Preparation of deuterium labelled *fackel* sterols, [5,6,6,7,7,11,11,12,12,15,16,16,17-^2H_n]-5α-cholesta-8,14-dien-3β-ol and its (24*R*)-24-methyl and -ethyl congeners

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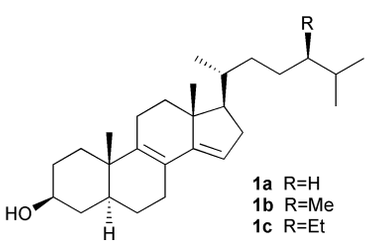
The reversible nature of the protonation and deprotonation sequence among the conjugated dienes implicated in the acid-catalysed double bond migration of steroid 5,7-dienes to 8,14-dienes allowed a high incorporation of deuterium atoms into the 5α-steroid 8,14-dienes. Treatment of cholesta-5,7-dien-3β-ol with a 37% deuterium oxide solution of deuterium chloride in refluxing *O*-deuterioethanol for 5 h afforded [5,6,6,7,7,11,11,12,12,15,16,16,17-^2H_n]-5α-cholesta-8,14-dien-3β-ol **3a** in 52% yield as a cluster of the d_n-congeners with the isotopic composition $d_5 : d_6 : d_7 : d_8 : d_9 : d_{10} = 4 : 17 : 33 : 31 : 12 : 3$. The average number of deuterium atoms (*n*D) incorporated was calculated as 7.3 D. Under the same conditions, the (24*R*)-24-methyl and -ethyl congeners of **3a** were prepared from campesta-5,7-dien-3β-ol and stigmasta-5,7-dien-3β-ol, with 7.0 D and 6.7 D, respectively. Prolongation of the reaction time increased the level of incorporation due to the primary kinetic isotope effect of the deuterium atom. With a 48 h reaction, the composition ratio of **3a** changed to $d_6 : d_7 : d_8 : d_9 : d_{10} : d_{11} : d_{12} : d_{13} = 2 : 8 : 22 : 32 : 24 : 9 : 2 : 1$, *i.e.*, the average incorporation increased to 9.1 D. Analysis of the ^{1}H NMR and MS spectra of **3a** implicated all possible conjugated dienes across the B, C, and D rings in this migration reaction, at least in the 5α-series.

Introduction

Recently, we determined that *fackel-J79* (*fk-J79*), a dwarf mutant of *Arabidopsis thaliana* with a defect in sterol C-14 reductase, accumulated three abnormal sterols: 5α-cholesta-8,14-dien-3β-ol **1a** and its (24*R*)-24-methyl and -ethyl congeners, *i.e.*, campesta-8,14-dien-3β-ol **1b** and stigmasta-8,14-dien-3β-ol **1c**, which were named *fackel* sterols.[1] These sterols, which have not been identified in wild-type *Arabidopsis*, are of considerable interest, since they could be one of the critical factors in causing the unique phenotype of *fk-J79* observed in embryonic and post-embryonic development. As part of our in-depth investigation into the roles of these sterols in embryogenesis, cell division, and cell expansion in plants, we are conducting an ongoing quantitative analysis of *fackel* sterols at different growth stages and in different organs of *fk-J79*. This requires *fackel* sterols with multiple deuterium atom labels as reference markers for gas chromatography-mass spectrometry (GC-MS) analysis.



There are many examples of isotope labelling of various 3β-hydroxysteroids using deuterium; these mainly rely on chemical manipulation of the 3β-hydroxy group.[2] A reaction sequence consisting of oxidation, deuterium exchange of the resulting 3-ketones with a base in an *O*-deuterated protic solvent, and reduction gives [2,2,4,4-$^2H_4$]-3β-hydroxysteroids, while reduction of the 3-ketones with lithium aluminium deuteride gives [3-^{2}H]-3β-hydroxysteroids. However, the levels and numbers of deuterium atoms incorporated onto the parent steroids achieved by these methods are insufficient for our purpose. The low incorporation causes the ion peaks due to the standard compounds to overlap with those of their parent compounds in regions around the molecular ion peak, affecting the precise quantification.

This paper reports the preparation of deuterium-labelled *fackel* sterol, [5,6,6,7,7,11,11,12,12,15,16,16,17-^2H_n]-5α-cholesta-8,14-dien-3β-ol **3a** † and its (24*R*)-24-methyl and -ethyl congeners **3b**,**c** from the corresponding 5,7-dienes **2a**–**c**. The simple method shown in Scheme 1, based on the reversible nature of the protonation and deprotonation sequence among the conjugated dienes implicated in the acid-catalysed double bond migration of steroid 5,7-dienes to 5α-steroid 8,14-dienes,[3–5] realized the introduction of striking numbers of deuterium atoms into the steroid B, C, and D rings with high levels of incorporation. The NMR analysis of **3a** clearly showed that 11- and 12-H_2 were replaced by deuterium, providing the first experimental proof that the 6,8-, 7,9(11)-, 8,11-, and 8(14),9(11)-dienes are involved in this well-known migration reaction, along with the other dienes previously verified: *i.e.*, 6,8(14)-, 7,14-, 8(14),15-, and 14,16-dienes.[4]

† The nomenclature of [5,6,6,7,7,11,11,12,12,15,16,16,17-^2H_n]-5α-cholest-8,14-dien-3β-ol is based on its formation mechanism, described in this paper. This is somewhat tentative, however, because absolute evidence for deuteration at the 17-position has not yet been obtained; judging from the ^{1}H NMR spectrum of **3a**, there seems to be very little, if any, deuteration at the 17-position; see text.

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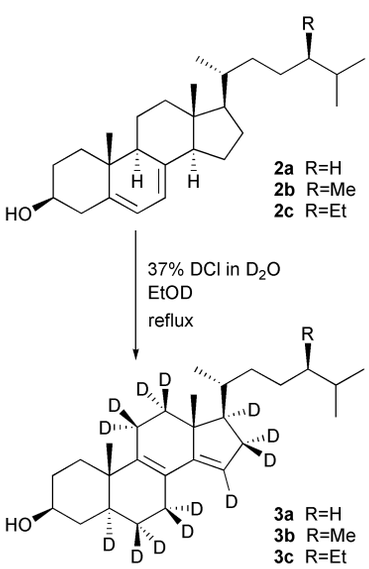
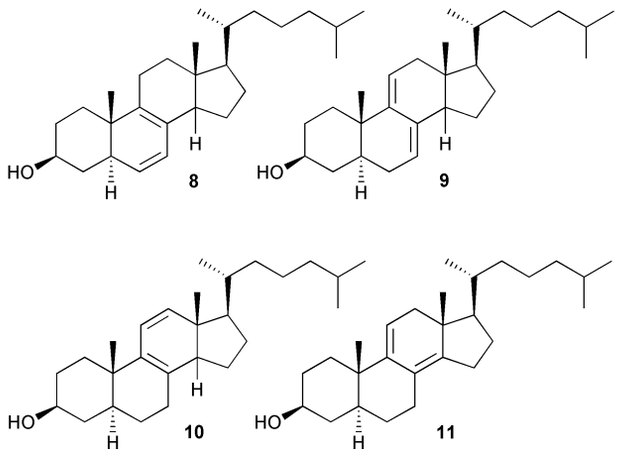
Results and discussion

In a previous work,[4] in connection with the preparation of *fackel* sterols **1a**–**c** from steroid 5,7-dienes **2a**–**c**, we detailed the stereo and chemical course of the acid-catalysed double bond migration of cholesta-5,7-dien-3β-ol **2a** to 5α-cholesta-8,14-dien-3β-ol **1a** with 36% hydrogen chloride in refluxing ethanol using product analysis and equilibrium experiments. This reaction proceeds through a pathway involving a sequence of protonation and deprotonation steps. Except for the step from 5,7-diene **2a** to 6,8(14)-diene **4**, all the steps are reversible; thus dienes **4**–**7** and **1a** equilibrate in the reaction mixture. This is shown in Scheme 2, where the reaction path to 5β-steroid dienes resulting from the minor 5β-protonation of **2a** is omitted.[4] Furthermore, although none have been identified yet, it is strongly expected that the 6,8-, 7,9(11)-, 8,11-, and 8(14),9(11)-dienes **8**–**11** are also implicated in this equilibration, as postulated by Boer *et al.* in their article on the approach to this migration reaction in terms of the heat of formation of the diene isomers.[5] Therefore, if the reaction is conducted in *O*-deuterioethanol (EtOD) using a 37% solution of deuterium chloride (DCl) in deuterium oxide (D_2O) as the acid catalyst, deuterium atoms should be introduced at multiple positions in **1a**, *i.e.*, the 5-, 6-, 7-, 11-, 12-,15-, 16-, and 17-positions, and the level of incorporation should increase with increasing reaction time due to the kinetic primary isotope effect of the deuterium atom *versus* the hydrogen atom.[6]

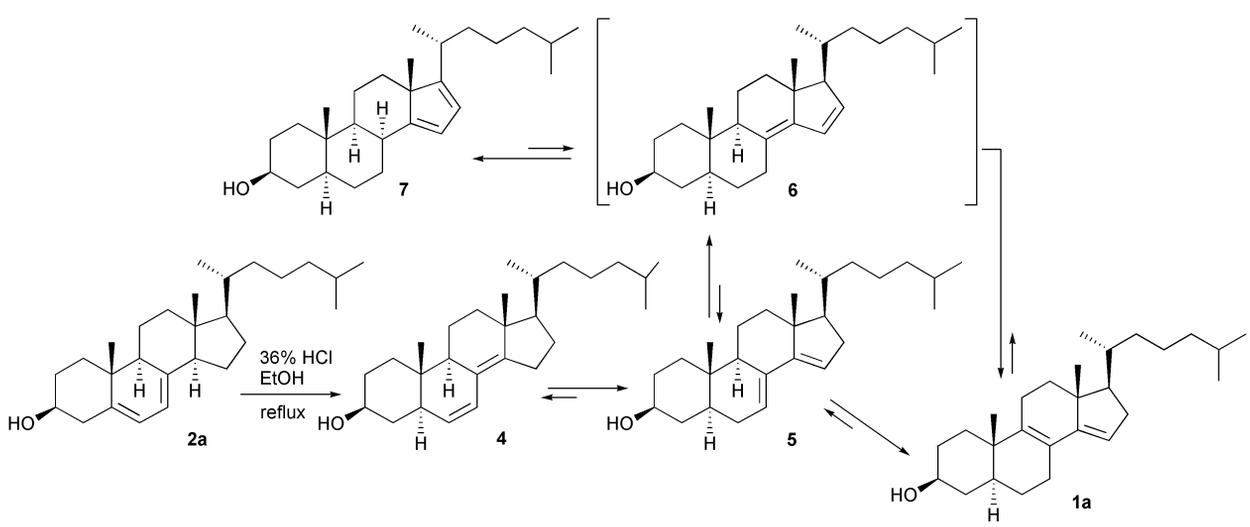


Scheme 1



With this expectation, the 5,7-dien-3β-ols **2a**–**c** were treated with a 37% D_2O solution of DCl (99.5 atom% D) in EtOD (99.5 atom% D) at refluxing temperature for 5 h. After the usual aqueous work-up and successive purification using flash chromatography and high-performance liquid chromatography (HPLC), [5,6,6,7,7,11,11,12,12,15,16,16,17-^2H_n]-5α-cholesta-8,14-dien-3β-ol **3a**, [5,6,6,7,7,11,11,12,12,15,16,16,17-^2H_n]-campesta-8,14-dien-3β-ol **3b**, and [5,6,6,7,7,11,11,12,12,15,16,16,17-^2H_n]-stigmasta-8,14-dien-3β-ol **3c** were isolated in 52, 49 and 51% yields, respectively. The retention times of **3a**–**c** on HPLC were identical to those of the authentic unlabeled compounds **1a**–**c**,[4] and in the ^1H NMR spectra of **3a**–**c**, the chemical shifts and coupling patterns of the 3α-proton and methyl protons (18-, 19-, 21-, 26-, and 27-H_3 of **3a**, 18-, 19-, 21-, 26-, 27-, and 28-H_3 of **3b**, 18-, 19-, 21-, 26-, 27-, and 29-H_3 of **3c**) were consistent with those observed in the spectra of **1a**–**c**, respectively.

Compounds **3a**–**c** and the corresponding unlabeled **1a**–**c** were subjected to GC-MS analysis after conventional *O*-trimethylsilylation by treatment with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide.[7] Table 1 shows the MS spectrum profiles of the trimethylsilyl derivatives of **3a**–**c**, *i.e.*, **13a**–**c**, in the region over *m*/*z* ($M^+ - 2$), where M^+ represents the respective molecular ion peaks of the trimethylsilyl derivatives of the unlabeled compounds **1a**–**c**, *i.e.*, **12a**–**c**. This suggests that **3a**–**c** are good standards in GC-MS analysis with respect to quantifying *fackel* sterols **1a**–**c** in plant materials, because the ion peaks of **13a**–**c** are completely separated from the molecular ion peaks of **12a**–**c**. Based on these data, the distribution of the d_n-congeners composing **3a**–**c** and the average numbers of deuterium atoms incorporated into them were calculated. Each of **3a**–**c** was a cluster of multi-deuterated congeners, with composition ratios



Scheme 2 Mechanism of the acid-catalysed double bond migration of cholesta-5,7-dien-3β-ol **2a** to 5α-cholesta-8,14-dien-3β-ol **1a** previously proposed by us,[4] where the reaction path to 5β-epimers resulting from the minor 5β-protonation of **2a** is omitted.

Table 1 Mass spectrum profiles of trimetylsilyl ethers **13a**–**c** over *m*/*z* ($M^+ - 2$)[a]

Compound	Intensity of ion peaks (%)[b]									
	$M^+ + 3$	$M^+ + 4$	$M^+ + 5$	$M^+ + 6$	$M^+ + 7$	$M^+ + 8$	$M^+ + 9$	$M^+ + 10$	$M^+ + 11$	$M^+ + 12$
13a	0	3	16	52	95	100	59	23	6	2
13b	1	5	22	63	100	79	38	13	4	1
13c	2	8	34	80	100	75	33	11	3	0

[a] M^+ indicates the respective molecular ion peak of the corresponding unlabeled trimethylsilylethers **12a**–**c; 12a** *m*/*z*: 454 ($M^+ - 2$, 1%), 455 ($M^+ - 1$, 20), 456 (M^+, 100), 457 ($M^+ + 1$, 43), 458 ($M^+ + 2$, 11), 459 ($M^+ + 3$, 2); **12b** *m*/*z*: 468 ($M^+ - 2$, 2%), 469 ($M^+ - 1$, 14), 470 (M^+, 100), 471 ($M^+ + 1$, 46), 472 ($M^+ + 2$, 13), 473 ($M^+ + 3$, 3); **12c** *m*/*z*: 482 ($M^+ - 2$, 1%), 483 ($M^+ - 1$, 15), 484 (M^+, 100), 485 ($M^+ + 1$, 48), 486 ($M^+ + 2$, 14), 487 ($M^+ + 3$, 3); the percent figures are relative intensities to each M^+ (100%). [b] The relative intensities to the maximum peak (100%) in this region. No other peak appeared in this region.

Table 2 Time course of the mass spectrum profiles of trimetylsilyl ether **13a** over *m*/*z* ($M^+ - 2$)[a]

Reaction time/h	Intensity of ion peaks (%)[b]									
	$M^+ + 4$	$M^+ + 5$	$M^+ + 6$	$M^+ + 7$	$M^+ + 8$	$M^+ + 9$	$M^+ + 10$	$M^+ + 11$	$M^+ + 12$	$M^+ + 13$
4	2	16	52	100	98	53	19	5	1	0
8	3	18	54	97	100	61	26	7	1	0
24	0	4	19	54	92	100	66	26	9	2
48	0	2	8	29	69	100	90	50	19	6

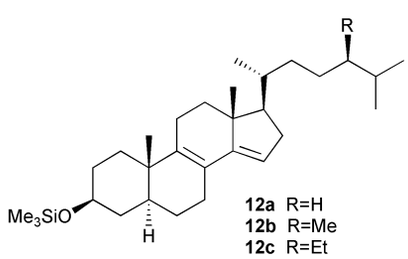
[a] M^+ indicates the molecular ion peak of the corresponding unlabeled trimethylsilyl ether **12a**: see footnote *a* of Table 1. [b] See footnote *b* of Table 1.

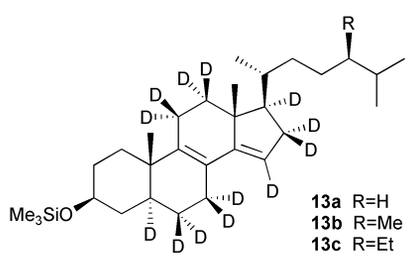
Table 3 Time course of the distribution of d_n congeners composing **3a** and average numbers of deuterium atoms incorporated[a]

Reaction time/h	Distribution of d_n-congeners (%)									Average number of deuterium atoms
	d_5	d_6	d_7	d_8	d_9	d_{10}	d_{11}	d_{12}	d_{13}	
4	5	17	37	30	9	2	0	0	0	7.3
8	5	17	33	30	12	3	0	0	0	7.5
24	1	5	17	30	30	14	2	1	0	8.4
48	0	2	8	22	32	24	9	2	1	9.1

[a] Calculated from MS data in Table 2.

of $d_5 : d_6 : d_7 : d_8 : d_9 : d_{10} = 4 : 17 : 33 : 31 : 12 : 3$ for **3a**, $d_4 : d_5 : d_6 : d_7 : d_8 : d_9 : d_{10} : d_{11} = 1 : 7 : 25 : 39 : 21 : 5 : 1 : 1$ for **3b**, and $d_4 : d_5 : d_6 : d_7 : d_8 : d_9 : d_{10} : d_{11} = 2 : 12 : 31 : 33 : 18 : 3 : 1 : 1$ for **3c**, and **3a**–**c** are labelled by 7.4, 7.0, and 6.7 deuterium atoms on average, respectively.





The effect of reaction time on the incorporation level of deuterium atoms was examined using **2a** as the reaction substrate. MS spectrum profiles of trimethylsilyl ether **13a** derivatized from **3a** after reaction times of 4, 8, 24, and 48 h, are shown in Table 2, and the distribution of d_n-congeners and the average numbers of deuterium atoms at each time are shown in Table 3. As expected from the kinetic primary isotope effect of the deuterium atom,[6] increasing the reaction time led to an increase in the level of incorporation. After 48 h, the average number of deuterium atoms increased to 9.1, where the composition ratio was $d_6 : d_7 : d_8 : d_9 : d_{10} : d_{11} : d_{12} : d_{13} = 2 : 8 : 22 : 32 : 24 : 9 : 2 : 1$. Such a high level of incorporation is worthy of comment, because it promises the preparation of tritium-labelled *fackel* sterols with high specific radioactivity when this diene migration reaction is carried out in a tritiated medium. These high-specific-radioactive *fackel* sterols should be useful for our future investigations on *fackel* sterols, *e.g.*, searching for their binding proteins and receptors.

Evidence for the labelling positions of **3a**–**c** was obtained by analysis of the ^{1}H NMR spectrum of **3a** obtained after a reaction time of 48 h, which is shown in Fig. 1 along with the spectrum of **1a** for comparison. Two signals based on 12β-H are observed at δ 1.98 (*ca.* 0.12H, s) and δ 2.01 (*ca.* 0.24H, d, J_{gem} 12.7 Hz). This indicates that *ca.* 64% of the atoms incorporated at the 12β-position are deuterium, as are at least 12% of the atoms incorporated at the 12α-position. Resonance due to 16a-H‡ appears at δ 2.05 (*ca.* 0.62H, dd, J_{gem} 16.1 and $J_{16a,17}$ 10.3 Hz); thus, *ca.* 38% of the atoms incorporated at the 16a-position are deuterium. Resonances due to 11α-H and 11β-H appear in the region δ 2.15–2.25 and the total integration of *ca.* 0.10H indicates *ca.* 95% incorporation of deuterium at both positions. Two signals based on 16b-H are observed at δ 2.32 (*ca.* 0.33H, d, $J_{16b,17}$ 7.3 Hz) and δ 2.35 (*ca.* 0.62H, dd, J_{gem} 16.1 and $J_{16b,17}$ 7.3 Hz). This indicates that *ca.* 5% of the

‡ The two protons of the methylenes at the 6-, 7-, or 16-position are differentiated by the suffixes a and b, respectively, because their stereochemistries (α or β) were not determined.

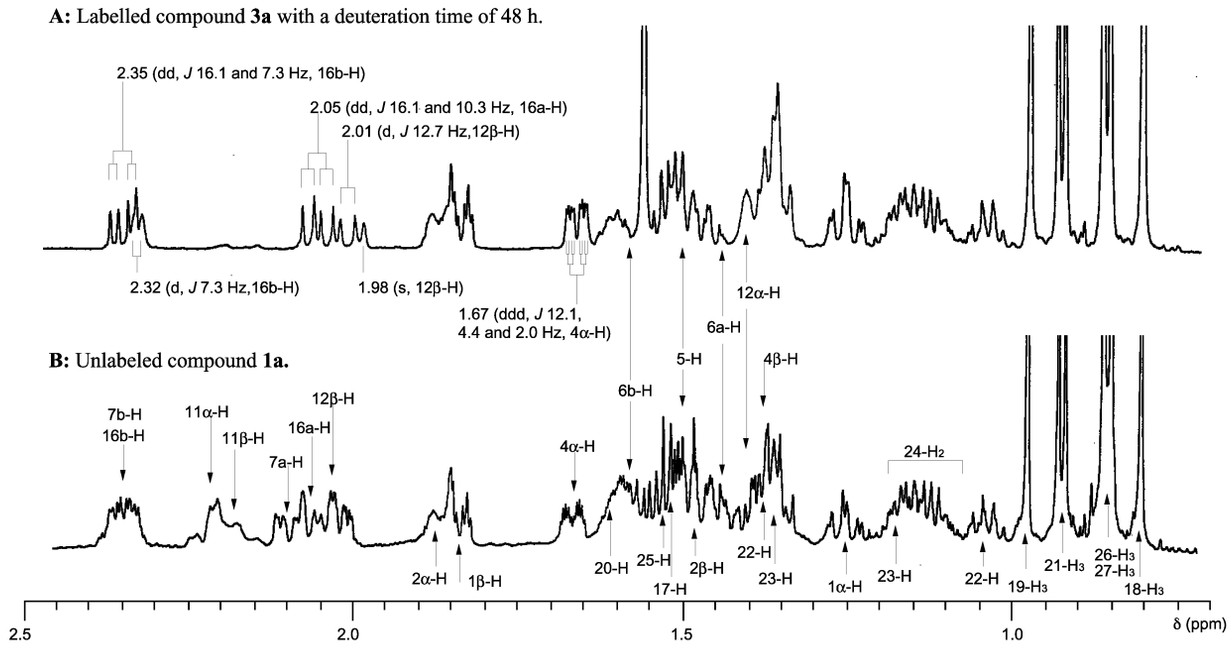
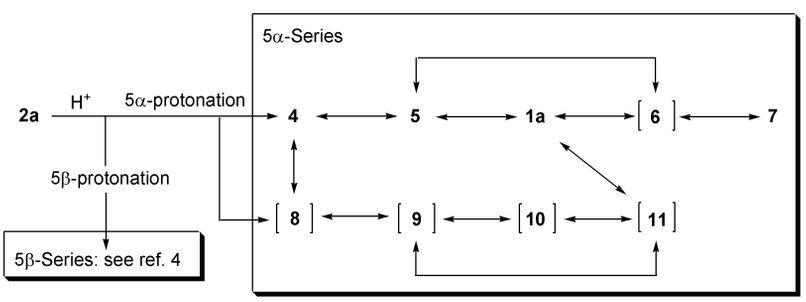


Fig. 1 600-MHz ^{1}H NMR spectra of labelled compound **3a** with a deuteration time of 48 h (**A**) and unlabeled authentic compound **1a** (**B**). Regions from δ 0.7 to 2.5 ppm are shown. Other resonances are observed at δ 3.63 (1H, tt, *J* 10.7 and 4.9 Hz, 3-H) and 5.36 (1H, dd, *J* 2.5 and 2.0 Hz, 15-H) for **1a**, and at 3.63 (1H, tt, *J* 10.7 and 4.9 Hz, 3-H) and 5.36 (0.08H, m, 15-H) for **3a**.



Scheme 3 Renewed mechanism of the acid-catalysed double bond migration of cholesta-5,7-dien-3β-ol **2a** to 5α-cholesta-8,14-dien-3β-ol **1a**, excluding the details of the reactions among the 5β-series.

atoms incorporated at the 16b-position are deuterium, while incorporation at the 17-position is less than 5%, if any. The resonance due to 15-H at δ 5.36 (*ca.* 0.08H, m) indicates *ca.* 92% incorporation of deuterium at the 15-position. No definite signal accounting for both 7a-H and 7b-H is observed in the regions around δ 2.10 and δ 2.35, which are assigned as the chemical shifts of 7a-H and 7b-H of **1a**, respectively. This indicates nearly complete deuteration at these positions.

By contrast, estimation of the incorporation at the 5-, 6a-, and 6b-positions was difficult from the features of the NMR spectrum alone, because the protons at these positions resonated in congested regions. However, the total incorporation of 9.1 deuterium atoms onto **3a**, calculated from the MS spectrum (*vide supra*), suggested nearly complete deuteration at these positions. This is endorsed by the absence of the cross peaks between 5-H and 4α-H/4β-H, and between 6a-H and 6b-H in the PFG-DQFCOSY of **3a**. Consequently, although absolute evidence for deuteration at the 17-position has not been obtained, deuteration at 5-, 6-, 7-, 11-, 12-, 15-, and 16-positions was clearly demonstrated. Therefore, our initial proposed mechanism for the acid-catalysed double bond migration of steroid 5,7-dienes to 5α-steroid 8,14-dienes shown in Scheme 2 should be altered to that depicted in Scheme 3, where all possible conjugated dienes across the B, C, and D rings are implicated in the 5α-series at least.

In conclusion, multi-deuterated *fackel* sterols **3a**–**c** were successfully prepared from the corresponding steroid 5,7-dien-3β-ols **2a**–**c** by simple treatment with a 37% D_2O solution of DCl in refluxing EtOD. In addition, the involvement of 6,8-, 7,9(11)-, 8,11-, and 8(14),9(11)-dienes in the acid-catalysed double bond migration of steroid 5,7-dienes to 5α-steroid 8,14-dienes was elucidated for the first time by analysis of the NMR spectrum of **3a**, leading to a profound understanding of the reaction mechanism. Since this reaction easily achieves a high incorporation of deuterium, it should also be applicable to the preparation of tritiated *fackel* sterols with high specific activities using *O*-tritiated protic media. These tritiated sterols will be useful tools for our future investigation of *fackel* sterols, which will identify their binding proteins, including the receptors. Quantitative analyses of *fackel* sterols **1a**–**c** at different growth stages and in different organs of *fk-J79* by GC-MS using **3a**–**c** as reference markers are now in progress.

Experimental

General

Melting points (mp) were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. NMR measurements were performed on a Bruker AC-300, JEOL JNM-A400, or JEOL JNM-A600 spectrometer in a $CDCl_3$ solution. Chemical shifts were recorded as δ values in parts per million (ppm) relative to tetramethylsilane (δ 0 ppm) for ^{1}H as an internal reference. All *J*-values are given in Hz. Analytical thin-layer chromatography was conducted on micro-slides coated with Merck Kieselgel KG60F-254; the developed plates were stained with 10% (w/v) vanillin in concentrated sulfuric acid at 180 °C. All reactions were carried out under a nitrogen atmosphere.

Flash chromatography was conducted using silica gel FL-60D [Fuji Silysia Chemical Ltd.] as the adsorbent. High-performance liquid chromatography (HPLC) was conducted with a Senshu Pak PG-S60–5251 (20 mm id × 25 cm; Senshu Scientific Co.) at a flow rate of 9.0 cm^3 min^{-1}; the peaks were detected using a refractive index detector. The ratios of mixed solvents were v/v.

[5,6,6,7,7,11,11,12,12,15,16,16,17-^2H_n]-5α-Cholesta-8,14-dien-3β-ol 3a

A solution of cholesta-5,7-dien-3β-ol **2a** (98 mg, 0.28 mmol: the reagent purchased from Sigma was used after purification by flash chromatography on silica gel) and 37% DCl [0.2 cm^3: 37 wt.% solution in D_2O, 99.5 atom% D (Aldrich)] in EtOD [4 cm^3: 99.5+ atom% D (Aldrich)] was refluxed for 5 h. After removing the solvent under reduced pressure, the residue was diluted with water and extracted with Et_2O. The extracts were successively washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated. The residue was subjected to flash chromatography using hexane–AcOEt (4 : 1) as the eluent. Fractions containing the major product eluted at around R_f 0.18 [hexane–AcOEt (4 : 1)] were collected and further purified by HPLC using hexane–AcOEt (4 : 1) as the mobile phase, affording [5,6,6,7,7,11,11,12,12,15,16,16,17-^2H_n]-5α-cholesta-8,14-dien-3β-ol **3a** (52 mg, 52%): R_t 31.6 min; colourless scales, mp 111–113 °C (MeOH) [lit.,[4] mp 111–113 °C (MeOH)]; δ_H (600 MHz) 0.82 (3H, s, 18-H_3), 0.868 (3H, d, *J* 6.8 Hz, 26-H_3), 0.871 (3H, d, *J* 6.8 Hz, 27-H_3), 0.94 (3H, d, *J* 6.4 Hz, 21-H_3), 0.99 (3H, s, 19-H_3), 3.63 (1H, tt, *J* 10.7 and 4.9 Hz, 3-H), 5.36 (0.09H, m, 15-H).

[5,6,6,7,7,11,11,12,12,15,16,16,17-^2H_n]-Campesta-8,14-dien-3β-ol 3b

A solution of campesta-5,7-dien-3β-ol **2b**[8] (50 mg, 0.13 mmol) and 37% DCl (0.1 cm^3) in EtOD (2 cm^3) was refluxed for 5 h. The work-up and purification procedure described above gave [5,6,6,7,7,11,11,12,12,15,16,16,17-^2H_n]-campesta-8,14-dien-3β-ol **3b** (25 mg, 49%): R_t 32.0 min; colourless scales, mp 111–113 °C (MeOH) [lit.,[4] mp 111–113 °C (MeOH)]; δ_H (300 MHz) 0.78 (3H, d, *J* 6.9 Hz, 26-H_3), 0.81 (3H, d, *J* 6.8 Hz, 27-H_3), 0.82 (3H, s, 18-H_3), 0.86 (3H, d, *J* 6.8 Hz, 28-H_3), 0.93 (3H, d, *J* 6.2 Hz, 21-H_3), 0.99 (3H, s, 19-H_3), 3.63 (1H, br tt, *J* 10.9 and 4.7 Hz, 3-H), 5.36 (0.09H, m, 15-H).

[5,6,6,7,7,11,11,12,12,15,16,16,17-^2H_n]-Stigmasta-8,14-dien-3β-ol 3c

A solution of stigmasta-5,7-dien-3β-ol **3c**[9] (50 mg, 0.13 mmol) and 37% DCl (0.1 cm^3) in EtOD (2 cm^3) was refluxed for 5 h. The work-up and purification procedure described above gave [5,6,6,7,7,11,11,12,12,15,16,16,17-^2H_n]-stigmasta-8,14-dien-3β-ol **3c** (26 mg, 51%): R_t 30.0 min; colourless scales, mp 105–106 °C (MeOH) [lit.,[4] mp 105–106 °C (MeOH)]; δ_H (300 MHz) 0.82 (3H, s, 18-H_3), 0.82 and 0.84 (each 3H, each d, each *J* 7.2 Hz, 26- and 27-H_3), 0.86 (3H, t, *J* 7.3 Hz, 29-H_3), 0.95 (3H, d, *J* 6.1 Hz, 21-H_3), 0.99 (3H, s, 19-H_3), 3.63 (1H, tt, *J* 10.8 and 4.9 Hz, 3-H), 5.36 (0.08H, m, 15-H).

Effect of reaction time on the incorporation rates of deuterium atoms into 3a

A solution of cholesta-5,7-dien-3β-ol **2a** (98 mg, 0.28 mmol) and 37% DCl (0.2 cm^3) in EtOD (4 cm^3) was refluxed. After 4, 8, 24 and 48 h, *ca.* 0.5 cm^3 aliquots were taken from the reaction mixture and were subjected to the work-up and purification procedure described above, giving deuterated 5α-cholesta-8,14-dien-3β-ol **3a** at each reaction time. A part of each sample was subjected to GC-MS analysis, as described below. The sample at 48 h was analysed by 600 MHz NMR spectroscopy using PFG-DQFCOSY, PFG-HMQC, PFG-HMBC, and NOE difference techniques, and the simple ^1H NMR spectrum is shown in Fig. 1 along with that of **1a**.

Gas chromatography-mass spectrometry (GC-MS) analysis

Before analysis, *ca.* 1 μg of 8,14-diene-3β-ol: **1a**–**c**,[4] **3a**–**c** with a reaction time of 5 h, or **3c** with a reaction time of 4, 8, 24, or 48 h, was derivatized to 3β-*O*-trimethylsilyl-8,14-diene-3β-ol: **12a**–**c** or **13a**–**c**, by treatment with 0.01 cm^3 of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (Aldrich) at 80 °C for 30 min. The mixture was subjected to analysis as it was. GC-MS was carried out in the electron impact mode (70 eV), on an Automass JMS-AM SUN (JEOL, Tokyo) fitted with a DB-5 column (0.25 mm × 15 m, 0.25 μm film thickness, J & W Scientific, Folsom, CA). The column oven temperature was programmed at 80 °C for 1 min, raised to 320 °C at a rate of 30 °C min^{-1}, and held at this temperature for 5 min. The carrier gas was He at a flow rate of 1 cm^3 min^{-1}; the injection port temperature was 280 °C and the samples were introduced by splitless injection. The MS spectrum profiles over *m*/*z* (M + 2) of **13a**–**c** with a 5 h reaction time, and **13c** with reaction times of 4, 8, 24, and 48 h are shown in Tables 1 and 2, respectively. Those of **12a**–**c** are shown in the footnote to Table 1.

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