Stereochemistry of Reduction of the 24,25-Double Bond in the Biosynthesis of Tigogenin in *Digitalis lanata*

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In tigogenin [(25*R*)-5α-spirostan-3β-ol] biosynthesized in *Digitalis lanata* a 4-*pro-R*-proton of mevalonic acid occupies the 24-*pro-S*-position, which indicates that reduction of Δ²⁴-biosynthetic intermediates occurs with *trans*-stereochemistry.

THE stereochemistry of the reduction of the 24,25-double bond of lanosterol or other similar Δ²⁴-precursors during the biosynthesis of cholesterol has recently been reported [1] to be ' *cis* ' in rat liver homogenates.

As cholesterol is a key intermediate in the formation of many steroidal components of plants, we decided to examine the reduction of the 24,25-double bond of cholesterol precursors in the biosynthesis of tigogegin [(25*R*)-5α-spirostan-3β-ol] in *Digitalis lanata*.[2]

The biosynthesis of tigogenin (I), is known [3] to proceed according to the pathway: lanosterol (or cyclo-artenol) ⟶ cholesterol ⟶ tigogenin (I), and this


(I)
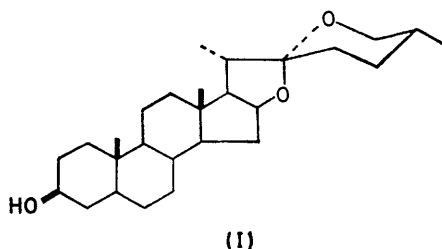
scheme necessarily involves, whatever the triterpenoid precursor may be, the reduction of the 24,25-double

chemistry of this reduction offers two main advantages: (i) the situation at C-25 could be inferred from published data; and (ii) the hydrogen atoms at C-24, one introduced during the reduction and the other one already present in the unsaturated intermediate, belong to a rigid system (ring F) and could be distinguished by simple chemical methods.

As far as the stereochemistry of the reduction at C-25 is concerned, the geometry [4] of the 24,25-double bond of Δ²⁴-precursors is represented in Scheme 1, in which the heavy dot indicates a carbon atom derived from C-2, and the heavy triangle indicates a carbon atom derived from C-3' of mevalonic acid (MVA).

Tamm *et al.*[5] showed that in the biosynthesis of tigogenin in *Digitalis lanata* the methyl group which is oxidized to $-CH_2O^-$ comes from C-3' of MVA. This, coupled with the *R* configuration at C-25 of tigogenin,[6] means that the hydrogen atom at C-25 entered from the top-side (Scheme 1).

Our approach to determining the stereochemistry of the introduction of hydrogen at C-24 was based on the use of (4*R*)-[4-³H]MVA, which is expected to give rise to a Δ²⁴-intermediate (II) of tigogenin in which a tritium atom is located at C-24.

SCHEME 1
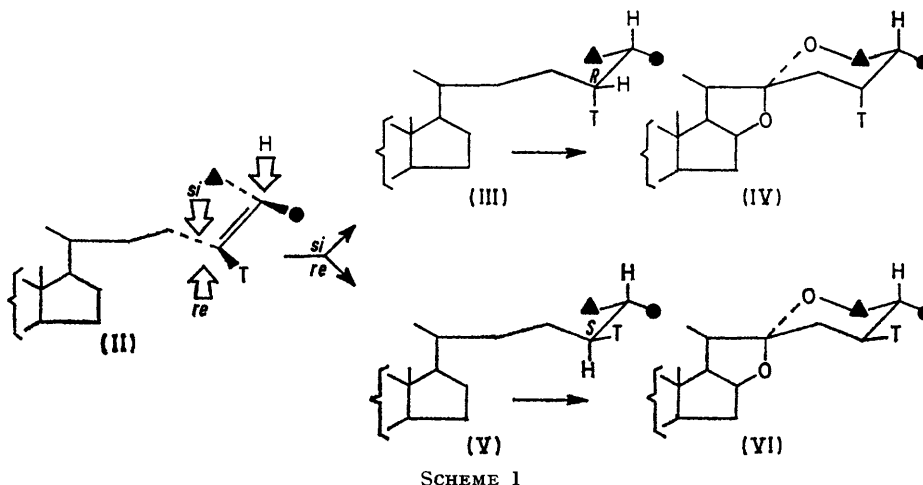
bond, *i.e.* the introduction of two hydrogen atoms, one at C-24 and the other at C-25.

The choice of tigogenin for the study of the stereo-

Attack of the hydrogen at C-24 on the *si*-face would produce the 24*R*-configuration (III) corresponding to a tigogenin (IV) in which the tritium atom at C-24 is

[1] M. Galli Kienle, R. K. Varma, L. J. Mulheirn, B. Yagen, and E. Caspi, *J. Amer. Chem. Soc.*, 1973, **95**, 1996.

[2] L. Canonica, F. Ronchetti, and G. Russo, *J.C.S. Chem. Comm.*, 1972, 1309.

[3] R. D. Bennett and E. Heftmann, *Phytochemistry*, 1965, **4**, 577.

[4] K. J. Stone, W. R. Roeske, and R. B. Clayton, *Chem. Comm.*, 1969, 530.

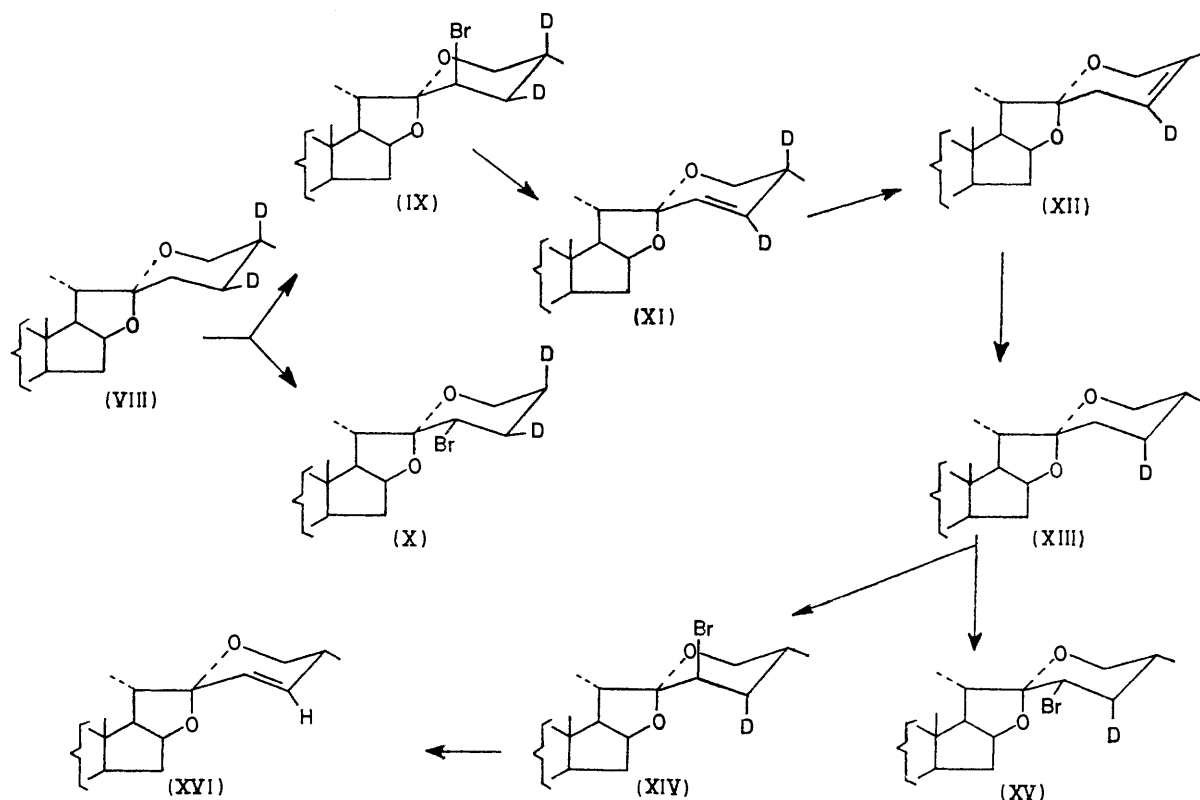[5] R. Joly and C. Tamm, *Tetrahedron Letters*, 1967, 3535.

[6] I. Scheer, R. B. Kostie, and E. Mosettig, *J. Amer. Chem. Soc.*, 1953, **75**, 4871; 1955, **77**, 641.

axial; * *vice-versa*, attack on the *re*-face would produce the 24S-configuration (V), corresponding to a tigogenin with an equatorial tritium at C-24 (VI).

Consequently, the stereochemistry at C-24, and thence that of the whole reduction of the 24,25-double bond, could be inferred by determining the orientation of the tritium on that carbon.
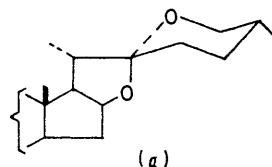

(VII)
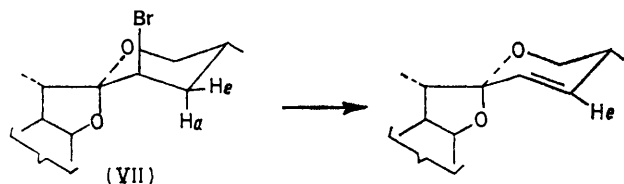
To solve this problem we used the dehydrobromination of the (23R)-bromo-derivative (VII) with Bu^{t}OK in

However, it was first necessary to ascertain whether the dehydrobromination of the (23R)-bromo-derivative was really an antiperiplanar process; more exactly, whatever the spatial arrangement of the atoms involved in the transition state might be, it was necessary to verify that the eliminated hydrogen atom is that one *trans* to the bromine in the ground state (H$_a$).

For this preliminary control we decided to dehydrobrominate the (23R)-bromo-derivatives (IX) and (XIV) containing a deuterium atom in the 24-equatorial or 24-axial position, respectively. These two compounds were synthesized according to Scheme 2, using procedures described by Faul *et al.*[8] on undeuteriated compounds: bromination with fast quenching of (24S,25R)-24,25-dideuterio-5α-spirostan [8] (VIII) yielded an equimolecular mixture of (23R,24R,25R)- (IX) and

SCHEME 2

Me$_2$SO at room temperature, which was expected to proceed in an antiperiplanar fashion with consequent selective loss of the 24-axial hydrogen.

* The equivalence between the 24R-configuration (III) and axial orientation of the 24-tritium atom in [24-³H]tigogenin (IV)

(a)

is based on the assignment to ring F of sapogenins of the chair conformation (a) on the basis of steric arguments [7a] and of X-ray and n.m.r. data.[7b]

(23S,24R,25R)-23-bromo-24,25-dideuterio-5α-spirostan (X).

The fact that (VIII) yields two monobromo-derivatives, while (25S)-sapogenins afford only one monobromo-derivative,[7b] confirms the 25R-configuration of (VIII).

The 24S-configuration of (VIII) and the equatorial and, respectively, axial orientation of the bromine

[7] (a) L. F. Fieser and M. Fieser, ' Steroids,' Reinhold, New York, 1959, pp. 824—825; (b) R. K. Callow, V. H. T. James, O. Kennard, J. E. Page, P. N. Paton, and L. Riva di Sanseverino, *J. Chem. Soc.* (C), 1966, 288.

[8] W. H. Faul, A. Failli, and C. Djerassi, *J. Org. Chem.*, 1970, 35, 2571.

atoms in (X) and (IX) were confirmed by the 23-H signal in the n.m.r. spectra of (X) and (IX); this signal appears as a doublet with J_{aa} 12 Hz in the first case and as a doublet with J_{ea} 3 Hz in the second.

The (23R)-bromo-derivative (IX) was dehydrohalogenated to (25R)-24,25-dideuterio-5α-spirost-23-ene (XI) which was, in turn, isomerized to 24-deuterio-5α-spirost-24-ene (XII); the position of the deuterium in the above compounds was confirmed by lack of the signal due to 24-H and by reduction of the multiplicity of the signal of 23-H (which appears as a singlet) in the n.m.r. spectrum of compound (XI), and by lack of the 24-H signal in that of compound (XII).

Homogeneous catalytic hydrogenation of (XII) yielded the spirostan (XIII) in which the hydrogens introduced from the less hindered top-side [8] had pushed down the deuterium at C-24 to the axial position. Bromination of (XIII) afforded the monobromo-derivatives (XIV) and (XV); the 23-H signal in the n.m.r. spectra of these compounds appears as a doublet with $J_{ae} = J_{ee} = 3$ Hz in both cases, and this is in agreement with the axial orientation of deuterium at C-24 and the equatorial and, respectively, axial orientation of the bromine atoms in (XV) and (XIV).
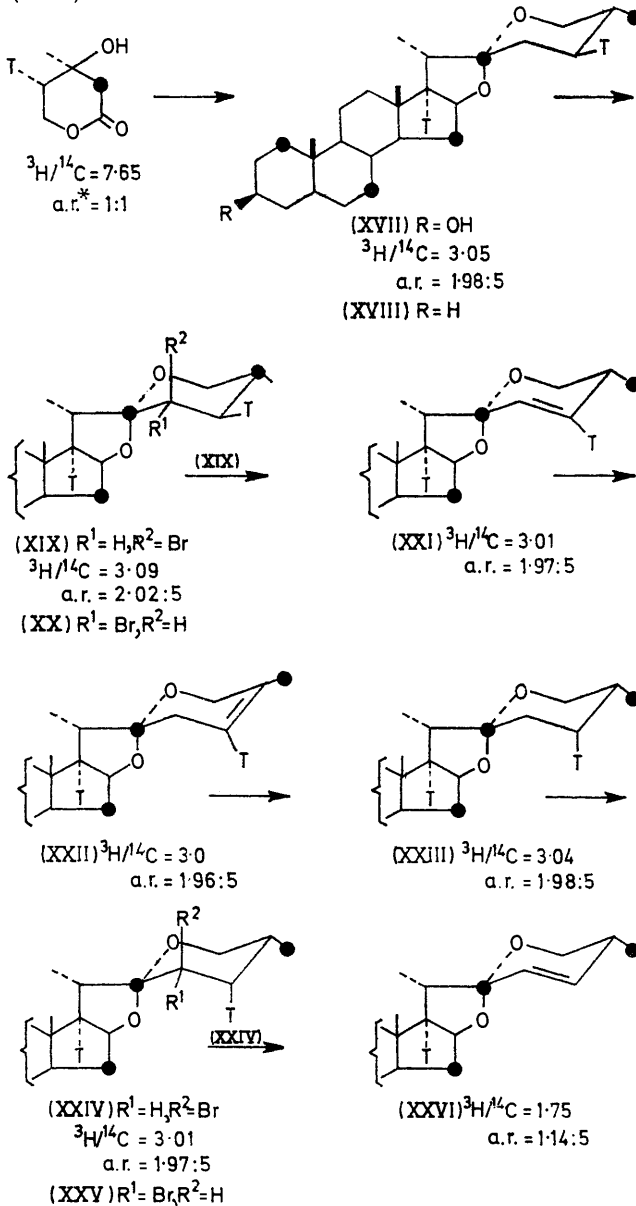
Dehydrohalogenation of the (23R)-bromo-derivative (XIV) occurred with loss of the axial deuterium at C-24 to yield (XVI); this result, together with the retention of the equatorial deuterium during the conversion of (IX) into (XI), confirmed that the dehydrobromination of the (23R)-bromo-derivative (XIV) occurs with *trans*-stereochemistry.

We could now start with tracer experiments. (4R)-[4-³H,2 ¹⁴C]MVA (0·1 mCi of ¹⁴C; ³H/¹⁴C ratio 7·65) was administered to five young *Digitalis lanata* plants, which were harvested after four weeks. The biosynthesized tigogenin (XVII) was isolated, purified, and, after dilution with carrier material, crystallized to constant specific activity (see the Table): its ³H/¹⁴C ratio (3·05) showed the presence of two tritium atoms in the molecule (7·65 × $\frac{2}{5}$ = 3·06).

Since cholesterol biosynthesized from (4R)-[4-³H]MVA in rat liver homogenates has three tritium atoms, one each at C-17, C-20, and C-24,[9] and tigogenin biosynthesized from the same MVA lacks tritium at C-20,[10] the two tritium atoms were assumed to be at C-17 and C-24. The presence of tritium at C-24 was essential to our approach, and was proved later.

The doubly labelled tigogenin (XVII) was transformed, by reduction of the corresponding tosyl ester with LiAlH₄, into 3-deoxytigogenin (XVIII); from the latter the corresponding Δ²³-olefin (XXI) was obtained as follows: bromination yielded a mixture of the bromides (XIX) and (XX), which were separated by chromatography. The equatorial (23S)-bromo-derivative (XX) could not be dehydrobrominated with Bu^{t}OK in Me₂SO–benzene at room temperature, whereas under

⁹ L. J. Mulheirn and E. Caspi, *J. Biol. Chem.*, 1971, **12**, 3948.
¹⁰ A. F. Wickramasinghe, B. P. Burrows, R. K. Sharma, J. B. Greig, and E. Caspi, *Phytochemistry*, 1969, **8**, 1433.

the same conditions the axial derivative (XIX) easily yielded the Δ²³-olefin (XXI) which exhibited the same ³H/¹⁴C ratio and specific activity as the starting bromide (XIX).

$^3H/^{14}C = 7.65$
a.r.* = 1:1

(XVII) R = OH
$^3H/^{14}C = 3.05$
a.r. = 1.98:5
(XVIII) R = H

(XIX) R¹= H, R²= Br
$^3H/^{14}C = 3.09$
a.r. = 2.02:5
(XX) R¹= Br, R²=H

(XXI) $^3H/^{14}C = 3.01$
a.r. = 1.97:5

(XXII) $^3H/^{14}C = 3.0$
a.r. = 1.96:5

(XXIII) $^3H/^{14}C = 3.04$
a.r. = 1.98:5

(XXIV) R¹= H, R²= Br
$^3H/^{14}C = 3.01$
a.r. = 1.97:5
(XXV) R¹= Br, R²=H

(XXVI) $^3H/^{14}C = 1.75$
a.r. = 1.14:5

*a.r = Atomic ratio

The previously proved loss of the axial hydrogen at C-24 during the dehydrohalogenation, together with the retention of tritium when this reaction was effected on the labelled compound (XIX) showed, provided that a tritium atom was located at C-24, that it was equatorial.

Finally we confirmed the presence of tritium at C-24. Our approach was based on the fact that the equatorial C-24 hydrogen of (25R)-5α-spirostan is retained during the transformations leading to 5α-spirost-24-ene, and inverted to the axial position by the catalytic homogeneous hydrogenation, occurring from the β-side (Scheme 2).

Thus, the Δ^{23}-olefin (XXI) was isomerized to (XXII) with Bu^{t}OK–Me$_2$SO at 110°; this was hydrogenated to the (25R)-5α-spirostan (XXIII), and the saturated compound brominated as before; the (23R)-bromo-spirostan (XXIV) was separated from its (23S)-isomer (XXV) and dehydrobrominated to a Δ^{23}-olefin (XXVI) with loss of 0·85 tritium atoms; this result proved, as expected, the presence of a tritium atom at C-24.

Our results demonstrate that in the biosynthesis of tigogenin in *Digitalis lanata* the hydrogen introduced at C-24 enters from the *re*-face and assumes the 24-*pro-R*-position. The whole process of reduction of the 24,25-double bond thus occurs, in a different manner to that found in rat liver, with *trans*-stereochemistry.

EXPERIMENTAL

N.m.r. spectra were determined on a Varian 100 Hz instrument. T.l.c. was carried out on unactivated silica gel on glass with a stationary phase thickness of 0·25 mm. The spray reagent was 50% sulphuric acid, whose application was followed by heating the plates at 100° for 5 min. All organic solutions were dried over anhydrous sodium sulphate. All the compounds yielded satisfactory elemental analyses.

(23R,24R,25R)- (IX) *and* (23S,24R,25R)-23-*Bromo*-[24,25-^{2}H$_2$]-5α-*spirostan* (X).—(24S,25R)-[24,25-^{2}H$_2$]-5α-Spirostan [8] (VIII) (850 mg) was brominated by the procedure of Faul *et al.*[8] to yield a mixture of (IX) and (X). The two compounds [t.l.c., hexane–benzene, 7 : 3 (v/v), 2 elutions, $R_{F(IX)}$ 0·57, $R_{F(X)}$ 0·35] were carefully separated by chromatography on SiO$_2$–Celite (1 : 1) (170 g); elution with light petroleum–benzene (95 : 5, v/v) afforded (IX) (412 mg), m.p. 222—225° (CHCl$_3$–MeOH), and (X) (430 mg), m.p. 192—194° (CHCl$_3$–MeOH).

(25R)-[24,25-^{2}H$_2$]-5α-*Spirost-23-ene* (XI).—The bromo-compound (IX) (400 mg) was dissolved into anhydrous benzene (28 ml) under stirring and, in a N$_2$ atmosphere, Bu^{t}OK (265 mg), dissolved in anhydrous Me$_2$SO (19 ml), was added. After 90 min, the mixture was poured into 10% aqueous HCl, the organic phase separated, and the aqueous phase extracted with ether. Evaporation of the combined organic layers *in vacuo* and chromatography of the residue over SiO$_2$–Celite (1 : 1) [eluant, light petroleum–benzene, 7 : 3 (v/v)] yielded the spirost-23-ene (XI) (322 mg), m.p. 174—176° (CHCl$_3$–MeOH).

The bromo-compound (X), submitted to the same conditions, was recovered unchanged.

[24-^{2}H]-5α-*Spirost-24-ene* (XII).—To the olefin (XI) (320 mg), suspended in dry Me$_2$SO (42·5 ml), was added Bu^{t}OK (722 mg) in Me$_2$SO (10·7 ml) under a N$_2$ atmosphere. The mixture was stirred at 110° for 20 min, and then poured into 10% aqueous HCl. The usual work-up and chromatography over SiO$_2$–Celite (1 : 1) [eluant, light petroleum–benzene, 8 : 2 (v/v)] yielded the spirost-24-ene (XII) (235 mg), m.p. 190—192° (CHCl$_3$–MeOH).

(24R,25R)-[24-^{2}H]-5α-*Spirostan* (XIII).—The spirostene (XII) (235 mg) was dissolved in acetone (47 mg) and added to a previously hydrogenated solution of Wilkinson's catalyst[11] (560 mg) in acetone (31 ml). After shaking under hydrogen for 24 h, the precipitate formed was filtered off, the solvent evaporated off *in vacuo*, and the residue chromatographed on SiO$_2$–Celite (1 : 1),

using hexane–ether (1 : 1, v/v), to give the spirostan (XIII) (220 mg), m.p. 168—172° (CHCl$_3$–MeOH).

(23R,24R,25R)- (XIV) *and* (23S,24R,25R)-23-*Bromo*-[24-^{2}H]-5α-*spirostan* (XV).—The spirostan (XIII) (220 mg), after bromination and work-up as above, yielded the bromo-compounds (XIV) (99 mg) and (XV) (111 mg).

(25R)-5α-*Spirost-23-ene* (XVI).—The (23R)-bromo-compound (XIV) (99 mg) was dehydrohalogenated using the preceding procedure, to yield (25R)-5α-spirost-23-ene (XVI) (68 mg).

Administration of (3RS,4R)-[4-^{3}H;2-^{14}C]*Mevalonic Acid to* Digitalis lanata.—The leaves of five, 3 month old plants of *D. lanata* were washed with AcOEt, and (3RS,4R)-[4-^{3}H; 2-^{14}C]MVA (0·1 mCi of ^{14}C; ^{3}H/^{14}C ratio 7·65) dissolved in acetone was applied to these once a week, for three weeks. After each administration the leaves were sprayed with a 10% solution of silicon oil in ligroin.

Extraction of Plants and Isolation of (24S,25R)-[17,24-^{3}H$_2$;1,7,15,22,27-^{14}C$_5$]-5α-*Spirostan-3β-ol* (XVII).—One week after the last treatment, the plants were harvested, dried at 60° for 12 h, and powdered. The dried residue (35 g) was extracted with 70% EtOH (350 ml) under reflux for 30 min. After cooling, the mixture was filtered and the residue washed with H$_2$O (100 ml); the aqueous washings were added to the filtrate. The combined aqueous alcoholic phase was extracted with ligroin (3 × 200 ml) and then 65% EtOH (1·5 l) was added. To this solution was added a 10% solution of Fe$_2$(SO$_4$)$_3$ (450 g) and, after 5 min, CaCO$_3$ (45 g) with stirring, to adjust pH to 7. The precipitate obtained was filtered off and the filtrate was extracted with CHCl$_3$ (3 × 450 ml) to eliminate the cardenolide fraction. The CHCl$_3$ extract gave a residue (490 mg). The filtrate was re-extracted with CHCl$_3$–EtOH (3 : 1; 3 × 450 ml), salted out with Na$_2$SO$_4$, and re-extracted with CHCl$_3$–EtOH (3 : 2; 3 × 450 ml). The combined CHCl$_3$–EtOH extracts were evaporated to yield 210 mg of residue which was combined with the 490 mg previously obtained from the CHCl$_3$ extract, and dissolved in Kiliani mixture (AcOH–conc. HCl–H$_2$O, 35 : 10 : 55) (20 ml) and refluxed. After 1·30 h the solution was concentrated *in vacuo*, H$_2$O (10 ml) was added and the solution obtained was extracted with CHCl$_3$–EtOH (1 : 3, 3 × 50 ml). The extracts were washed with H$_2$O, 2N-NaOH, and H$_2$O and then evaporated *in vacuo* to yield a residue (240 mg), which was refluxed with 5% methanolic KOH (5 ml). After 1 h, H$_2$O was added, MeOH was evaporated off, and the solution was extracted with CHCl$_3$–EtOH (1 : 3; 3 × 50 ml); the organic layers were washed with H$_2$O until neutral and evaporated. The crude residue (160 mg) was then chromatographed on SiO$_2$–Celite (1 : 1) (16 g). PhH–AcOEt (9 : 1, v/v), eluted radioactive tigogenin (XVII) (13·5 mg) which was diluted to 506 mg with carrier tigogenin, and repeatedly crystallized from MeOH and counted (see the Table).

(24S,25R)-[17,24-^{3}H$_2$;1,7,15,22,27-^{14}C$_5$]-5α-*Spirostan* (XVIII).—Radioactive tigogenin (XVII) (506 mg), tosylated and reduced with LiAlH$_4$ using a standard procedure,[12] yielded the labelled spirostan (XVIII) (495 mg) which was crystallized from CHCl$_3$–MeOH to constant specific activity (see the Table).

(23R,24R,25R)- (XIX) *and* (23S,24R,25R)-23-*Bromo*-[17,24-^{3}H$_2$;1,7,15,22,27-^{14}C$_5$]-5α-*spirostan* (XX).—The spirostan (XVIII) (395 mg) was brominated by the procedure

[11] A. J. Birch and K. A. M. Walker, *J. Chem. Soc.* (*C*), 1966, 1894.

[12] M. E. Wall and S. Serota, *J. Amer. Chem. Soc.*, 1956, **78**, 1747.

used for the deuteriated sample to yield, after chromatography on SiO_2–Celite (1 : 1), (XIX) (195 mg), (XX) (125 mg), and a mixture of the two (60 mg). Compound (XIX) was repeatedly crystallized from $CHCl_3$–MeOH and counted (see the Table).

Incorporation of $(3RS,4R)$-[4-^{3}H; 2-^{14}C]MVA into tigogenin in *Digitalis lanata*

Products	^{14}C specific activity × 10^{-5} (disint. min^{-1} mmol^{-1})	^{3}H/^{14}C Activity ratio
Tigogenin (XVII)	3·07	3·05
3-Deoxytigogenin (XVIII)	2·98	3·07
$(23R, 25R)$-23-Bromo-5α-spirostan (XIX)	2·95	3·09
$(25R)$-5α-Spirost-23-ene (XXI)	3·08	3·01
5α-Spirost-24-ene (XXII)	3·10	3·0
3-Deoxytigogenin (XXIII)	3·04	3·04
$(23R, 25R)$-23-Bromo-5α-spirostan (XXIV)	3·06	3·01
$(25R)$-5α-Spirost-23-ene (XXVI)	3·02	1·75

$(25R)$-[17,24-^{3}H$_2$;1,7,15,22,27-^{14}C$_5$]-5α-*Spirost*-23-*ene* (XXI).—The bromo-compound (XIX) (185 mg) was (dehydrobrominated as for (IX) by using Me_2SO–Bu^{t}OK to yield, after chromatography on SiO_2–Celite (1 : 1), the olefin (XXI) (151 mg), which was crystallized from $CHCl_3$–

MeOH to constant specific activity and counted (see the Table).

$(25R)$-[17,24-^{3}H$_2$;1,7,15,22,27-^{14}C$_5$]-5α-*Spirost*-24-*ene* (XXII).—The olefin (XXI) (151 mg) was isomerized as for (XI) to give (XXII) (105 mg) which was crystallized from $CHCl_3$–MeOH and counted (see the Table).

$(24R,25R)$-[17,24-^{3}H$_2$;1,7,15,22,27-^{14}C$_5$]-5α-*Spirostan* (XXIII).—The 24-ene (XXII) (105 mg) was hydrogenated on Wilkinson's catalyst as for (XII) to yield (XXIII) (103 mg) which was crystallized from $CHCl_3$–MeOH and counted (see the Table).

$(23R,24S,25R)$- (XXIV) *and* $(23S,24S,25R)$-23-*Bromo*-[17,24-^{3}H$_2$;1,7,15,22,27-^{14}C$_5$]-5α-*spirostan* (XXV).—The spirostan (XXIII) (103 mg) was brominated by the procedure previously described to yield (XXIV) (40 mg) (crystallized and counted), (XXV) (25 mg), and a mixture (10 mg).

$(25R)$-[17-^{3}H;1,7,15,22,27-^{14}C$_5$]-5α-*Spirost*-23-*ene* (XXVI). —The bromo-compound (XXIV) (40 mg) was dehydrobrominated as for (IX) to yield (XXVI) (31 mg) (crystallized and counted, see Table).