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ORIGINAL ARTICLE

The Liebermann–Burchard Reaction: Sulfonation, Desaturation, and Rearrangment of Cholesterol in Acid

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Abstract In the Liebermann–Burchard (LB) colorimetric assay, treatment of cholesterol with sulfuric acid, acetic anhydride, and acetic acid elicits a blue color. We studied the reactivity of cholesterol under LB conditions and provide definitive NMR characterization for approximately 20 products, whose structure and distribution suggest the following mechanistic picture. The major reaction pathways do not involve cholestadienes, i-steroids, or cholesterol dimers, as proposed previously. Instead, cholesterol and its acetate and sulfate derivatives undergo sulfonation at a variety of positions, often with skeletal rearrangements. Elimination of an SO_3H group as H_2SO_3 generates a new double bond. Repetition of this desaturation process leads to polyenes and ultimately to aromatic steroids. Linearly conjugated polyene cations can appear blue but form too slowly to account for the LB color response, whose chemical origin remains unidentified. Nevertheless, the classical polyene cation model is not excluded for Salkowski conditions (sulfuric acid), which immediately generate considerable amounts of cholesta-3,5-diene. Some rearrangements of cholesterol in H_2SO_4 resemble the diagenesis pathways of sterols and may furnish useful lipid biomarkers for characterizing geological systems.

Keywords Sterol · Colorimetric test · NMR · Mass spectrometry · Sultone · Rearrangement · Diagenesis

Abbreviations

ES	Electrospray
FAB	Fast-atom bombardment
HPLC	High-performance liquid chromatography
LB	Liebermann–Burchard
NBA	3-Nitrobenzyl alcohol
TLC	Thin-layer chromatography

Introduction

Numerous colorimetric tests have been devised for the identification and quantitation of steroids [1]. Perhaps the foremost of these methods is the Liebermann–Burchard (LB) reaction, which was the leading assay for serum cholesterol in clinical laboratories during most of the twentieth century [2–4] and is still recommended by the USA Food and Drug Administration as a standard against which new methods are compared [5]. The LB reaction was first described in 1885 by Liebermann [6] and was later investigated extensively by Burchard [7]. Burchard developed the reaction into a quantitative test for cholesterol and used this assay to confirm the widespread presence of sterols in animal and plant tissues. The LB reaction has been studied for many other sterols; the color response varies markedly

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depending on the double bond system [8], other functional groups, and the presence of a nonpolar side chain [9] (Fig. 1).

The mechanism that generates the LB color response has intrigued chemists for over 100 years, and much work was carried out even before the structure of sterols was established [10]. During the past 50 years, the color response has been attributed to polyene cations formed under LB conditions. Following Watanabe's [11] 1959 proposal of a tetraene dimer complexed with acid, Brieskorn and Hofmann [9] suggested the formation of a Δ4,6,8(14),15,17(20) steroid complex from cholesterol by a series of sulfonations with SO_3, coupled with elimination of SO_2. Key mechanistic analyses in 1974 [12, 13] supported and refined the polyene cation hypothesis, but extensive kinetic measurements failed to correlate SO_2 evolution from the LB reaction with the color response. These and other mechanistic studies were summarized by Zuman [14] in 1991 in a comprehensive review covering the LB reaction and similar colorimetric assays.

A major weakness of the mechanistic work has been the lack of knowledge about the LB reaction pathways. Very few LB products have been characterized beyond UV and combustion analyses, and no polar intermediates have been reported apart from steryl sulfates and perchlorates. Investigators variously postulate cholesterol dimers [11, 15], cholestadienes [9, 12, 13], or epicholesterol derivatives [9] as pivotal intermediates without any evidence of their quantitative importance. As a result, the behavior of cholesterol in strongly acidic solution is still poorly understood.

We have now investigated the LB reaction with modern chromatographic and spectral methods. In situ NMR analysis and experiments with ^{14}C-labeled cholesterol were used to trace the increasing levels of polar products as the reaction progresses. Preparative high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) led to the isolation of numerous reaction products, whose structures were determined by 2D NMR in conjunction with quantum-mechanical NMR calculations. The results indicated that cholesterol is rapidly converted to acetate and sulfate derivatives, which are slowly desaturated via sulfonic acids, eventually rearranging to aromatic steroids. These new insights into the behavior of cholesterol under LB conditions are discussed with regard to the chemical origin of the color response.

	1 min	30 min	1 h
LB conditions: conc. H_2SO_4, Ac_2O, AcOH			
cholesterol	colorless	blue	green
7-dehydrocholesterol	pink	blue	pale green
5α-cholesta-8,14-dien-3β-ol	blue	intense blue	
19-norcholesta-5,7,9-trien-3β-ol	no color response		
C_{19} steroids and bile acids	no color response		
Salkowski conditions: conc. H_2SO_4			
cholesterol	pink	red	red

Fig. 1 Colorimetric behavior of sterols under acidic conditions

Experimental Procedures

Materials

Solvents and reagents were chromatography or reagent grade; chloroform was amylene-stabilized from bottles less than 6 months old unless otherwise specified. Deuterated reagents (acetic acid, acetic anhydride, and sulfuric acid) were obtained from Aldrich (Milwaukee, WI, USA). Commercial cholesterol (**1**) was purified via the dibromide and then recrystallized from methanol to remove oxysterol contaminants. [4-^{14}C]Cholesterol was obtained from Amersham, and its purity was confirmed by radio-TLC (ethyl acetate/hexanes 2:7). A cholesteryl sulfate standard was purchased from Steraloids (Newport, RI, USA).

LB Reaction

The LB color reagent was prepared freshly, as described by Abell et al. [16], by adding concentrated H_2SO_4 to acetic anhydride at 0 °C, stirring for 10 min, adding acetic acid, and warming to room temperature. The proportion of H_2SO_4, acetic anhydride, and acetic acid is specified for each experiment, varying from the classic 1:20:10 (v/v) ratio to 8:20:10. Whereas the LB reagent is usually added directly to a dry residue in clinical protocols [16], we followed Burchard [7] in adding the reagent to a solution of cholesterol in chloroform; this facilitated in situ NMR studies and mixing in low-temperature experiments without qualitatively altering the color response.

Spectral Methods

^1H NMR and ^{13}C NMR spectra of steroids were acquired at 25 °C in relatively dilute solution (1–20 mM) with Bruker AMX and Avance 500-MHz spectrometers. Proton spectra were referenced to tetramethylsilane; ^{13}C spectra were referenced to $CDCl_3$ at 77.0 ppm or CD_3OD at 49.0 ppm. Chemical shift reproducibility was about ±0.001 ppm for ^1H (except for protons near functional groups in CD_3OD solution)

and about ±0.03 ppm for ^{13}C. Mass spectra were obtained by direct probe using a ZAB-HF spectrometer with electron-impact ionization (70 eV) or fast-atom bombardment (FAB) in a matrix of glycerol or 3-nitrobenzyl alcohol (NBA). Higher-resolution mass spectra were acquired by infusing methanol solutions into a Waters (Micromass) Q-TOF Ultima with positive (ES+) or negative (ES–) electrospray ionization. Spectrophotometric analysis of LB reactions was done in 1.4- or 3.5-mL quartz cuvettes (10-mm path length) using a Shimadzu 1601 spectrophotometer. Radioactivity was measured with a Packard model 1500 liquid scintillation analyzer using ScintiVerse or toluene/2,5-diphenyloxazole.

Quantum-Mechanical NMR Calculations

NMR shieldings were calculated with Gaussian 03 [17] by the gauge independent atomic orbital method at the B3P91/6-311G(d,p)//B3LYP/6-31G(d) level. The shieldings were converted to chemical shifts using empirical adjustments (L.-W. Guo, W. K. Wilson, and C. H. L. Shackleton, unpublished results). The structures of **10–20** were confirmed by comparing the observed ^{1}H and ^{13}C NMR chemical shifts with predicted shieldings, as described previously [18]. The agreement was generally within 0.1 ppm for ^{1}H and within 1 ppm for ^{13}C, except for systematic deviations affecting nuclei influenced by sulfonate groups. Sulfonates were modeled as sulfonic acids to compensate for hydrogen bonding; the model did not include solvation. The predicted shieldings greatly facilitated signal assignments, spectral interpretation, and positioning of sulfonate groups.

Results

Preliminary Studies of the LB Reaction

We initially explored many variations of the LB reaction. Under nearly all conditions, the reaction progressed from colorless, to pale blue, blue, bluish green, and green. A modified LB reagent with a 4:20:10 ratio (elevated amount of H_2SO_4) increased the absorbance and was used in some studies. LB reactions with ethanol-stabilized chloroform were more sluggish than reactions with amylene-stabilized chloroform, producing the same sequence of colors but with lower absorbance. Omitting both acetic acid and anhydride, i.e., Salkowski conditions [19, 20], produced a red color, but other variations of LB conditions had no qualitative effect on color formation. Intermediate stages of the LB reaction could be preserved for kinetic and NMR studies by maintaining the temperature below –20 °C. Substituting dichloromethane for chloroform reduced the reaction viscosity at low temperatures without affecting the color response. Quenching was explored by dropwise addition of cold 24% NaOH, ethanol, methanol, or pyridine, the last three reactions requiring subsequent neutralization with aqueous NaOH. Even nucleophilic quenching reagents generated only modest amounts of adducts with the nucleophile. The aim of quenching was to obtain stable products that reveal the structure of intermediates. Nevertheless, some workup conditions may have resulted in ester hydrolysis or further sulfonation.

Studies of LB Reaction Products with [4-^{14}C]Cholesterol

Reaction of [4-^{14}C]cholesterol (25 mg; 500,000 dpm) in chloroform (15 mL) with LB reagent (2.5 mL, 1:20:10 ratio) at room temperature produced a bluish-green color after 30 min. The mixture was immediately chilled to –70 °C and quenched by dropwise addition of cold 24% NaOH (10 mL). Scintillation counting of the resulting aqueous and organic layers and the precipitate indicated a 70:22:8 distribution of radioactivity. Chilling the aqueous layer to –20 °C produced more precipitate, corresponding to almost half the aqueous-layer radioactivity. A duplicate experiment gave similar results. With a more concentrated solution of cholesterol (250 mg) in chloroform (15 mL) and LB reagent (2.5 mL, 4:20:10 ratio), the distribution of the ^{14}C label was 3:33:64 (aqueous layer, organic layer, precipitate). The precipitate was predominantly cholesteryl sulfate (see later). These results indicate that most of the cholesterol is converted to highly polar products that are insoluble in chloroform.

Sterols Produced by LB Reaction A

To a solution of cholesterol (250 mg) in chloroform (25 mL; stabilized with 0.75% ethanol) was added LB reagent (1:20:10 ratio, 10 mL). The solution gradually turned blue and, before any appearance of green color, the reaction was quenched with 24% aqueous NaOH (3 mL) followed by water (20 mL). The precipitate that formed between the chloroform and the aqueous layers was identified as cholesteryl sulfate (**2**, 81 mg, above 99% purity). Evaporation of the chloroform layer gave a residue (201 mg) comprising cholesteryl acetate (**3**), with traces of i-steroids (3α,5α-cyclocholestanes) and other sterols. This residue was subjected

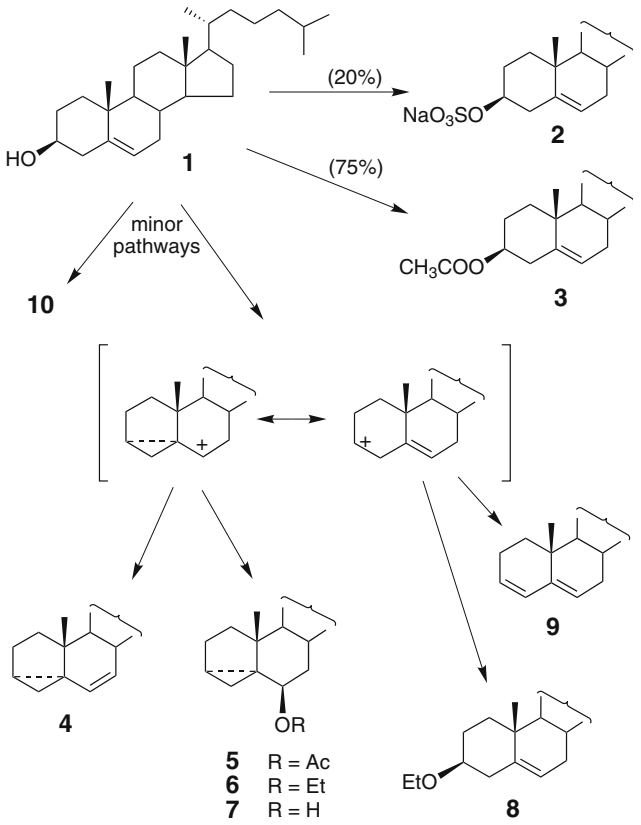

Fig. 2 Products from Liebermann–Burchard (*LB*) reactions quenched at the blue stage. The initial major products **2** and **3** are subsequently converted to polar steroids

to repeated column chromatography and TLC (silica gel; ethyl acetate/hexane 1:5, 1:8, and 1:40); the products in order of increasing polarity were the Δ6 i-steroid **4** (0.2 mg), **3** (130 mg), 6*β*-acetoxy i-steroid **5** (1 mg), 6*β*-ethoxy i-steroid **6** (1 mg), cholesteryl ethyl ether (**8**; 1 mg), and 6*β*-hydroxy i-steroid **7** (0.5 mg). These compounds, which were identified by NMR, are shown in Fig. 2.

NMR data: **2** (CD_3OD, δ_H) 0.718 (s), 0.877 (d, 6.6 Hz), 0.881 (d, 6.6 Hz), 0.943 (d, 6.6 Hz), 1.031 (s), 2.341 (ddddd, 13.4, 11.7, 3.4, 2.8, 2.1 Hz), 2.530 (ddd, 13.4, 5.0, 2.4 Hz), 4.129 (tt, 11.5, 4.7 Hz), 5.385 (dt, 5.4, 2.1 Hz); **4** ($CDCl_3$, δ_H) 0.435 (dd, 8.0, 5.0 Hz), 0.718 (s), 0.863 (d, 6.6 Hz), 0.867 (d, 6.6 Hz), 0.897 (s), 0.903 (d, 6.6 Hz), 5.185 (dd, 9.8, 2.6 Hz), 5.522 (dd, 9.8, 1.9 Hz); **5** ($CDCl_3$, δ_H) 0.413 (dd, 8.3, 5.3 Hz), 0.499 (dd, 5.1, 3.8 Hz), 0.726 (s), 0.863 (d, 6.6 Hz), 0.867 (d, 6.6 Hz), 0.912 (d, 6.6 Hz), 1.009 (s), 2.048 (s), 4.508 (t, 3.0 Hz); **6** ($CDCl_3$, δ_H) 0.377 (dd, 8.3, 5.3 Hz), 0.616 (dd, 5.0, 3.7 Hz), 0.720 (s), 0.863 (d, 6.6 Hz), 0.868 (d, 6.6 Hz), 0.910 (d, 6.6 Hz), 1.013 (s), 1.148 (t, 7.0 Hz), 2.863 (t, 2.9 Hz), 3.377 (dq, 9.5, 7.0 Hz), 3.600 (dq, 9.5, 7.0 Hz); **7** ($CDCl_3$, δ_H) 0.291 (ddd, 8.1, 4.8, 0.8), 0.523 (dd, 4.8, 3.8 Hz), 0.721 (s), 0.863 (d, 6.6 Hz), 0.867 (d, 6.6 Hz), 0.912 (d, 6.6 Hz), 1.057 (s), 1.999 (dt, 12.7, 3.5 Hz), 3.261 (t, 3.0 Hz); **8** ($CDCl_3$, δ_H) 0.677 (s), 0.863 (d, 6.6 Hz), 0.867 (d, 6.6 Hz), 0.914 (d, 6.6 Hz), 1.002 (s), 1.200 (t, 7.0 Hz), 3.155 (tt, 11.3, 4.4 Hz), 3.52 (dq, 9.2, 7.0 Hz), 3.53 (dq, 9.2, 7.0 Hz), 5.345 (dt, 5.2, 2.1 Hz).

Similar LB reactions, carried out and quenched under a variety of mild conditions, also generated mainly **2** and **3**, often with traces of i-steroids, sultone **10** (see later), and Δ3,5 diene **9**. NMR of **9**: ($CDCl_3$, δ_H) 0.704 (s), 0.864 (d, 6.6 Hz), 0.869 (d, 6.6 Hz), 0.921 (d, 6.6 Hz), 0.953 (s), 5.388 (br dd, 5, 2 Hz), 5.588 (br dd, 10, 5 Hz), 5.926 (dd, 9.8, 2.7 Hz). No other hydrophobic sterols were observed in LB reactions, apart from **11** and artifactual products like cholesteryl chloride [δ_H 0.675 (s), 1.027 (s), 3.766 (tt), 5.370 (dt)] and esters of 7-hydroxycholesterol [e.g., δ_H 0.689 (s), 1.055 (s), 5.606 (t, 2 Hz)].

Sterols Produced by LB Reaction B

To a solution of cholesterol (2 g) in chloroform (40 mL) was added LB reagent (4:20:10 ratio, 20 mL). After 30 min at room temperature, the bluish-green solution was chilled and quenched with ethanol (60 mL) at –78 °C. The resulting yellowish-green mixture was evaporated in vacuo at 40 °C until there was no odor of acetic acid. Water (15 mL) and 24% aqueous NaOH (2–3 mL) were added to adjust the pH to 8. This solution was extracted with chloroform (2 × 20 mL); no precipitate was observed. The organic phase was evaporated to a brown residue (0.37 g); ^1H NMR indicated a complex mixture comprising mainly **1**, **3**, **10**, and **11**, with traces of i-steroids but no **9** or backbone rearrangement products [21]; reversed-phase chromatography (C_{18} column, elution with 70–100% methanol in water) gave sultone **10** and (after further purification by C_8 HPLC) the phenanthrene derivative **11**, which were characterized by NMR (Tables 1, 2).

The aqueous phase was evaporated in vacuo to a solid, suspended in methanol (40 mL) with sonication, and centrifuged at 3,000 rpm for 10 min. The methanolic supernatant was evaporated to a residue (5.3 g), which was chromatographed on a 200-g C_{18} column (50–100% methanol in water) to give, in order of elution, **15**, **18**, **12**, **17**, **19**, **20**, **13**, **14**, and **16**. Compounds **12**–**14** crystallized as colorless needles, but most other steroids were eluted as mixtures that were further purified by HPLC or TLC on C_{18} media. Apart from **2**, the polar steroids comprised a multiplicity of minor products. In addition to compounds **12**–**20** (roughly 5–10 mg each), at least ten unidentified polar steroids were noted, together with a larger number of less abundant products.

Table 1 ^{1}H NMR chemical shifts of Liebermann–Burchard (LB) reaction products from cholesterol

	10	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
H-1α	2.269	8.569	2.449	2.430	2.529	1.434	1.893	2.331	2.356	8.080	7.942
H-1β	1.741		2.288	2.275	2.219	2.298	1.302		2.615		
H-2α	2.678	7.501	1.467	1.530	1.524	3.233	2.138	6.988	7.190	7.471	7.349
H-2β	1.909		2.196	1.737	1.844		2.21				
H-3α	5.056	7.393	4.651	3.977	5.052	4.359	5.787	7.004		7.489	7.297
H-4α	1.910		1.596	1.699	1.544	5.598	7.230	7.127		7.970	
H-4β	2.084		3.127	2.714	3.019						
H-6α		7.903							7.200		7.813
H-6β	3.206		2.789	2.791	2.782	3.619		5.089			
H-7α	1.509	7.751	2.079	1.941	1.928	1.260	2.008	3.202	6.505	8.406	8.543
H-7β	2.229		1.940	2.083	2.086	2.439	2.652				
H-8β	2.105		2.07	2.068	2.084	2.192	1.653	2.451			
H-9α						0.755	0.977	3.067	2.42		
H-11α	2.519	8.342	2.554	2.542	2.549	1.568	1.597	2.443	1.71	3.20[a]	3.23[a]
H-11β	1.960		1.979	1.973	1.991	1.454	1.455	1.268	1.68	3.22[a]	3.26[a]
H-12α	1.177		1.113	1.096	1.116	1.155	1.213	1.490	1.301	1.993	2.005
H-12β	1.960		2.051	2.053	2.059	2.035	2.065	2.014	2.126	2.255	2.259
H-14α	1.042		1.460	1.438	1.463	0.952	1.092	2.086			
H-15α	1.650	3.24[a]	1.761	1.764	1.761	1.653	1.712	2.142	2.50[a]		
H-15β	1.170	3.26[a]	1.158	1.158	1.162	1.245	1.175	1.234	2.36[a]		
H-16α	1.867	2.20[a]	1.871	1.871	1.875	1.854	1.888	1.940	1.964	3.120	3.122
H-16β	1.310	2.22[a]	1.292	1.294	1.299	1.290	1.319	1.312	1.529	2.666	2.678
H-17α	1.084	3.462	1.135	1.123	1.134	1.110	1.141	1.287	1.230	1.811	1.823
H-18	0.770	2.577	0.853	0.850	0.856	0.738	0.741	0.805	0.958	0.961	0.969
H-19	1.614	2.748	1.457	1.439	1.425	1.343	1.030	2.331	0.900	2.646	2.659
H-20	1.395	2.046	1.418	1.420	1.423	1.397	1.413	1.46	1.533	1.715	1.725
H-21	0.903	1.083	0.927	0.927	0.927	0.934	0.949	0.966	0.992	1.079	1.083
H-22R	1.335	0.95[a]	1.37	1.38	1.37	1.38	1.39	1.40	1.40	1.52	1.52
H-22S	0.988	0.99[a]	1.010	1.010	1.012	1.013	1.028	1.05	1.11	1.19	1.19
H-23R	1.33	1.28[a]	1.37	1.38	1.37	1.38	1.38	1.40	1.38	1.47	1.47
H-23S	1.14	0.95[a]	1.17	1.182	1.18	1.18	1.19	1.18	1.19	1.29	1.29
H-24	1.10	0.93	1.11	1.11	1.11	1.11	1.11	1.12	1.12	1.21	1.21
H-24	1.14	0.93	1.15	1.15	1.15	1.15	1.16	1.15	1.15	1.21	1.21
H-25	1.515	1.337	1.526	1.526	1.526	1.526	1.531	1.539	1.526	1.576	1.577
H-26	0.861	0.721[a]	0.875	0.875	0.875	0.876	0.879	0.885	0.886	0.909[a]	0.909[a]
H-27	0.865	0.733[a]	0.879	0.879	0.879	0.880	0.883	0.888	0.889	0.910[a]	0.910[a]

Chemical shifts were measured at 25°C in $CDCl_3$ (**10**, **11**) or CD_3OD (**12**–**20**)

[a] Assignments of geminal or stereoisomeric pairs that may be interchanged. For ring A signals of **10**, α denotes the sultone side. Coupling constants: H18 and H19 were singlets, H21, H26, and H27 were doublets (approximately 6.6 Hz), and H25 nonets (6.6 Hz); other notable couplings: **10**, H-1α, dddd, 14.1, 12.5, 4.0, 1.6 Hz; H-1β, dddd, 14.1, 9.2, 5.3, 1.8 Hz; H-2α, dddd, 13, 9, 4, 3 Hz; H-3β, ddd, 4.8, 3.1, 1.1 Hz; H-4β, dd, 13.5, 3.0 Hz; H-6β, ddd, 12.7, 2.2, 1.5 Hz; H-7α, td, 12.9, 11.0 Hz; H-7β, ddd, 13.1, 6.1, 2.3 Hz; H-8β, br t, 11 Hz; H-11α, ddd, 14.8, 4.6, 1.7 Hz; H-15α, dddd, 12.2, 9.9, 7.1, 2.9 Hz; H-16α, dtd, 13.4, 9.7, 6.1 Hz; **11**, H1, d, 8.4 Hz; H2, dd, 8.4, 7.0 Hz; H-3, dt, 7.0, 1.0 Hz; H6, br d, 9.2 Hz; H7, br d, 9.2 Hz; H-15, ddd, 16, 7, 6 Hz and 16, 9, 8 Hz; H17, ddd, 7, 4, 4 Hz; H20, dqdd, 10, 6.9, 3.3 Hz; **12**, H-1α, dt, 14.2, 3.5 Hz; H-1β, br t, 14.4 Hz; H-2α, br d, 12.5 Hz; H-3α, quintet, 3.6 Hz; H-4α, dd, 14.9, 3.8 Hz; H-4β, ddd, 14.9, 3.9, 2.4 Hz; H-6α, dd, 12.9, 2.2 Hz; H-7β, td, 13.6, 6.7 Hz; H-11α, dt, 13.5, 3.7, 2.8 Hz; H-11β, td, 13.6, 3.9 Hz; H-15β, dddd, 12.1, 9.8, 7.2, 2.5 Hz; H-16β, dddd, 12.9, 9.4, 8.7, 5.8 Hz; **13** and **14** had couplings similar to **12** (±0.5 Hz); **15**, H-1α, t, 13.4 Hz; H-1β, dd, 12.9, 3.1 Hz; H-2β, ddd, 13.5, 9.2, 3.0 Hz; H-3α, dd, 9.2, 2.6 Hz; H-4, d, 2.6 Hz; H-6α, d, 7.2 Hz; H-7β, dd, 14.2, 3.7 Hz; H-9α, ddd, 12.5, 10.7, 4.3 Hz; **16**, H3, br d, 10.4 Hz; H4, ddd, 10.4, 2.8, 1.5 Hz; H7α, dd, 19, 11 Hz; H7β, dd, 19.4, 5.5 Hz; H8β, qd, 10.6, 5.5 Hz; **17**, H2, dd, 7.6, 2.1 Hz; H3, t, 7.4 Hz; H4, dd, 7.0, 2.1 Hz; H-6α, dd, 3.3, 1.7 Hz; H-8β, td, 10.9, 3.4 Hz; H-9α, td, 11.5, 3.2 Hz; H-12β, td, 12.8, 3.2 Hz; **18**, H-1α, dd, 16.6, 3.1 Hz; H-1β, d, 16.7 Hz; H-2, d, 3.1 Hz; H6, d, 10.0 Hz; H7, d, 9.9 Hz; **19**, H1, dd, 8, 2 Hz; H2, ddd, 8.0, 6.8, 1.7 Hz; H3, ddd, 8.1, 6.9, 1.7 Hz; H4, dd, 8, 2 Hz; H12α, ddd, 13.3, 10.0, 7.4 Hz; H12β, ddd, 13.3, 5.8, 3.8 Hz; H16α, dd, 15.6, 7.9 Hz; H16β, dd, 16, 11 Hz; **20**, H1, d, 8.8 Hz; H2, dd, 8.5, 6.9 Hz; H3, d, 6.9 Hz; H6, d, 9.1 Hz; H7, d 9.0 Hz; H12β, ddd, 13, 5, 4 Hz; H16α, dd, 15.5, 7.9 Hz; H16β, dd, 16, 12 Hz

Structure Elucidation of Compounds **10**–**20** by NMR and Mass Spectrometry

Because of the extensive rearrangements and/or presence of sulfonate groups, the structures of **10**–**20** were not readily established by comparisons with NMR spectra of known compounds. Each of these steroids was analyzed by heteronuclear single quantum coherence, heteronuclear multiple bond correlation, correlation spectroscopy with F1 decoupling, and

Table 2 ^{13}C NMR chemical shifts of LB reaction products from cholesterol

	10	11	12	13	14	15	16	17	18	19	20
C-1	28.74	120.99	20.85	20.79	21.03	41.27	34.76	137.17	35.35	125.41	123.28
C-2	30.47	125.83	33.20	34.96	32.52	57.25	23.82	131.53	125.08	126.31	126.34
C-3	88.88	127.05	76.36	67.53	71.64	76.18	129.59	126.03	137.92	126.77	127.76
C-4	42.33	134.76	45.68	47.44	44.84	130.87	126.41	130.15	134.15	125.30	135.55
C-5	49.69	130.40	40.30	40.25	40.15	143.60	142.18	137.80	145.58	134.08	133.72
C-6	65.86	121.67	65.83	65.36	65.82	65.55	136.60	71.11	122.69	131.53	121.82
C-7	24.57	123.41	25.29	25.30	25.23	34.18	35.11	64.53	131.96	131.19	130.55
C-8	37.41	126.38	38.56	38.55	38.59	32.21	32.98	36.95	125.84	129.06	129.06
C-9	136.22	129.71	135.16	134.91	135.82	54.72	49.13	41.95	45.93	132.94	135.19
C-10	123.85	130.48	133.30	133.80	132.67	38.72	37.66	141.95	40.91	132.88	132.75
C-11	25.95	122.29	27.72	27.71	27.78	22.09	22.05	29.21	20.46	24.87	25.28
C-12	39.50	132.98	43.70	43.79	43.72	41.18	41.05	41.76	37.71	38.88	38.88
C-13	42.25	143.75	44.87	44.87	44.88	43.64	43.55	45.05	45.11	50.79	50.69
C-14	56.83	140.99	56.93	56.99	56.93	58.12	58.25	53.36	153.10	147.56	147.54
C-15	24.21	31.25	24.75	24.77	24.72	25.15	25.19	24.72	25.89	136.59	136.76
C-16	28.18	25.80	29.41	29.40	29.41	29.33	29.35	29.44	28.34	41.01	40.98
C-17	55.89	51.44	57.58	57.65	57.59	57.60	57.50	57.73	57.42	55.48	55.51
C-18	11.30	20.25	12.39	12.37	12.39	12.60	12.40	12.99	18.93	16.06	16.08
C-19	13.65	20.01	21.29	21.73	21.10	20.77	18.89	22.18	15.86	19.37	19.69
C-20	35.64	36.09	37.10	37.10	37.10	37.13	37.12	37.24	35.93	35.35	35.34
C-21	18.58	19.19	19.08	19.08	19.08	19.20	19.23	19.18	19.42	19.70	19.70
C-22	36.02	30.51	37.23	37.23	37.68	37.35	37.36	37.34	37.07	37.11	37.12
C-23	23.76	25.59	24.89	24.89	24.89	24.93	24.94	24.87	24.88	24.87	24.87
C-24	39.44	39.09	40.67	40.67	40.67	40.68	40.69	40.72	40.68	40.68	40.68
C-25	27.98	27.88	29.13	29.13	29.14	29.15	29.15	29.17	29.15	29.19	29.19
C-26	22.53	22.35[a]	22.93	22.93	22.93	22.93	22.94	22.95	22.93	22.97	22.97
C-27	22.78	22.68[a]	23.16	23.16	23.17	23.18	23.19	23.18	23.17	23.20	23.20

Chemical shifts were measured at 25 °C in $CDCl_3$ (**10**, **11**) or in CD_3OD (**12**–**20**). Ethoxy signals for **15**: δ 15.89, 63.36

[a] Assignments that may be interchanged

distortionless enhanced polarization transfer experiments. Signal assignments were aided by quantum-mechanical NMR shielding predictions. These results, together with chemical shift comparisons, established the carbon skeleton and double-bond positions. The presence of $–SO_3Na$ and $–OSO_3Na$ substituents was established by mass spectrometry, and their location was inferred from NMR shielding patterns. In aromatic systems, $–SO_3Na$ substitution deshields the *ipso* carbon by 10–20 ppm, and resonance-related carbons (e.g., *ortho* and *para*) are deshielded by a few parts per million. In aliphatic systems, the *ipso* carbon is strongly deshielded (by approximately 30–40 ppm), and nearby carbons are modestly deshielded. The exact position of sulfonate substitution in ring A could not be deduced for **18**, and the stereochemistry at C17 and C20 was not established for **11** (a single stereoisomer).

Mass spectral analyses were consistent with the structures shown in Fig. 3: **10** ($C_{27}H_{44}O_3S$), electron impact m/z 448 (97, M^+), 433 (22), 384 (46), 366 (48), 335 (100), 253 (52), 159 (91); FAB (NBA) 449 (43, M + 1), 367 (100, M – HSO_3); **12** ($C_{27}H_{44}O_7Na_2S_2$), ES+ 613.216 (M + Na), ES- 567.229 (M – Na); **13** ($C_{27}H_{45}O_4NaS$), ES+ 511.285 (M + Na), ES- 465.292 (M – Na); **14** ($C_{29}H_{47}O_5NaS$), ES+ 553.294 (M + Na), ES- 507.291 (M – Na); **15** ($C_{29}H_{48}O_7Na_2S_2$), FAB (glycerol) 641 (30, M + Na), 537 (100, M – HSO_3); **16** ($C_{27}H_{43}O_3NaS$), FAB (NBA) 493 (100, M + Na); **17** ($C_{27}H_{41}O_4NaS$), FAB (NBA) 507 (100, M + Na); **18** ($C_{27}H_{38}O_6Na_2S_2$), FAB (glycerol) 591 (M + Na); **19** ($C_{27}H_{35}O_3NaS$), FAB (NBA) 485 (100, M + Na).

Reactivity of Cholesterol Under Salkowski Conditions

To cholesterol (1 mg) in $CDCl_3$ (0.6 mL) in an NMR tube was added D_2SO_4 (1 μL). The tube was inverted several times (the solution becoming pale pink), placed in the magnet, and maintained at 15 °C for 2 h while 25 ^1H NMR spectra were measured at 2–10-min intervals. NMR analysis indicated rapid formation of Δ3,5 diene **9**. The ratio of Δ5, Δ3,5, and other olefins was 74:25:1 after 4 min and 70:24:6 after 15 min, with little change thereafter. In contrast, cholesterol in LB reactions mainly underwent acetylation and sulfation, with little or no Δ3,5 diene formation. Under both LB and Salkowski conditions, NMR showed no aromatic signals until later stages of the reaction, well after the blue or red color had fully developed.

Another Salkowski reaction was performed by adding H_2SO_4 (512 μL) to a solution of cholesterol (1 g) in chloroform (50 mL). The mixture was shaken until a dark red color persisted, followed by chilling to –78 °C and quenching with ethanol (25 mL; –78 °C). To the resulting colorless solution was added 24% aqueous NaOH (10 mL). The precipitate contained **2**;

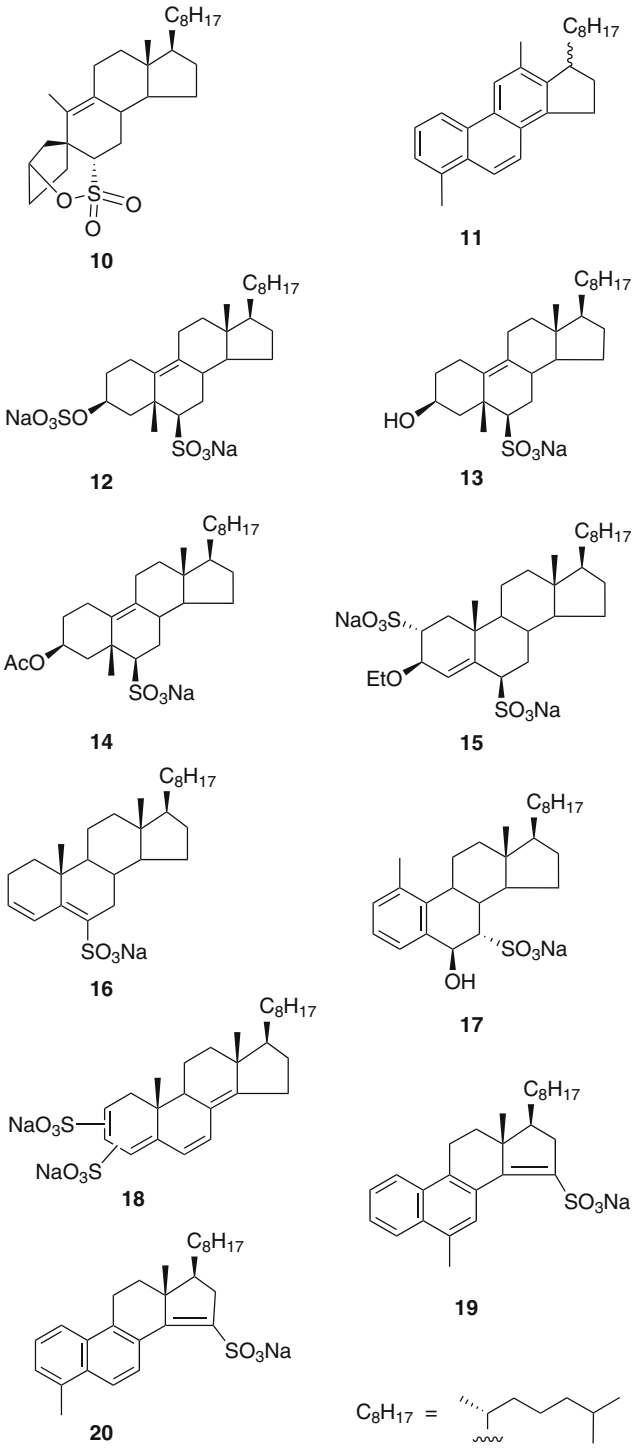

Fig. 3 Products from a LB reaction quenched at the bluish-green stage (reaction B) and worked up under conditions permitting further reaction. The position of the sulfonate groups in **18** was not determined, but 2,4 or 3,4 substitution appeared likely

the organic phase was separated by preparative TLC (developed with $CHCl_3$) into cholesterol (main component) and two backbone-rearrangement products that were characterized by NMR: **21** ($CDCl_3$, δ_H) 0.833 (d, 6.6 Hz), 0.838 (d, 6.6 Hz), 0.897 (s), 0.949 (d, 6.6 Hz), 1.080 (s), 4.093 (quintet, 2.9 Hz); **22** ($CDCl_3$, δ_H) 0.851 (d, 6.6 Hz), 0.857 (d, 6.6 Hz), 0.897 (s), 0.903 (d, 6.6 Hz), 1.080 (s), 4.093 (quintet, 2.9 Hz). These products were identified, except for C10 stereochemistry, from NMR data reported for the 3-deoxy analogs [21]. The 10α-H stereochemistry was assigned as shown in Fig. 4 on the basis of the H3 shieldings (matching calculated values much better for the 10α-H than the 10β-H structure) and H3 coupling constants (indicating an axial 3β-hydroxy group and thus an AB-*trans* ring junction).

Discussion

We have investigated the fate of cholesterol under strongly acidic conditions, with the ultimate goal of elucidating the origin of the color response in the LB reaction. The isolated products shown in Figs. 2 and 3 indicate the operation of several reaction pathways, summarized in Fig. 5. The LB mixture of sulfuric acid, acetic acid, and acetic anhydride in chloroform rapidly converts cholesterol to its acetate and sulfate derivatives, accompanied by small amounts of i-steroids, cholesta–3,5-diene, and other unsaturated species. The predominant Δ5 species are then slowly converted to monounsaturated, diunsaturated, and polyunsaturated sulfonic acids. The polyene species gradually rearrange to aromatic steroids that are likely devoid of blue/green color, even as cations. We argue that none of these mainstream unsaturated species are responsible for the LB color response and that its chemical origin remains unknown.

The nature of the color-generating species in the LB reaction has received much attention. A neutral polyunsaturated steroid would not be colored, as judged by UV spectra of conjugated tetraenes [22], although only five C=C bonds are needed to generate the blue color of azulene. More likely candidates are linearly conjugated polyene cations; linear tetraenylic and pentaenylic cations (from a pentaene and hexaene) absorb in

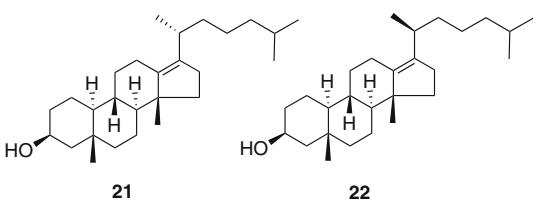

Fig. 4 Structures for backbone rearrangement products formed under Salkowski conditions

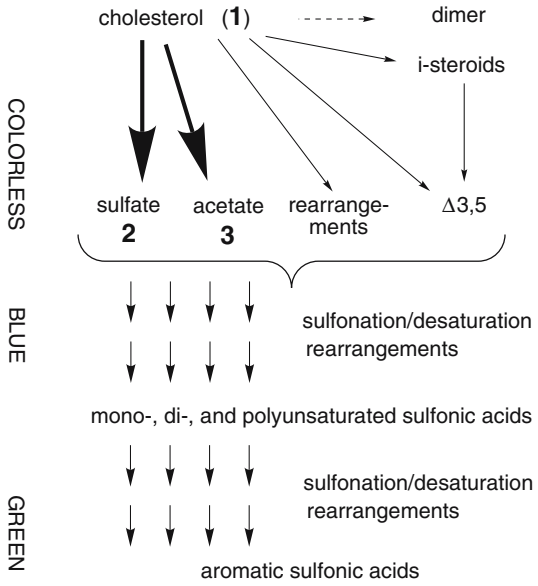

Fig. 5 Major and minor pathways occurring in the LB reaction. The initial Δ5 species **1**–**3** are slowly sulfonated and desaturated (oxidized) to form polyene sulfonic acids and aromatic steroids, as exemplified by **10**–**20**. The blue color appears at the nascent stage of polyene formation

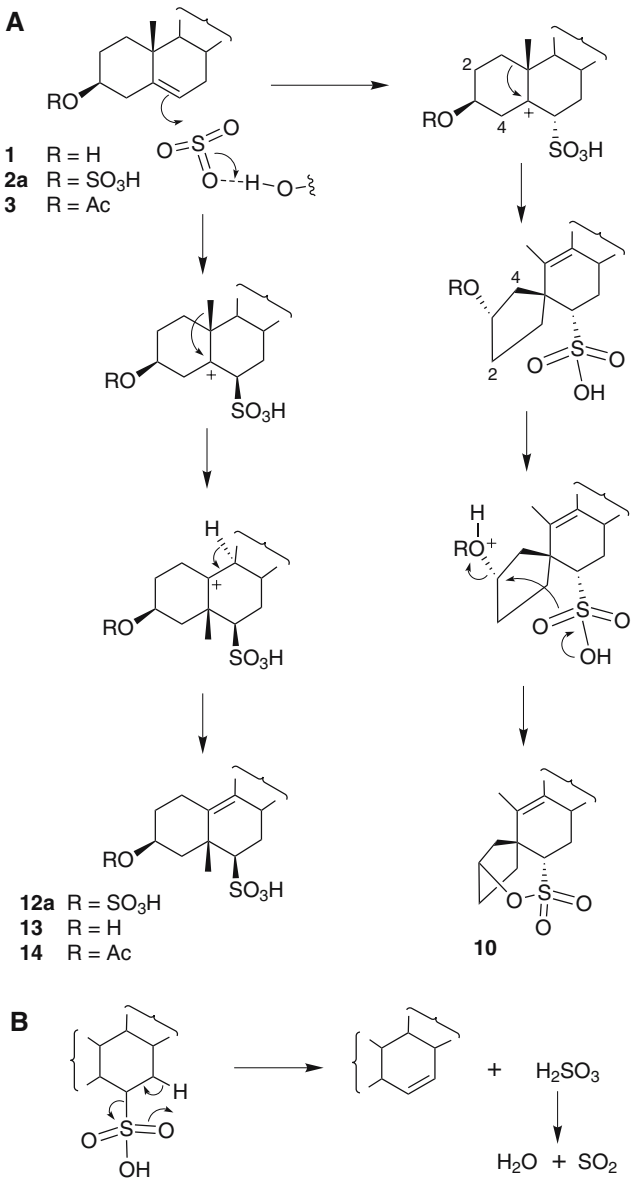

Fig. 6 Suggested mechanisms for **A** the sulfonation of Δ5 sterols and **B** oxidative elimination of H_2SO_3 to give an olefin. Compounds **2** and **12** were isolated as sodium salts but are shown here as the sulfonic acids (**2a** and **12a**) that would exist in the LB reaction. For simplicity, the oxidative elimination in **B** is shown as a cyclic process, although the new O–H bond formation would normally proceed through an intervening solvent molecule

the vicinity of 550 and 620 nm [23], respectively, whereas a tetraenylic cation containing a benzene ring absorbs at only 389 nm [24].

Desaturation of cholesterol to a polyene involves sulfuric acid, which is present in the reagents for the LB, Salkowski, and Zak colorimetric assays. The ability of concentrated H_2SO_4 or $ClSO_3H$ to convert sterols to polyunsaturated sulfonic acids with evolution of SO_2 has long been known [25]. Brieskorn and Hofmann [9] noted that the dehydrating effects of the LB reagent would convert some H_2SO_4 to SO_3, and their observation of SO_2 evolution during the LB reaction confirmed the role of H_2SO_4 in the desaturation of cholesterol.

Our characterization of numerous sulfonic acids from the LB reaction provides the first detailed insights into how the desaturation of cholesterol proceeds. Possible mechanisms for the conversion of Δ5 species **1**–**3** to sulfonic acid derivatives are suggested in Fig. 6a. The modest concentration of SO_3 and the unfavorable activation energy for attack of an isolated C=C on SO_3 are consistent with the sluggishness of the LB color response. Moisture or alcohol (from ethanol-stabilized chloroform) would reduce the SO_3 concentration and further retard the reaction, as is observed.

Our NMR analysis of hydrophobic sterol intermediates (Fig. 2) indicates that Δ5 species are the major substrates in the LB reaction, as shown in Fig. 5. In contrast, Salkowski conditions produce substantial amounts of cholesta-3,5-diene (**9**), which would more readily attack SO_3, since an allylic cation would be formed. This is consistent with the rapid Salkowski color response. The Zak reaction (1:1.5 H_2SO_4/AcOH with Fe^{3+}) may similarly benefit from the absence of acetic anhydride, which favors 3-acetate formation over dehydration to **9**.

Cholesterol dimers have been proposed as the initial species in colorimetric reactions [11, 15]. As pointed

out by others [9, 12–14], the selectivity of the LB reaction (Fig. 1), the UV behavior, and the reaction kinetics (first order in cholesterol) indicate this to be a very minor pathway. We did not detect any sterol dimers in our analyses.

Our results raise serious doubts about the prevailing hypothesis that the LB color response is derived from linearly conjugated polyene cations. Our first concern with this hypothesis is the lack of intermediate colors in the LB reaction. Whereas the Zak reaction proceeds through distinct UV absorbance maxima separated by well-defined isosbestic points [13], our time-course UV analysis of the LB reaction (partially shown in Fig. 3 in [8]) and those of Burke et al. [12] indicated no intermediate species that absorb in the 300–500-nm range. Yellow and red colors are absent in the LB reaction, whereas sequentially yellow, red, blue, and green colors would be observed if double bonds are added one at a time to generate a polyene. Secondly, our NMR analysis showed only traces of sulfonic acids and polyenes at the stage of blue color. Thus, the formation of hexaunsaturated steroids seems too slow to account for the blue color response, even if the LB reagent is sufficiently acidic to protonate a linearly conjugated hexaene effectively. Finally, previous studies [12, 13] correlated the consumption of the oxidant (Fe^{3+} or SO_3) with the color response in the Zak reaction but not the LB reaction. Taken together, these findings indicate that the LB color response does not stem from linearly conjugated polyene cations, although this mechanism may apply to the Zak reaction.

Some conceivable alternatives to the polyene cation model include charge-transfer complexes, phenol/quinone systems (e.g., a polycyclic analog of phenol red), and azulene systems (Fig. 7). Although these proposals are speculative and no such structures have been isolated, some of these systems contain structural features of **10**–**20** and might produce the observed color at trace levels. It is notable that the LB reaction produces a blue or green color, whereas the Zak and Salkowski assays are red.

Despite our advances in elucidating the mechanistic pathways of the LB reaction, the story is far from complete. We have not studied the elimination of H_2SO_3 from sulfonic acids to generate olefins (Fig. 6b). This critical step appears to be slow under LB conditions, thus perhaps accounting for the paucity of polyunsaturated hydrocarbons and the predominance of sulfonic acid intermediates. Many more polar intermediates remain to be characterized and quantified; a comprehensive analysis would permit ranking the many sulfonation and rearrangement pathways in order of importance.

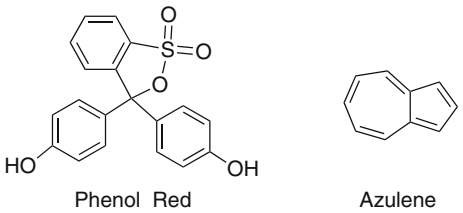

Fig. 7 Polyunsaturated structures that produce intense color at low concentrations. Steroidal polyenes with some of these structural features might conceivably be responsible for the color response in the LB reaction

In summary, we have established the structures of many novel products of the LB reaction. In the presence of colorimetric reagents, cholesterol can variously undergo acetylation, i-steroid formation, backbone rearrangement, dimerization, sulfonation, oxidation/desaturation, and aromatization. Different kinetics among these pathways results in the different color responses observed for the LB, Zak, and Salkowski reactions. Although we have not identified the elusive color-producing species, our mechanistic insights and advances in methodology lay a sound foundation for future work. A resurrection of colorimetric assays is unlikely in clinical laboratories, but rapid and simple steroid assays continue to draw interest [26]. Some of the rearrangements observed for the LB and Salkowski reactions are similar to degradation pathways inferred from studies of sterol diagenesis under various geological conditions [27–29]. Modification of the LB reaction may provide conditions for accelerated ageing of sterols to produce lipid biomarkers that mimic specific geological processes. The behavior of sterols under acidic conditions is a fundamental, enduring topic of research, with many potential applications.

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