*J. Pharm. Pharmac.*, 1973, **25**, 143–149 *Received July* 24, 1972

# Mass spectrometry in the analysis of steroid drugs and their metabolites: electron-impact-induced fragmentation of ring D

B. S. MIDDLEDITCH,* PAUL VOUROS* AND C. J. W. BROOKS**

* *Institute for Lipid Research, Baylor College of Medicine, Houston, Texas 77025, U.S.A.* ** *Chemistry Department, University of Glasgow, Glasgow G12 8QQ, U.K.*

Certain steroid drugs, their metabolites, and related model compounds which possess a 17-hydroxy group can be converted to trimethylsilyl ethers which give characteristic ions of type $[128 + R]^+$ in their mass spectra, where R is the mass of the other 17-substituent. Extensive deuterium labelling studies are carried out to verify the modes of formation of these and related ions which are of diagnostic value in analytical work.

The technique of combined gas chromatography-mass spectrometry (GC-MS) provides a powerful method of identifying synthetic drugs and their metabolites. Recent examples of its application include studies of barbiturates (Bonnichsen, Maehly & others, 1970a; Gilbert, Millard & Powell, 1970; Law, Aandahl & others, 1971), sympathomimetic amines (Bonnichsen, Maehly & others, 1970b; Brandenberger, 1970), catecholamines and related β-hydroxyamines (Horning, Moss & others, 1968b; Anthony, Brooks & Middleditch, 1970), and synthetic steroid drugs (Brooks & Middleditch, 1971).

The use of GC-MS in the characterization of steroids is well documented (Horning, Brooks & VandenHeuvel, 1968a; Brooks & Middleditch, 1973). Hydroxylic steroids are frequently examined as their derived trimethylsilyl (TMS) ethers, since these derivatives confer upon the molecule greater thermal stability, an increase in volatility, a decrease in polarity, and useful mass spectrometric properties. The TMS group often directs the electron-impact-induced fragmentation of a molecule in a manner characteristic of structural features of the molecule. The most fully substantiated example is that of the formation of ions of *m/e* 129 and $[M-129]^+$ in the spectra of $\Delta^5$-3β-trimethylsilyloxy steroids as shown in Fig. 1a (Eneroth, Hellström & Ryhage, 1964; Brooks, Chambaz & others, 1966; Diekman & Djerassi, 1967). [The fragmen-

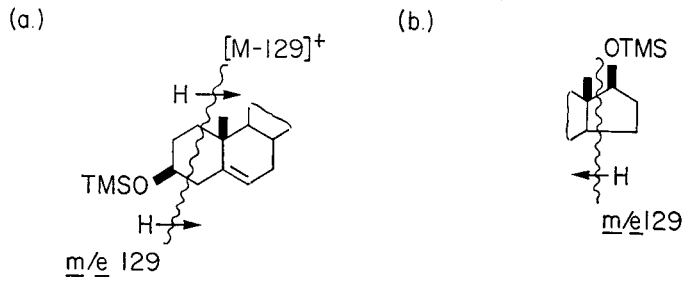


FIG. 1. (a) Formation of ions of *m/e* 129 and $[M-129]^+$ in the spectra of $\Delta^5$-3β-trimethylsilyloxy steroids. (b) Formation of the ion of *m/e* 129 in the spectra of 17β-trimethylsilyloxy steroids.

tation of the $\Delta^4$-isomers is different (Brooks, 1967; Anthony & Brooks, 1968) and of unestablished mechanism.] Ions of *m/e* 129 have been observed in the spectra of the derived TMS ethers of testosterone and analogous steroids with a 17$\beta$-hydroxyl group (Sjövall & Vihko, 1966; Diekman & Djerassi, 1967; Anthony & Brooks, 1968), as shown in Fig. 1b. No significant ion at $[M-129]^+$ was observed in the spectra of these compounds (*cf.* Brooks, Horning & Young, 1968). Moreover, we have found by examining the spectra of a large number of TMS ethers of 17$\alpha$-substituted 17$\beta$-hydroxysteroids, that ions of *m/e* (128 + R), where R is the mass of the 17$\alpha$-substituent (hydrogen, methyl, ethyl, allyl), are invariably formed from these compounds (Brooks, Thawley & others, 1971; Brooks & Middleditch, 1973). The specificity of this mode of fragmentation is well illustrated in Fig. 2. The ion of

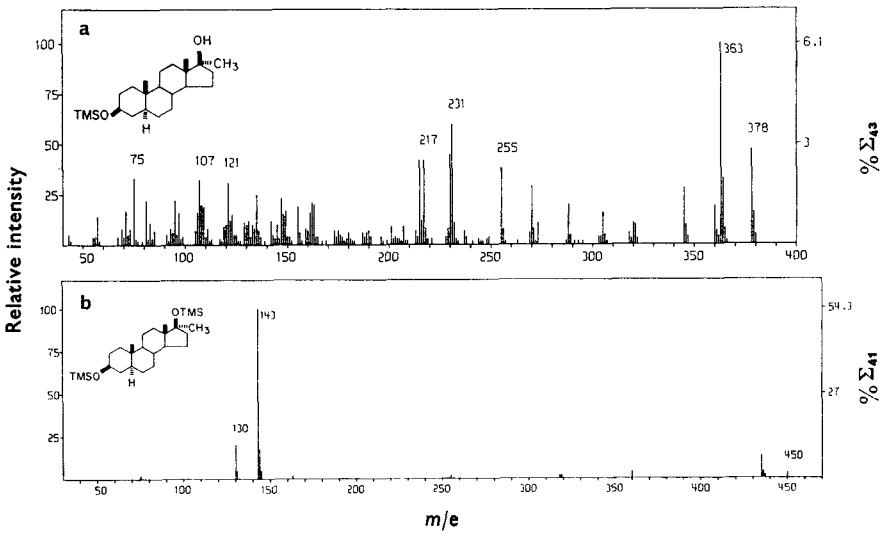


FIG. 2. Mass spectra (22·5 eV) of mono and di TMS ethers of 17$\alpha$-methyl-5$\alpha$-androstane-3$\beta$,17$\beta$-diol.

*m/e* 143 accounts for more than 50% of the total ion current of the spectrum of fully trimethylsilylated 17$\alpha$-methyl-5$\alpha$-androstane-3$\beta$,17$\beta$-diol, whereas fragmentation of the mono-(3-) TMS ether is more random in nature.

The practical significance of these observations is that it permits one to infer the presence of structural features in steroid drug metabolites for which no reference compounds are available. One can conclude that $\Delta^5$-3$\beta$-[and 3$\alpha$-] trimethylsilyloxy steroids give rise to ions of *m/e* 129 and $[M-129]^+$ or corresponding ions bearing substituents on C-1 to C-3, whereas 17$\beta$-trimethylsilyloxy steroids provide an ion of *m/e* 129, but none of type $[M-129]^+$ (or corresponding ions bearing substituents on C-15 to C-17). We have now performed extensive deuterium labelling studies to substantiate the validity of this potentially useful conclusion.

## METHODS

Mass spectra were obtained using LKB 9000 instruments fitted with 10 ft × 0·25 inch glass columns packed with 1% OV-1 or OV-17 on Gas Chrom Q (100 to 120 mesh) under the following conditions: molecular separator, 250°; ion source, 270°; electron energy, 22·5 or 70 eV; accelerating voltage, 3·5 kV.

TMS ethers were prepared by dissolving the steroids in bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (10:1 ratio) and heating at 100° overnight.

Perdeuteriotrimethylsilyl ($d_9$-TMS) ethers were prepared using $d_{18}$-*N*,*O*-bis(trimethylsilyl)acetamide (McCloskey, Stillwell & Lawson, 1968).

$d_1$-Sterols were prepared by reduction of the appropriate keto steroids with lithium aluminium deuteride.

$d_2$- and $d_4$-Sterols were prepared by reduction of the appropriate $d_2$- and $d_4$-keto steroids with lithium aluminium hydride: the $d_2$ and $d_4$-keto steroids were prepared from 17- and 16-keto steroids by exchange of α-hydrogen atoms in a mixture of $D_2O$, dioxan, and NaOD.

## RESULTS AND DISCUSSION

Diekman & Djerassi (1967) found that the ion of *m/e* 129 in the spectrum of the TMS ether of 5α-androstan-17β-ol was of low abundance, and showed that it had the composition $C_6H_{13}OSi$, suggesting that it comprised C-15 to C-17 with all but one of the attached hydrogen atoms. We have found, however, that the ions of *m/e* 129 are base peaks of the TMS ethers of 5α-androstan-17β-ol and 5β-androstan-17β-ol (Table 1). This discrepancy is apparently due to instrumental differences. That

Table 1. *Relative abundances of ions of* m/e 129 *in 70 eV spectra of TMS ethers of various steroids and of the corresponding ions in the spectra of deuterium-labelled analogues.* (Peaks mainly due to naturally-occurring heavy isotopes in parentheses.)

| *m/e* | 129 | 130 | 131 | 138 |
|---|---|---|---|---|
| 5α-Androstan-17β-ol TMS | 100 | (35) | (10) | 0 |
| $d_9$-TMS | 0 | 0 | 2 | 100 |
| 17-$d_1$ | 0 | 100 | (37) | 0 |
| 16,16-$d_2$ | 0 | 100 | (33) | 4 |
| 5β-Androstan-17β-ol TMS | 100 | (35) | (10) | 0 |
| $d_9$-TMS | 0 | 0 | 0 | 100 |
| 5α-Androstane-3α,17β-diol di TMS | 100 | (35) | (15) | 0 |
| $d_{18}$-di TMS | 0 | 0 | 5 | 100 |
| 17-$d_1$ | 15 | 100 | (35) | 0 |
| 16,16-$d_2$ | 15 | 100 | (30) | 0 |
| 5α-Androstane-3β,16α-diol di TMS | 18 | 10 | 19 | 0 |

these ions contain an intact TMS group was confirmed by the presence of analogous ions of *m/e* 138 in the spectra of the $d_9$-TMS ethers. The corresponding 17-$d_1$, and 16,16-$d_2$ derivatives of 5α-androstan-17β-ol both gave spectra with base peaks at *m/e* 130 (Table 1), confirming the suggestion by Diekman & Djerassi (1967) that only one of the hydrogen atoms at C-16 is retained in these ions, as illustrated in Fig. 3.

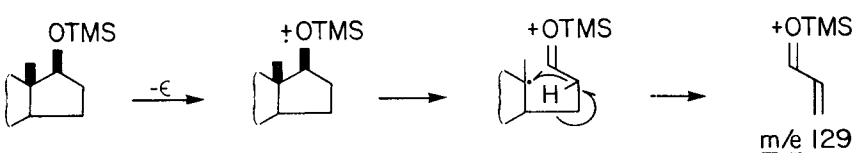


FIG. 3. Postulated mechanism of formation of the ions of *m/e* 129 in the spectra of the TMS ethers of 5α- and 5β-androstan-17β-ol.

Table 2. *Relative abundances of ions of* m/e *215 and 217 in 70 eV spectra of TMS ethers of various steroids and of the corresponding ions in the spectra of deuterium-labelled analogues.* (Peaks mainly due to naturally occurring heavy isotopes in parentheses.)

| *m/e* | 215 | 217 | 218 | 221 |
|---|---|---|---|---|
| 5α-Androstan-17β-ol TMS .. .. .. | 6 | 36 | (8) | 0 |
| $d_9$-TMS .. .. .. .. .. | 9 | 52 | (11) | 1 |
| 17-$d_1$ .. .. .. .. .. | 7 | 33 | (9) | 0 |
| 16,16-$d_2$ .. .. .. .. .. | 10 | 52 | (18) | 0 |
| 5β-Androstan-17β-ol TMS .. .. .. | 8 | 45 | (10) | 0 |
| $d_9$-TMS .. .. .. .. .. | 10 | 42 | (10) | 0 |
| 5α-Androstane-3α,17β-diol di TMS .. .. | 52 | 38 | (6) | 0 |
| $d_{18}$-di TMS .. .. .. .. .. | 66 | 51 | (11) | 0 |
| 17-$d_1$ .. .. .. .. .. | 41 | 29 | (11) | 8 |
| 16,16-$d_2$ .. .. .. .. .. | 40 | (12) | 18 | 0 |
| 5α-Androstane-3β,16α-diol di TMS .. .. | 21 | 32 | (6) | 1 |
| $d_{18}$-di TMS .. .. .. .. .. | 15 | 18 | (4) | 0 |
| 16-$d_1$ .. .. .. .. .. | 12 | (3) | 18 | 1 |
| 15,15,17,17-$d_4$ .. .. .. .. .. | 28 | (7) | (4) | 35 |

There is no significant ion of type $[M-129]^+$ in the spectra of these compounds. It should be noted that the spectra of the androstan-17β-ols, their TMS ethers, and the deuterated analogues of their TMS ethers all contain relatively intense ions (Table 2) of *m/e* 217 which are apparently formed by elimination of C-15 to C-17 with substituents and an additional hydrogen atom. This hydrogen atom may originate from C-8, by analogy with similar ions observed in the spectra of 17-keto steroids (Tökés, LaLonde & Djerassi, 1967).

Ions of *m/e* 129 in the spectra of TMS ethers of androstane-3,17-diols may originate from either ring A or ring D (Sjövall & Vihko, 1966). Examination of the spectra of the TMS ethers of 5α-androstane-3α,17β-diol and its 17-$d_1$ and 16,16-$d_2$ analogues, however, showed that more than 85% of the ions of *m/e* 129 originated from ring D (Table 1). This confirms that a $\Delta^5$ bond is required to direct strongly this fragmentation of ring A (Horning & others, 1968a). Also, the ions of *m/e* 215 and *m/e* 217 were both found to arise by cleavage of ring D. The former ion was apparently formed by elimination of C-15 to C-17 (together with substituents and an additional hydrogen atom) from the ion $[M-90]^{+\bullet}$ arising by elimination of trimethylsilanol from ring A. The ion of *m/e* 217 was found to be formed *via* similar loss of trimethylsilanol from ring A and cleavage of ring D, but with transfer of a hydrogen atom from C-16 to the charge-retaining fragment. No ions were observed in the spectrum of the di-TMS ether of 5α-androstane-3α,17β-diol at *m/e* 307 or *m/e* 305 which would correspond to $[M-129]^+$ or $[M-131]^+$ formed by D-ring cleavage of the molecular ion.

The ion of *m/e* 129 was of low relative abundance in the spectrum of the di-TMS ether of 5α-androstane-3β,16α-diol. Moreover, deuterium labelling studies showed that the ion of *m/e* 215 $[M-90, 131]^+$ arose *via* cleavage of the D-ring, whereas the ion of *m/e* 217 $[M-90, 129]^+$ was formed by fragmentation of ring A (Table 2). In this case, an ion of type $[M-131]^+$ was observed at *m/e* 305 (14%), but none of type $[M-129]^+$ was seen.

The mass spectrum of the TMS ether of 17α-methyl-5β-androst-3-en-17β-ol (Fig. 4A) contained an intense ion of *m/e* 143. The spectra of the TMS ethers of estr-4-en-17β-ol and its 17α-ethyl and allyl analogues exhibited abundant ions of *m/e* 129, 157 and

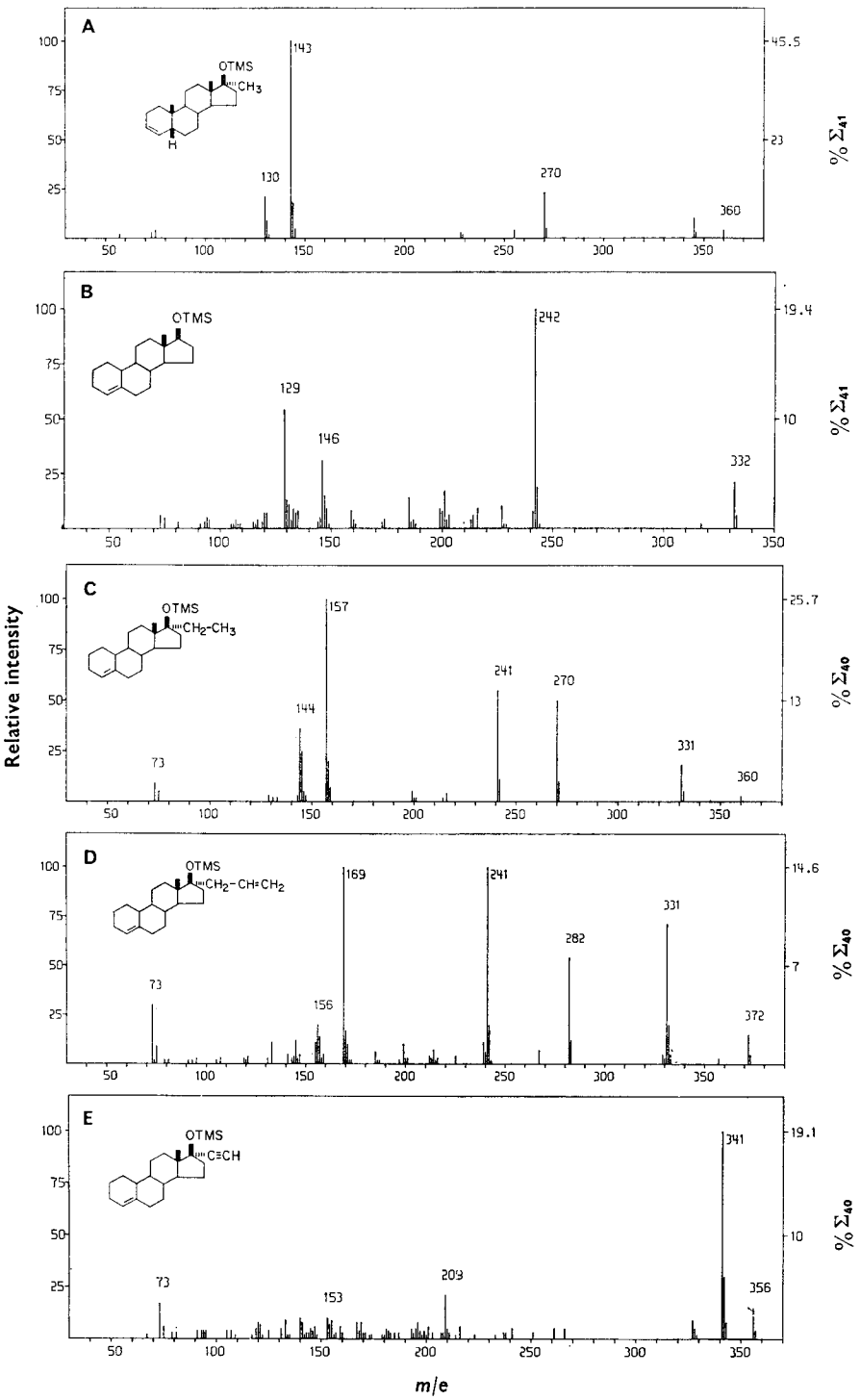


FIG. 4. Mass spectra (22·5 eV) of TMS ethers of (A) 17α-methyl-5β-androst-3-en-17β-ol, (B) estr-4-en-17β-ol, (C) 17α-ethylestr-4-en-17β-ol, (D) of 17α-allylestr-4-en-17β-ol, and (E) 17α-ethynylestr-4-en-17β-ol.

169, respectively (Figs. 4 B-D). These ions are unmistakably due to fragmentations of the D rings. None of the spectra of these compounds contained any significant ions of the type $[M-(128 + R)]^+$ at *m/e* 203 (where R is the mass of the 17α-substituent), and only the spectrum of the TMS ether of estr-4-en-17β-ol contained an ion of type $[M-(130 + R)]^+$ at *m/e* 201. The mass spectrum of the TMS ether of 17α-ethynylestr-4-en-17β-ol (Fig. 4E) did not contain a strong ion of type $[128 + R]^+$ at *m/e* 153. However, TMS ethers of 17α-ethynyl steroids contain characteristic fragment ions of type $[M-29]^+$, formation of which may be rationalized as in Fig. 5.

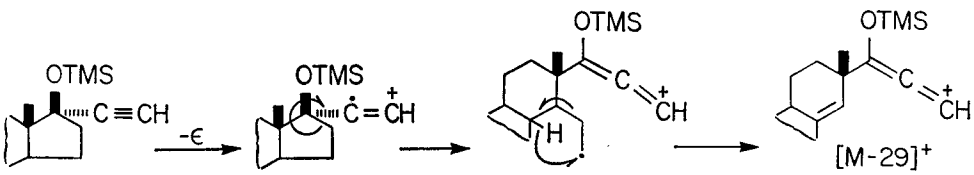


FIG. 5. Postulated mechanism of formation of the ions of type $[M-29]^+$ in the spectra of TMS ethers of 17α-ethynyl-17β-hydroxysteroids.

*Conclusion*

The results confirm the suggested origins of ions of type $[128 + R]^+$ in the mass spectra of TMS ethers of 17β-hydroxy steroids. Ions of this type have been found in spectra of a large number of such steroids, and their formation is apparently independent of the stereochemistry at C-17 (Macdonald, Sykes, & others, 1971).* Similar ions occur in the spectra of Δ⁵-3β-trimethylsilyloxy steroids, but these compounds are readily distinguishable from the 17-trimethylsilyloxy steroids since only the former (Horning & others, 1968a) gave rise also to ions of type $[M-(128 + R)]^+$. The spectra of 17-trimethylsilyloxy steroids with no other 17-substituent contain ions of type $[M-131]^+$.

These features serve to distinguish TMS ethers of 17-hydroxy steroids from those of Δ⁵-3β-hydroxy steroids. It is often necessary to make this distinction when attempting to identify derivatives of natural steroids, even though reference spectra of many such compounds are available. The occurrence of intense ions of type $[128 + R]^+$ in the spectra of TMS ethers of metabolites of 17α-alkyl-17β-hydroxy steroids in which this moiety is unchanged has facilitated the location of metabolites by selective ion monitoring during GC-MS (Brooks, Thawley & others, 1971; Lawson & Brooks, 1971). This characteristic serves to distinguish these synthetic drugs and many of their metabolites from natural steroids. It is also envisaged that steroids or their metabolites which possess a Δ⁵-3β-hydroxy grouping and which contain an alkyl group at C-1, C-2, or C-3 may be distinguished from the 17α-alkyl-17β-hydroxy steroids by virtue of the ions of type $[128 + R]^+$ *and* $[M-128, R]^+$ in the spectra of their TMS ethers.

*Acknowledgements.* This work was partially supported by grants from the MRC (to CJWB), the SRC (B/SR/2398 to CJWB and G. Eglinton), and the NIH (GM-13901 and GM-16216). We thank Dr. G. F. Woods (Organon Laboratories Ltd.) for gifts of steroids.

* TMS ethers of 17α-ethynyl-17β-hydroxy steroids give rise to spectra containing ions of type $[128 + R]^+$ at *m/e* 153 in lower abundance, but these spectra contain characteristic intense fragment ions of type $[M-29]^+$.

REFERENCES

ANTHONY, G. M. & BROOKS, C. J. W. (1968). In C. Cassano *et al.*, *Research on Steroids*, Vol. III, pp. 131–141. Amsterdam: North Holland Publishing Co.

ANTHONY, G. M., BROOKS, C. J. W. & MIDDLEDITCH, B. S. (1970). *J. Pharm. Pharmac.*, **22**, 205–213.

BONNICHSEN, R., MAEHLY, A. C., MÅRDE, Y., RYHAGE, R. & SCHUBERT, B. (1970a). *Zacchia*, **6**, 1–15.

BONNICHSEN, R., MAEHLY, A. C., MÅRDE, Y., RYHAGE, R. & SCHUBERT, B. (1970b). *Z. Rechtsmedizin*, **67**, 19–26.

BRANDENBERGER, H. (1970). *Pharm. Acta Helv.*, **45**, 394–413.

BROOKS, C. J. W. (1967). *Process Biochem.*, (2) 27–29.

BROOKS, C. J. W., CHAMBAZ, E. M., GARDINER, W. L. & HORNING, E. C. (1966). *Excerpta med.*, **132**, 366–372.

BROOKS, C. J. W., HORNING, E. C. & YOUNG, J. S. (1968). *Lipids*, **3**, 391–402.

BROOKS, C. J. W., THAWLEY, A. R., ROCHER, P., MIDDLEDITCH, B. S., ANTHONY, G. M. & STILLWELL, W. G. (1971). *J. Chromatog. Sci.*, **9**, 35–43.

BROOKS, C. J. W. & MIDDLEDITCH, B. S. (1971). *Clinica chim. Acta*, **34**, 145–157.

BROOKS, C. J. W. & MIDDLEDITCH, B. S. (1973). *In* E. Heftmann, *Modern Methods of Steroid Analysis*. New York: Academic Press, in press.

DIEKMAN, J. & DJERASSI, C. (1967). *J. org. Chem.*, **32**, 1005–1012.

ENEROTH, P., HELLSTRÖM, K. & RYHAGE, R. (1964). *J. Lipid Res.*, **5**, 245–255.

GILBERT, J. N. T., MILLARD, B. J. & POWELL, J. W. (1970). *J. Pharm. Pharmac.*, **22**, 897–901.

HORNING, E. C., BROOKS, C. J. W. & VANDENHEUVEL, W. J. A. (1968a). *Adv. Lipid Research*, **6**, 273–392.

HORNING, M. G., MOSS, A. M., BOUCHER, E. A. & HORNING, E. C. (1968b). *Analyt. Letters*, **1**, 311–321.

LAW, N. C., AANDAHL, V., FALES, H. M. & MILNE, G. W. A. (1971). *Clinica chim. Acta*, **32**, 221–228.

LAWSON, A. M. & BROOKS, C. J. W. (1971). *Biochem. J.*, **123**, 25–26P.

MCCLOSKEY, J. A., STILLWELL, R. N. & LAWSON, A. M. (1968). *Analyt. Chem.*, **40**, 233–236.

MACDONALD, B. S., SYKES, P. J., ADHIKARY, P. M. & HARKNESS, R. A. (1971). *Steroids*, **18**, 753–766.

SJÖVALL, J. & VIHKO, R. (1966). *Steroids*, **7**, 447–457.

TÖKÉS, L., LALONDE, R. T. & DJERASSI, C. (1967). *J. org. Chem.*, **32**, 1012–1019.