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Clemmensen reduction of diosgenin and kryptogenin: synthesis of [16,16,22,22,23,23-$^2H_6$]-(25*R*)-26-hydroxycholesterol

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Abstract

A new experimental protocol has been established for the Clemmensen reduction of diosgenin and kryptogenin with the aim to prepare deuterated isotopomers of (25*R*)-26-hydroxycholesterol. Uncontrolled deuteration has been achieved from diosgenin, whereas [16,16,22,22,23,23-$^2H_6$]-(25*R*)-26-hydroxycholesterol (**1**) can be synthesized from kryptogenin.

Keywords: Deuterated isotopomers; 26-Hydroxycholesterol; Clemmensen reduction; Kryptogenin; Diosgenin; Cholesterol metabolism

1. Introduction

The biological importance of (25*R*)-26-hydroxycholesterol (**1**), a major intermediate in the metabolic pathway from cholesterol to bile acids [1,2], is well established and is related to its role in the regulation of cholesterol homeostasis [3,4] or inhibition of HMG-CoaA reductase [5]. In connection with studies concerning the evaluation of serum levels of 26-hydroxycholesterol (**1**) in patients with primary biliary cirrhosis [6], it became necessary to prepare deuterated **1** for intravenous infusions aimed to evaluate the 26-hydroxylation pathway in bile acid production [7]. In a previous paper, we have prepared a tetradeuterated 26-hydroxycholesterol, [7,7,21,21-$^2H_4$]-26-hydroxycholesterol (**1-d$_4$**), from the deuterated steroid intermediate, (20*S*)-[7,7,21,21-$^2H_4$]-20-methyl-pregn-5-en-3β,21-diol [8] coupled to a suitable C-5 synthon [9] (Fig. 1).

However, for studies on the metabolism of cholesterol involving the position 7, sterols labeled in the side chain or in ring D are much more suitable. For this purpose, synthesis of deuterated isotopomers of **1** could be realized starting from two natural isoprenoids, diosgenin (**2**) and kryptogenin (**3**) using the classical Clemmensen reduction [10-13] (Fig. 2), although the non-reproducibility of the Clemmensen reaction may constitute a limitation of the approach. Furthermore, kryptogenin (**3**) is no longer commercially available and the reported harsh conditions required for the Zn/HCl reduction cause extensive enolization of the carbonyl groups. As a result, five to nine deuterium atoms were incorporated into the deuterated 26-hydroxycholesterol (**1**) starting from **3** [14].

A recently described modification of the Clemmensen reduction for the synthesis of (25*R*)-26-hydroxycholesterol (**1**) from diosgenin (**2**) [15] renewed our interest for the classical procedure that, if adequately controlled, could still represent an useful method for the introduction of deuterium label into the side chain and at position 16 as well. We present here our results on the study carried out to establish experimental condition for a controlled Clemmensen reduction of diosgenin (**2**) that could be applied to the preparation of deuterated (25*R*)-cholest-5-en-3β,16β,26-triol (**4**) (Scheme 1). This compound can be transformed into a deuterated isotopomer of 25(*R*)-26-hydroxycholesterol (**1**) through the procedure described by Williams et al. [15]. Application of our protocol to kryptogenin diacetate (**5**) has then led directly to (25*R*)-26-hydroxycholesterol (**1**) and related isotopomers.

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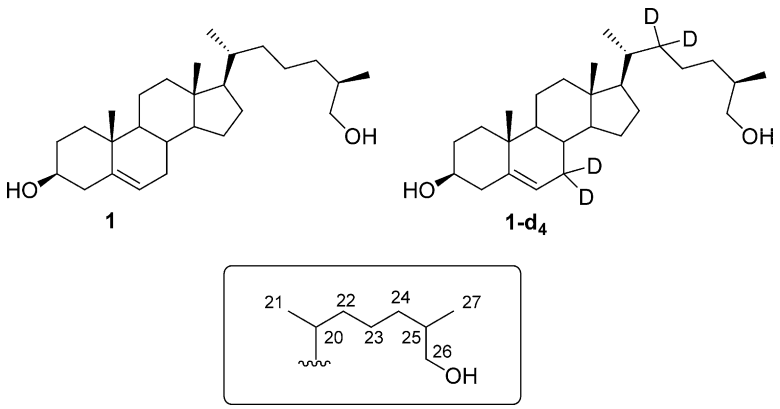


Fig. 1. Structure of unlabeled and labeled (25*R*)-26-hydroxycholesterol (**1**) and (**1-d$_4$**).

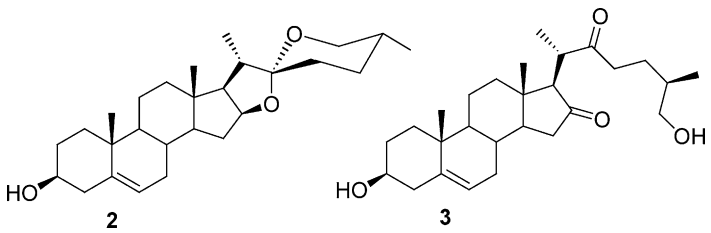


Fig. 2. Structure of diosgenin (**2**) and kryptogenin (**3**).

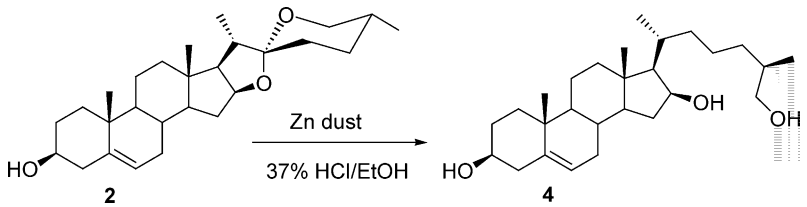
2. Experimental

2.1. General

Melting points were recorded on Stuart Scientific SMP3 instrument and are uncorrected. All reagents were purchased from Sigma-Aldrich. All reactions were monitored by TLC on silica gel 60 F_{254} precoated plates with a fluorescent indicator (Merck). Flash chromatography [16] was performed using silica gel 60 (230–400 mesh, Merck). Mass spectra were carried out using a Hewlett Packard 5988A instrument set at 70 eV ion energy, 0.1 mA emission current and 280 °C transfer line temperature. All synthetic compounds were analyzed after derivatisation with trimethylsilylimidazole:piperidine (1:1) for 10 min at room temperature to obtain the trimethylsilyl (TMS) ethers. GC–MS analyses were performed on an SPB5 (Supelco Inc.) capillary column 0.32-mm i.d., 0.25 μm film thickness, 30-m long, operating at 20 ml/min helium flow rate. Column temperature was programmed from 180 to 300 °C at 10 °C/min. Mass spectral data are given as *m/z* (relative abundance).

2.2. NMR experiments

All NMR spectra were recorded on a Bruker AM-500 spectrometer operating at 500.13 and 125.76 MHz for ^{1}H and ^{13}C, respectively, in $CDCl_3$ solutions. The sample temperature was 303 K. Chemical shifts are reported as δ (ppm) relative to $CHCl_3$ fixed at 7.24 ppm for ^{1}H NMR spectra and to $CDCl_3$ fixed at 77.00 ppm for ^{13}C NMR; coupling constants (*J*) are given in hertz. NMR signals were assigned by a combination of 1D ^{1}H, ^{13}C NMR and standard COSY and HSQC experiments. For overlapped signals of hydrogen atoms important for the identification of deuterium position, the 1D HOHAHA technique [17] was used to obtain the chemical shifts and coupling constants.



Scheme 1. Synthesis of (25*R*)-cholest-5-en-3β,16β,26-triol (**4**) from diosgenin (**2**).

2.3. (25R)-Cholest-5-en-3β,16 β,26-triol (4)

A mixture of diosgenin (**2**) (0.10 g; 0.24 mmol), Zn dust (2.25 g, 34.4 mmol) and ethanol (15.0 ml) was refluxed for 15 min and then 37% HCl (7.0 ml) was added during a period of 15 min. At the end of the addition, the reaction, monitored by TCL (CH_2Cl_2/MeOH, 97:3, v/v), was cooled to room temperature and filtered to remove Zn dust. CH_2Cl_2 (20.0 ml) was then added to the solution and the organic layer was washed with water, aqueous saturated $NaHCO_3$ and water. The organic phase was dried with Na_2SO_4 and the solvent evaporated at reduced pressure. The residue was purified by flash chromatography (CH_2Cl_2/MeOH, 99:1, v/v) to give the product **4** as a white solid (0.050 g, 50%). Mp 175–177 °C (lit. [15], Mp 172–174 °C); ^{1}H NMR ($CDCl_3$), (: 0.86 (3H, s, 18-CH_3), 0.89 (3H, d, J = 7.0 Hz, 27-CH_3), 0.96 (3H, d, J = 7.0 Hz, 21-CH_3), 0.99 (3H, s, 19-CH_3), 3.42 (1H, dd, J = 5.6, 10.5 Hz, 26-H_a), 3.46 (1H, dd, J = 6.3, 10.5 Hz, 26-H_b), 3.50 (1H, tt, J = 4.9, 11.2 Hz, 3α-H), 4.33 (1H, dt, J = 4.2, 7.3, 16α-H), 5.33 (1H, dd, J = 1.4, 4.9 Hz, 6-H). MS: see Table 1.

2.4. Deuterated (25R)-cholest-5-en-3β,16β,26-triol

The reaction was performed following the same condition described in the previous procedure using deuterated reagents. From diosgenin (**2**) (0.1 g, 0.24 mmol) reacted with Zn dust (2.25 g, 34.4 mmol), EtOD (15.0 ml) and 37% DCl (7.0 ml), the deuterated compound **4** was obtained as a white solid (0.051 g, 50%). All ^{1}H NMR signals of hydrogen atoms are identical to those reported for non-deuterated compound **4**. Only the integration of the complex signals between 0.90 and 2.30 ppm indicates the presence of deuterium atoms. For MS fragmentation data see Table 1.

2.5. Cholest-5-en-3β,26-diol (1)

Kryptogenin diacetate (**5**) was prepared by an overnight reaction from kryptogenin (**3**) (0.2 g, 0.46 mmol), acetic anhydride (0.4 ml) and pyridine (0.8 ml) in 82% yield (0.206 g). To a solution of kryptogenin diacetate (**5**) (0.1 g, 0.18 mmol) in EtOH (14.0 ml), Zn dust (1.64 g, 25.0 mmol) was added and the mixture stirred and refluxed for 15 min. Then 37% HCl (3.0 ml) was added dropwise during a period of 15 min and the reaction, monitored by TLC (petroleum ether/AcOEt, 70:30, v/v), was slowly cooled to room temperature. After filtration of Zn dust, CH_2Cl_2 (20.0 ml) was added and the organic layer was washed with water, aqueous saturated $NaHCO_3$ and water. The organic solution was dried with Na_2SO_4 and the solvent evaporated at reduced pressure. The residue was purified by flash chromatography (petroleum ether/AcOEt, 80:20, v/v) to give the product **1** as a white solid (0.055 g, 76%). Mp 169–170 °C (from AcOEt; lit. [15] Mp 168–170 °C).^{1}H NMR ($CDCl_3$), δ: 0.65 (3H, s, 18-CH_3), 0.89 (6H, d, J = 7.0 Hz, 27-CH_3 and 21-CH_3), 0.98 (3H, s, 19-CH_3), 0.99–1.04 (1H, m, 24-H_a), 1.24–1.30 (1H, m, 24-H_b), 1.54–1.60 (1H, m, 25-H), 3.40 (1H, dd, J = 6.3 and 10.5 Hz, 26-H_a), 3.48 (1H, dd, J = 5.6 and 10.5 Hz, 26-H_b), 3.49 (1H, tt, J = 4.9, 11.2 Hz, 3α-H), 5.33 (1H, dd, J = 2.8 and 5.6 Hz, 6-H). ^{13}C NMR ($CDCl_3$), δ: 12.5 (C 18), 17.2 (C 27), 19.4 (C 21), 20.1 (C 19), 21.8 (C 11), 24.1 (C 23), 25.0 (C 15), 28.9 (C 16), 32.4 (C 2), 32.6 (C 7), 32.6 (C 8), 34.2 (C 24), 36.1 (C 20), 36.5 (C 25), 36.8 (C 22), 37.2 (C 10), 37.9 (C 1), 40.4 (C 12), 42.8 (C 4), 42.9 (C 13), 50.8 (C 9), 56.6 (C 17), 57.5 (C 14), 69.2 (C 26), 72.5 (C 3), 122.4 (C 6), 141.4 (C 5). MS: see Table 2.

Table 1
EI spectra of undeuterated and deuterated (25*R*)-cholest-5-en-3β,16 β,26-triol (**5**)[a]

m/z	Ion	Relative intensity[b]
Undeuterated		
634	$[M]^+$	15
544	[M–Me_3SiOH]	40
529	[544-Me]	25
454	[M–$(Me_3SiOH)_2$]	100
439	[454-Me]	20
364	[M–$(Me_3SiOH)_3$]	10
253	[454-silylated side chain]	20
Deuterated		
639	$^2H_5[M]^+$	9
638	$^2H_4[M]^+$	20
637	$^2H_3[M]^+$	20
636	$^2H_2[M]^+$	18
549	$^2H_5$[M–Me_3SiOH]	20
548	$^2H_4$[M–Me_3SiOH]	27
547	$^2H_3$[M–Me_3SiOH]	30
546	$^2H_2$[M–Me_3SiOH]	26
534	$^2H_5$[549-Me]	10
533	$^2H_4$[548-Me]	18
532	$^2H_3$[547-Me]	14
531	$^2H_2$[546-Me]	16
459	$^2H_5$[M–$(Me_3SiOH)_2$]	50
458	$^2H_4$[M–$(Me_3SiOH)_2$]	82
457	$^2H_3$[M–$(Me_3SiOH)_2$]	90
456	$^2H_2$[M–$(Me_3SiOH)_2$]	100
444	$^2H_5$[459-Me]	12
443	$^2H_4$[458-Me]	15
442	$^2H_3$[457-Me]	14
441	$^2H_2$[456-Me]	10
369	$^2H_5$[M–$(Me_3SiOH)_3$]	7
368	$^2H_4$[M–$(Me_3SiOH)_3$]	9
367	$^2H_3$[M–$(Me_3SiOH)_3$]	25
366	$^2H_2$[M–$(Me_3SiOH)_3$]	14
253	[459-silylated side chain][c]	40

[a] Derivatized as trimethylsilyl ether.
[b] Percentage of total ionisation.
[c] The same fragmentation would be obtained from *m/z* 456, 457 and 458.

2.6. [16,16,22,22,23,23-$^2H_6$]-cholest-5-en-3β,26-diol (1-d_6)

The reaction was performed following the same conditions described in the previous procedure using deuterated reagents. Treatment of kryptogenin diacetate (**5**) (0.1 g, 0.18 mmol) with Zn dust (1.64 g, 25.0 mmol), EtOD (15.0 ml) and 37% DCl (3.0 ml) afforded the compound **1-d_6** as white solid (0.056 g, 76%). ^{1}H NMR ($CDCl_3$), δ: 0.65 (3H, s, 18-

Table 2
EI spectra of undeuterated (**1**) and deuterated (25*R*)-cholest-5-en-3β,26-diol (**7**)[a]

m/z	Ion	Relative intensity[b]
Undeuterated		
546	$[M]^+$	43
531	[M–Me]	10
456	[M–Me_3SiOH]	100
441	[456-Me]	27
417	[M-129]	30
255	[456-silylated side chain]	20
Deuterated		
552	$^2H_6[M]^+$	45
537	$^2H_6$[M–Me]	10
462	$^2H_6$[M–Me_3SiOH]	100
447	$^2H_6$[462-Me]	25
423	$^2H_6$[M-129]	30
257	$^2H_6$[462-silylated side chain]	20

[a] Derivatized as trimethylsilyl ether.
[b] Percentage of total ionisation.

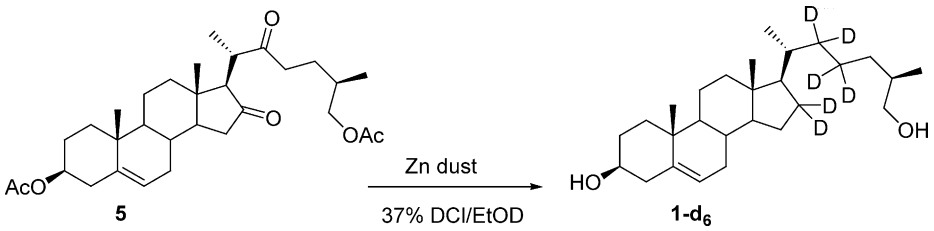
CH_3), 0.89 (6H, d, $J = 7.0$ Hz, 27-CH_3 and 21-CH_3), 0.99 (3H, s, 19-CH_3), 1.02 (1H, t, $J = 13.3$ Hz, 24-H_a), 1.35 (1H, dq, $J = 7.0$ and 9.8 Hz, 20-H), 1.27 (1H, dd, $J = 4.9$ and 13.3 Hz, 24-H_b), 1.54–1.60 (1H, m, 25-H), 3.40 (1H, dd, $J = 6.3$ and 10.5 Hz, 26-H_a), 3.48 (1H, dd, $J = 5.6$ and 10.5 Hz, 26-H_b), 3.49 (1H, dddd, $J = 4.9$, 4.9, 11.2, 11.2 Hz, 3α-H), 5.33 (1H, dd, $J = 2.8$ and 5.6 Hz, 6-H). ^{13}C NMR ($CDCl_3$), δ: 12.5 (C 18), 17.2 (C 27), 19.4 (C 21), 20.1 (C 19), 21.8 (C 11), 25.0 (C 15), 32.4 (C 2), 32.6 (C 7), 32.6 (C 8), 34.2 (C 24), 36.1 (C 20), 36.5 (C 25), 37.2 (C 10), 37.9 (C 1), 40.4 (C 12), 42.8 (C 4), 42.9 (C 13), 50.8 (C 9), 56.6 (C 17), 57.5 (C 14), 69.2 (C 26), 72.5 (C 3), 122.4 (C 6), 141.4 (C 5). MS: see Table 2.

3. Results and discussion

The recent report on a new experimental protocol for the Clemmensen reduction of diosgenin (**2**) [15] prompted us to repeat the procedure prior to application to the preparation of deuterated isotopomers of 26-hydroxycholesterol (**1**). The ratio of ethanol and concentrated HCl versus diosgenin (**2**) (ca. 100 ml and 75 ml per mmol) seemed too high especially in view of a deuteration process, but we repeated the reaction as described [15]. We obtained a mixture of products that was too complicated to be purified by crystallization and isolation of low yield of pure **5** required a careful purification by silica gel chromatography. Therefore, we monitored the formation of the product by TLC during the dropwise addition of HCl as described in the original procedure and found that, after addition of 30 ml/mmol, diosgenin (**2**) was cleanly converted to **4** in 50% yield. Additional HCl transformed the remaining diosgenin (**2**), but yield of **4** were only marginally increased and several side products were formed, including chlorinated sterols. In our hands, therefore, the Clemmensen reduction worked well with addition of concentrated HCl under controlled monitoring of the transformation of diosgenin (**2**) and it was preferable to stop at 50–60% conversion in order to avoid undesired products that would complicate the final purification.

In order to obtain the deuterated derivatives of compound **4**, we performed the Clemmensen reduction under our conditions using deuterated reagents. NMR spectra of the product could only suggest that labeling was located in the side chain and the integration of the complex signals between 0.90 and 2.30 ppm indicated involvement of positions going from C21 to C24. Analysis of mass spectra shows that a variable number of deuterium atoms were randomly incorporated into the structure. Table 1 reports the fragmentation pattern of unlabeled and labeled (25*R*)-cholest-5-en-3β,16β,26-triol (**4**) derivatized as trimethylsilyl ether (mol. wt. 634). The most intense peak in the non-deuterated compound is at *m/z* 454 representing the [M-($Me_3SiOH)_2$] ion that becomes *m/z* 456 in deuterated **4** corresponding to an enrichment of two deuterium atoms and is accompanied by close ions at *m/z* 457, 458, 459 of variable intensities. All other peaks show a similar pattern and it can be concluded that a mixture of deuterated isotopomers have been prepared from diosgenin (**2**). The fragment *m/z* 253 in the spectra of unlabeled and labeled **4** confirms the indications obtained by NMR about the presence of the label in the side chain, since this ion can be derived from M^+ by loss of three Me_3SiOH and of the side chain.

In order to find a more reliable deuteration procedure, we have also investigated the reduction of a sample of crude kryptogenin (**3**) available to us as a gift from Prof. Javitt and purified as the diacetate **5**. This compound was efficiently transformed (76% of purified product) into 26-hydroxycholesterol (**1**) under controlled conditions that use a lower amount of concentrated HCl (16 ml/mmol instead of 30 ml/mmol). Furthermore, using deuterated ethanol and HCl, a deuterated isotopomer of **1** was cleanly obtained. Comparing the mass spectra of the above deuterated with unlabeled compound **1**, it was possible to establish that six deuterium atoms were present in the structure (Table 2). In fact the most abundant ions present in the spectrum of unlabeled **1** [546(43), 531(10), 456(100), 441(27), 417(30)] became [552 (45), 537(10), 462(100), 447(25), 423 (30)] with nearly the same relative intensities. Moreover, the fragment at *m/z* 255 (loss of one Me_3SiOH and the silylated side chain) in non-deuterated **1** becames 257 in the deuterated isotopomer. These data highlight that four deuterium atoms were inserted in the side chain and two in the D ring. In order to assign the position of deuterium atoms, a detailed NMR study was performed. In the ^{1}H NMR spectrum of **1** the 16-H atoms resonate within a complex range between 1.76–1.86 and 1.45–1.60 ppm and the presence of deuterium atoms in position 16 could be clearly evidenced in the ^{13}C NMR spectrum that showed the absence of the resonance at 28.9 ppm corresponding to the C16. Assignment of the position of deuterium atoms in the side chain was achieved by the following experiments. Decoupling at 0.89 ppm (21-CH_3) simplified the double quartet



Scheme 2. Synthesis of [16,16,22,22,23,23-$^2H_6$]-cholest-5-en-3β,26-diol (**1-d$_6$**) from kryptogenin diacetate (**5**).

(dq) at 1.35 ppm that corresponds to the 20-H to a doublet ($J_{20,17}$ = 9.8 Hz), showing that no protons are present in position 22.

By decoupling the 26-H (3.40 and 3.48 ppm), it was possible to assign the 25-H resonance between 1.54 and 1.60 ppm that was used to identify one of position 24-H at 1.27 ppm. The splitting pattern of this hydrogen is a doublet (J_{gem} = 13.3 Hz), which suggests that no proton in position 23 was present. Furthermore, by 1D HOHAHA experiment [17] on 25-H the other 24-H was detected for which the splitting pattern is a t (1.02 ppm, J_{gem} = 13.3 and $J_{24,25}$ = 13.3 Hz) confirming the absence of proton atoms in position 23. Additional evidence of label at positions 22 and 23 was furnished by the ^{13}C NMR spectrum of deuterated compound **1-d$_6$** where the lack of peaks at 24.1 ppm (C23) and 36.8 ppm (C22) was observed. It can be therefore concluded that Clemmensen reduction of kryptogenin (**3**) allows a clean preparation of [16,16,22,22,23,23-$^2H_6$] isotopomer **1-d$_6$** (Scheme 2). We have, therefore, observed no introduction of label into positions C 15, C 17 and C 20 during the strongly acidic Clemmensen reduction conditions. This is probably due to the fact that enolization into these positions is slow compared to the rates of enolization at C 23 and the reduction process.

Results so far obtained may constitute a general approach to the synthesis of deuterium-labeled 26-hydroxycholesterol (**1**) required for biomedical applications. One drawback may be related to the reproducibility of the Clemmensen reduction that depends, among other factors, on the form of zinc to be used in the reaction. In fact, when zinc was activated by washing with 37% HCl, water and acetone, a heptadeuterated isotopomer of **1-d$_6$** was obtained. Additionally, it should be pointed out that, although samples of kryptogenin (**3**) are no longer available, it is still possible to rely on the method described by Barton for the conversion of diosgenin (**2**) into kryptogenin (**3**) [18]. We have repeated this procedure and confirmed the reported yield of 35–40% that could not be improved by attempted modifications of the experimental protocol. Nonetheless, the overall 30% yield for the conversion of diosgenin (**2**) to kryptogenin (**3**) and the following controlled Clemmensen reduction of kryptogenin diacetate (**5**) to hexadeuterated **1-d$_6$** make our procedure an easy three-step preparation of labeled 26-hydroxycholesterol. Application of controlled conditions for the Clemmensen reaction to other steroid ketones is under current investigation, in order to verify whether the observed selectivity for kryptogenin (**3**) is a more general feature of the reaction.

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