Marine Sterols with a New Pattern of Side-chain Alkylation from the Sponge *Aplysina* (= *Verongia*) *aerophoba*

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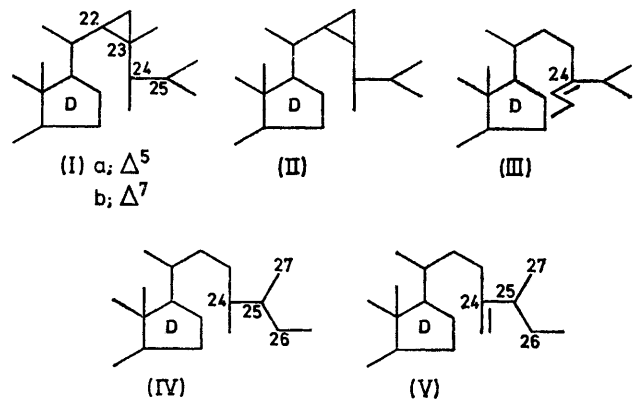
The principal sterols of the marine sponge *Aplysina* (= *Verongia*) *aerophoba*, aplysterol and 24,28-didehydroaplysterol are characterized as 26-methyl homologues of 24-methyl- and 24-methylene-cholesterol, respectively.

It is well known that natural sterols with the conventional C_{19} tetracyclic nucleus may have the normal C_8 side-chain modified by the addition of one or two carbon atoms at C-24. The biosynthetic origin of the ' extra ' carbon atoms of these C_{28} and C_{29} sterols has been extensively investigated and is well understood.[1]

Recently, other side-chain patterns have been discovered. In the marine sterols gorgosterol [part structure (Ia)],[2] acansterol [part structure (Ib)],[3] and 23-demethylgorgosterol [part structure (II)],[4] the attachment of ' extra ' carbon atoms to the normal cholesterol skeleton at C-22 and C-23 is exemplified. In 29-methylisofucosterol [part structure (III)],[5] isolated from a scallop *Placopecten magellanicus*, a propylidene group has been elaborated at C-24. We now report the isolation of sterols with the normal cholesterol nucleus and the side-chains as shown in (IV) and (V), involving a ' normal ' alkylation at C-24 and the unprecedented addition of an extra methyl group at C-26.

The two new sterols were isolated from the marine sponge *Aplysina* (= *Verongia*) *aerophoba*, from which we



have also obtained a series of brominated substances,[6] without a saponification procedure; a free sterol fraction was obtained by chromatography of the crude extract

[1] E. Lederer, *Biochem. J.*, 1964, **93**, 449; *Quart. Rev.*, 1969, **23**, 453.

[2] R. L. Hale, L. Leclercq, B. Tursch, C. Djerassi, R. A. Gross, jun., A. J. Weinheimer, K. Gupta, and P. J. Scheuer, *J. Amer. Chem. Soc.*, 1970, **92**, 2179; N. C. Ling, R. L. Hale, and C. Djerassi, *ibid.*, p. 5281.

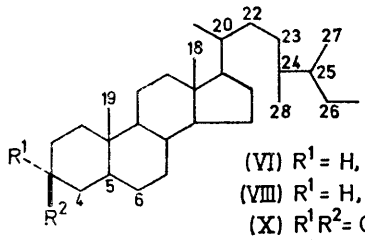
[3] C. Djerassi, Y. M. Sheikh, and B. M. Tursch, *Chem. Comm.*, 1971, 217.

[4] F. J. Schmitz and T. Pattabhiraman, *J. Amer. Chem. Soc.*, 1970, **92**, 6073.

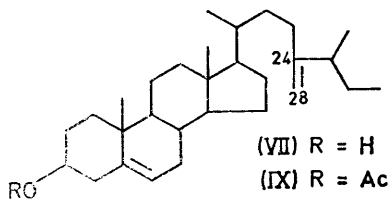
[5] D. R. Idler, L. M. Safe, and E. F. Macdonald, *Steroids*, 1971, **18**, 545.

[6] E. Fattorusso, L. Minale, and G. Sodano, *J.C.S. Perkin I*, 1972, 16; K. Moody, R. H. Thomson, E. Fattorusso, L. Minale, and G. Sodano, *ibid.*, p. 18.

on silica and was further resolved by acetylation and chromatography on alumina impregnated with silver nitrate. Recrystallization gave two products, both 98% pure by g.l.c., accounting for *ca.* 45 and 25% of the total sterols respectively, which we have designated aplysterol (VI) and 24,28-didehydroaplysterol (VII). 24-Methyl-



(VI) R^1 = H, R^2 = OH, $\Delta^{5(6)}$
(VIII) R^1 = H, R^2 = OAc, $\Delta^{5(6)}$
(X) R^1R^2 = O, Δ^4
(XI) R^1 = H, R^2 = OH
(XII) R^1 = H, R^2 = OAc
(XIII) R^1R^2 = O
(XIV) R^1 = R^2 = H



(VII) R = H
(IX) R = Ac

enecholesterol was observed as a minor component. Physical properties of the new products and certain derivatives are given in the Table.

Physical properties of aplysterol, 24,28-didehydroaplysterol, and derivatives

	M.p. (°C) [a]	$[\alpha]_D$ (°) [b]	G.l.c. R_t [c]
Aplysterol (VI)	135—136	−25	
Aplysteryl acetate (VIII)	119—120	−33	1·72
24,28-Didehydroaplysterol (VII)	128—130	−37	
24,28-Didehydroaplysteryl acetate (IX)	113—114	−50	1·65
Aplystanol (XI) [d]	135·5—136·5	+21	
Aplystanyl acetate (XII) [d]	115—116		1·77
Aplystane (XIV)	69—70		0·65

[a] Determined on a Kofler hot-stage apparatus. [b] Solutions in chloroform (*c* 1—3). [c] Relative to cholesteryl acetate; 2 m column of internal diam. 3 mm packed with 1% OV-1 on gaschrom (100—120 mesh), 245 °C, carrier gas (N_2) 32 ml min^{-1}; relative R_t values: β-sitosteryl acetate 1·64, 24-methylenecholesteryl acetate 1·27, cholestane 0·36. [d] Obtained from aplysterol.

Elemental analysis combined with accurate mass measurements indicated molecular formulae $C_{29}H_{50}O$ and $C_{29}H_{48}O$ for aplysterol and 24,28-didehydroaplysterol, respectively. The presence of a normal 3β-hydroxy-Δ^5-sterol system in both substances is indicated by the following data. In the mass spectra of the acetates the base peaks arise by loss of $CH_3{\cdot}CO_2H$, as expected; the n.m.r. spectra of both sterols include a 1H broad signal at δ 3·4 p.p.m. (C*H*·OH), a signal for one olefinic proton at δ 5·3, and two methyl singlets at δ 0·65 and 1·0 (13-Me and 10-Me, respectively). The optical rotations of both sterols and of their acetates (Table 1) are in the range characteristic of 3β-hydroxy-Δ^5-sterols. Oppenauer oxidation of aplysterol gave an αβ-unsaturated ketone (aplyst-4-en-3-one) (X), *m/e* 412 (M^+), $\nu_{max.}$ 1675 cm^{-1}, the o.r.d. spectrum of which was virtually identical with that of cholest-4-en-3-one; the u.v. absorption of this ketone [$\lambda_{max.}$ 241 nm (log ε 4·23)] excludes the possibility of a methyl substituent at C-4.

The mass spectra of the two sterols show that in both substances the additional carbon atoms indicated by the molecular formulae are in the side-chain rather than on the cholesterol nucleus. In particular, the spectrum of aplysterol shows peaks at *m/e* 273 (loss of C-10 side-chain), 255 (loss of C-10 side-chain plus H_2O), 231 (loss of C-10 side-chain plus 42 mass units), and 213 (loss of C-10 side-chain plus H_2O and 42 mass units);[7] in the spectrum of 24,28-didehydroaplysterol, besides fragments of *m/e* 255 and 213 there is a major peak at *m/e* 271 (loss of C-10 side-chain plus two hydrogen atoms from the steroid nucleus), diagnostic for the presence of a double bond in the side-chain.[8]

Hydrogenation of both aplysterol (VI) and 24,28-didehydroaplysterol (VII) gave the same product, aplystanol (XI), M^+ 416. In principle, the aplystanol from 24,28-didehydroaplysterol should be a mixture of C-24 epimers, but the two samples of aplystanol gave identical n.m.r. and mass spectra and the derived acetates (XII) moved as a single peak on g.l.c. when co-chromatographed. Consequently, the carbon skeletons of the two new sterols are identical. Moreover, aplystanol proved to be distinguishable by g.l.c. from known C_{29} 3β-hydroxy-5α-stanols, *viz.* stigmastanol (24α-Et), poriferastanol (24β-Et) and sargastanol (20α-Me; 24-epimeric Et). Aplystanol (XI) was converted *via* aplystanone (XIII) into the parent hydrocarbon aplystane (XIV), whose mass spectrum, with the base peak at *m/e* 217 (elimination of C_{10} side-chain + 42 mass units)[7] further confirms the location of both 'extra' carbon atoms in the side-chain rather than for example at C-14.

24,28-Didehydroaplysterol (VII) has a 24-methylene group. In the n.m.r. spectrum the $=CH_2$ signal appears as a broad singlet, δ 4·66 p.p.m.; in the i.r. spectrum there is a strong band at 885 cm^{-1}; and the t.l.c. R_F value (SiO_2–$AgNO_3$) is identical with that of 24-methylenecholesterol. In the mass spectrum there is a prominent peak at *m/e* 314, characteristic[8] of unsaturation at C-24 and ascribed to McLafferty rearrangement (cleavage of the 22,23-bond and H transfer from C-20). The presence of a corresponding peak at *m/e* 296 in the spectrum of didehydroaplysteryl acetate (IX) obtained by combined g.l.c.–mass spectrometry eliminated the possibility that this was due to traces of 24-methylenecholesterol in the 24,28-didehydroaplysterol. A peak at *m/e* 314 is also given by both gorgosterol and 23-demethylgorgosterol,[2,4] together with one at *m/e* 328 which

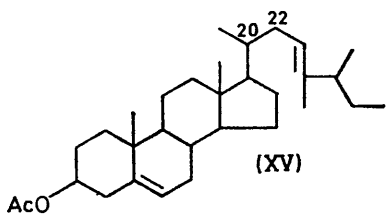
[7] K. Biemann, 'Mass Spectrometry,' McGraw-Hill, New York, 1962; J. A. McCloskey, *Methods Enzymol.*, 1969, **14**, 382.
[8] S. G. Wyllie and C. Djerassi, *J. Org. Chem.*, 1968, **33**, 305.

is equally apparent in the mass spectrum of didehydroaplysterol; however, the presence of a cyclopropane system in our material is excluded by other evidence, in particular by the absence of any n.m.r. signals in the region δ 0—0·6 p.p.m.

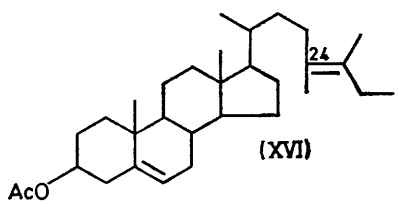
At this point our concern was to establish the position of the 'extra' methyl group. This was solved by treatment of the 24,28-didehydroaplysteryl acetate (IX) with iodine in benzene to effect migration of the double bond in the side-chain,[9] and subsequent exhaustive ozonolysis of the products.

T.l.c. (SiO_2–$AgNO_3$) of the iodine rearrangement mixture indicated the absence of the starting material, and the n.m.r. spectrum showed a new olefinic proton signal at δ 5·09 (0·8H, t, J 6 Hz) and vinylic methyl signals at 1·58 and 1·47 p.p.m., the broad singlet at δ 4·66 (CH_2=) having disappeared. Thus the iodine isomerization mixture consisted mainly of one isomer with a trisubstituted double bond in the side chain. Ozonolysis of the foregoing mixture yielded 3-methylpentan-2-one as the main component, with some butan-2-one; these ketones were isolated as their 2,4-dinitrophenylhydrazones and characterized by direct comparison with authentic samples and with a range of alternative possible products.

Therefore, the iodine rearrangement mixture consisted mostly of compounds (XV) (main component) and (XVI). Under our conditions, 24-methylenecholesteryl acetate similarly rearranged to give the corresponding trisubstituted olefin (24-methylcholesta-5,23-dien-3β-yl acetate) as the major product (60%) and the $\Delta^{24(25)}$-tetrasubstituted isomer as a minor product.



(XV)



(XVI)

In agreement with structures (XV) and (XVI), the mass spectrum of the mixture shows, besides M^+ (m/e 454) and $M^+ - CH_3{\cdot}CO_2H$ (m/e 394, base peak) signals, prominent peaks at m/e 296, associated with (XVI) and originating from $M^+ - CH_3CO_2H$ by the McLafferty rearrangement,[8] and at m/e 283, associated with (XV) and arising from $M^+ - CH_3{\cdot}CO_2H$ by allylic cleavage of the 20,22-bond.[8] The structures of the rearrangement products (XV) and (XVI) establish that of 24,28-didehydroaplysterol as (VII), and consequently that of aplysterol as (VI).

It is important to recognize the possibility of encountering completely new types of sterol in marine organisms. Many reports of marine sterols actually refer to mixtures of substances,[8,10] and we feel that the application of more refined techniques to a reinvestigation of marine species is warranted. This is particularly relevant when chemical data are to be applied in taxonomic considerations, and in this respect the sponges, which occupy a peculiar phylogenetic position in the animal kingdom, and which contain sterols in considerable variety,[11] offer a particularly promising area of research.

EXPERIMENTAL

Sponges [*Aplysina* (= *Verongia*) *aerophoba*], collected in the Bay of Naples, were obtained from the supply department of the Zoological Station, Naples. U.v. and i.r. spectra were recorded on a Bausch and Lomb Spectronic 505 and a Perkin-Elmer 257 Infracord spectrophotometer. N.m.r. spectra were taken at 100 MHz on a Varian HA-100 for solutions in carbon tetrachloride unless otherwise indicated (tetramethylsilane as internal standard). Mass spectra were recorded on an A.E.I. MS-9 instrument with a direct inlet system (ionizing energy 70 eV). G.l.c. was performed with a Carlo Erba Fractovap model GV instrument fitted with a glass column (see Table). Analyses were performed by Mr. S. De Rosa in our laboratory with a Perkin-Elmer 240 instrument.

Isolation of Sterols.—Fresh sponge (600 g dry weight after extraction) was extracted four times with acetone at room temperature for 3 days; after concentration the aqueous residue was extracted with ether (4 × 1 l). The combined ethereal extracts were taken to dryness and the gummy mass (130 g) was treated with light petroleum (b.p. 40—70°). The light petroleum-soluble material (11 g) was chromatographed on a column of silica gel (400 g; Merck) to give, on elution with chloroform, a crude sterol fraction (3·5 g), which was crystallized from methanol (1·6 g). Treatment with acetic anhydride–pyridine under reflux for 1 h gave the acetates, which were chromatographed on a column of alumina (180 g; neutral Merck) impregnated with silver nitrate (60 g) [eluant light petroleum (b.p. 40—70°) containing increasing amounts of benzene]. Initial elution with light petroleum gave *aplysteryl acetate* (VIII) (690 mg), m.p. 119—120° (from methanol), $[\alpha]_D$ −33° (Found: C, 81·4; H, 11·3. $C_{31}H_{52}O_2$ requires C, 81·5; H, 11·5%), $\nu_{max.}$ ($CHCl_3$) 1730 cm^{-1}, δ 5·34 (1H, m, CH=C), 4·55 (1H, m, C*H*·OAc), 1·94 (3H, s, $MeCO_2$), 1·00 (3H, s, 19-H_3), and 0·67 p.p.m. (3H, s, 18-H_3), m/e 456 (M^+, 1·2%), 396 ($M^+ - CH_3{\cdot}CO_2H$, 100), 381 (16), 255 (21), and 213 (13). Elution with light petroleum–benzene (9 : 1) afforded 24,28-*didehydroaplysteryl acetate* (IX) (386 mg), m.p. 113—114° (from methanol), $[\alpha]_D$ −50° (Found: C, 81·9; H, 11·0. $C_{31}H_{50}O_2$ requires C, 81·9; H, 11·1%), $\nu_{max.}$ (CS_2) 1725, 1220, 1025, and 885 cm^{-1}, δ 5·32 (1H, m, CH=C), 4·66br (2H, s, =CH_2), 4·53 (1H, m, C*H*·OAc), 1·94 (3H, s, $MeCO_2$), 1·00

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[10] J. Austin, *Adv. Steroid Biochem. Pharmacol.*, 1970, **1**, 73.

[11] C. S. Hammen and M. Florkin in 'Chemical Zoology,' vol. II, eds. M. Florkin and B. T. Scherer, Academic Press, New York and London, 1968, p. 55.

(3H, s, 19-H_3), and 0·67 p.p.m. (3H, s, 18-H_3), *m/e* 454 (M^+, 0·6%), 394 (M^+ − $CH_3{\cdot}CO_2H$, 100), 379 (10), 310 (3), 296 (7), 281 (4), 255 (7), 253 (7), 213 (7). *Aplysterol* (24,26-*dimethylcholest*-5-*en*-3β-*ol*) (VI) (prepared by treatment of the acetate with methanolic 10% potassium hydroxide at reflux for 2 h) crystallized from methanol as prisms, m.p. 135—136°, $[\alpha]_D$ −25° (Found: C, 83·85; H, 12·05%; M^+, 414·38611. $C_{29}H_{50}O$ requires C, 84·0; H, 12·15%; *M*, 414·38614), δ 5·30 (1H, m, CH=C), 3·47 (1H, m, C*H*·OH), 1·00 (3H, s, 19-H_3), and 0·69 p.p.m. (3H, s, 18-H_3), *m/e* 414 (M^+, 100%), 399 (25), 396 (30), 329 (25), 303 (31), 273 (25), 255 (34), 231 (18), and 213 (28%). 24,28-*Didehydroaplysterol* (26-*methyl*-24-*methylenecholest*-5-*en*-3β-*ol*) (VII) (obtained similarly) crystallized from methanol as prisms, m.p. 128—130°, $[\alpha]_D$ −37° (Found: C, 84·6; H, 11·8%; M^+, 412·37050. $C_{29}H_{48}O$ requires C, 84·4; H, 11·7%; *M*, 412·37049), $\nu_{max.}$ (CS_2) 3300br, 1040, and 885 cm^{-1}, δ 5·32 (1H, m, CH=C), 4·68br (1H, s, =CH_2), 3·48 (1H, m, C*H*·OH), 1·00 (3H, s, 19-Hz), and 0·69 p.p.m. (3H, s, 18-H_3), *m/e* 412 (M^+, 100%), 397 (12), 379 (13), 328 (13), 314 (36), 299 (30), 271 (34), 255 (12), 253 (7), and 213 (19).

Aplyst-4-*en*-3-*one* (24,26-*Dimethylcholest*-4-*en*-3-*one*) (X).—A mixture of aplysterol (VI) (100 mg), freshly distilled cyclohexanone (4·5 ml) and dry toluene (40 ml) was distilled until the moisture had been azeotropically removed. A solution of aluminium isopropoxide (210 mg) in dry toluene (10 ml) was added and the mixture was refluxed for 2 h. After removal of volatile solvents by steam distillation, the aqueous residue was extracted with chloroform. The solvent was removed and the residue was chromatographed on silica gel with benzene as eluant to give the α,β-*unsaturated ketone* (X), which crystallized from ethanol in prisms (57 mg), M^+ 412, m.p. 75—77° (Found: C, 84·25; H, 11·65. $C_{29}H_{48}O$ requires C, 84·4; H, 11·7%), $\lambda_{max.}$ (MeOH) 241 nm (log ε 4·23), $\nu_{max.}$ ($CHCl_3$) 1675 and 1620 cm^{-1}, o.r.d. (*c* 0·2 in MeOH; 22·5°) $[\phi]_{400}$ +618°, $[\phi]_{350-336}$ −410°.

Aplystanol (24,26-*Dimethylcholestan*-3β-*ol*) (XI).—Aplysterol (VI) (100 mg), dissolved in ethyl acetate—acetic acid (1 : 1; 5 ml), was hydrogenated at room temperature and atmospheric pressure over platinized carbon (10%; 100 mg) for 6 h. *Aplystanol* crystallized from methanol, m.p. 135·5—136·5°, M^+ 416, $[\alpha]_D$ +21° (Found: C, 83·4; H, 12·4. $C_{29}H_{52}O$ requires C, 83·6; H, 12·6%), δ ($CDCl_3$) 3·59 (1H, m, C*H*·OH), 0·79 (3H, s, Me), and 0·64 p.p.m. (3H, s, Me).

24,28-Didehydroaplysterol, when similarly hydrogenated, afforded a stanol, M^+ 416, the n.m.r. spectrum of which was identical with that of aplystanol; its acetate was indistinguishable (g.l.c. and mass spectrometry) from *aplystanyl acetate*, m.p. 115—116° (from methanol) (Found: C, 81·25; H, 11·5. $C_{31}H_{54}O_2$ requires C, 81·15; H, 11·85%), *m/e* 458 (M^+, 90%), 398 (M^+ − $CH_3{\cdot}CO_2H$, 90), 383 (25), 276 (40), 275 (30), 257 (8), and 215 (100%).

Aplystanone (24,26-*Dimethylcholestan*-3-*one*) (XIII).—Oppenauer oxidation of aplystanol (XI) (80 mg) as already described, followed by silica gel column chromatography in benzene, gave the *ketone* (XIII) (33 mg), m.p. 153·5°—154·5° (from ethanol), M^+ 414, $[\alpha]_D$ +44° (Found: C, 83·65; H, 12·05. $C_{29}H_{50}O$ requires C, 84·0; H, 12·15%), $\nu_{max.}$ ($CHCl_3$) 1710 cm^{-1}.

Aplystane (24,26-*Dimethylcholestane*) (XIV).—Aplystanone (XIII) (20 mg) on modified Wolff–Kishner reduction [12] gave *aplystane* (XIV) (12 mg), which was chromatographed on a silica gel column with light petroleum (b.p. 40—70°) as eluant, and then crystallized from methanol; m.p. 69—70° (Found: C, 86·75; H, 12·95. $C_{29}H_{52}$ requires C, 86·9; H, 13·1%), *m/e* 400 (M^+, 82%), 385 (26), 232 (20), 217 (100), and 149 (40).

Reaction of 24,28-*Didehydroaplysteryl Acetate* (IX) *with Iodine and Ozonolysis of the Products*.—A solution of 24,28-didehydroaplysteryl acetate (IX) (100 mg) and iodine (7 mg) in benzene (5 ml) was refluxed for 16 h. After cooling, benzene (5 ml) was added and the solution was washed with 1% sodium thiosulphate solution and water. The benzene layer was dried (Na_2SO_4) and evaporated to give the crude product (95 mg). T.l.c. (SiO_2–$AgNO_3$) showed the absence of starting material; g.l.c. analysis showed only one peak: R_t (relative to cholesteryl acetate) 1·67; δ 5·34 [1H, m, C(5)=CH] 5·08br (0·8H, t, *J* 7 Hz), 4·48 (1H, m, C*H*·OAc), 1·94 (3H, s, $MeCO_2$), 1·58 and 1·46 (each s, MeC=C), 1·01 (3H, s, 19-H_3), and 0·70 p.p.m. (3H, s, 18-H_3); *m/e* 394 (M^+ − $CH_3{\cdot}CO_2H$, 100%), 379, 296, 283, 255, 253, and 213.

A solution of the crude product (90 mg) in methylene chloride (5 ml) was treated with ozone (1 l min^{-1} flow rate of 2% O_3) for 30 min at −15°. After treatment with zinc (10 mg) and acetic acid (1 ml), a saturated solution of 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid was added, and the mixture was stirred for 15 min, then extracted with methylene chloride. The products were purified by silica gel preparative t.l.c. (benzene as developer). Extraction (with chloroform) of the band of R_F 0·7, gave 3-methylpentan-2-one dinitrophenylhydrazone (6 mg), M^+ 280, single peak on g.l.c. (1% OV 1; 220°), single spot on t.l.c. [silica gel; cyclohexane–benzene (6 : 4)], δ ($CDCl_3$) 9·13, 8·31, and 7·95 (aromatic protons), 2·49 (1H, m, CH), 2·01 (3H, s, CH_3C=N), 1·60 (2H, m, CH_2), 1·17 (3H, d, *J* 7 Hz, Me), and 0·92 p.p.m. (3H, t, *J* 7 Hz, *Me*CH_2), identified by direct comparison with an authentic sample and with the alternative possible products: 4-methylpentan-2-one, 2-methylpentan-3-one, and 3,3-dimethylbutan-2-one hydrazones.

Extraction of the band of R_F 0·65 gave a mixture (3 mg) shown by t.l.c. and g.l.c. to contain dinitrophenylhydrazones of acetone and butan-2-one.

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