Identification of 17α, 20α-Dihydroxyprogesterone in Testicular extracts after Incubation with Ketoconazole

W. F. J. Lauwers,† L. Le Jeune, H. Vanden Bossche and G. Willemsens
Department of Analytical Research and Comparative Biochemistry, Research Laboratories, Janssen Pharmaceutica, Beerse, Belgium

The antifungal ketoconazole affects testosterone synthesis in dispersed rat testicular cells. In the presence of ketoconazole an accumulation of 17α,20α-dihydroxyprogesterone has been observed. This steroid was isolated from the testis of Wistar rats after a [4-^{14}C]progesterone incorporation in the presence of ketoconazole. Its identification was achieved from the gas chromatographic/mass spectrometric analysis of the isolated radioactive fraction. A chemical derivization of the fraction with butylboronic acid followed by mass spectrometric analysis confirmed the presence of 17α,20α-dihydroxyprogesterone.

INTRODUCTION

Ketoconazole (*cis*-1-acetyl-4-[4-[[2-(2,4-dichlorophenyl) -2- (1,*H*-imidazol-1-ylmethyl-1,3-dioxolan - 4- yl]-methoxy]phenyl] piperazine), is an orally active azole derivative with broad spectrum activity against a variety of yeasts, dermatophytes and dimorphous fungi.[1]

Besides the inhibitory effect of ketoconazole and other nitrogen heterocycle-containing antifungals on the cytochrome P-450 dependent (P-450) ergosterol biosynthesis in yeasts and fungi,[2-4] a transient decrease in serum testosterone levels has been observed in humans treated with doses from 200 to 600 mg ketoconazole.[5,6] It has also been observed that ketoconazole suppresses testosterone production by up to 50% when incubated at a concentration of 2×10^{-7} mol l^{-1} in dispersed rat testicular cells.[7]

Previous studies had shown an effect of ketoconazole on the microsomal P-450 of dog testis[4] at a concentration $>10^{-7}$ mol l^{-1}. Therefore it was tempting to speculate that decreased testosterone synthesis might originate from an interaction with one of the P-450 dependent steps in the metabolism of pregnenolone or progesterone. In view of this hypothesis the effects of ketoconazole on testosterone synthesis in the subcellular fraction of dog testis were studied.[8]

These studies suggest an effect of ketoconazole on the P-450 dependent C17,20-lyase. The aim of the present study was to give details on the steroid that accumulated in the microsomal suspension incubated in the presence of ketoconazole and progesterone. A clean up of the accumulated fraction followed by a gas chromatographic/mass spectrometric analysis has allowed identification of the unknown steroid as 17α,20α-dihydroxyprogesterone, the substrate of the C17,20-lyase.

A preliminary gas chromatographic separation before ionization was inevitable due to the remaining complexity of the fraction, even after purification by two-dimensional thin layer chromatography. The electron impact mass spectrum of the unknown steroid allowed identification of the latter in all probability as 17α,20α-dihydroxyprogesterone. Full evidence for the structure was obtained by gas chromatographic/mass spectrometric analysis of the fraction after treatment with butylboronic acid.

The chemical derivatization of 1,2- or 1,3-diol groups can be easily achieved with boronic acids.[9-11] This procedure has obtained widespread recognition for the confirmation of the occurrence of diol systems in molecules. The results clearly indicate the formation of such a corresponding butylboronate derivative.

EXPERIMENTAL

Tissue preparation

Wistar rats (body weight 250–300 g) were sacrificed by decapitation. The testes were collected in ice-cold potassium phosphate buffer (0.1 M, pH 7.4) and homogenized by a Potter-Elvehjem homogenizer with teflon pestle. The homogenate was centrifuged at 500 *g* for 10 min and the supernatant centrifuged at 10 000 *g* for 20 min. The resulting supernatant, called the S10 fraction, was used for the incubation.

Incubation and sample collection

The S10 fraction was brought to a protein concentration of 5 mg ml^{-1} as determined by the Bio-Rad method (Bio-Rad protein assay, München).

The incubation mixture consisted of 8 ml of the S10 fraction, 1.6 mM NADPH, 5 mM ATP, 3 mM $MgCl_2.6H_2O$ and 0.2 μCi [4-^{14}C] progesterone (sp. act. 56 mCi mmol^{-1}), in a final volume of 10 ml. The incubations were carried out in a reciprocating shaker at 37 °C and constantly gassed with air.

The reaction was stopped after 3 h by adding 50 ml of ethyl acetate and the steroids were extracted by the method of Wilcox and Engel.[12] Separation of the steroids

† Author to whom correspondence should be addressed.

CCC-0306-042X/85/060296-06 $03.00

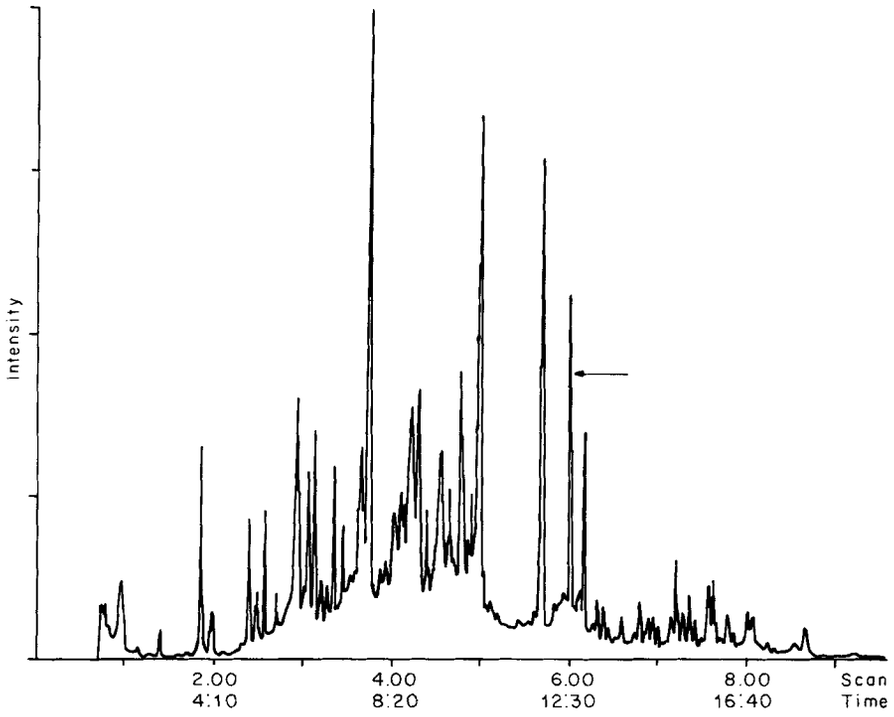


Figure 1. Total ion chromatogram of the gas chromatographic/mass spectrometric analysis of the radioactive fraction.

was accomplished with two-dimensional thin layer chromatography (TLC) on precoated silica gel 60 F_{254} plates as previously described.[8]

The first solvent system was benzene + ethyl acetate (1 : 1, v/v), and the second solvent was composed of two solvent systems, i.e. (A) isooctane + isopropyl ether + acetic acid (2 : 1 : 1, v/v/v) and (B) benzene + ethyl acetate (4 : 3, v/v) with A/B = 1.

The R_f values of the fractions were compared with those of available standards. The accumulated radioactive fraction which deviated from the standards was scraped off from the TLC plate.

The silica gel was extracted with 50 ml of ethyl acetate. The solution was filtered and the solvent evaporated under dry nitrogen. The residue was dissolved in 50 μl methanol and put into a 60 μl vial (Pierde Chemicals).

Derivatization

The butylboronate ester derivative was formed by adding 20 μl of a solution of boronic acid in 2,2-dimethylpropane (1 mg ml^{-1}) to the dry residue in the reaction vial. The reaction mixture was kept for 15 min at 60 °C. The solution was evaporated to dryness and the residue dissolved in 50 μl of 2,2-dimethoxypropane. An aliquot of about 1 μl was injected on the capillary column.

Gas chromatography/mass spectrometry

The gas chromatographic /mass spectrometric analysis was performed on a Finnigan 4500 mass spectrometer coupled to the Incos data system. An aliquot of the methanol of 2,2-dimethoxypropane solution was injected in the splitless mode on the SP Sil-5 capillary column (10 m × 0.32 mm i.d.; Chrompack).

Helium was used as the carrier gas with a flow of 1.5 ml min^{-1}. The temperature of the injector and the separator was 260 °C. The temperature of the injector and the separator was 260 °C. The temperature of the column oven was raised from 50 °C to 290 °C at a rate of 12 °C min^{-1}.

The mass spectrometer was operated in the electron impact mode at an ionization energy of 70 eV. The ion source temperature was maintained at 240 °C. The mass spectra were registered with a repetitive scan of one second and collected on a magnetic disc.

RESULTS AND DISCUSSION

Several attempts to analyse reference steroidal compounds indicated that the best results in respect of the gas chromatographic properties were obtained with a SP Sil-5 short capillary column. The analysis was performed on subnanogram quantities of steroids. The injection of 1 μl of the purified fraction yielded the total ion chromatogram shown in Fig. 1.

The major compounds separated on the column corresponded to fatty acids and their related compounds and also to some contaminants (softeners).

In spite of this contamination a compound was detected at a column temperature of 235 °C which appeared to have a steroidal skeleton. The mass spectrum is shown in Fig. 2(a). A library search for the mass spectrum with the aid of the available NBS library[13] yielded 17-hydroxy-pregn-4-ene-3,20-dione (17-hydroxyprogesterone) as the best fit (Fig. 2(b)).

The similarity of the two spectra clearly indicates that the two compounds have a great part of the structure in common (see **1**).

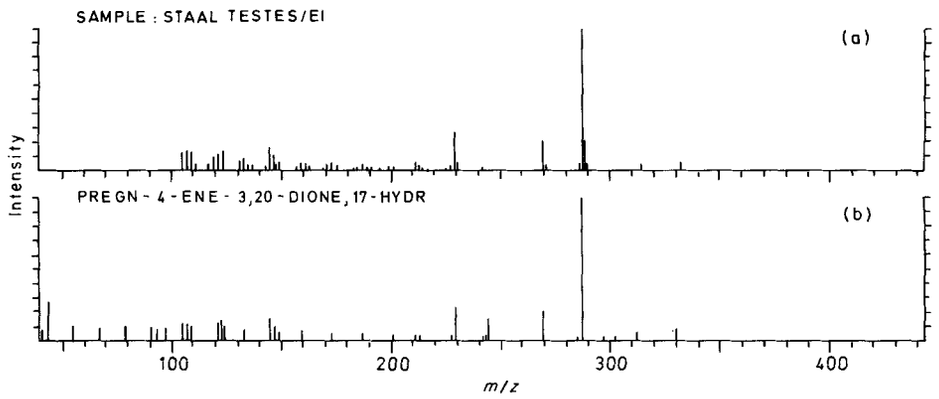
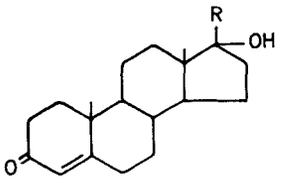


Figure 2 (a) Electron impact mass spectrum of the unknown steroid. (b) Electron impact reference mass spectrum of 17-hydroxyprogesterone.



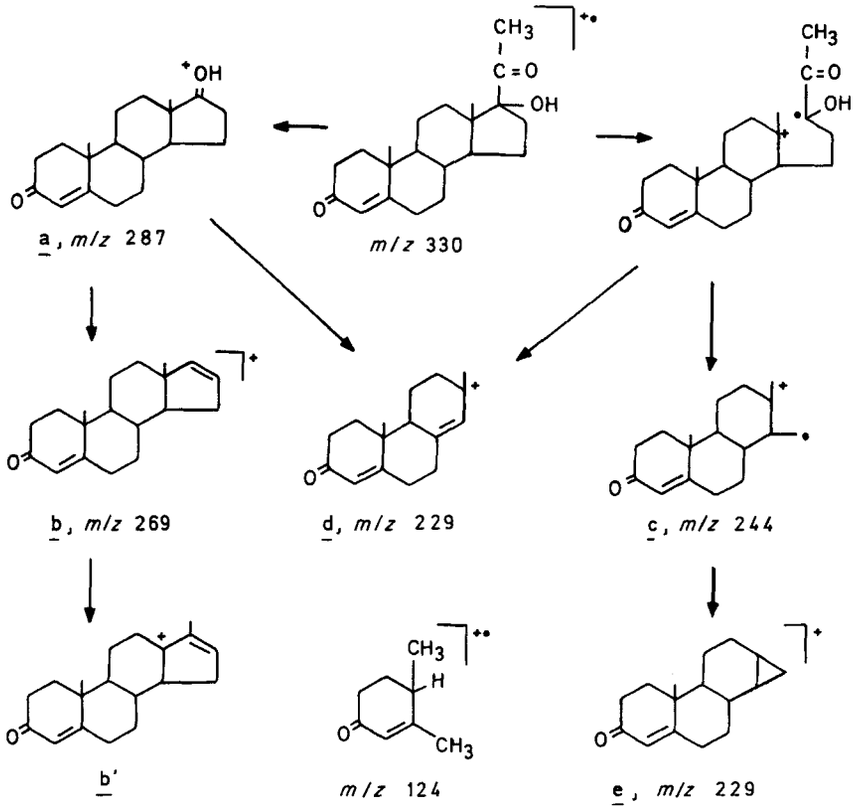
R = $COCH_3$: 17-hydroxyprogesterone

1

The ions displayed in the reference mass spectrum of 17-hydroxyprogesterone are rationalized by loss of the C-17 substituent and cleavage of ring D.[12-15]

The major fragmentation pathways are predicted in Scheme 1. The base peak of the mass spectrum is formed by loss of CH_3CO(*a*). This fragment ion *a* further decomposes by the expulsion of water. The loss of water gives rise to hydrogen scrambling and skeleton rearrangement (C-18 methyl migration; b→b'). The fragment ion *a* also decomposes by loss of the fragments C_2H_3 (*c*) and C_3H_6O (*d*). These ions are also formed directly from the molecular ion when an initial cleavage of ring D is involved. For instance, the cleavage of C_{13}—C_{17} results in the loss of the neutral 2,3-butadione. The generated ion *c* is further stabilized by expulsion of the C-13 methyl group (*e*). In addition, the simultaneous loss of ring D and expulsion of a hydrogen atom is a well-known fragmentation of steroids with a C_{17} side chain.

This fragmentation is also valid for 17-hydroxyprogesterone and yields fragment ion *e*, which



Scheme 1

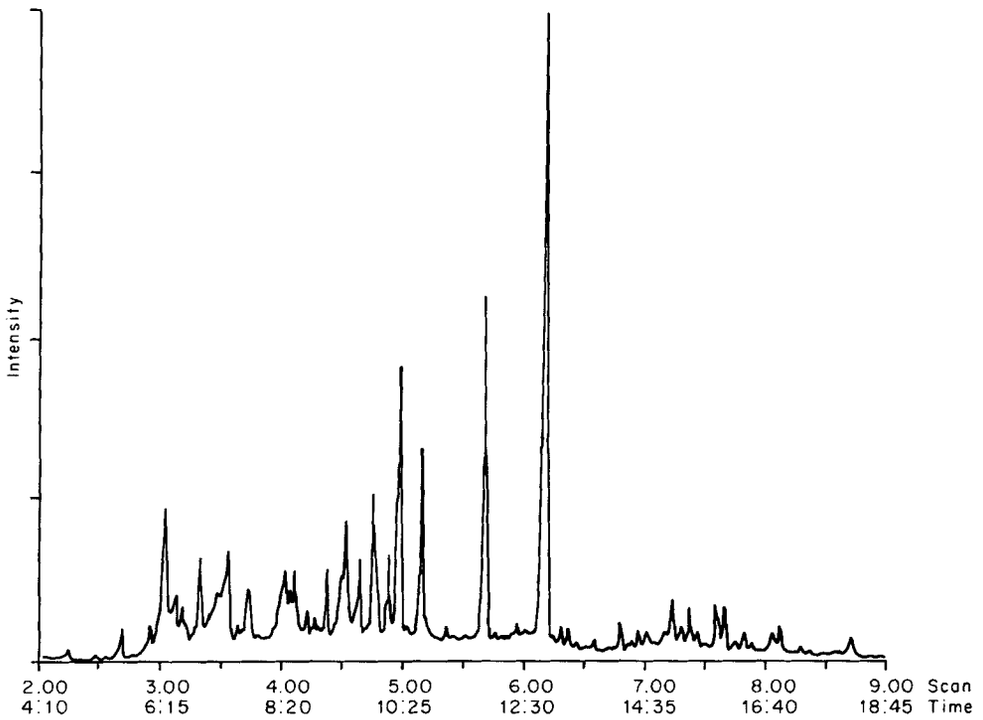


Figure 3. Total ion chromatogram of the gas chromatographic/mass spectrometric analysis of the radioactive fraction after treatment with butylboronic acid.

is also derived from the precursor *c*. In contrast to the fragmentation of progesterone, the cleavages of the bonds 6–7 and 9–10 are suppressed by the hydroxyl substituent at C-17. This implies the absence of the ion m/z 124, which is usually related to the steroidal Δ^4-3-ketones.[16,17]

The described ions are related to the structure of 17-hydroxyprogesterone. The same ions are present in the mass spectrum of the unknown steroid in almost comparable abundance, and one may therefore assume that the structure displays the same steroidal skeleton.

A weak molecular ion is found at m/z 332 in the mass spectrum of the unknown (Fig. 2(a)). The most prominent fragment ion induces the loss of the C-17 substituent with formation of fragment ion *a*, which implies that R (45 u) is consistent with the following structural

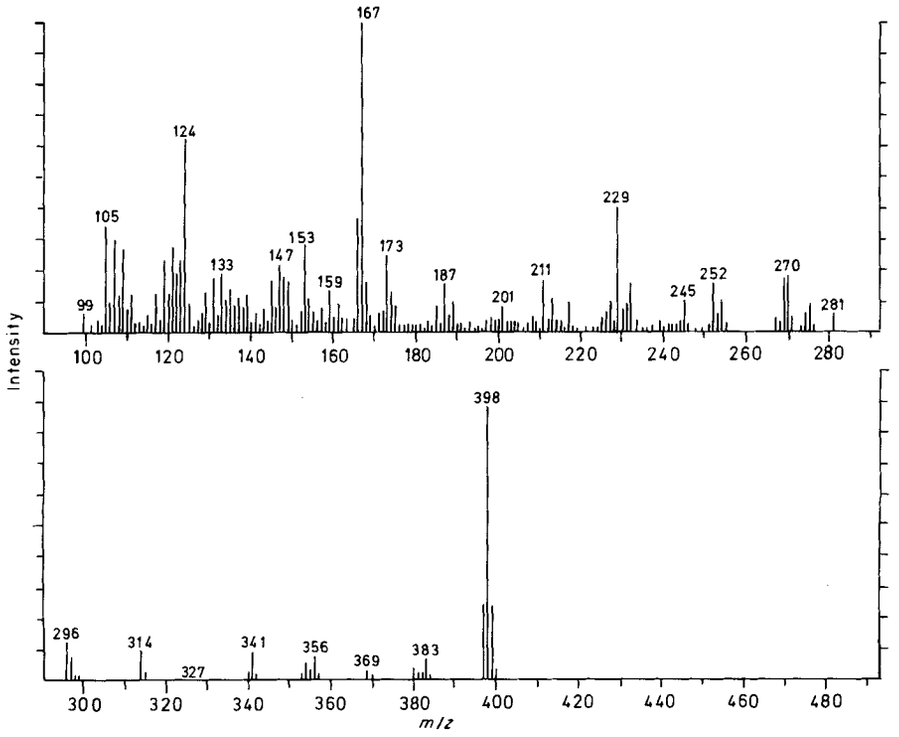
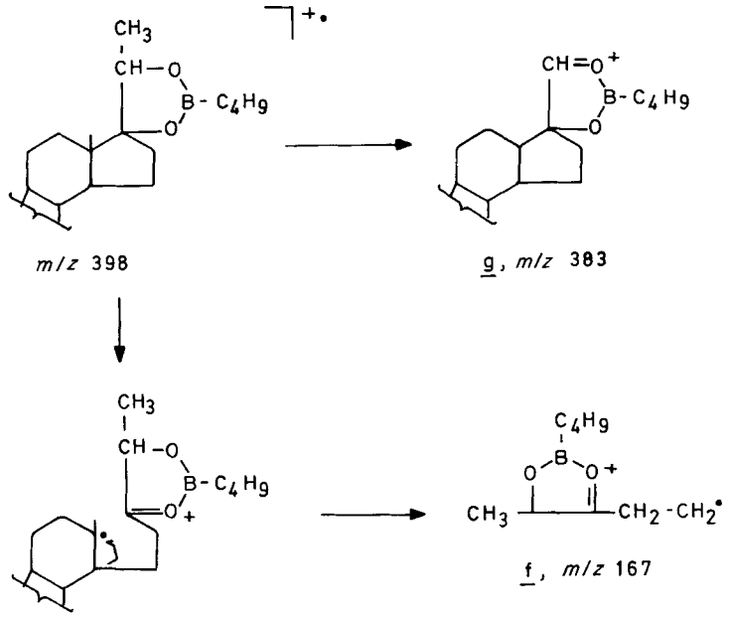


Figure 4. Electron impact mass spectrum of the cyclic boronate derivative of the unknown steroid.



Scheme 2

proposals: CH_2OHCH_2— and CH_3CHOH—. The loss of 31 mass units from the molecular ion was not observed, which indicates that a 1,3-diol system is much less likely.

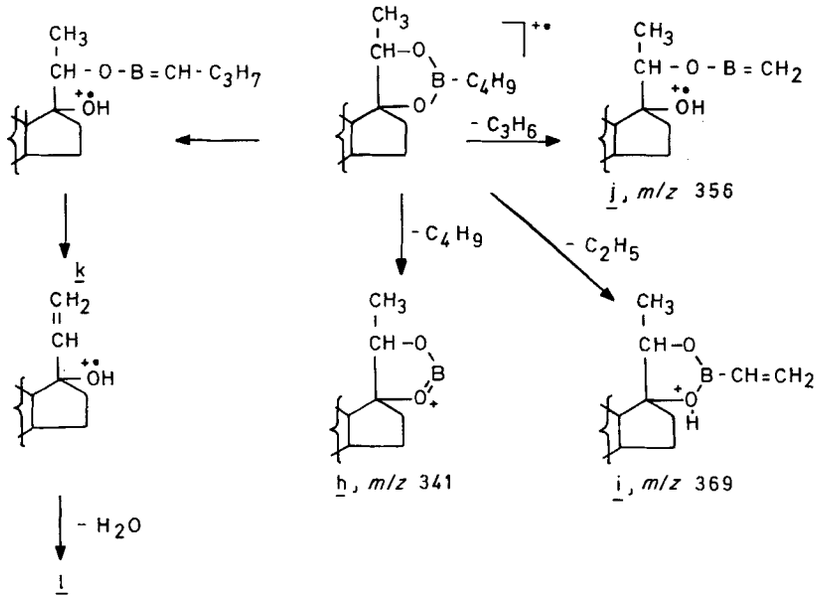
More evidence of a 1,2-diol or 1,3-diol system was obtained from the corresponding butylboronate derivative. It is known that derivatization of 1,2- or 1,3-diol groups can be achieved with boronic acids; $RB(OH)_2$. This prompted us to derivatize the compound with butylboronic acid. The injection of an aliquot of the reaction mixture under identical conditions yielded a total ion chromatogram as presented in Fig. 3.

The peak detected in the previous analysis (Fig. 1) disappeared and was replaced by the occurrence of a prominent peak which was detected at a column temperature of 241 °C. The mass spectrum corresponding to the cyclic boronate derivative is represented in Fig. 4.

The molecular ion shifted 66 u as expected for the boronate ester. The pronounced $[M-1]^+$ ion was mainly attributed to the incorporation of the isotope ^{10}B and confirmed the presence of a boron atom in the molecule. The fragmentation of the compound was consistent with a 17-hydroxyprogesterone derivative.[18]

A major fragment ion *c* (Scheme 1) formed by cleavage of ring D was still present. A prominent ion m/z 124 characterizes unambiguously the steroidal Δ^4-3-one moiety. This ion was almost absent in the underivatized steroid due to the generation of the oxonium ion *a*.

Charge retention on the ring oxygen induced an initial cleavage of the bond 13-17 and gave rise to the base peak (*f*, *m/z* 167, Scheme 2). A similar α-cleavage generated the cyclic oxonium ion *g* after expulsion of a methyl radical. This fragment ion of moderate abundance implied a terminal methyl group for the C-17 substituent



Scheme 3

Table 1

	$[M-H_2O]^+/[M]^{+\cdot}$	$[M-2H_2O]^+/[M-H_2O]^+$	$[M-CH_3CHOH]^+/[M]^{+\cdot}$
17α,20α	0.7	0.6	13.4
17α,20β	0.4	0.3	3.5
Steroid	0.6	0.14	16

and permitted identification of the compound as a 1,2-diol boronate derivative.

The $[M\text{-}15]^+$ ion originated almost exclusively from the α-cleavage, as the latter was much less pronounced in the mass spectrum of 17-hydroxyprogesterone and almost absent in the spectrum of the underivatized steroid (Figs. 2(a) and 2(b)).

More evidence of the structure was gained from the ions observed in the high mass range. The fragment ions *h*, *i* and *j* resulted from cleavages at the butyl side chain (Scheme 3). Ion *k* was most probably formed by a double hydrogen shift and further decomposed by loss of water yielding ion *l* of moderate abundance.

The stereochemical configuration of the C-20 hydroxyl group can be tentatively determined as α. The spectra of 17α,20α-dihydroxyprogesterone and the 20β-epimer exhibited considerably divergent values for the ratios $[M-H_2O^+]/[M]^{+\cdot}$, $[M-2H_2O]^+/[M-H_2O]^+$ and $[M-CH_3CHOH]/[M]^{+\cdot}$[19] and therefore the two epimers could be easily differentiated on account of these values (Table 1).

The ratios calculated from the mass spectrum 2(a) (Table 1) showed that the position of the hydroxyl function at C-20 was probably α if the values for $[M-H_2O]^+/[M]^{+\cdot}$ and $[M-CH_3CHOH]^+/[M]^{+\cdot}$ were taken into account. Conversely, the ratio $[M-2H_2O]^+/[M-H_2O]^+$ referred to 20β. However the ion $[M-H_2O]^+$ was of negligible abundance (<1% RI) and included the possibility of considerable intensity variations due to experimental registration conditions. On the grounds that the ratio $[M-2H_2O]^+/[M-H_2O]^+$ was unreliable, the unknown steroid was identified as 17α,20α-dihydroxyprogesterone.

Acknowledgements

We are grateful to Paul A. Janssen for his constant interest. Grateful appreciation is also expressed to C. Sprangers for typing the text.

REFERENCES

1. H. B. Levine, *Ketoconazole in the Management of Fungal Disease*. ADIS Press, New York.
2. H. Vanden Bossche, W. Lauwers, G. Willemsens, P. Marichal, H. Cornelissen and W. Cools, *Pestic. Sci.*, **15**, 188 (1984).
3. H. Vanden Bossche, G. Willemsens, P. Marichal, W. Cools and W. Lauwers, in *Mode of Action of Antifungal Agents*, edited by J. Ryley and A. Trinci. Cambridge University Press, Cambridge, in press.
4. H. Vanden Bossche, G. Willemsens, W. Cools. P. Marichal and W. Lauwers, *Biochem.* Soc. Transc. **11**, 665 (1984).
5. A. Pont, P. L. Williams, S. Azhar, R. E. Reitw, C. Bochra, E. R. Smith and D. A. Stevens, *Arch. Intern. Med.* **142**, 2137 (1982).
6. R. J. Santen, H. Vanden Bossche, J. Symoens, J. Brugmans and R. De Coster, *J. Clin. Endocrinol. Metab.* **57**, 732 (1983).
7. R. De Coster, *7th International Congress of Endocrinology*, Quebec, July (1984) abstract No. 001517.
8. H. Vanden Bossche, W. Lauwers, G. Willemsens and W. Cools, in *Proceedings of the 6th International Symposium on Microsomes and Drug Oxidations*, edited by A. R. Boobis, J. Caldwell, F. de Matteis and C. R. Elcombe. Taylor & Francis, London, in press.
9. C. J. W. Brooks and D. J. Harvey, *J. Chromatogr.* **54**, 193 (1971).
10. J. Gaskell and C. J. W. Brooks, *J. Chromatogr.* **158**, 331 (1978).
11. C. J. W. Brooks. B. S. Middleditch and D. J. Harvey, *Org. Mass Spectrom.* **5**, 1429 (1971).
12. B. Wilcox and L. L. Engel, *Steroids* **5**, Suppl. I, 49 (1965).
13. Library of the National Bureau of Standards, spectrum No. 24052.
14. C. Djerassi, *Pure Appl. Chem.* **50**, 171 (1978).
15. S. Hammerum and C. Djerassi, *Steroids* **25**, 817 (1975).
16. R. H. Shapiro and C, Djerassi, *J. Am. Chem. Soc.* **86**, 2825 (1964).
17. F. U. Crostic and K. L. Rinehart, *J. Org. Chem.* **33**, 1740 (1968).
18. G. M. Anthony, C. J. W. Brooks, J. Maclean and I. Sangster, *J. Chromatogr. Sci.* **7**, 623 (1969).
19. Z. V. Zaretskii, *Mass Spectrometry of Steroids*, p. 75. Wiley, New York (1976).

Received 25 September 1984; accepted 8 February 1985