The ozonation of cholesterol: separation and identification of 2,4-dinitrophenylhydrazine derivatization products of 3β-hydroxy-5-oxo-5,6-secocholestan-6-al

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The ozonation products of cholesterol, which are of interest as possible biomarkers of O_3 exposure, were studied by derivatization with 2,4-dinitrophenylhydrazine (DNPH). The DNPH derivatization of 3β-hydroxy-5-oxo-5,6-secocholestan-6-al **(2)** *produces the expected* trans **(3b)** *and* cis **(3c)** *derivatives of 3β-hydroxy-5-oxo-5,6-secocholestan-6-al, and the unexpected DNPH derivative of 3,5-dihydroxy-B-norcholestane-6-carboxyaldehyde* **(3a)**. *The structures of* **3a**, **3b**, *and* **3c** *were identified with ^1H nuclear magnetic resonance (NMR), ^{13}C NMR, DEPT, COSY, and H-C correlation two-dimensional NMR techniques, and by comparison with the spectra of known compounds. A possible mechanism involving an enamine functionality is proposed for the formation of* **3a**. *The ratio of* **3a**/(**3b** + **3c**) *depends on the concentration of acid used and the reaction time.* (*Steroids* **58**:225–229, 1993)

Keywords: DNPH derivatization; sterol; cholesterol; 3β-hydroxy-5-oxo-5,6-secocholestan-6-al; ozonation; enamine functionality

Introduction

Because of our interest in identifying biomolecules that can be used to measure exposure to inhaled ozone in smog,[1–4] we are developing analytical methods to detect the products formed when animals are exposed to ozone. We have proposed that the lung lining fluid layer may be a primary target for ozone reaction.[1,3] Cholesterol **(1)**, which occurs in the lung lining fluid layer in relatively high concentrations in almost all mammals,[5,6] reacts rapidly with ozone in both organic and aqueous solvents.[7,8] A number of groups have studied the ozonation of cholesterol and identified 3β-hydroxy-5-oxo-5,6-secocholestan-6-al **(2)** as the major product after the reduction of the ozonation mixture[7–10] (Scheme 1).

In our quest for a sensitive analytical method for **2**, we prepared the 2,4-dinitrophenylhydrazine (DNPH) derivatives of **2** using reported conditions[10] (Scheme 1). However, instead of the expected DNPH derivatives of 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (**3b** and/or **3c**), we found the main product to be the DNPH derivative of 3,5-dihydroxy-B-norcholestane-6-carboxaldehyde **(3a)** (Figure 1). As discussed below, **3b** and/or **3c** can become the major products if the reaction conditions are controlled, especially with regard to the concentration of acid used and the reaction time.

Experimental

Melting points (mp) were determined on a Mel-Temp melting point apparatus and were uncorrected. Mass spectra in plasma desorption were obtained on a Bio Ion 20 mass spectrometer. The high-performance liquid chromatography (HPLC) analyses were conducted with a Phase Sep 25 cm × 4.6 mm S5 ODS2 HPLC column and methanol/water as the mobile phase, and detected at 360 nm. The ultraviolet (UV) spectra were obtained on a Hewlett Packard 8451A Diode Array spectrophotometer. The infrared (IR) spectra were obtained on a Perkin-Elmer FT-IR 1700X instrument. The nuclear magnetic resonance spectra were obtained on either a Bruker AM-400 MHz or AC- 200

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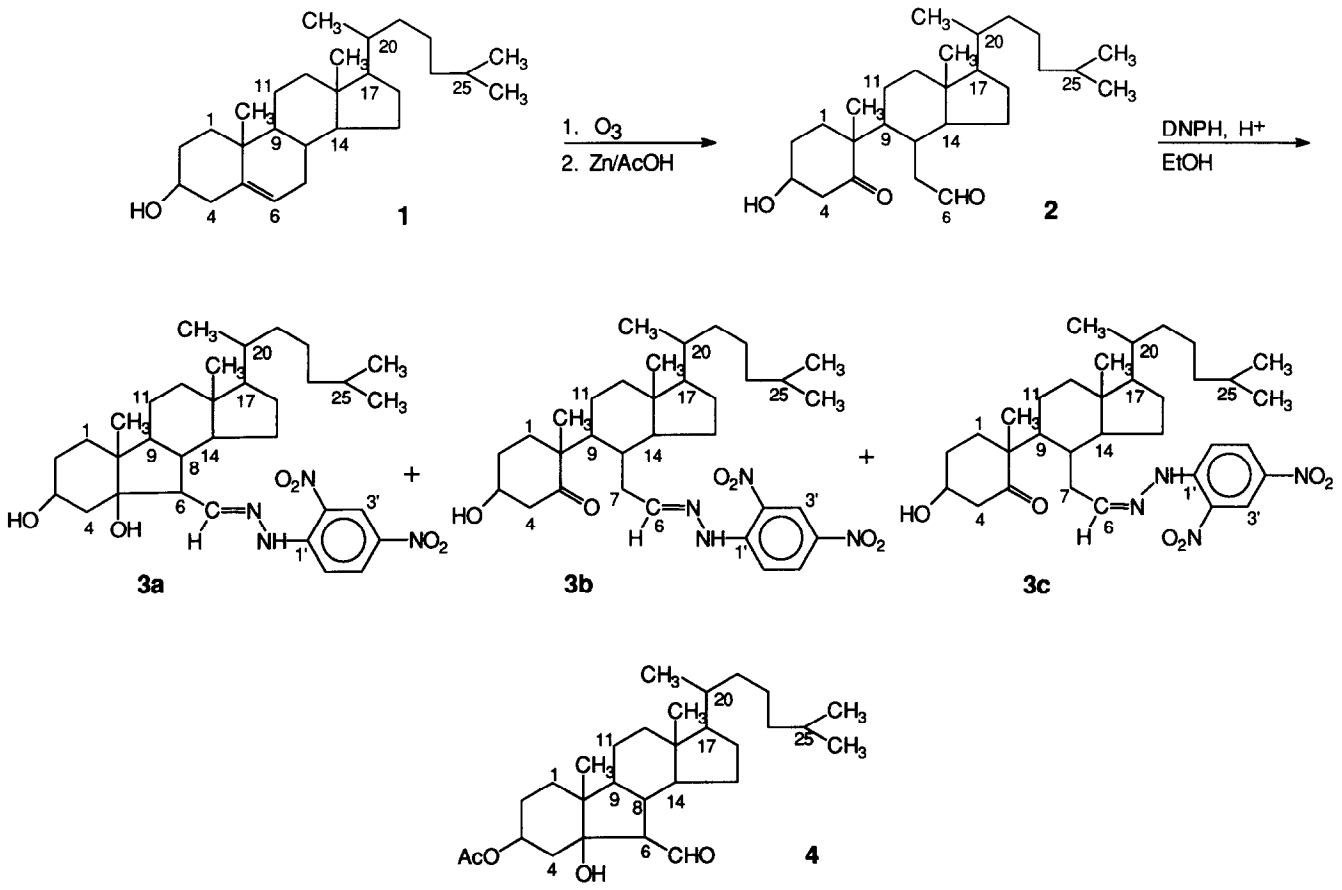

Scheme 1

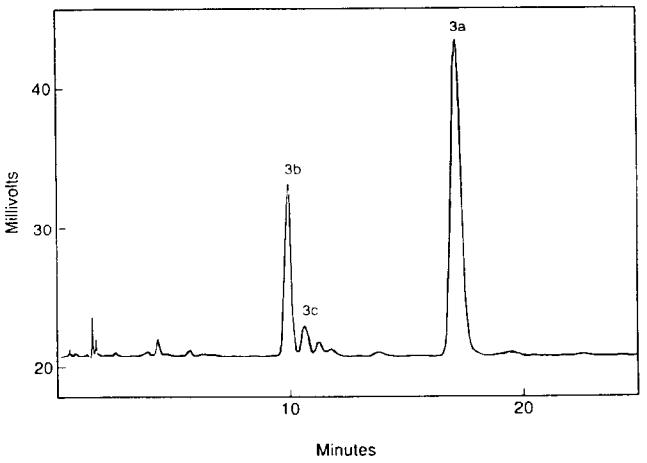

Figure 1 HPLC separation of **3a**, **3b**, and **3c**. Column, 25 cm × 4.6 mm S5 ODS2; mobile phase, water/MeOH (7 : 3), detected at 360 nm. The elution times for **3b**, **3c**, and **3a** are 9.9, 10.6, and 17.2 minutes, respectively. (Peaks before 3 minutes are due to solvent impurities. Small peaks between **3c** and **3a** were not identified.)

MHz NMR spectrometer. Silica gel used for flash column chromatography (60–200 mesh, grade 62) and thin-layer chromatography (TLC) plate (silica gel 2–25 μm, F-254, 250-μm layer) were purchased from Aldrich. Ozone was generated with an Enmet ozone generator, measured by the indigo method.[11] Cholesterol and DNPH were purchased from Aldrich. DNPH (0.010 M) solution was made by dissolving 1.42 g 70% DNPH with 4 ml concentrated HCl in ethanol in a 500-ml volumetric flask.

Preparation of 3β-hydroxy-5-oxo-5, 6-secocholestan-6-al (2)

A flow of 1.2 L/min of 100 ppm ozone in air was bubbled through the solution of 200 mg cholesterol in 50 ml dichloromethane for 4 hours. The reaction mixture was evaporated and stirred with 130 mg Zn powder and 1.0 ml water/20 ml acetic acid for 2 hours. The reduced mixture was diluted with 100 ml dichloromethane, then washed by 3 × 100 ml H_2O, and dried over sodium sulfate. After the evaporation on a rotary evaporator, the residue was chromatographed on a silica gel column, and eluted with ethyl acetate/hexane. The fraction of **2** was monitored by TLC with the DNPH derivatization method.[12] The evaporation of the fraction gave 96 mg (44%) of **2** as a viscous oil: IR (neat) 2,724, 1,720 (CHO), 1,702 cm^{-1} (CO) (lit.[7]: 2,730, 1,730, 1,710 cm^{-1}); ^1H NMR ($CDCl_3$) δ 9.601 (broad s, 1 H, CHO), 4.462 (m, 1 H, H-3), 3.105 (dd, J = 13.8, 3.9 Hz, 1 H, H-4e), 1.015 (s, 3 H, CH_3-19), 0.898 (d, J = 6.4 Hz, 3 H, CH_3-21), 0.853 (d, J = 6.7

Hz, 6 H, CH_3-26,27), 0.676 (s, 3 H, CH_3-18) [lit.[7]: δ 9.62 (broad s), 4.48 (m), 3.12 (dd), 1.02 (s), 0.68 (s)].

Synthesis of 2,4-dinitrophenylhydrazones of 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (3b and 3c)

Cholesterol (200 mg) was ozonized, reduced, extracted, and evaporated as before. The DNPH solution in ethanol (55 ml, 0.010 M) was added to the residue and stirred for 1 hour at room temperature. The reaction mixture was immediately diluted with H_2O, and extracted with 3 × 30 ml dichloromethane. The extract was evaporated to dryness and separated on a silica gel column with ethyl acetate/hexane. The evaporation of the fraction of **3b** plus **3c** gave 150 mg (48%) yellow crystals (75% **3b** and 25% **3c** by HPLC): mp 134–136 C. IR (neat) 1,700 (CO); 1,619, 1,594 (C═N—NH); 1,521, 1,335 cm^{-1} (Ar—NO_2). UV (in CH_3OH): λ_{max} = 358 nm, ε = 22,000 M^{-1} cm^{-1}. HRMS (FAB) calculated for $C_{33}H_{51}N_4O_6$ (M + H) 599.3809, found m/z 599.3796. Furthermore, the mixture of 75% **3b** and 25% **3c** was separated by HPLC to fraction A (95% **3b** + 5% **3c**) and fraction B (25% **3b** + 75% **3c**). The proton and carbon NMR spectra were obtained for each fraction. The proton NMR spectrum ($CDCl_3$) of **3b** shows δ 10.981 (s, 1 H, NH), 9.100 (d, J = 2.5 Hz, 1 H, H-3′), 8.313 (dd, J = 9.6, 2.5 Hz, 1 H, H-5′), 7.921 (d, J = 9.6 Hz, 1 H, H-6′), 7.423 (dd, J = 6.6, 4.4 Hz, 1 H, H-6), 2.989 (dd, J = 13.4, 3.9 Hz, 1 H, H-4e), 4.433 (m, 1 H, H-3), 1.084 (s, 3 H, CH_3-19), 0.922 (d, J = 6.4 Hz, 3 H, CH_3-21), 0.858 (d, J = 6.6 Hz, 6 H, CH_3-26,27), 0.718 ppm (s, 3 H, CH_3-18). The ^1H NMR spectrum ($CDCl_3$) of **3c**: δ 10.910 (s, 1 H, NH), 9.144 (d, J = 2.5 Hz, 1 H, H-3′), 8.300 (dd, J = 9.6, 2.5 Hz, 1 H, H-5′), 7.873 (d, J = 9.6 Hz, 1 H, H-6′), 6.927 (dd, J = 6.6, 4.4 Hz, 1 H, H-6), 4.456 (m, 1 H, H-3), 3.043 (dd, J = 13.6, 3.8 Hz, 1 H H-4e), 1.062 (s, 3 H, CH_3-19), 0.922 (d, J = 6.4 Hz, 3 H, CH_3-21), 0.858 (d, J = 6.6 Hz, 6 H, CH_3-26,27), 0.737 (s, 3 H, CH_3-18).

Synthesis of 2,4-dinitrophenylhydrazone of 3,5-dihydroxy-B-norcholestan-6-carboxaldehyde 3a

To the Zn-reduced cholesterol (200 mg) ozonation products, 55 ml 0.01 M DNPH solution and an additional 0.5 ml of concentrated HCl were added and the reaction mixture was stirred for 15 hours, diluted with water, and extracted with dichloromethane. The extract was evaporated to dryness and separated on a silica gel column with ethyl acetate/hexane. The evaporation of the fraction containing **3a** gave 131 mg (42%) yellow crystals: mp 221–223 C; IR (neat) 1,617, 1,587 (C═N—NH); 1,515, 1,380 cm^{-1} (Ar—NO_2); UV (in CH_3OH) λ_{max} = 359 nm, ε = 24,000 M^{-1} cm^{-1}; ^1H NMR ($CDCl_3$) δ 11.041 (s, 1 H, NH), 9.100 (d, J = 2.2 Hz, 1 H, H-3′), 8.277 (dd, J = 9.6, 2.6 Hz, 1 H, H-5′), 7.899 (d, J = 9.6 Hz, 1 H, H-6′), 7.563 (d, J = 7.0 Hz, 1 H, N═CH), 4.210 (m, 1 H, H-3), 2.366 (dd, J = 9.0, 7.0 Hz, 1 H, H-6), 0.966 (s, 3 H, CH_3-19), 0.918 (d, J = 6.6 Hz, 3 H, CH_3-21), 0.848 (d, J = 6.4 Hz, 6 H, CH_3-26,27), 0.715 ppm (s, 3 H, CH_3-18). HRMS (FAB) calculated for $C_{33}H_{51}N_4O_6$ (M + H) 599.3809, found m/z 599.3799.

Yields of products as a function of time and HCl concentration

Cholesterol (8.0 mg) was ozonized using two equivalents of ozone and reduced with Zn in acetic acid. To the reduction residue, 2.4 ml 0.01 M DNPH solution was added and diluted with methanol to 20.0 ml. The solution was divided into two flasks and 50 μl of concentrated HCl was added to one, the flasks stoppered, and the reaction mixtures stirred at room temperature. Aliquots (1.0 ml) were taken at 0.5, 2, 4, and 24 hours, diluted with 10 ml dichloromethane, washed with three 5-ml portions of water, and dried and separated on a TLC plate with ethyl acetate/hexane (1 : 5). The bands of **3a**, **3b**, and **3c** were scraped off, extracted with ethyl acetate, brought to 2.0 ml with more ethyl acetate, and analyzed by HPLC. The yields of **3a**, **3b**, and **3c** are shown in Table 1.

Table 1 Percent yields of **3a**, **3b**, and **3c** as a function of time and acidity

Time (h)	1.2 mM HCl			60 mM HCl[a]		
	3a	**3b**	**3c**	**3a**	**3b**	**3c**
0.5	2.2	74.4	23.4	10.8	68.6	20.6
2.0	2.2	74.9	22.9	49.3	39.6	11.1
4.0	3.4	77.9	18.7	79.2	17.0	3.8
24.0	16.3	73.9	9.8	96.9	2.4	0.7

Yields are calculated from HPLC peak areas, corrected for the 40% cholesterol that is recovered unchanged.
[a] To 10 ml DNPH solution, 50 μl concentrated HCl was added at the beginning of the DNPH derivatization reaction.

Table 2 Percent yields of **3a**, **3b**, and **3c** after treatment with HCl in methanol.

Time (h)	**3a**	**3b**	**3c**
0.0	0	88	12
2.0	29	59	12
24.0	93	6	1
48.0	100	0	0

To the solution of 1.7 mg **3b** + **3c** in 10.0 ml methanol, 50 μl HCl was added at the beginning of the reaction.

Treatment of 3b and 3c in methanol with aqueous HCl

To the solution of 1.7 mg **3b** + **3c** in 10.0 ml methanol in a 25-ml flask, 50 μl concentrated HCl was added at the beginning of the reaction. The flask was stoppered and the reaction mixtures were stirred at room temperature. Aliquots (1.0 ml) were taken at 2, 24, and 48 hours. Each aliquot was diluted with 10 ml dichloromethane, and washed with three 5-ml portions of water, dried, and separated on a TLC plate with ethyl acetate/hexane (1 : 5). The bands of **3a**, **3b**, and **3c** were scraped off, extracted with ethyl acetate, brought to 2.0 ml with more added ethyl acetate, and analyzed by HPLC. The results are shown in Table 2.

Results and discussion

We have repeated the DNPH derivatization reaction of **2** originally reported by Cornforth *et al.*[10] The major DNPH derivatization product **(3a)** was separated and purified by flash column chromatography. Both IR and ^{13}C NMR data fail to show a carbonyl group in **3a**, so **3a** is not the DNPH derivative of 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (**3b** or **3c**). The reaction of **2** with two DNPH was ruled out by the observation of just one set of peaks for the DNPH group by ^1H NMR (δ 11.041, 9.100, 8.277, 7.899, and 7.563 ppm) and ^{13}C

Table 3 ^{13}C NMR data on cholesterol derivatives

C	1[a]	2	3a[b]	3b	3c	4[c]
1	37.14	34.03	28.10	34.16	33.85	28.1
2	31.47	27.67	27.78	27.92	27.42	24.5
3	71.52	71.00	67.53	71.04	70.90	70.3
4	42.12	46.85	43.65	47.37	47.35	42.2
5	140.62	217.50	83.40	215.93	215.99	83.6
6	121.44	202.73	56.38	150.88	148.39	51.5
7	37.75	44.18	—	32.71	32.70	—
8	35.80	34.73	42.62	37.04	37.01	39.3
9	50.01	42.19	55.15	42.66	42.63	64.5
10	36.33	52.63	45.53	52.61	52.57	45.6
11	20.96	23.01	21.66	23.16	23.05	21.6
12	39.64	39.84	39.70	39.88	39.86	39.6
13	42.12	42.53	44.80	42.63	42.63	44.6
14	56.63	54.24	55.59	54.61	54.22	56.0
15	24.15	25.28	24.70	25.36	24.73	24.5
16	28.10	27.79	28.51	27.95	27.80	28.5
17	56.05	56.08	56.38	56.12	56.10	55.8
18	11.71	11.50	12.49	11.70	11.67	12.5
19	19.26	17.54	18.68	17.80	17.76	18.2
20	35.64	35.69	35.60	35.69	33.66	35.7
21	18.60	18.51	18.75	18.59	18.56	18.7
22	36.07	35.94	36.19	35.99	35.98	36.2
23	23.72	23.70	23.81	23.75	23.73	23.9
24	39.38	39.40	39.45	39.44	39.43	39.5
25	27.84	27.97	27.98	27.97	27.94	28.0
26	22.68	22.49	22.53	22.54	22.50	22.6
27	22.43	22.75	22.79	22.80	22.76	22.8
1′	—	—	145.14	144.96	145.37	—
2′	—	—	128.70	128.82	129.44	—
3′	—	—	123.54	123.47	123.46	—
4′	—	—	137.57	137.83	138.00	—
5′	—	—	129.89	130.20	129.92	—
6′	—	—	116.36	116.53	116.25	—

The assignment of the peaks is based on the DEPT, COSY, and H-C correlation 2D NMR techniques.
[a] Data our own and from Jaworski and Smith.[8]
[b] The absorbance for N=CH occurs at δ 155.24 ppm.
[c] Data from Boswell *et al.*[19]

NMR spectra [just one N=CH signal (at δ 155.24 ppm) and just six aromatic carbon signals (C-1′ to C-6′); Table 3]. Another possible product that would not have a carbonyl is a hydrate. If the carbonyl group were hydrated during the derivatization process, the yield of **3a** should increase with increasing concentrations of water; however, when more water was added to the solvent, no increase in the yield of **3a** was observed.

Compounds **3b** and/or **3c** become the major products when the concentration of acid is decreased and the temperature for the derivatization reaction is lowered to room temperature, as shown in Table 1. Neither **3b** nor **3c** are detected if the reaction mixture is heated to 78 C, the temperature used by Cornforth *et al.*[10] Compounds **3b** and **3c** were identified as the *trans* and *cis* isomers of the 2,4-dinitrophenylhydrazone of 3β-hydroxy-5-oxo-5,6-secocholestan-6-al by comparison with the Sadtler Standard NMR spectra of known DNPH derivatives.[13–15] Although products **3b** and **3c** can be separated by HPLC, they slowly isomerize in the solution at room temperature (the new equilibrium of 75% **3b** and 25% **3c** was reached after 3 days). Therefore, an attempt to obtain a two-dimensional (2D) NMR spectrum for **3c** failed.

Other investigators have shown that B-norsterol can be formed through an intramolecular aldol condensation of secoaldehyde **(2)**.[16–19] However, in our system, **3b** and **3c** are formed directly from the reaction between **2** and DNPH, and **3a** is produced in a subsequent acid-catalyzed reaction of **3b** and **3c** (Table 1). A possible mechanism for the rearrangement of the hydrazones (**3b** and/or **3c**) involves conversion to an enamine functionality that attacks the carbonyl group (C-5) to give the cyclized tertiary alcohol **3a** (Scheme 2). The mechanism was further confirmed by the treatment of a mixture of **3b** and **3c** in methanol with aqueous HCl; **3a** is indeed formed from **3b** and/or **3c** (Table 2).

The structure assigned to **3a** fits the spectroscopic data. The ^{13}C NMR spectrum shows a quaternary carbon at δ 83.40 ppm for C-5, a CH peak at δ 56.38 ppm for C-6, and a CH peak at δ 155.24 ppm for N=CH; the remaining carbons in the B-norcholestane ring have absorbances that are similar[20] to those of 3β-(acetyloxy)-5β-hydroxy-B-norcholestane-6β-carboxyaldehyde **(4)**, as shown in Table 3. (The structure of **4** is given at the bottom of Scheme 1.) The proton NMR spectrum shows a doublet at δ 7.56 ppm assigned to N=CH; this proton correlates with the carbon signal at δ 155.24 ppm in the H-C correlation 2D NMR. The doublet of doublets at δ 2.37 ppm for H-6 correlates with the carbon signal at δ 56.38 (C-6) in the H-C correlation 2D NMR and couples with the signals at δ 7.56 ppm and δ 2.05 ppm in the COSY 2D NMR spectra. It is noteworthy that the *cis* 2,4-dinitrophenylhydrazone of 3,5-dihydroxy-B-norcholestan-6-carboxyaldehyde is not observed under our experimental conditions.

The spectroscopic data reflect the fact that **3b** has a carbonyl function. Table 1 shows the ^{13}C NMR spectrum for **3b** with absorbances at δ 215.93 ppm for C-5, at δ 32.71 ppm for the CH_2 peak at C-7, and at δ 150.88 ppm for the CH in N=CH. The proton NMR of **3b** shows a doublet of doublets at δ 7.423 ppm for N=CH that correlates with the signal at δ 150.88 ppm in H-C correlation 2D NMR, and couples with the signals at δ 2.3–2.4 ppm in COSY 2D NMR, and a doublet of doublets at δ 2.99 ppm for H-4e (which also implies the existence of an α-carbonyl group).

Although the proton NMR of **3b** shows a typical *trans*-hydrazone N=CH absorption at 7.423 ppm, that of **3c** shows a *cis*-hydrazone signal at 6.927 ppm.[13] Thermodynamically, the *trans*-isomer **3b** is more stable than the *cis*-isomer **3c**, because of steric hinderance between the steroid group and the aromatic ring. Therefore, the *trans*-isomer **3b** predominates in the equilibrium mixture of **3b** and **3c**.

The production of **3a** in our experiments is unexpected, because DNPH derivatization is a standard method to characterize aldehydes and ketones[21] and rearrangements are not expected.

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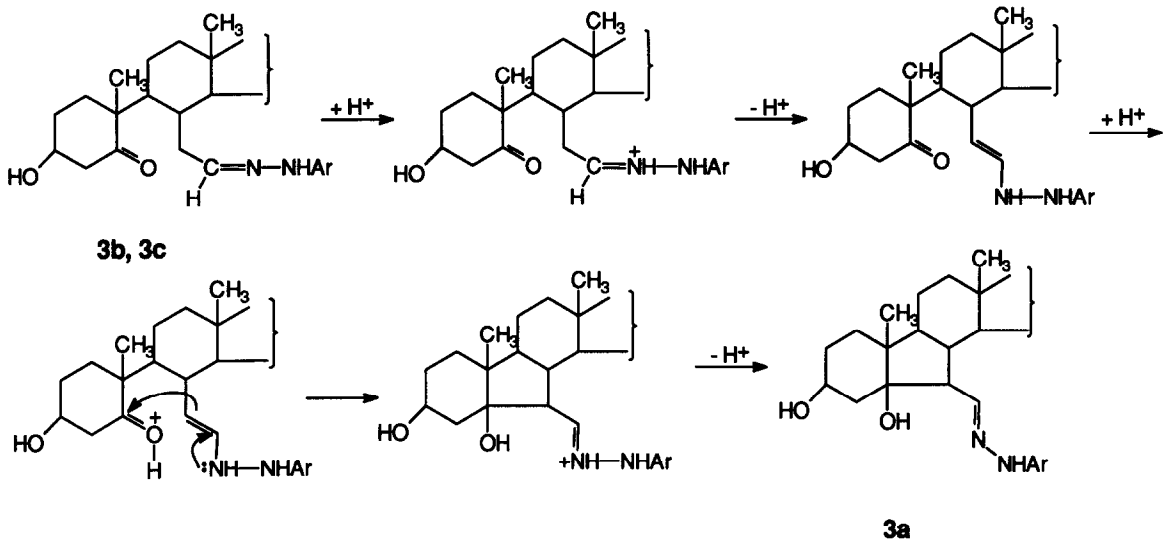

Scheme 2

tion mass spectral determinations were made at the Midwest Center for Mass Spectrometry at the University of Nebraska.

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