.7	—	0.7	0.8	0.7	0.6	—

Key: F = cortisol, T = testosterone, ET = epitestosterone, ΔA = androstenedione, P = progesterone.

EXPERIMENTAL

The radioimmunoassay and fluorimetry of testosterone or testosterone with epitestosterone and androstenedione, the fluorimetry of their acetates, and the radioimmunoassay of progesterone were performed after the third TLC run on Si gel

Table 2. Amounts of corticosteroids found in pine pollen by various reactions

	μg/10 g pollen				
	F	E	B	S	DOC
UV spectra	0.7	0.4	0.03	0.3	0.1
Tetrazolium salt reaction	0.3	0.2	0.05	0.1	0.05
Porter-Silber reaction	0.6	0.2	—	0.5	—
Fluorescence at 598, 615 nm	—	—	0.04	0.2	0.05
Fluorescence at 470, 520 nm	0.7	—	0.02	—	—

Key: F = cortisol, E = cortisone, B = corticosterone, S = 11-deoxycortisol, DOC = 11-deoxycorticosterone.

* Part III in the series "Steroid Hormones in Pollen of Pines" [5, 6]. For Part II see ref. [6].

HF_{254} (TLC isolation of pollen steroids in each run was made as described previously [5, 6]; material to be used for radioimmunological determination of testosterone and androstenedione was repurified by one additional run on a chromatographic column [7]. These steroids were determined by the same procedure as in plasma using biokits from Biolab, Bruxelles. Antiserum to testosterone had very little cross reactivity to epitestosterone. Fluorescence was measured in a spectrofluorimeter with a xenon light source; activation wavelength was 595 nm, emission wavelength 615 nm. Cortisol was measured by the solid phase cortisol RIA kit NEN, New England Nuclear Corporation. Since we attempted to detect corticosteroids in pollen, the previously described procedure [5] for testosterone, epitestosterone and androstenedione in the pollen was modified in order to detect the corticosteroids. From the suspension of pollen in H_2O, the steroids were extracted with Et_2O or CH_2Cl_2, before and after hydrolysis with β-glucuronidase and after hydrolysis with H_2SO_4 at room temp. The extracts were first washed with 0.1 N NaOH then with 0.1 N HOAc, and finally freed from lipids by addition of 70% MeOH. Ketonic and non-ketonic fractions were separated by Girard T reagent. The ketonic fraction was chromatographed on Si gel HF_{254}, and the zones containing the corticosteroids were rechromatographed, along with authentic standards using: $CHCl_3$–Me_2CO (9:1), $CHCl_3$–MeOH (19:1), $CHCl_3$–EtOH (9:1) and CH_2Cl_2–Me_2CO (4:1). The steroids were located and identified under a UV 254 lamp, and the corresponding zones were eluted with MeOH. After chromatographic separation the identities of each corticosteroid were confirmed and their amounts were estimated by semiquantitative or fully quantitative methods, in comparison to known amounts of standard substances.

1. Zones containing Δ4-3-keto-corticosteroids were eluted with 5.5 ml MeOH and the extract was used for recording the absorption spectra between 225 and 255 nm. An equally sized clean zone from the same chromatogram was eluted with 5.5 ml of the same solvent and the spectrum of this eluate was subtracted from the sample spectra. The resulting curves were spectra corresponding to those of corticosteroids: all had maxima at 240–241 nm. By comparison with corresponding spectra recorded with methanolic solutions of the corticosteroid standards (6 μg/5.5 ml), the contents were calculated per 10 g pollen mass.

2. MeOH extracts were evapd to dryness and the residues were taken up in 0.5 ml 96% EtOH. These solns were treated with 0.25 ml 0.5% tetrazolium salt (3,3' dianisole-*bis*-4,4'-(3.5-diphenyl)tetrazolium chloride) in 96% EtOH and 0.25 ml 4% aq. Me_4NOH. After standing 25 min in the dark, 1 ml HOAc was added, whereupon a purple colour developed which was characteristic of steroids with —CO–CH_2OH at C-17. The intensity of this colour was estimated by eye.

3. Cortisol, cortisone and 11-deoxycortisol, steroids with >COH—CO—CH_2OH at C-17 were characterized by the Porter-Silber reaction resulting in a yellow colour with max at 410–415 nm. Eluates from these zones were divided into 2 equal parts and each of these was evapd. One part (a) was mixed with 3 ml 64% H_2SO_4 in EtOH (2:1) to serve as the blank; the other part (b), was mixed with the same amount of H_2SO_4 in EtOH containing phenylhydrazine hydrochloride. Samples and standards were measured in a spectrophotometer at 390, 415 and 440 nm; the readings were corrected, and results calculated, according to Allen. At the same time the entire spectra were recorded from 380 to 450 nm, and a similar spectrum was recorded with eluate from a clean spot of the chromatogram. The spectra from samples (a) were compared to those from samples (b) corrected for Si gel absorption by subtracting the spectrum of the clean zone. Both the spectra from (a) and corrected spectra from (b) samples had the same maximum, at 415 nm, as the spectra from corresponding standards, which indicated substances with the dihydroxyacetone group.

4. Samples with corticosterone, 11-deoxycortisol and 11-deoxycorticosterone developed a red fluorescence when treated by the following procedure. Dry residues from the eluates were taken up in 0.1 ml 96% EtOH, then carefully mixed with 0.5 ml of conc H_2SO_4, whereupon the vessel containing this mixture was immersed in boiling H_2O for 30 sec. After rapid cooling, 2.4 ml 1:3 ethanolic H_2SO_4 was added, and the final mixture kept for 30 min at 30°. The fluorescence was excited at 598 nm and the emission was measured at 615 nm. No other corticosteroids developed fluorescence under the conditions described.

5. The zones and standards of cortisol and corticosterone developed fluoresence at 520 nm (exc. at 470 nm). This fluorescence was also measured in a spectrofluorimeter with a xenon arc.

Acknowledgement—We are indebted to Prof. M. Vidaković and Ž. Borzan (Faculty of Forestry, University of Zagreb) for kindly supplying the pollen.

REFERENCES

1. Knights, B. A. (1973) *Chem. Br.* **9**, 106.
2. Caspi, E., Wickramasinghte, J. A. F. and Lewis, D. O. (1968) *Biochem. J.* **108**, 499.
3. Bennett, R. D. and Heftmann, E. (1970) *Phytochemistry* **9**, 807.
4. Geuns, J. M. C. (1977) *Z. Pflanzenphysiol.* **81**, 1.
5. Šaden-Krehula, M., Tajić, M. and Kolbah, D. (1971) *Experientia* **27**, 108.
6. Šaden-Krehula, M., Tajić, M. and Kolbah, D. (1976) *Biol. Zbl.* **95**, 223.
7. Furuyama, S., Mayes, D. and Nugent, C. A. (1970) *Steroids* **16**, 415.
8. Mattingly, D. (1962) *J. Clin. Pathol.* **15**, 374.