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# FORMATION OF PENTAFLUOROPHENYLDIMETHYLSILYL ETHERS AND THEIR USE IN THE GAS CHROMATOGRAPHIC ANALYSIS OF STEROLS

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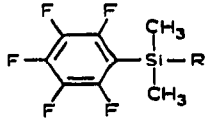
## SUMMARY

The pentafluorophenyldimethylsilyl group is an excellent protecting group for steroid alcohols, giving volatile ethers, detectable at picogram levels in gas chromatography with an electron capture detector. By the use of five reagents, increasingly hindered hydroxyl groups can be protected, so that structural information about unidentified sterols can be obtained. Quantitative derivative formation for a wide range of hydroxyl groups is possible by selection of the correct reagent combination, without affecting unprotected enolizable ketones.

## INTRODUCTION

We have already described three fluorocarbonsilyl ether groups as suitable for making volatile derivatives of sterols for gas chromatography (GC)[1]. The one giving the best combination of volatility and sensitivity to electron capture detection (ECD) was the pentafluorophenyldimethylsilyl ether, for which we suggest the abbreviated name flophemesyl (I). We have now investigated further the preparation of a number of derivatives of the flophemesyl group and studied their reactivity towards sterol hydroxyl groups.




I

The strongly electronegative pentafluorophenyl group alters reactivity at the silicon atom, so that silylating reagents could not be prepared by methods used for simpler compounds such as trimethylsilylimidazole. New methods have therefore been found for preparing a number of reagents containing the flophemesyl group, of these, the flophemesyl chloride, amine, diethylamine, and imidazole and also 1,3-bis(pentafluorophenyl)-1,1,3,3-tetramethyldisilazane are useful reagents for silylating sterol hydroxy groups.

This study was initiated by the need for volatile derivatives of sterols that could be determined at picogram levels by gas chromatography, particularly the polyhydroxy sterols, such as the insect moulting hormones ecdysone and 20-hydroxyecdysone. We have already shown that the currently used heptafluorobutyryl group is unstable in at least one case[2] (*i.e.*, heptafluorobutyryl cholesterol).

## EXPERIMENTAL

The preparations of flophemesylamine (I, R = $NH_2$), flophemesyl chloride (I, R = Cl) and 1,3-bis(pentafluorophenyl)-1,1,3,3-tetramethyldisilazane were outlined in an earlier paper[1].

Flophemesylimidazole (I, R = $C_3H_4N_2$) was prepared in two stages. To dichloromethyldimethylchlorosilane (Pierce and Warriner, Chester, Great Britain) in dry diethyl ether was added slowly a solution of pentafluorophenyllithium[3] in diethyl ether at −70°. The mixture was allowed to reach room temperature and filtered. Removal of solvent and distillation of the residue gave dichloromethylpentafluorophenyldimethylsilane (I, R = $CHCl_2$, yield 45%). This product was dissolved in dry dimethylformamide, and cooled to −10° under nitrogen. Dropwise addition of a solution of imidazolyllithium in dimethylformamide, followed by filtration and distillation, gave flophemesylimidazole (yield 30% by GC).

Flophemesyldiethylamine (I, R = $N(C_2H_5)_2$) was prepared by addition of a solution of flophemesyl chloride in hexane to a two-molar excess of dry, redistilled diethylamine, dissolved in hexane and cooled to −70° under nitrogen. The mixture

TABLE I

PHYSICAL AND SPECTRAL DATA FOR FLOPHEMESYL REAGENTS

| *Compound* | *B.p. (°C/mm Hg)* | *GC elution temperature (°C)** | *NMR shift (δ)*** | | *IR frequency ($cm^{-1}$)**** | | |
|---|---|---|---|---|---|---|---|
| | | | *$SiCH_3$* | *Other* | $\nu_{Si-C_6F_5}$ | $\nu_{N-H}$ | $\nu_{Si-N-Si}$ |
| $C_6F_5Si(CH_3)_2$–H | 144/575 | 83 | 0.46[d] | Si–H 4.64[dd] | 1641 | †† | |
| $C_6F_5Si(CH_3)_2$–Cl | 96/30 | 109 | 0.92 | | 1644 | | |
| $C_6F_5Si(CH_3)_2$–Br | 79/50 | | 0.99 | | 1640 | | |
| $C_6F_5Si(CH_3)_2$–$CH_2Cl$ | 110/72 | 134 | 0.55 | $CH_2X$ 3.05 | | | |
| $C_6F_5Si(CH_3)_2$–$CHCl_2$ | 122/50 | 145 | 0.67[s] | $CHX_2$ 5.60 | | | |
| | | | | | | 3485 | |
| $C_6F_5Si(CH_3)_2$–$NH_2$ | 52/6 | | 0.57 | | 1640 | 3410 | |
| | | | | | | | 1180 |
| $C_6F_5Si(CH_3)_2$–$N(C_2H_5)_2$ | 81/10 | 127 | 0.66 | $C_2H_5$ {1.19[ddd] / 3.13[q]} | 1642 | | 930 |
| $[C_6F_5Si(CH_3)_2]_2$–NH | 151/125 | 131 | 0.60 | | 1630 | 3370 | |
| $[C_6F_5Si(CH_3)_2]_2$–O | — | 181 | 0.65 | | 1645 | | ††† |

* Flow-rate, 60 ml $min^{-1}$; programmed at 16° $min^{-1}$ from 60° to 210°.
** At 60 MHz, in ppm from TMS, taken on neat liquid with 5% benzene as internal standard.
*** Strong bands at 800 and 845 $cm^{-1}$ are diagnostic of disubstituted dimethylsilanes.
[s] Doublet.
[dd] Septet.
[ddd] Triplet.
[q] Quartet.
†† $\nu_{Si-H}$ is strong at 2170 $cm^{-1}$.
††† $\nu_{Si-O-Si}$ is in the region 1000–1100 $cm^{-1}$, unresolved from several C–F bands.

was allowed to reach room temperature, filtered, and the filtrate distilled under vacuum. A lower yield (32%) was obtained when the bromosilane was used in place of the chlorosilane (67%). The colourless product darkened on standing to a pale orange, with precipitation of some polymeric material but this did not impair its properties as a silylating reagent.

Spectral data and boiling points for some of the compounds prepared are given in Table I. The corresponding siloxane, produced as a by-product when moisture was not rigorously excluded, is also given in the table.

The sterols 2$\beta$,3$\beta$-dihydroxy-5$\alpha$-cholestane, 2$\beta$,3$\beta$-dihydroxy-5$\beta$-cholest-7-en-6-one, 2$\beta$,3$\beta$,14$\alpha$-trihydroxy-5$\beta$-cholest-7-en-6-one, 3$\beta$,5$\alpha$,6$\beta$-trihydroxycholestane and 3$\beta$,5$\alpha$-dihydroxycholestan-6-one were synthesized in our laboratory. Other sterols were purchased from Koch-Light (Colnbrook, Great Britain) and Sigma (St. Louis, Mo., U.S.A.).

For forming the sterol ethers, typically, flophemesylamine (20 $\mu$l) was added to the sterol (1–5 mg) in pyridine (20 $\mu$l, dried over barium oxide) at room temperature in a Reacti-vial (Pierce and Warriner) and allowed to stand for 15 min before GC using flame ionization detection (FID). For ECD the solvent was removed under vacuum and the residue redissolved in purified benzene or hexane.

Steroid methoximes were prepared by treating the appropriate keto-steroid in an anhydrous solvent, usually pyridine, with excess methoxyamine hydrochloride at room temperature overnight or at 65° for 3 h. Solvent was removed under vacuum and the residue partitioned between ethyl acetate and a solution of sodium chloride (10%) and hydrochloric acid (5%) in water. The ethyl acetate was washed with a solution of sodium chloride (10%) and sodium bicarbonate (5%) in water, dried over molecular sieves and evaporated to give the methoximes.

Gas–liquid chromatography (GLC) was carried out with a Pye Series 104 chromatograph fitted with FID and ECD. For ECD, a flow-rate of 60 ml $min^{-1}$ of nitrogen was used, a pulse width of 0.75 $\mu$sec, a pulse period of 50 $\mu$sec, a pulse height of 47–60 V, and a detector oven temperature of 300°, without using a purge or quench gas. Mass spectra were obtained with an Hitachi-Perkin Elmer RMU-6 spectrometer, trap current 60 $\mu$A, electron energy 80 eV, and accelerating voltage 1.8 kV. Combined GC–MS was performed with a Pye Series 104 chromatograph coupled through a Watson-Bieman separator, maintained at 300°, to the mass spectrometer. The carrier gas was helium at a flow-rate of 18 ml $min^{-1}$.

Typically, androst-4-ene-3,17-dione dimethoxime 11$\beta$-flophemesyl ether had a retention time of 4 min when chromatographed on a 54-cm column of 1% OV-101 on CQ at 216° with 60 ml $min^{-1}$ nitrogen; and pregn-4-ene-3,20-dione dimethoxime 17$\alpha$-flophemesyl ether had a retention time of 3.7 min at 230° on the same column with 60 ml $min^{-1}$ nitrogen.

## RESULTS AND DISCUSSION

Considerable difficulty was encountered in preparing flophemesylimidazole, but in the expectation that this would be a very active silylating reagent, it was pursued actively until a method involving the displacement of a dichloromethyl group from I (R = $CHCl_2$) by imidazolyllithium was found. However, comparison of the several silylating reagents containing the pentafluorophenyl group showed that the reactivity

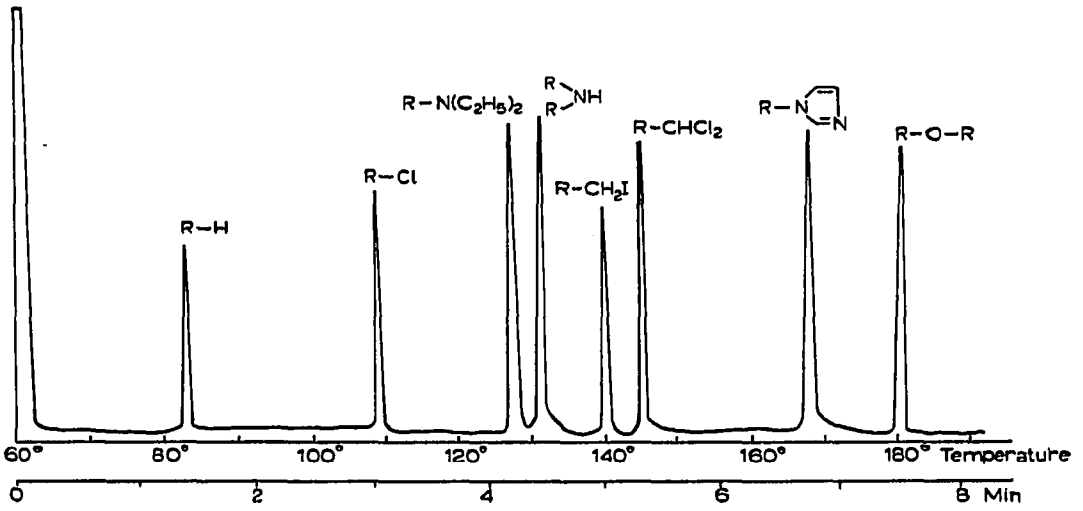



Fig. 1. Separation of a mixture of flophemesyl compounds ($R = C_6F_5Si(CH_3)_2$–) by temperature-programmed GC. Conditions: 5-ft. column of 3% QF-1 on CQ programmed at 16° $min^{-1}$ from 60° to 210°; nitrogen flow-rate, 60 ml $min^{-1}$.

did not parallel that of trimethylsilyl donors[4]. In pyridine solution, the order of reactivity of flophemesyl reagents is: silylamine > chlorosilane > silyldiethylamine > disilazane ≫ silylimidazole. When catalysed by a little flophemesyl chloride, the most reactive reagents are: silyldiethylamine ≫ silylamine > disilazane.

The silylating reagents were purified by distillation under vacuum through a lagged 12-in. Vigreux column. In the preparation of the purified reagents it was found that GC on a 5-ft. column of 3% QF-1 (J. J.'s Chromatography, Kings Lynn, Great Britain) on support CQ gave excellent separation of the reagent mixtures encountered. By temperature programming, each of the reagents described here could be recognized by its elution temperature (see Fig. 1). Satisfactory mass spectra were obtained, with the exception of flophemesylamine, bromide and iodide, which are unstable in GC and did not give good spectra by the GC–MS technique.

Trimethylsilylimidazole is the strongest silylating reagent for sterols so far prepared[4]. The weak amphoteric properties of the imidazole ring make it a good leaving group and hydroxyl groups react readily in the presence of enolizable ketones. Flophemesylimidazole, in contrast, failed to react completely with cholesterol after heating at 85° for 12 h. This lack of reactivity is presumably due to electron withdrawal of the pentafluorophenyl ring, preventing the development of a negative charge on nitrogen in the transition state between breaking a Si–N and formation of an Si–O bond.

The uncatalysed reactions of flophemesyldisilazane and flophemesyldiethylamine with cholesterol parallel those of the trimethylsilyl derivatives, giving 85% reaction in 24 h at room temperature in pyridine. Flophemesyl chloride and flophemesylamine, however, in pyridine, rapidly react with unhindered secondary hydroxyl groups to give the silyl ethers. Thus, cholesterol and 2$\beta$,3$\beta$-dihydroxy-5$\alpha$-cholestane gave quantitative yields of the mono- and diethers, respectively, in 15 min with a 1:1

mixture of flophemesylamine and pyridine. The reagent did not cause formation of enol ethers. For example, in 12 h at 80° flophemesylamine had no effect on steroid 3-, 6-, 11-, and 17-keto groups and 4-en-3-one and 7-en-6-one groups. The more hindered hydroxy groups do not react; thus 17$\alpha$-methylandrost-4-en-17$\beta$-ol-3-one, the 14-hydroxy group of 2$\beta$,3$\beta$,14$\alpha$-trihydroxy-5$\beta$-cholest-7-en-6-one, 11$\beta$-hydroxyandrost-4-ene-3,17-dione or 17$\alpha$-hydroxypregn-4-ene-3,20-dione are all unaffected by these conditions. Direct comparison with trimethylsilylamine is not possible because the latter disproportionates to hexamethyldisilazane spontaneously. Flophemesylamine therefore provides a simple reagent for making silyl ethers of unhindered secondary hydroxy groups in the presence of enolizable ketones.

*Catalysed silylation reactions*

To extend the range of hydroxyl groups which could be protected by the flophemesyl group, the effect of catalysts was studied. Flophemesyl chloride or bromide had a marked catalytic effect on reactivity; boron trifluoride etherate or *p*-toluenesulphonyl chloride were useful but tended to give some by-products. Ammonium sulphate was also useful but limited by its solubility in organic solvents.

Using flophemesyl chloride as catalyst, the reactivity of the disilazane, and particularly the diethylamine, increased markedly. The catalysed disilazane gave a quantitative yield of flophemesyl cholesterol in 3 h at 65° in pyridine and the diethylamine gave a quantitative yield in less than 15 min at room temperature. The catalysed reaction probably involves an intermediate with quaternized nitrogen, the disilazane nitrogen is both more hindered and much less basic than the amine because of electron withdrawal by fluorine.

We were particularly interested in finding conditions which would enable the derivatization of hydroxyl groups in the presence of unprotected ketones. Silylating reagents tend to give incomplete conversion to enol ethers with ketone groups; this must be either avoided or the ketone must be protected, involving another step in preparation. It was found that a 10:1 mixture of flophemesyldiethylamine and flophemesyl chloride in pyridine could give complete conversion of cholesterol to the silyl ether without affecting 5$\alpha$-cholestan-3-one, even after 6 h at 80°. This reagent mixture could also convert the 17$\beta$-hydroxy group of 17$\alpha$-methylandrost-4-en-17$\beta$-ol-3-one to a silyl ether, but did not affect the hindered 11$\beta$-hydroxy group in 11$\beta$-hydroxyandrost-4-ene-3,17-dione.

A 1:1 mixture of flophemesyldiethylamine and flophemesyl chloride was a very potent silylating reagent, causing extensive enol ether formation. Ketone groups were first protected by methoxime formation. The 1:1 reagent in pyridine completely converted the 11$\beta$-hydroxy groups of 11$\beta$-hydroxyandrost-4-ene-3,17-dimethoxine in 3 h at 60°. Complete reaction of the hydroxyl group in 17$\alpha$-hydroxypregn-4-ene-3,20-dimethoxime required 6 h at 85° or 1 h at 150°. The 14$\alpha$-hydroxy group of 2$\beta$,3$\beta$,14$\alpha$-trihydroxy-5$\beta$-cholest-7-ene-6-methoxime was unaffected. Satisfactory mass spectra, consistent with their structure, were obtained for all these derivatives.

Two interesting steric effects due to the larger bulk of the flophemesyl group were observed. The 17$\alpha$-hydroxy group in a pregnane could be converted to its silyl ether efficiently with a 20-methoxime as a near neighbour. When a 21-hydroxy group was also present, it presumably reacted more quickly, hindering access to the 17-hydroxy group and only 30% silylation of the latter group was obtained. When a

17,20-dihydroxypregnane was studied, reaction took place at the 20-hydroxy group and completely prevented silylation of the 17-hydroxy group. All reactions were carried out under the same experimental conditions.

Again, in a 3$\beta$,5$\alpha$,6$\beta$-trihydroxycholestane, the 5$\alpha$ position is completely inhibited by more rapid ether formation at the secondary 3$\beta$ and 6$\beta$ positions. However, when the compound is converted to the 3$\beta$,5$\alpha$-dihydroxycholestan-6-one methoxine, partial reaction of the 5$\alpha$-hydroxy group is achieved. Using flophemesylamine under mild conditions, the 3$\beta$-hydroxy group of this compound could be completely protected selectively.

A number of solvents were studied in both the catalysed and the uncatalysed reaction. Unhindered hydroxyl groups react smoothly in a variety of solvents. In more difficult cases, pyridine was generally the best solvent. The effect of solvent on rate of reaction of the 17-hydroxy group in 17$\alpha$-hydroxypregn-4-ene-3,20-dimethoxime, with a 1:1 mixture of flophemesyldiethylamine and flophemesyl chloride, is illustrated in Table II. Generally, a more polar solvent is preferred. The nature of the effect of pyridine is not clear, though it suggests some more specific interaction than solvation. Formation of an N-flophemesylpyridinium salt might be expected, but in the case of trimethylchlorosilane and pyridine, Wannagat *et al.*[5] found no evidence of complex formation.

TABLE II

EFFECT OF SOLVENT ON EXTENT OF REACTION BETWEEN 17$\alpha$-HYDROXYPREGN-4-ENE-3,20-DIMETHOXIME AND A 1:1 MIXTURE OF FLOPHEMESYLDIETHYLAMINE AND FLOPHEMESYL CHLORIDE
4 h at 120°.

| *Solvent* | *Silyl ether formation (%)* |
|---|---|
| Pyridine | 100 |
| Acetonitrile | 85 (complete in 7 h) |
| Dimethylformamide | Decomposition |
| Dimethyl sulphoxide | 35 |
| Tetrahydrofuran | 30 |
| Ethyl acetate | 25 |

Several examples of the decomposition of flophemesyl ethers in dimethylformamides have been observed and dimethyl sulphoxide is a poor solvent for the reagents, as two layers are formed.

*Formation of mixed derivatives*

To improve volatility or resolution, or to aid structural elucidation it may be desirable to form mixed derivatives. We found the methoximes of keto-steroids were, in all cases studied, stable to derivatization with silylating reagents. Similarly an *n*-butylboronate of 2$\beta$,3$\beta$-dihydroxy-5$\alpha$-cholestane was not cleaved by the silylating reagents. A trimethylsilyl ether of cholesterol is stable toward flophemesyl reagents, but flophemesyl cholesterol was easily cleaved by trimethylsilylimidazole, to give trimethylsilyl cholesterol. Similar replacement was found with other sterols. Advantage can

be taken of this in identification of sterols by MS. The flophemesyl derivatives tend to give strong molecular ions; the compound can then be converted to the trimethylsilyl derivative, and its fragmentation pattern compared with published mass spectra.

*Electron capture properties*

The technique of determining steroids by ECD is valuable, but the range of derivatives available is limited, and they have their disadvantages[6]. Heptafluorobutyrates are probably the most useful, but hindered hydroxyl groups do not always react quantitatively, and in at least one case, that of heptafluorobutyryl cholesterol,[2] we have shown the compound decomposes thermally in the gas chromatograph. We find the flophemesyl group has excellent sensitivity to the ECD combined with moderate volatility and stable derivatives can be formed for a wide range of hydroxyl groups.

A crystalline sample of flophemesyl cholesterol was stable in the laboratory atmosphere for at least 48 h. It could be chromatographed unchanged on columns of neutral alumina (activity I) eluting with hexane–ethyl acetate, or Sephadex LH-20 (swollen in hexane–ethyl acetate) or by reversed-phase chromatography on Amberlite XAD-2. It could be run on silica gel thin-layer plates and visualized with iodine or 20% sulphuric acid with heating to 110°, but recovery from thin-layer plates, with diethyl ether, dichloromethane or ethyl acetate led to approximately 10% hydrolysis to cholesterol. Recovery by shaking the silica gel with a mixture of benzene and water produced only 3% hydrolysis. The ECD response *versus* sample size for flophemesyl cholesterol is given in Fig. 2.

Table III records the least detectable amounts expressed as nanograms of the parent steroid required to give a signal to noise ratio of two for some of the compounds studied. The instrument was set to the optimum operating conditions for cholesterol.

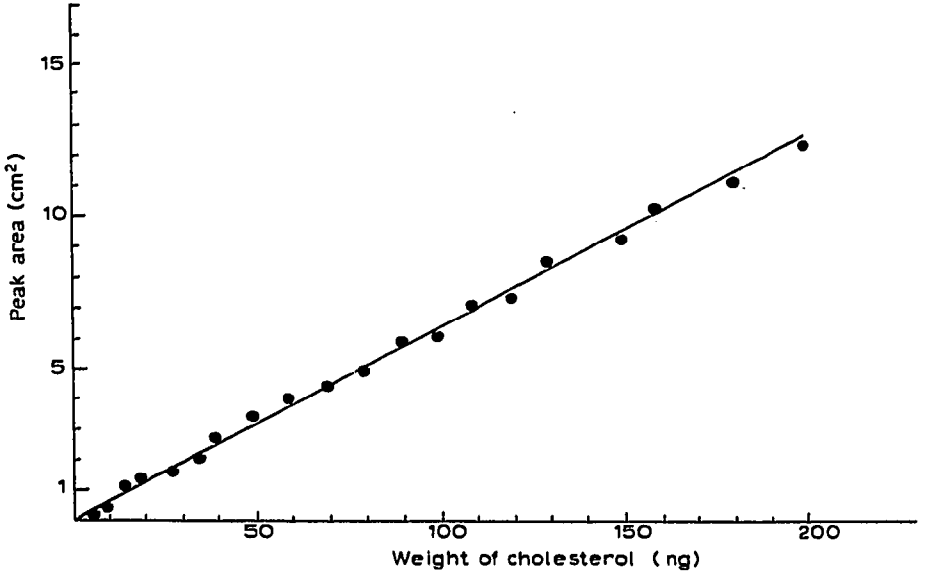



Fig. 2. Linearity of response of ECD to flophemesyl cholesterol expressed as peak area in cm² against weight of cholesterol in ng.

TABLE III

LIMIT OF DETECTION OF SOME STEROIDS BY ECD AS THEIR FLOPHEMESYL DERIVATIVES

| *Steroid* | *Number of pentafluorophenyl groups* | *Least detectable amount (ng)* |
|---|---|---|
| 17α-Hydroxypregn-4-ene-3,20-dimethoxime | 1 | 8.0 |
| 17(α)-Methylandrost-4-en-17β-ol-3-one | 1 | 5.0 |
| Cholesterol | 1 | 4.0 |
| Ergosterol | 1 | 1.0 |
| 3α,20α-Dihydroxy-5β-pregnane | 2 | 0.20 |
| 2β,3β,14α-Trihydroxy-5-cholest-7-en-6-one | 2 | 0.08 |
| 3β,5α,6β-Trihydroxycholestane | 2 | 0.07 |
| 2β,3β-Dihydroxy-5α-cholestane | 2 | 0.03 |

* Limit expressed as weight of parent sterol detectable with a signal to noise ratio of 2:1. For operating conditions of detector see Experimental.

## CONCLUSION

Pentafluorophenyldimethylsilyl ethers can be formed with a wide range of hydroxyl groups by selection of the appropriate reagent mixtures. These derivatives have moderate volatility in GLC with slightly longer retention times than the chloromethyldimethylsilyl ethers but much shorter than the bromomethyldimethylsilyl ethers of steroids. A combination of volatility and sensitivity to detection by electron capture make them the reagents of choice for steroids which do not form stable heptafluorobutyrates. The introduction of one pentafluorophenyl group allows detection at the nanogram ($\times 10^{-9}$ g) level whereas introduction of two or more groups allows picogram ($\times 10^{-12}$ g) detection. Use of the correct reagents permits derivatisation of hydroxyl groups in the presence of unprotected ketone groups thus simplifying the analysis of many steroids. The derivatives show characteristic mass spectra usually accompanied by a strong molecular ion.

## ACKNOWLEDGEMENTS

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