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STRUCTURAL ANALYSIS OF STEROIDS BY ASSOCIATION OF THIN-LAYER CHROMATOGRAPHY TECHNIQUES WITH GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

The association of thin-layer chromatographic techniques with gas chromatography–mass spectrometry was employed for the separation and identification of steroids. The application of this method in two different situations has been discussed for steroids with similar polarities by both thin-layer and gas chromatography, but with different mass spectrometric fragmentation, and for steroids with similar pattern of mass fragmentation but different chromatographic mobilities.

INTRODUCTION

Thin-layer chromatography (TLC) is one of the fractionation methods most employed for the resolution of compounds of similar polarity. Usually the identification of the substances separated on the layers can be achieved by using specific colour reactions or by the formation of derivatives prior, during or after the chromatography[1,2].

The characterisation of compounds in microgram amounts has been improved in the last years by the combination of the separation capacity of an adsorbent sheet with the high sensitivity of gas–liquid chromatography (GLC). These chromatographic procedures have been associated in two different ways.

The first was described independently by several groups of investigators—CASU AND CAVALLOTTI[3], NIGAM *et al.*[4], JANÁK[5] and KAISER[6]—for the further separation of a group of compounds which remains unresolved on GLC. By the technique developed by CURTIUS AND MÜLLER[7], the materials eluted from the GLC columns are directly adsorbed at the starting line of a thin-layer plate. Trimethylsilyl ether (TMSi ether) derivatives usually employed in GLC are poorly resolved by TLC. Therefore in this technique[7] they are cleaved directly on the adsorbent by treating the starting line with a methanolic hydrochloric acid solution.

In the second way GLC is employed for the identification of compounds after previous separation by TLC. First applied by IKEDA and co-workers[8,9] in the analysis

of essential oils, this procedure was advantageously used in analytical methods for steroid determination (*cf.* ref. 10) and in recent studies on the metabolism of steroids by tissue preparations[11–15].

The retention behaviour of an unknown material in GLC—even on two different phases—is not necessarily a proof of identity for an isolated material of biological origin. A definite characterisation of an isolated material requires an analysis of its structure, for instance, by means of mass spectrometry, as in the metabolic studies referred to above.

The purpose of this communication is to show some examples in which the combination of TLC and GC–MS is indispensable for the complete identification of steroids.

MATERIALS AND METHODS

The material and methods employed in this investigation have been described in detail elsewhere. Ascending TLC was carried out on Silica Gel G (Merck) or boric acid-treated Silica Gel G layers according to the specifications of LISBOA and co-workers[1,2,16]. GC–MS analyses were carried out as described previously[13], using an LKB 9000 gas chromatograph–mass spectrometer (LKB-Beckman Produkter AB, Stockholm, Sweden).

The steroids used in this investigation were kindly supplied by Dr. O. A. DE BRUIN (6-dehydro-progesterone = pregna-4,6-diene-3,20-dione; 16-dehydro-progesterone = pregna-4,16-diene-3,20-dione), Prof. E. DICZFALUSY (retro-androstenedione = 9β,10α-androst-4-ene-3,17-dione; retro-progesterone = 9β,10α-pregn-4-ene-3,20-dione; 16-dehydro-retro-progesterone = 9β,10α-pregna-4,16-diene-3,20-dione), Dr. F. GALLETTI (5α-androstane-3β,16α,17β-triol; 5α-androstane-3β,16β,17β-triol), Dr. J. THIJSSEN (11-dehydro-progesterone = pregna-4,11-diene-3,20-dione), Dr. J. UFER (nor-testosterone = 17β-hydroxy-oestr-4-en-3-one; nor-androstenedione = oestr-4-ene-3,17-dione; testosterone = 17β-hydroxy-androst-4-en-3-one; androstenedione = androst-4-ene-3,17-dione; progesterone = pregn-4-ene-3,20-dione), and Prof. M. E. WOLFF (5α-androstane-2β,3α,17β-triol; 5α-androstane-2β,3β,17β-triol).

RESULTS AND DISCUSSIONS

Two different situations will be presented in which the association of TLC techniques with GC–MS is very useful for an unambiguous identification of steroids.

First steroids having a close similarity on TLC and/or GLC but having different mass spectrometric fragmentation will be considered. In this regard a mixture of steroids differing only by the presence of one double bond or an angular methyl group, or by the configuration at positions C-9/C-10, are sometimes very difficult to resolve both by TLC and by paper chromatography.

Progesterone has chromatographic properties similar to its 6-, 11- and 16-dehydro derivatives on silica gel layers. The separation of progesterone from 6- and 11-dehydro-progesterones (but not from 16-dehydro-progesterone) can only be achieved on silica gel layers after impregnation with silver nitrate[17] (Table I). The identification of progesterone and 16-dehydro-progesterone can be achieved by GC–MS (Fig. 1), using an SE-30 phase (t_R related to cholestane: 0.86 and 0.77, respectively).

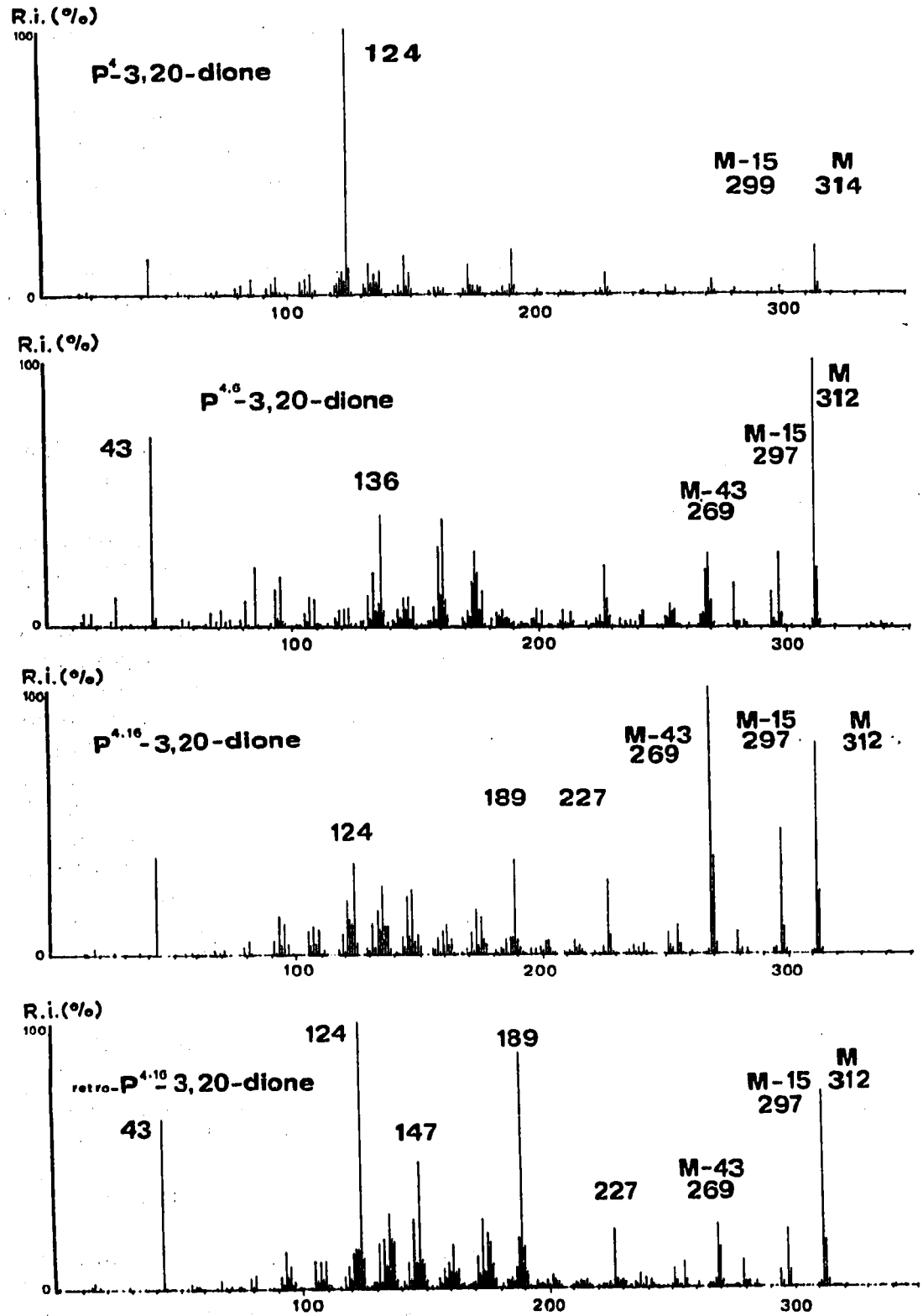

Fig. 1. Mass spectra of progesterone, 6-dehydro-progesterone, 16-dehydro-progesterone and 16 dehydro-retro-progesterone. The analysis was carried out with the LKB 9000 GC–MS using a 1% SE-30 column (238°). Temperatures of the molecule separator and the ion source: 250° and 290°, respectively. Energy of the bombarding electrons: 22.5 eV; ionising current: 60 μA. R.i. = relative intensity.

TABLE I

hR_F VALUES OBTAINED FOR PROGESTERONE AND ITS 6-DEHYDRO, 11-DEHYDRO AND 16-DEHYDRO DERIVATIVES BY ASCENDING TLC

Adsorbents: (a) Silica Gel G (Merck); (b) Silica Gel G + silver nitrate. Solvent systems: (I) cyclohexane–ethyl acetate (50:50); (II) *n*-hexane–ethyl acetate (75:25); (III) benzene–ethyl acetate (50:50).

P^4-*dione*	*Solvent systems*			
	Ia	*Ib*	*IIa*	*IIIb*
P^4-3,20	38	39	12	46
$P^{4,6}$-3,20	34	35	10	40
$P^{4,11}$-3,20	39	28	15	33
$P^{4,16}$-3,20	38	40	14	46

Steroids differing by the presence of a 19-angular methyl group, *e.g.* testosterone/nor-testosterone and androstenedione/nor-androstenedione, are difficult to separate by TLC (Table II). The complete identification of such steroids can be achieved by means of GC–MS. Fig. 2 shows the differences between mass spectra of androstenedione and nor-androstenedione (t_R related to cholestane on SE-30 columns: 0.56 and 0.48, respectively).

As shown in Table III, steroids differing in the configuration at positions C-9/C-10 are inseparable by TLC on silica gel under the conditions employed in this work. The studies of WULFSON and co-workers[18,19] and ZARETSKII *et al.*[20] have shown that aromatic steroids with 9α/9β-stereoisomerism[19], 8β,9α,10β-, 8α,9α,10β- and 8β,9β,10α-isomers of nor-testosterone[18] as well as Δ^4-3-oxo-steroids of both C_{19}- and C_{21}-series with 9α,10β/9β,10α-configuration[20] have different mass spectra. These differences should be useful for establishment of the stereoconfiguration at C-9 and C-10. It must be pointed out that the differences observed by ZARETSKII *et al.*[20] by comparing steroids of the 9β,10α- and 9α,10β-series were only quantitative. These authors have indicated that the peak intensity of the ions at *m/e* 124, 147 and 191 were considerably greater in the spectra of iso-progesterone (9β,10α-progesterone, retro-progesterone) than in that of the corresponding steroid with 9α,10β-structure. As can be seen in Fig. 1, the same can be concluded for the ions at *m/e* 124, 147 and 189 for the isomeric

TABLE II

hR_F VALUES OBTAINED FOR ANDROSTENEDIONE, NOR-ANDROSTENEDIONE, TESTOSTERONE AND NOR-TESTOSTERONE BY ASCENDING TLC

Adsorbent: Silica Gel G (Merck). Solvent systems: (I) cyclohexane–ethyl acetate (50:50); (II) cyclohexane–ethyl acetate–ethanol (45:45:10); (III) chloroform–ethanol (95:5); (IV) benzene–ethanol (90:10).

	Solvent systems			
	I	*II*	*III*	*IV*
Androstenedione	29	53	65	55
Nor-androstenedione	26	51	61	52
Testosterone	23	48	43	39
Nor-testosterone	19	42	39	37

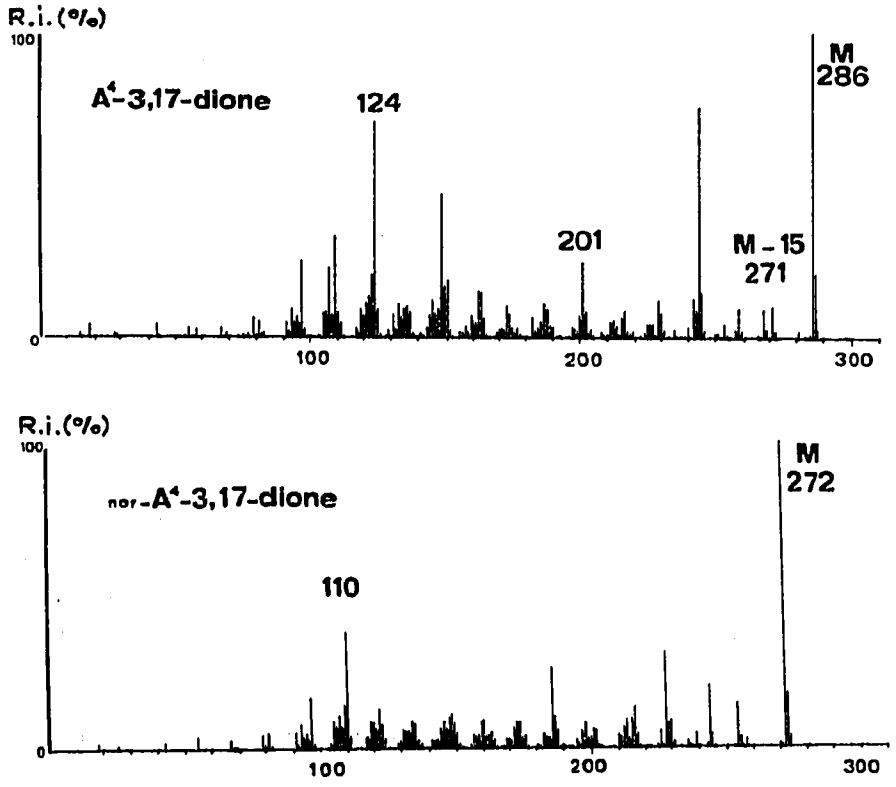

Fig. 2. Mass spectra of androstenedione and nor-androstenedione. For particulars, see legend to Fig. 1.

pair 16-dehydro-progesterone/16-dehydro-retro-progesterone (t_R related to cholestane on SE-30 phase: 0.77/0.83).

The second group of examples to be considered here is that of epimeric steroids. As pointed out in several reports[13,21,22], steroids presenting epimeric hydroxyl groups show very similar fragmentation patterns and their identification must be achieved by means of other physical constants. Two examples will be presented in which the combination of the chromatographic behaviour on TLC and GLC with the mass spectrometric data was necessary for the identification of epimeric steroids.

The tris(trimethylsilyl ether) derivative of the steroid pairs 5α-androstane-

TABLE III

hR_F VALUES OBTAINED FOR PROGESTERONE, ANDROSTENEDIONE, RETRO-PROGESTERONE AND RETRO-ANDROSTENEDIONE BY ASCENDING TLC

Adsorbent: Silica Gel G (Merck). Solvent systems: (I) cyclohexane–ethyl acetate–ethanol (45:45:10); (II) cyclohexane–ethyl acetate (50:50); (III) chloroform–ethanol (90:10); (IV) ethyl acetate–*n*-hexane–glacial acetic acid (75:20:5).

	Solvent systems			
	I	*II*	*III*	*IV*
Progesterone	51	39	62	57
9β,10α-Progesterone	51	35	63	55
Androstenedione	49	32	62	55
9β,10α-Androstenedione	49	31	61	55

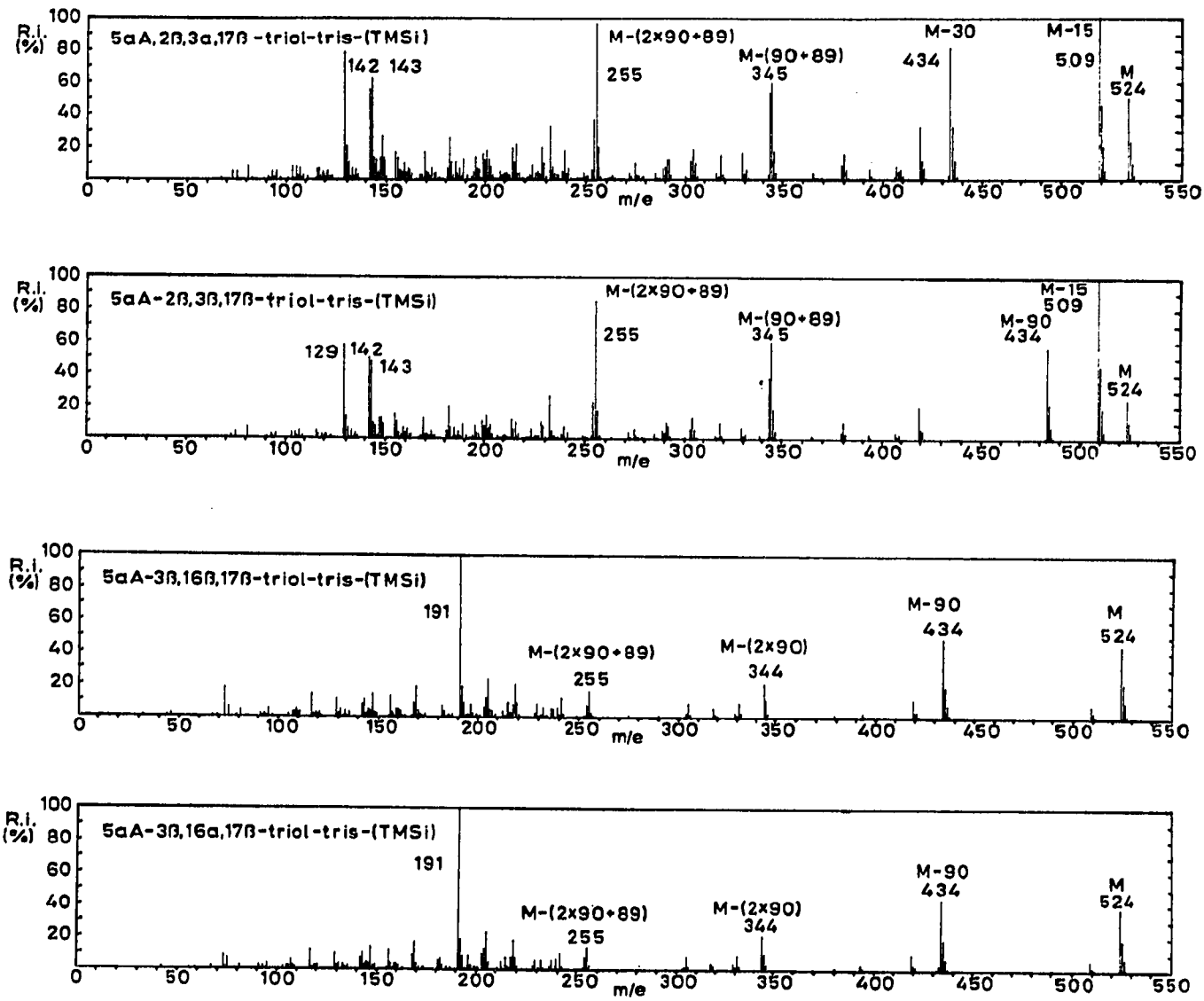

Fig. 3. Mass spectra of the tris(trimethylsilyl ether) derivatives of 5α-androstane-2β,3α,17β-triol, 5α-androstane-2β,3β,17β-triol, 5α-androstane-3β,16β,17β-triol and 5α-androstane-3β,16α,17β-triol. For particulars, see legend to Fig. 1.

TABLE IV

hR_S (S = TESTOSTERONE) VALUES OBTAINED FOR FOUR ISOMERIC 5α-ANDROSTANETRIOLS BY ASCENDING TLC

Adsorbent: Silica Gel G (Merck). Solvent systems: (I) ethyl acetate–*n*-hexane–glacial acetic acid (75:20:5); (II) ethyl acetate–*n*-hexane–glacial acetic acid–ethanol (72:13.5:10:4.5); (III) cyclohexane–ethyl acetate–ethanol (45:45:10); (IV) chloroform–ethanol (90:10).

5α-Androstanetriols	*Solvent systems*			
	I	*II*	*III*	*IV*
2β, 3α, 17β	53	80	55	31
2β, 3β, 17β	66	83	68	54
3β, 16α, 17β	44	74	45	36
3β, 16β, 17β	77	92	85	67

3β,16β,17β/3β,16α,17β-triols and 5α-androstane-2β,3α,17β/2β,3β,17β-triols each have similar pattern fragmentation (Fig. 3). In each group the *cis* epimer is much less polar than the *trans* in all the solvent systems employed (Table IV). An improvement of their separation can be accomplished by TLC on boric acid-impregnated silica gel layers[23]. With this technique, also used for further characterisation of steroid glycols, the compound with a *cis* configuration forms much less polar borate complexes[24] whereas the *trans* epimer remains unchanged.

In these two examples MS is mainly useful to prove that the GLC peaks detected are of a steroidal nature and that they are not due to product degradation or contaminants.

Finally, it is important to mention that the formation of steroid methoximes[25,26] and dinitrophenylhydrazones[27] often gives two geometrical isomers with an *anti* and *syn* configuration. Studies of the TMSi ethers of steroid methoximes[14,26,28,29] formed with 3-(5α-H)-, 3-(Δ^4)- and 16-oxo groups showed that the geometrical isomers had different retention behaviour on some GLC phases (*e.g.* OV-17, SE-30, QF-1) but the same pattern of mass spectrometric fragmentation. Since *anti* and *syn* isomers of both dinitrophenylhydrazones[27] and TMSi ether methoximes[25,26] can be well separated on TLC, it is useful to combine this technique with GC–MS when these derivatives are used in structural studies of steroids.

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