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Ovine Metabolism of Lithogenic Sapogenins. Synthesis of [2,2,4,4-$^2H_4$]Sarsasapogenone, [2,2,4,4-$^2H_4$]Sarsasapogenin, and [2,2,4,4-$^2H_4$]Episarsasapogenin and Evaluation of Deuterium Retention in a Sheep-Dosing Trial

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The suitability of [2,2,4,4-$^2H_4$]sarsasapogenone (**1b**), [2,2,4,4-$^2H_4$]sarsasapogenin (**2b**), and [2,2,4,4-$^2H_4$]episarsasapogenin (**3b**) as isotopically labeled dosing substrates to determine the levels of free and conjugated sapogenins present in feces from sheep grazing saponin-containing plants implicated in the development of ovine heptagenous photosentization diseases was investigated. A 1:4 mixture of [2,2,4,4-$^2H_4$]sarsasapogenin (**2b**) and [2,2,4,4-$^2H_4$]episarsasapogenin (**3b**), obtained by reduction of [2,2,4,4-$^2H_4$]sarsasapogenone (**1b**), was found to retain 94% of incorporated deuterium, when dosed to one sheep. The recovery of the dosed mixture of genins **2b** and **3b** was calculated to be 85%. Considerable loss of deuterium and a lower recovery of genin material were observed when [2,2,4,4-$^2H_4$]sarsasapogenone (**1b**) was dosed.

KEYWORDS: Deuterated sapogenins; dosing trial; sheep; fecal material; *Narthecium ossifragum*; episarsasapogenin; sarsasapogenin; sarsasapogenone

INTRODUCTION

Hepatogenous photosensitization of sheep associated with grazing of plants containing steroidal saponins is both economically important and an animal welfare problem in many parts of the world (*1*). At least nine plants containing steroidal saponins have been reported to cause hepatogenous photosensitizations of sheep (*2*). Common features of all of the diseases associated with the grazing of saponin-containing plants are their sporadic occurrence, the difficulty in reproducing symptoms during dosing experiments (*1*, *3*), and the appearance of calcium salts of episapogenin glucuronides in the liver and in the bile ducts (*4*–*10*). The sporadic occurrence of hepatogenous photosensitization diseases, and the difficulties of reproducing them, may be related to variations in the saponin content of the plants and the proportion of saponin-containing plants in the diet.

Bog asphodel (*Narthecium ossifragum*), a member of the lily family, is one of the saponin-containing plants known to cause severe problems to the sheep industry in Norway and on the British Isles (*1*, *2*). Photosensitization has been reproduced in sheep by dosing them with crude saponins from *N. ossifragum* (*11*, *12*), but the doses necessary for causing the typical symptoms have been unrealistically large and far beyond those that probably could have been ingested by grazing. It has been shown that dosing with relatively large (but not unrealistically so) doses of *N. ossifragum* daily for 21 days did not cause liver damage in lambs (*13*). An attempt to reproduce the typical lesions in the liver by dosing with pure sarsasapogenin and diosgenin has also failed (*14*).

Improved knowledge of the normal uptake of saponins by sheep grazing pastures possessing saponin-containing plants and of saponin doses that induce photosensitization under field conditions is required if the etiology of saponin-associated photosensitization diseases is to be better understood. We have proposed that by dosing a known amount of deuterium-labeled sapogenin once per day to sheep that were additionally dosed with *N. ossifragum* and determining the ratio of labeled sapogenin to unlabeled sapogenins in feces, it would be possible to estimate the amount of sapogenins originating from the ingested plant material (*15*). This proposal presupposed that no deuterium, or a very small amount of deuterium, was lost during ovine metabolism of the labeled sapogenin and that the concentrations of sapogenins in feces were reasonably constant, regardless of time of feces collection or the time of dosing and ingestion of saponins. Flåøyen et al. (*15*) showed that although consistent fecal sapogenin levels were present during days 4–9 of a dosing trial in which [20,23,23-$^2H_3$]sarsasapogenin and *N.*

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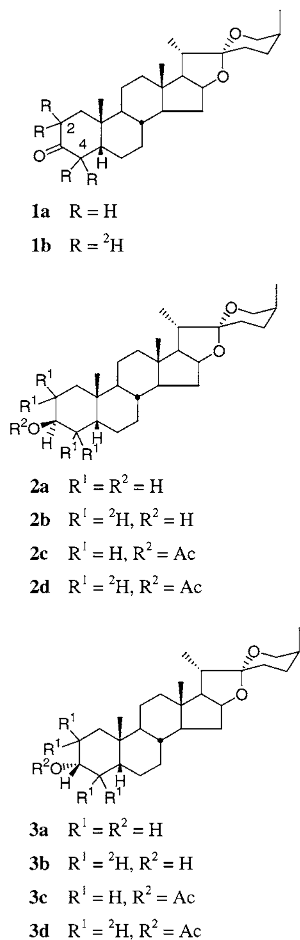

Figure 1. Structures of sapogenins referred to in this investigation.

ossifragum plants were administered once and three times daily, respectively, to two sheep, [20,23,23-$^2H_3$]sarsasapogenin was not a suitable dosing substrate because the 20- and 23-deuterium atoms were lost during the dosing experiment and/or subsequent chemical analysis.

We now report the synthesis of [2,2,4,4-$^2H_4$]sarsasapogenone (**1b**), [2,2,4,4-$^2H_4$]sarsasapogenin (**2b**), and [2,2,4,4-$^2H_4$]episarsasapogenin (**3b**) (**Figure 1**) and the use of **1b** and a 1:4 mixture of **2b** and **3b** in dosing experiments performed using one sheep.

MATERIALS AND METHODS

General Experimental Procedures. One- and two-dimensional ^1H and ^{13}C NMR spectra were obtained from $CDCl_3$ solutions using a Bruker DRX-400 instrument fitted with a 5 mm inverse probe head operating at 400.13 (^1H) and 100.62 (^{13}C) MHz. Chemical shifts are reported relative to internal tetramethylsilane (TMS), where δ (^1H) TMS + δ (^1H) $CHCl_3$ = 7.26 ppm and δ (^{13}C) TMS + δ (^{13}C) $CDCl_3$ = 77.06 ppm or δ (^1H) TMS + δ (^1H) C_5D_4HN = 8.72 ppm and δ (^{13}C) TMS + δ (^{13}C) C_5D_5N = 123.52 ppm. Radial chromatography was performed using a Chromatotron 7924T and a 22.5 cm diameter × 2 mm thick silica gel (60 G, Merck 7731) plate. GC-MS analyses were performed using a 25 m × 0.22 mm i.d. HP-5 column installed in an HP5890 GC instrument interfaced to an HP5970B mass selective detector, operated in selected ion mode.

Sarsasapogenone (1a). In a typical preparation, sodium dichromate (8 g) in water (50 mL) and concentrated sulfuric acid (10 mL) were added to a stirred solution of 95% sarsasapogenin (**2a**) (Sigma Chemical Co.) (10 g) in acetone (700 mL) and stirred for 24 h. Water (1 L) was added, and the mixture was extracted with $CHCl_3$ (1 × 400, 2 × 200, and 1 × 100 mL). The extracts were combined and dried over anhydrous Na_2SO_4 (24 h); solvent was removed using a rotary evaporator, and the product material was redissolved in $CHCl_3$ (150 mL) and filtered through an alumina column (Al_2O_3, Brockman grade II). The column was washed with a further 50 mL of $CHCl_3$, and the solvent was evaporated using a rotary evaporator to afford crystalline material, which was dried for 3 h under high vacuum. Sarsasapogenone (**1a**) (8.13 g, 81.8%): mp 194−198 °C [lit. mp 212−214 °C (*16*)]; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.79 (3H, s, H-18), 1.00 (3H, d, J = 6.7 Hz, H-21), 1.04 (3H, s, H-19), 1.08 (3H, d, J = 7.1 Hz, H-27), 2.29 (1H, ddd, J = 5.3, 14.5, 14.5 Hz, H-2α), 2.68 (1H, dd, J = 13.6, 14.9 Hz, H-4α), 3.29 (1H, br d, J = 11.0 Hz, H-26α), 3.94 (1H, dd, J = 2.6, 11.0 Hz, H-26β), 4.39 (1H, m, H-16); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 14.4 (C-21), 16.1 (C-27), 16.5 (C-18), 21.1 (C-11), 22.7 (C-19), 25.8 (C-24), 26.0 (C-23), 26.1 (C-7), 26.6 (C-6), 27.1 (C-25), 31.8 (C-15), 35.1 (C-10), 35.2 (C-8), 37.0 (C-1), 37.2 (C-2), 40.2 (C-12), 40.7 (C-13), 41.0 (C-9), 42.2 (C-20), 42.4 (C-4), 44.3 (C-5), 56.3 (C-14), 62.2 (C-17), 65.2 (C-26), 81.0 (C-16), 109.7 (C-22), 213.0 (C-3). MS (70 eV), m/z 414 (M^+, 5%), 300 (8), 285 (5), 271 (24), 139 (100).

[2,2,4,4-$^2H_4$]Sarsasapogenone (1b). In a typical preparation, sarsasapogenone (**1a**) (10 g), dioxane (300 mL), D_2O (25 mL), and sodium metal (0.35 g) were refluxed for 24 h with an anhydrous $CaCl_2$ water trap fitted to the condenser. Distilled water (500 mL) was added to the cooled solution, and the precipitate was recovered by filtration using a Büchner funnel. Product material was washed with distilled water and dried under high vacuum for 3 h. GC-MS data showed that the product material (9.42 g, 93.7%, mp 208−214 °C) consisted mainly of [^2H]-, [$^2H_2$]-, and [$^2H_3$]sarsasapogenone: MS (70 eV), m/z 418 (2%), 417 (5), 416 (7), 415 (5), 414 (0.6), 302 (5), 287 (3), 273 (12), 139 (100). Product material from several deuteration reactions (21.2 g) in dioxane (400 mL) was subjected to a second deuteration cycle using D_2O (20 mL) and sodium metal (0.8 g). Workup as described above afforded [2,2,4,4-$^2H_4$]sarsasapogenone (**1b**) (deuterated ketone) (19.88 g, 93.3%): mp 208−214 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.80 (3H, s, H-18), 1.01 (3H, d, J = 6.7 Hz, H-21), 1.05 (3H, s, H-19), 1.09 (3H, d, J = 7.1 Hz, H-27), 3.31 (1H, br d, J = 11.0 Hz, H-26α), 3.96 (1H, dd, J = 2.6, 11.0 Hz, H-26β), 4.43 (1H, m, H-16); ^{13}C ($CDCl_3$, 400 MHz) δ 14.4 (C-21), 16.1 (C-27), 16.5 (C-18), 21.1 (C-11), 22.7 (C-19), 25.9 (C-24), 26.0 (C-23), 26.1 (C-7), 26.6 (C-6), 27.2 (C-25), 31.8 (C-15), 35.1 (C-10), 35.3 (C-8), 37.0 (C-1), 40.2 (C-12), 40.8 (C-13), 41.0 (C-9), 42.2 (C-20), 44.2 (C-5), 56.3 (C-14), 62.2 (C-17), 65.2 (C-26), 81.0 (C-16), 109.8 (C-22), 213.3 (C-3); MS (70 eV), m/z 418 (M^+, 3%), 304 (6), 289 (5), 275 (20), 139 (100).

[2,2,4,4-$^2H_4$]Sarsasapogenin (2b) and [2,2,4,4-$^2H_4$]Episarsasapogenin (3b). In a typical preparation [2,2,4,4-$^2H_4$]sarsasapogenone (**1b**) (10 g) and $NaBH_4$ (2 g) in EtOH/MeOH (1:1) (400 mL) were stirred at room temperature for 24 h, after which time the solvent was removed using a rotary evaporator to afford a 1:4 mixture of [2,2,4,4-$^2H_4$]sarsasapogenin (**2b**) and [2,2,4,4-$^2H_4$]episarsasapogenin (**3b**) (deuterated alcohol mixture): mp 194−200 °C. Separation of a portion of the mixture using radial chromatography, with 20 mL portion mixtures of petroleum spirits and diethyl ether (9:1, 4:1, 1:1, 3:7, and 1:9) as eluents afforded specimens of [2,2,4,4-$^2H_4$]sarsasapogenin (**2b**) and [2,2,4,4-$^2H_4$]episarsasapogenin (**3b**).

[2,2,4,4-$^2H_4$]sarsasapogenin (**2b**): mp 180−184 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.76 (3H, s, H-18), 0.98 (3H, s, H-19), 1.00 (3H, d, J = 6.7 Hz, H-21), 1.08 (3H, d, J = 7.1 Hz, H-27), 3.30 (1H, br d, J = 11.0 Hz, H-26α), 3.96 (1H, dd, J = 2.6, 11.0 Hz, H-26β), 4.09 (1H, br s, H-3), 4.41 (1H, m, H-16); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 14.4 (C-21), 16.1 (C-27), 16.5 (C-18), 21.0 (C-11), 24.0 (C-19), 25.9 (C-24), 26.0 (C-23), 26.6 (C-6, C-7), 27.2 (C-25), 29.9 (C-1), 31.8 (C-15), 35.3 (C-10), 35.4 (C-8), 36.5 (C-5), 40.0 (C-9), 40.4 (C-12), 40.8 (C-13), 42.2 (C-20), 56.6 (C-14), 62.2 (C-17), 65.2 (C-26), 67.0 (C-3), 81.1 (C16), 109.8 (C-22). Acetylation of a subsample of **2b** in a GC vial using pyridine/acetic anhydride (1:1) (0.5 mL) afforded [2,2,4,4-$^2H_4$]sarsasapogenin acetate (**2d**): MS (70 eV), m/z 462 (M^+, 8%), 348 (7), 333 (14), 319 (18), 288 (15), 273 (9), 259 (11), 139 (100).

[2,2,4,4-$^2H_4$]episarsasapogenin (**3b**): mp 193−197 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.76 (3H, s, H-18), 0.95 (3H, s, H-19), 1.00 (3H, d, J = 6.7 Hz, H-21), 1.08 (3H, d, J = 7.1 Hz, H-27), 3.31 (1H, br d, J = 11.0 Hz, H-26α), 3.61 (1H, br s, H-3), 3.96 (1H, dd, J = 2.6, 11.0 Hz, H-26β), 4.42 (1H, m, H-16); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 14.4

Table 1. Dry Matter (DM) and Free and Conjugated (conj) Sapogenins Determined for Fecal Material Recovered from a Sheep Administered Single Doses of **1b** on Day 0 and a 1:4 Mixture of **2b** and **3b** on Day 9

		feces DM		sapogenins (mg/kg of DM)				
day	extract[a]	%	kg	**1a** + **1b**	**2a** + **2b**	**3a** + **3b**	subtotal[b]	total[b]
				Ketone Dose (1000 mg)				
1	free	39.9	0.239	136	25	67	228	
	conj			5.7	7.1	20	33	261
2	free	39.4	0.217	459	110	254	823	
	conj			21	33	106	160	983
3	free	37.0	0.238	152	95	246	593	
	conj			8.4	19	75	102	695
4	free	36.8	0.220	40	37	125	202	
	conj			6.4	9.1	33	49	251
5	free	38.2	0.298	9.1	13	34	56	
	conj			1.5	2.5	5.4	9.4	65
6	free	39.3	0.266		1	26	37	
	conj				2	3.6	5.6	42
				Alcohol Dose (284 mg)				
10	free	43.6	0.184	3.5	10	54	68	
	conj				1	2.3	3.4	71
11	free	47.0	0.189	27	70	427	524	
	conj			6.6	12	55	74	598
12	free	41.8	0.221	19	52	213	284	
	conj			4.4	9.4	34	48	332
13	free	42.8	0.197	10	28	88	126	
	conj			3	6.4	20	29	155
14	free	41.2	0.224		11	32	43	
	conj				0.7	1.9	2.5	46

[a] Free = genin material obtained by CH_2Cl_2 extraction and acetylation; conj = genin material obtained by MeOH extraction, hydrolysis, and acetylation. [b] Subtotal = total free or conjugated genins; total = free + conjugated genins.

(C-21), 16.1 (C-27), 16.5 (C-18), 20.7 (C-11), 23.4 (C-19), 25.9 (C-24), 26.0 (C-23), 26.8 (C-7), 27.1 (C-6), 27.2 (C-25), 31.8 (C-15), 34.7 (C-10), 35.3 (C-1), 35.4 (C-8), 40.3 (C-12), 40.7 (C-9), 40.8 (C-13), 42.0 (C-5), 42.2 (C-20), 56.4 (C-14), 62.2 (C-17), 65.2 (C-26), 71.5 (C-3), 81.1 (C16), 109.8 (C-22). Acetylation of a subsample of **3b** in a GC vial using pyridine/acetic anhydride (1:1) (0.5 mL) afforded [2,2,4,4-$^2H_4$]episarsasapogenin acetate (**3d**): MS (70 eV), *m/z* 462 (M^+, 11%), 348 (4), 333 (13), 319 (19), 288 (19), 273 (8), 259 (12), 139 (100).

Dosing Trial. One 7-month-old male sheep, Dala breed, was dosed intraruminally (day 0) with 1000 mg of deuterated ketone (**1b**), suspended in 100 mL of water/EtOH (80:20) and washed down with 100 mL of water. Nine days later (day 9) the same sheep was dosed in the same fashion with 284 mg of the 1:4 mixture of **2b** and **3b**. Hay was fed ad-libitum for 1 week before and throughout the dosing experiment. The sheep was kept in a cage allowing collection of all fecal output. Fecal material was collected once per day, weighed (wet weight), and frozen at −20 °C (**Table 1**). Liquid nitrogen was added to a representative subsample from each day's collection. The subsamples were then finely chopped using a Robot Coupe R301 Ultra chopper (Robot Coupe, S.A., Montceau en Bourgogne, France) and freeze-dried overnight. Percent dry matter (DM) was calculated as 100 × freeze-dried weight/wet weight (**Table 1**). A sample of the hay that was fed was also freeze-dried and prepared for chemical analysis.

Sapogenin Analyses. Soxhlet extraction of freeze-dried fecal material using dichloromethane (free sapogenin extract), followed by methanol (conjugated sapogenin exttract), hydrolysis of the conjugated sapogenin extract for 90 min at 95 °C using 0.5 mol/L hydrochloric acid, and GC-MS analysis of acetylated sapogenin extracts were performed as previously reported (*17*, *18*) except that SIM GC-MS analyses were modified to include *m/z* 285−288, 315−320, 345−348, and 459−462 ions (deuterated sapogenin acetates) and *m/z* 271−275 ions (deuterated sarsasapogenone). Quantitation of the acetylated sapogenins was performed by integrating *m/z* 139 ion profiles extracted for SIM GC-MS chromatograms. The level of deuterium retention in sapogenin substrates was determined by assessment of the *m/z* 315−320 ion contributions determined for sapogenin acetates. Allowance was made for ^{13}C contributions, but not ^{18}O contributions, to these ions. Sapogenin concentrations (**Table 1**) were calculated relative to sarsasapogenin propionate as the internal standard using response factors (R_F) determined by comparison of the integrated peak areas recorded for a reference solution containing known weights of sarsasapogenin propionate and sarsasapogenin acetate. All calculations were performed using purpose-written Excel spreadsheets.

RESULTS AND DISCUSSION

Synthesis of Deuterated Sapogenins. Oxidation of sarsasapogenin (**2a**) using sodium dichromate and concentrated sulfuric acid afforded sarsasapogenone (**1a**). Thereafter, two cycles of deuteration afforded material (**1b**) that exhibited an M^+ ion at *m/z* 418 compared to *m/z* 414 in **1a**. Similarly, the fragment ions that occurred at *m/z* 271, 285, and 300 in the mass spectrum of **1a** occurred at *m/z* 275, 289, and 304, respectively, in the mass spectrum of **1b**. Reduction of **1b** using sodium borohydride afforded a 1:4 mixture of [2,2,4,4-$^2H_4$]sarsasapogenin (**2b**) and [2,2,4,4-$^2H_4$]episarsasapogenin (**3b**). The relative contributions of the deuterated genins **2b** and **3b** in the synthetic mixture are comparable to those of the natural genins **2a** and **3a** detected in fecal samples following the dosing, in previous trials, of *N. ossifragum* plant material (*15*, *17*). Complete ^1H and ^{13}C NMR assignments (see Supporting Information) of **1b**, **2b**, and **3b** were derived from detailed analyses of one- and two-dimensional NMR spectral data, including COSY, TOCSY, ROESY, g-HSQC, and g-HMBC spectra. The presence of deuterium atoms at C-2 and C-4 suppressed the ^{13}C signals attributable to these atoms. Comparison of the g-HSQC spectra of **1a** and **1b** revealed the absence of signals attributable to H-2α, H-2β, H-4α, and H-4β in the g-HSQC spectrum of **1b**. In **1a** these signals occurred at 2.29, 2.13, 2.65, and 1.99 ppm, respectively. Similarly, the H-2α, H-2β, H-4α, and H-4β signals of sarsasapogenin (**2a**) and episarsasapogenin (**3a**) were not seen in the HSQC spectra of **2b** and **3b**, respectively.

Deuterium Retention Calculations prior to Dosing. Because, in dosing trials, the levels of **2a** and **3a** are determined by GC-MS analyses of the corresponding acetylated genins, that is, sarsasapogenin acetate (**2c**) and episarsasapogenin acetate (**3c**), respectively (*18*), deuterium incorporation was assessed using specimens of the acetylated deuterated genins **2d** and **3d**, respectively. The incorporation of one, two, three, or four deuterium atoms afforded M^+ ions at *m/z* 459, 460, 461, and 462, respectively, whereas the presence of an *m/z* 463 ion can be attributed to a natural abundance ^{13}C contribution, rather than a pentadeuterated contribution. Nondeuterated and deuterated M^+ ion contributions were therefore calculated as the sum of the *m/z* 458 and 459 ions and the *m/z* 460−463 ion clusters, respectively. This approach, which includes the natural ^{13}C isotope contribution to the *m/z* 458 (M^+) and 462 ([2,2,4,4-$^2H_4$]-M^+) ions, respectively, also assumes a negligible level of monodeuteration and ignores minor ^{18}O contributions. The percent deuterium enrichment, expressed as the sum of the $^2H_2$, $^2H_3$, $^2H_4$ and corresponding ^{13}C isotope contributions were calculated as 100 × $\sum$(*m/z* 460−463)/$\sum$(*m/z* 458−463). Two deuteration cycles afforded deuterated sapogenin material, which when acetylated and analyzed by SIM GC-MS showed 99.9% deuterium incorporation.

Because of the low relative intensity of the molecular ion cluster of deuterated sapogenin acetates, deuterium retention during the two dosing periods was determined by monitoring *m/z* 315−320 ion cluster contributions. This ion cluster was the most intense of six diagnostic ion clusters that occur at *m/z* 344−349, 329−334, 315−320, 284−289, 269−274, and 255−259 in the mass spectra of the deuterated acetates **2d** and **3d**. Because

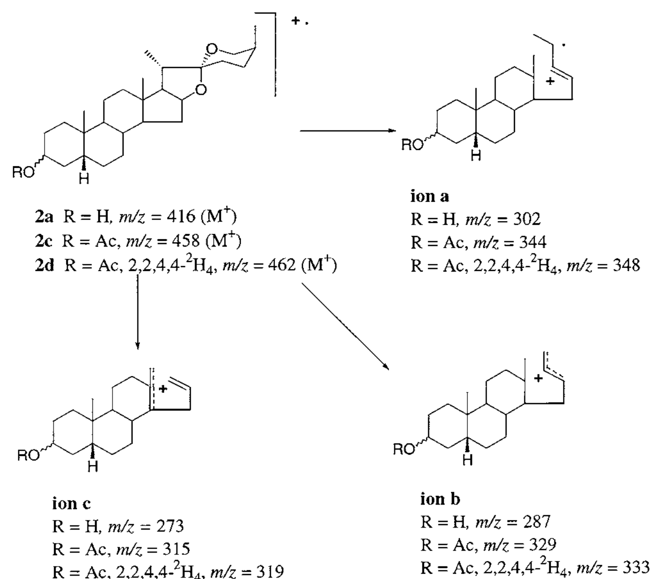

Figure 2. Postulated mass spectral fragmentation pathways for sapogenins **2a**, **2c**, **2d**, **3a**, **3c**, and **3d** leading to the ions referred to in the text.

the *m/z* 284−289, 269−274, and 255−259 ion clusters arise by loss of HOAc or [^{2}H]OAc from the *m/z* 344−349, 329−334, and 315−320 ion clusters, respectively, it was not appropriate to use the *m/z* 284−289, 269−274, and 255−259 ion clusters for quantitative deuterium retention calculations. The percent deuteration levels of sapogenins prior to dosing were calculated as 100 × ∑(*m/z* 317−320)/∑(*m/z* 315−320), on the basis of the ion intensities determined for subsamples of the acetylated variants of the parent genins. The average deuterium content of the *m/z* 315−320 ion cluster of an acetylated subsample of episarsasapogenin was determined to be 94.6%, compared to 99.9% for the *m/z* 458−463 (M^+) ion cluster. This difference can be attributed to a small part of the *m/z* 315−320 ion current originating from fragmentation ions other than those arising from the isotopically labeled variants of ion c (**Figure 2**). Experimentally determined deuterium retention levels were corrected (see below) for this discrepancy.

Dosing Trial Results. Previous investigations have shown that in the rumen of a dosed sheep sapogenins are partly oxidized and reduced to afford episapogenins, which, along with sapogenins and low levels of oxidized sapogenins, are ultimately secreted in fecal material but not in urine (*15*, *18*). Fecal samples were collected daily before and during the trial period. Representative subsamples were freeze-dried, extracted, and analyzed using previously reported SIM GC-MS methodology (*19*), modified to included *m/z* 271−275 (deuterated ketogenins) and *m/z* 315−320 ion clusters (deuterated acetates). Total genin levels were determined using *m/z* 139 ion responses (*19*), whereas the *m/z* 271−275 and 315−320 ion data were used to determine the deuterium retention levels of ketogenins and genins, respectively. The hay-fed ad libitum throughout the dosing trial was shown to contain no sapogenins. Similarly, no sapogenins were detected in feces collected prior to the commencement of the two dosing trials, that is, on day 0, prior to the dosing of the deuterated ketone, and on day 9, prior to the dosing of the deuterated alcohol mixture.

In each of the dosing trials, maximum genin levels were found in fecal material 2−3 days after the respective deuterated genins were dosed (**Table 1**). Thereafter, genin levels declined to negligible levels 5−6 days after dosing. Appreciable levels of ketogenins and genins were detected in day 2−3 fecal samples (**Table 1**). SIM GC-MS analyses showed that although episarsasapogenin and sarsasapogenin retained predominantly three or four deuterium atoms, as evidenced by strong *m/z* 318 and 319 ion contributions, recovered sarsasapogenone predominantly exhibited *m/z* 271, 272, and 273 ion responses corresponding to the presence of none, one, or two deuterium atoms, respectively. Deuterium retention analyses of stored batches of the deuterated ketone (**1b**), performed several months after the dosing trial, unexpectedly showed the presence of only one or two deuterium atoms; however, analyses of the genin alcohols derived by sodium borohydride reduction of the stored ketone material showed that the reduction products predominantly retained three or four deuterium atoms. These observations can be interpreted as indicating that although the majority of deuterium atoms incorporated into deuterated ketone (**1b**) were retained during ovine metabolism of **1b** to **2b** and **3b**, deuterium loss from unmetabolized ketone may have occurred during the extraction, hydrolysis (conjugate extracts only), and/or derivatization steps during the GC-MS analysis protocol.

Table 2. Uncorrected and Corrected Percent $^2H_2$–$^2H_4$[a] Retention Levels Determined for the *m/z* 315–320 Ion Cluster of Acetylated Episarsasapogenin Recovered from Fecal Samples Collected during the Dosing Experiments

day	uncorrected % $^2H_{2c}$–$^2H_4$ free	uncorrected % $^2H_{2c}$–$^2H_4$ conjugated	corrected % $^2H_2$–$^2H_4$ free[b]	corrected % $^2H_2$–$^2H_4$ conjugated[b]
		Ketone[c] Experiment		
2	90.1	90.9	95.2	96.1
3	88.4	87.8	93.4	92.8
		Alcohol[d] Experiment		
11	91.5	90.5	96.7	95.6
12	87.4	85.6	92.4	90.4

[a] Uncorrected % $^2H_2$–$^2H_4$ = 100 × ∑(*m*/*z* 317–320)/∑(*m*/*z* 315–320); corrected % $^2H_2$–$^2H_4$ = 100 × ∑(*m*/*z* 317–320)/∑(*m*/*z* 315–320) ÷ 0.946, where the average % $^2H_4$ incorporation in the dosed substrates was 94.6%. [b] Free = genin material obtained by CH_2Cl_2 extraction and acetylation; conjugated = genin material obtained by MeOH extraction, hydrolysis, and acetylation. [c] Ketone = [2,2,4,4-$^2H_4$]sarsasapogenone. [d] Alcohol = 4:1 mixture of [2,2,4,4-$^2H_4$]episarsasapogenin and [2,2,4,4-$^2H_4$]sarsasapogenin.

Our results showed that some loss of deuterium from the deuterated genin alcohols **2b** and **3b** occurs during ovine metabolism and/or extraction and chemical analyses, as evidenced by a moderate reduction in the relative $^2H_4$ contribution and an increased $^2H_3$ contribution (see Supporting Information), but the total $^2H_2$−$^2H_4$ contribution remains essentially unchanged because the reduced $^2H_4$ contribution is offset by an increased $^2H_3$ contribution. When corrected for an average $^2H_2$−$^2H_4$ content of 94.6%, calculated using the *m/z* 315−320 ion cluster of [2,2,4,4-$^2H_4$]episarsasapogenin, the recovered $^2H_2$−$^2H_4$ contribution was ∼90−96% (average = 94%) of that dosed (**Table 2**). Although the principle objective of this trial was to assess the extent to which deuterium was retained in the deuterated genin substrates during ovine metabolism, and subsequent workup and analysis procedures, the results showed there to be a satisfactory overall recovery (241 mg, ∼85%) of dosed sapogenin material, calculated as the sum of total sapogenins (mg/kg of DM) multiplied by the daily weight of fecal material (kg of DM) (**Table 1**). Because only a single sheep was dosed in the trials reported in this paper, care must be exercised when the results presented in **Tables 1** and **2** are interpreted. The results are, however, consistent with levels of free and conjugated sapogenins and episapogenins detected in previous dosing trials (*15*, *17*). Prior to the use of the methodology in a field trial, there is a need to validate the

methodology in a multiple-sheep trial (e.g., $n = 3$) in which the deuterated subtrate is dosed over an extended time period.

During the present and previous dosing trials (*15*, *17*) we have observed (but not reported or discussed) that ruminal metabolism of β-sitosterol (a constituent of many plants grazed by animals) affords three isomeric dihydroanalogues of β-sitosterol, which, when acetylated, exhibit significant m/z 458 (M^+) and m/z 315 ions. The GC-MS retention times of two of the three dihydro-β-sitosterol acetate isomers detected in the extracts are similar to those of sarsasapogenin and episarsasapogenin acetates; however, provided care was taken to adjust GC-MS acquisition conditions to minimize peak overlap, interference was not significant.

Our results show that the deuterated alcohol mixture consisting of **2b** and **3b** is a suitable dosing material for use in determining the percentage deuterium content of sapogenins recovered from sheep fecal material during a dosing trial. On the other hand, the lower recovery of [2,2,4,4-$^2H_4$]sarsasapogenone (**1b**) (~55%), possibly attributable to more extensive rumen metabolism, including complete degradation, and considerable loss of deuterium from **1b** during the dosing trial show that this substrate is not a suitable dosing material.

ABBREVIATIONS USED

COSY, correlated spectroscopy; GC-MS, gas chromatography−mass spectrometry; g-HMBC, pulsed field gradient heteronuclear multiple bond correlation; g-HSQC, pulsed field gradient heteronuclear single quantum coherence; m/z, mass to charge ratio; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; ROESY, rotating frame Overhauser effect spectroscopy; SIM, selected ion mode; TOCSY, total correlation spectroscopy.

Supporting Information Available: Table of ^{1}H and ^{13}C NMR assigments determined for sarsasapogenin, [2,2,4,4-$^2H_4$]-sarsasapogenin, and [2,2,4,4-$^2H_4$]episarsasapogenin (δ in $CDCl_3$) and figures showing the relative percent contributions of ions contributing to ion a, ion a-HOAc (or [^{2}H]OAc), ion b, ion b-HOAc (or [^{2}H]OAc), ion c, and ion c-HOAc (or [^{2}H]OAc) clusters determined for sarsasapogenin acetate (**2c**) and [2,2,4,4-$^2H_4$]episarsasapogenin acetate (**3d**).

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