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Intestinal and Fecal Sterols in Germfree and Conventional Rats

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The predominant sterols in feces and intestinal contents of germfree and control rats have been studied by combined gas chromatography-mass spectrometry.

Cholesterol, lathosterol, methostenol, campesterol, stigmasterol, and β-sitosterol were the predominant sterols in feces from germfree rats. The latter three sterols were derived from the diet. The same six sterols were found in the small intestine and the cecum, cholesterol being by far the major sterol in the small intestine. No coprostanol or coprostanol analogues of the plant sterols were found in the germfree rats.

Coprostanol, cholesterol, lathosterol, methostenol, 24α-ethyl-coprostanol, and β-sitosterol were the predominant sterols in the feces from the control animals. Only small amounts of coprostanol were seen in the small intestine whereas in the cecum and feces this was the major sterol.

The conversion of cholesterol * to coprostanol in the intestine is carried out by bacteria. In 1934 Dam [1] succeeded in obtaining an *in vitro* hydrogenation of cholesterol by human feces and by colon contents. This reaction has been further studied in different systems by Snog-Kjaer *et al.*[2] and by Coleman and Baumann [3] and the reaction mechanism has been investigated, *e.g.* by Rosenfeld *et al.*[4–6]

The composition of the fecal sterols in conventional rats has been extensively studied by Wells, Coleman and Baumann.[3,7–11] Danielsson and Gustafsson [12] found that in the feces of germfree rats on a steroid free diet at least 82 % of the neutral sterols consisted of cholesterol. Evrard *et al.*,[13–14] applying

* The following systematic names are given to steroids referred to in this report by trivial names: cholesterol, cholest-5-en-3β-ol; coprostanol, 5β-cholestan-3β-ol; lathosterol, 5α-cholest-7-en-3β-ol; methostenol, 4α-methyl-5α-cholest-7-en-3β-ol; β-sitosterol, 24α-ethyl-cholest-5-en-3β-ol; campesterol, 24α-methyl-cholest-5-en-3β-ol; stigmasterol, 24α-ethyl-cholest-5,22-dien-3β-ol.

gas chromatographic techniques, have confirmed the predominance of cholesterol among the fecal sterols of germfree rats and have shown that coprostanol is absent from the feces of germfree rats.

The use of germfree animals gives an excellent opportunity to study the influence of the intestinal microorganisms on the composition of the intestinal and fecal sterols. The present paper describes the identification by combined gas chromatography-mass spectrometry of the major intestinal and fecal sterols of germfree rats, and the results are compared with the corresponding findings in the conventional animals.

MATERIALS AND METHODS

Animals and diet. Rats were reared germfree according to the technique of Gustafsson,[15] and fed a standard diet *ad libitum*.[16] This diet is semisynthetic with 10 % peanut oil as source of fat. The sterols in the diet were investigated as described below. Control animals of the same stock were reared outside the germfree isolators on the same sterilized diet. Male rats, 3—6 months old, were used. The animals were kept in metabolism cages and feces collected every 24 h. Pooled feces from 3 day periods were used for sterol analyses. Rats were also killed and the small intestine and cecum were removed. The intestinal contents were obtained by washing with saline.

Isolation of fecal sterols. Feces were homogenized in chloroform-methanol, 1:1, with an Ultra-Turrax homogenizer (Janke and Kunkel, KG, Staufen i. Br., West Germany). The filtered chloroform-methanol extract was evaporated under reduced pressure and 0.1 ml of 4 N KOH and 0.1 ml of methanol per mg dry weight of the extract were added. After saponification for 2 h at 60° the hydrolysate was extracted three times with petroleum ether, the extracts were combined, washed with redistilled water until neutral, acidified with 0.1 M HCl and again washed with redistilled water until neutral. The petroleum ether phase was dried over Na_2SO_4, concentrated under reduced pressure and transferred to a test tube, where it was taken to dryness under a stream of nitrogen.

Isolation of intestinal sterols. The saline washings were homogenized with chloroform-methanol, 1:1, with an Ultra-Turrax homogenizer. The precipitate was filtered off and the phases were separated in a separatory funnel. The chloroform phase was collected, evaporated under reduced pressure and saponified and treated as described for the fecal sterols.

Isolation of dietary sterols. 20 g of diet (*i.e.* the approximate daily ration for a rat) was homogenized in chloroform-methanol as described for the feces. Water was added to the concentrated extract which was extracted continuously with petroleum ether for 12 h. The water phase was evaporated under reduced pressure and subjected to acid hydrolysis (see Results). The petroleum ether extract was subjected to alkaline hydrolysis. The hydrolysates were worked up essentially as described above for the feces. The sterols from the alkaline hydrolysate were purified on a silicic acid column. The samples were applied to the column in benzene and after washing with benzene the sterols were eluted with 2 % ethyl acetate in benzene.

Analysis of the sterol samples. The neutral steroids were analyzed by gas-liquid chromatography. The following stationary phases were used: 2 % F-60, 3 % QF-1, and 0.5 % CNSi.[17] The sterols were analyzed before and after preparation of their corresponding trimethylsilyl (TMSi) ethers.[17]

For further identification the TMSi ethers were analyzed in a gas chromatography-mass spectrometry combination instrument (GC-MS instrument) using the conditions described in the paper by Eneroth *et al.*[18]

RESULTS

Dietary sterols. The dietary sterols were analyzed as TMSi ethers in the GC-MS instrument on a 2 % F-60 column at 240°C. Four major peaks were found. The retention times and the mass spectral fragmentation pattern

indicated that the compounds were cholesteryl, campesteryl, stigmasteryl, and β-sitosteryl TMSi ethers, respectively. These identifications were verified by comparison with the authentic compounds. Two minor peaks on each side of cholesteryl TMSi ether were shown by mass spectrometric analysis not to contain any steroids.

In the preparation of the diet, solutions of tocopheryl acetate and of vitamins A, D, and K were added.[16] Since these compounds might give rise to peaks in the gas chromatograms an aliquot corresponding to about a daily ration was taken from each of the above mentioned solutions and carried through the procedure for sterol analysis. When these amounts were analyzed only the tocopheryl acetate sample produced any peaks.

On a 2 % F-60 column at 210° these peaks had RRT:s (retention times relative to cholesterol) of 0.90, 1.21, and 1.56. These peaks usually did not interfere in the sterol analysis of feces and intestinal contents since they were small compared with the sterol peaks.

In order to investigate the possible presence of sterol glycosides the aqueous solution remaining after extraction of the saponified lipids with petroleum ether was subjected to acid hydrolysis with 1 % H_2SO_4 in methanol. After refluxing for 13 h the solution was concentrated, diluted with water and extracted with petroleum ether. No sterols, however, were found upon GLC analysis of this extract.

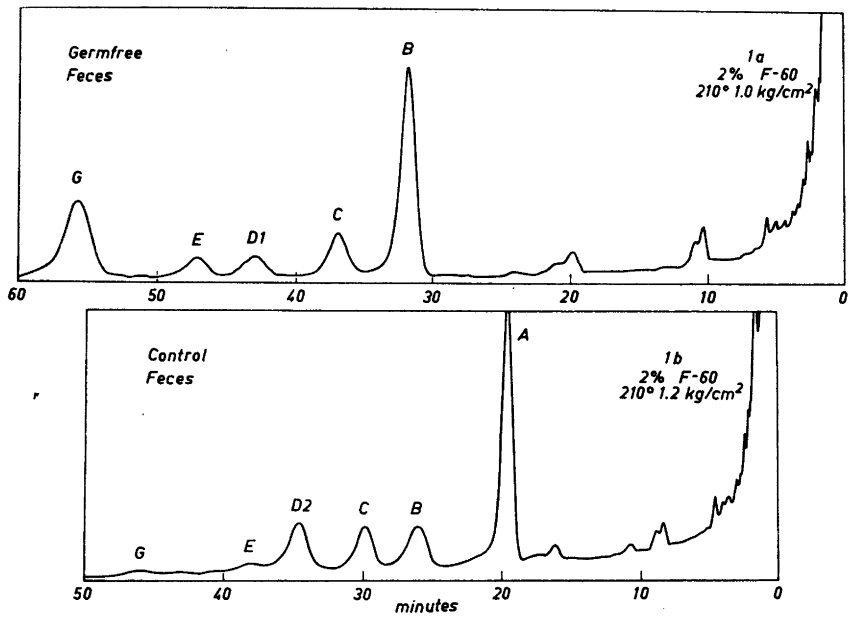

Fig. 1. Gas chromatograms of the TMSi ethers of fecal sterols from germfree (1a) and control (1b) rats. For identification of the peaks, see text. Note that the argon inlet pressure was different in the two analyses.

Fecal sterols. Fig. 1 shows the gas chromatograms of the TMSi derivatives of the fecal sterols on 2 % F-60. The major components were identified by GC-MS analysis.

Fecal sterols of the germfree animals. The peaks referred to are those shown in the chromatogram of Fig. 1a.

Peak B. Gas chromatographic and mass spectrometric data were the same as those found for cholesteryl TMSi ether.

Peak C. The retention time of this compound was the same as that found for lathosteryl TMSi ether. The mass spectrum is shown in Fig. 2. The cracking pattern is in good agreement with that of lathosteryl TMSi ether, which is also shown in Fig. 2. Peaks are found at *m/e* 458 (molecular ion: M), 443 (M − 15), 368 (M − 90), 353 (M − (90 + 15)), 255 (M − (side chain + 90)), 229, 213 (ABC-rings). The peak at *m/e* 129 has a low relative intensity in striking contrast to the high intensity of this peak in the mass spectra obtained for 3-TMSi ethers of Δ^5-sterols.[18] The relative intensity of the peak at *m/e* 108 is low. This peak is pronounced in spectra of 3-TMSi ethers of 5β-sterols.[18] The peak at *m/e* 229 (of unknown origin) is very prominent which is not the case in spectra of 3-TMSi ethers of saturated sterols or mono-unsaturated sterols with the double bond in the 5—6 position.

Peak D1. Gas chromatographic and mass spectrometric data were the same as those found for campesteryl TMSi ether.[18]

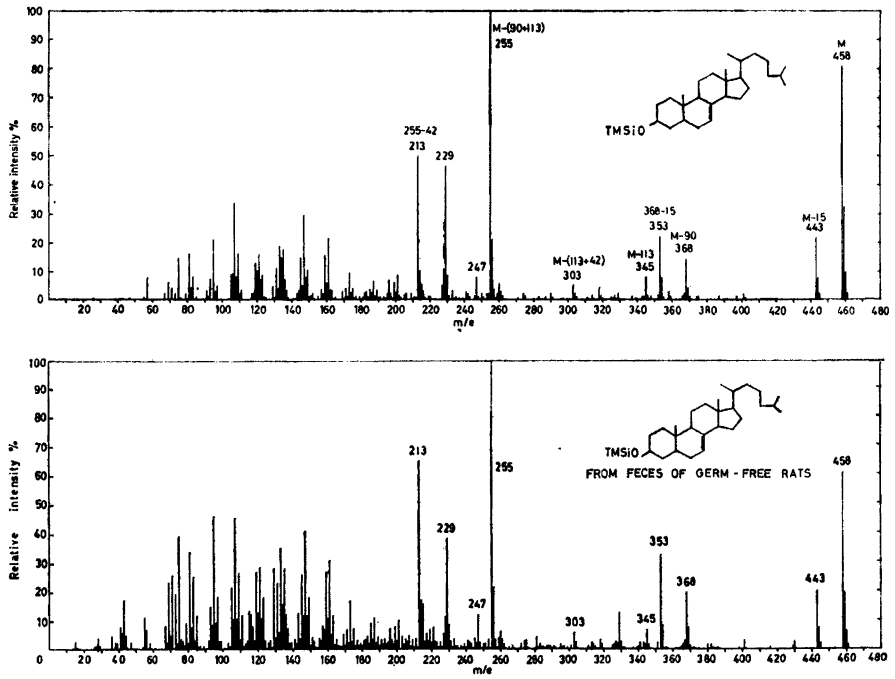

Fig. 2. Mass spectrum of compound C shown in Fig. 1a (lower panel) and of authentic lathosteryl TMSi ether (upper panel).

Peak E. The retention time of this peak was the same as that of a mixture of the TMSi ethers of stigmasterol and methostenol.

The mass spectrum recorded from the effluent constituting peak E is shown in Fig. 3. It is seen to consist of the fragmentation patterns of two compounds: stigmasteryl and methostenyl TMSi ethers. The characteristic peaks at *m/e* 484, 394, 343, 255, 213, 129, and 83 all belong to the stigmasterol component.[19] The peaks at *m/e* 472, 457, 382, 367, 269, 243, and 227 are due to the presence of methostenyl TMSi ether. The mass spectrum of the latter compound is also shown in Fig. 3.

The cracking pattern of methostenyl TMSi ether in the region below *m/e* 190 resembles that of the TMSi ether of lathosterol. This is also true of the high mass range except that the methostenol fragments are increased by 14 mass units as compared to the lathosterol fragments because of the presence of a 4α methyl group in the former compound. The considerations concerning the intensities of the peaks at *m/e* 108 and *m/e* 129 in the mass spectrum of lathosteryl TMSi ether can also be applied to the spectrum of methostenyl TMSi ether.

Peak G. Gas chromatographic and mass spectrometric data were the same as those found for β-sitosteryl TMSi ether.

Further support for the identifications described was provided by the analyses of the sterols and their TMSi ethers on both the F-60 and the QF-1

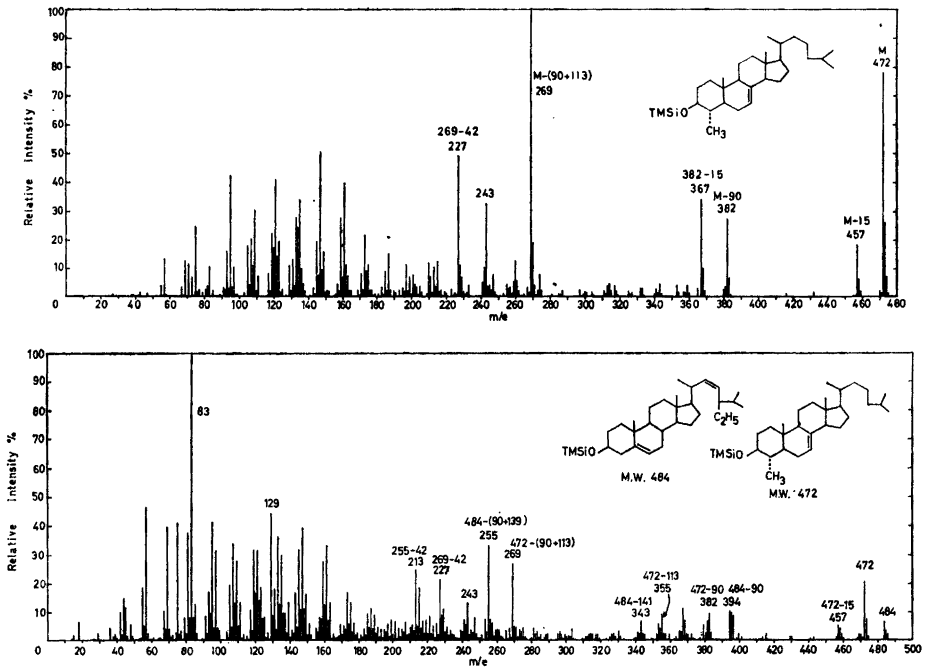

Fig. 3. Mass spectrum of the compounds constituting peak E shown in Fig. 1a (lower panel) and of authentic methostenyl TMSi ether (upper panel).

columns. The results are summarized in Table 1. It is seen that methostenol and campesterol appeared as a compound peak before stigmasterol when the free sterols were analyzed on the F-60 column. This was confirmed by mass spectrometry. As already mentioned the TMSi ether of campesterol appeared before the compound peak of the TMSi ethers of methostenol and stigmasterol when the TMSi ethers were analyzed.

A small peak with an RRT of 1.27 was seen when the free sterols were analyzed on the F-60 column (*cf.* Fig. 4). This compound was not further studied.

Fecal sterols of the control animals. The peaks referred to are those shown in the chromatogram of Fig. 1b.

Gas chromatographic (see Table 1) and mass spectrometric data gave the following information concerning the identity of the major compounds: Peak A, coprostanyl TMSi ether; Peak B, cholesteryl TMSi ether; Peak C, lathosteryl TMSi ether; Peak D2, 24α-ethylcoprostanyl TMSi ether; Peak E, methostenyl TMSi ether; Peak G, β-sitosteryl TMSi ether.

Although the compounds identified were the predominant sterols the mass spectra indicated that some peaks also contained minor components. Some of these were seen as separate peaks in the gas chromatograms of the free sterols. They were not further studied.

Sterols in the intestinal contents of germfree and control animals. Fig. 4 shows the gas chromatograms of the sterols in the small and large intestines

Table 1. Retention times relative to cholesterol of the fecal sterols and their trimethylsilyl ethers on F-60 and QF-1 columns.

Germfree animals	2 % F-60		3 % QF-1	
	OH*	TMSi*	OH*	TMSi*
Cholesterol	1.00	1.26	1.00	0.71
Lathosterol	1.14	1.46	1.14	0.80
Campesterol	1.37	1.70	1.30	0.92
Methostenol		1.87		
Stigmasterol	1.50			0.97
β-Sitosterol	1.78	2.21	1.57	1.11
Control animals	**2 % F-60**		**3 % QF-1**	
	OH	TMSi	OH	TMSi
Coprostanol	0.89	0.95	0.88	0.55
Cholesterol	1.00	1.26	1.00	0.71
Lathosterol	1.14	1.45	1.15	0.79
Methostenol	1.37	1.85	1.26	0.86
24α-Ethylcoprostanol	1.56	1.68	1.38	
β-Sitosterol	1.78	2.22	1.58	—

* OH = sterols with free hydroxyl group;
TMSi = trimethylsilyl ethers.

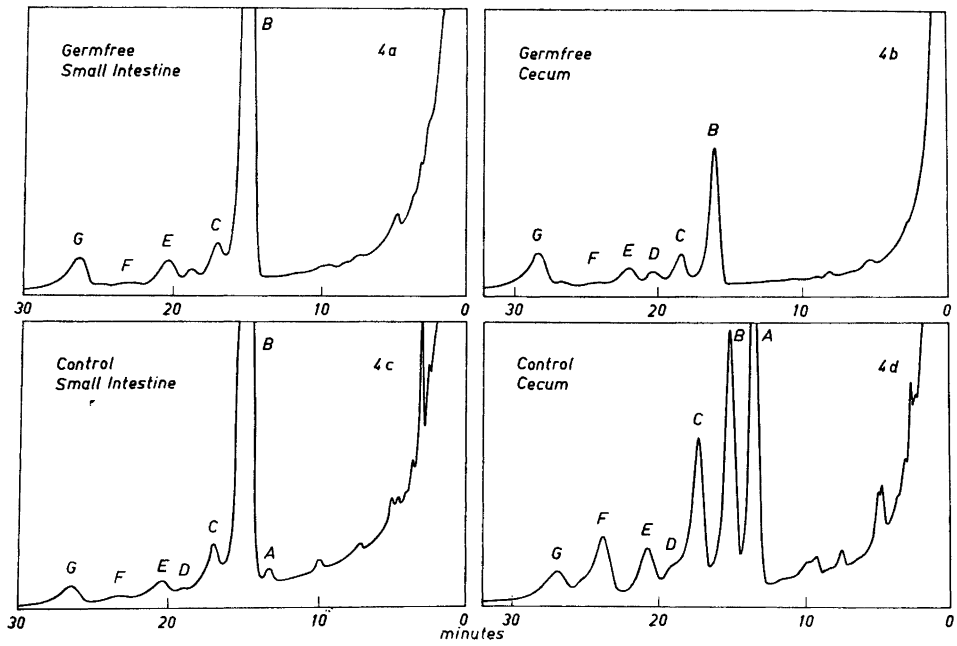

Fig. 4. Gas chromatograms of sterols in the small intestinal contents (4a) and cecum (4b) of germfree rats and control rats (4c and 4d). Column: 2 % F-60, 210°, argon inlet pressure 1.8 kg/cm² except for Fig. 4b where it was 1.7 kg/cm².

of germfree and control animals. The RRT:s and the tentative identifications of the compounds are given in Table 2. The analyzes were made at different times and the variations in the RRT:s are within the limits of error of the determinations. Peak D, present in all the chromatograms, was not identified. Peak F which has the RRT of 24α-ethylcoprostanol or stigmasterol might

Table 2. Retention times relative to cholesterol of sterols in the intestinal contents analyzed on a 2 % F-60 column at 210°.

Peak (as indicated in Fig. 4)	Compound	Germfree animals		Control animals	
		Small intest.	Cecum	Small intest.	Cecum
A	Coprostanol	—	—	0.88	0.89
B	Cholesterol	1.00	1.00	1.00	1.00
C	Lathosterol	1.13	1.15	1.13	1.15
D	Unknown	1.25	1.26	1.27	1.26
E	Campesterol and/or methostenol	1.36	1.37	1.36	1.39
F	24α-Ethylcoprostanol	—*	—*	—*	1.58
G	β-Sitosterol	1.76	1.76	1.76	1.79

* See text.

partly represent the product from tocopheryl acetate mentioned under "Dietary sterols". However, the relatively large peak F seen in the chromatogram of cecal sterols from the control animals probably represents mainly 24α-ethylcoprostanol.

As seen in Fig. 4a and b there is no coprostanol in the germfree rats. The amount of coprostanol present in the small intestine of the control animals is extremely small whereas in the cecum it is the predominant sterol (Fig. 4d).

As is evident from Fig. 4a-d the amount of lathosterol, methostenol, and the plant sterols relative to that of cholesterol (and coprostanol) is much larger in the cecum than in the small intestine. Quantitative determinations of the ratios between these sterols in the small intestine are difficult because of the very large proportion of cholesterol present. In the cecum the ratio of cholesterol-coprostanol on one hand to lathosterol, methostenol, and the plant sterols on the other was roughly 1.0 and 1.5 in the germfree and control animals, respectively.

DISCUSSION

The purpose of the present investigation was to study the nature of the predominant sterols in feces and intestinal contents of germfree rats. It should therefore be pointed out that no attempts were made to identify minor components, *e.g.* cholestanol which has been shown to be formed in germfree pigs.[20]

Cholesterol, lathosterol, methostenol, and the dietary plant sterols, β-sitosterol, campesterol, and stigmasterol, were the predominant sterols in feces from germfree rats. No coprostanol could be found which is in agreement with the studies by Evrard *et al.*[14] and with the general view [1-4] that this sterol is formed by the action of intestinal microorganisms on cholesterol.

In contrast to the germfree animals the control animals excreted predominantly coprostanol and 24α-ethylcoprostanol in the feces. Common to both groups of animals was the presence in feces of the two Δ^7-stenols lathosterol and methostenol.

Lathosterol was found by Fieser [21] in a commercial cholesterol preparation. Later it has been found in rodent skin,[22,23] in sebum of rats and mice,[24] and in rat feces.[7] The isolation of methostenol from rat feces was first described by Wells *et al.*[7,8] and its structure was studied by Neiderhiser and Wells.[25] It is likely that these two sterols are intermediates in one metabolic pathway between lanosterol and cholesterol.[26-28] The present study has shown that an intestinal flora is not required for their appearance in the feces. Our data, however, do not permit any conclusions as to the origin of the fecal Δ^7-sterols. They might be synthesized in the intestinal wall [27] but it is equally possible that they originate in the skin of the animals [27] and are ingested when the animals lick their fur. In fact Miettinen (personal communication) has found that if conventional rats are put in restraining cages which prevent their licking of the fur there is a pronounced decrease in the amount of lathosterol and methostenol in the feces.

The proportions between lathosterol, methostenol, and the plant sterols at different levels of the intestinal tract were of the same order of magnitude in the control and in the germfree rats. Thus the ratios in the germfree rats

between β-sitosterol and lathosterol (which could be measured as single compounds) were about 1.5, 1.8, and 2.5 in the small intestine, cecum, and feces, respectively. This indicates that lathosterol is poorly absorbed or is secreted into the intestinal lumen since it is known that very little β-sitosterol is absorbed. The ratio between cholesterol and coprostanol on the one hand and the Δ^7-stenols and plant sterols on the other hand was much higher in the small intestine than in the cecum and feces. This is certainly due to the absorption of cholesterol in the small intestine. In the cecum the latter ratio was about 1.5 in the control animals and 1.0 in the germfree animals. It is possible that this difference between germfree and control rats is due to the poor absorption of coprostanol in the control animals (see discussion in Ref. 29). Further studies of the quantitative aspects of sterol excretion in germfree and normal animals are, however, needed to clarify this point and the question of the absorption of Δ^7-stenols.

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