5-Z) was prepared by a modification of the procedure used to prepare the corresponding ethyl esters.[20] The isomers were separated by column chromatography (5% ether–95% pentane, silica gel); 5-E elutes before 5-Z. For 5-E: ^{1}H NMR (100 MHz, C_6D_6)[21] δ 5.95 (m, 1 H), 3.23 (s, 3 H), 2.25 (br s, 3 H); IR (pentane) 1735, 1640 cm^{-1}. For 5-Z: ^{1}H NMR (100 MHz, C_6D_6) δ 5.70 (m, 1 H), 3.33 (s, 3 H), 1.61 (br s, 3 H); IR (pentane) 1750, 1642 cm^{-1}.

A solution of $[(CH_3)_3SiCH_2]_2CuLi$ prepared from $(CH_3)_3SiCH_2Li$ (0.64 g, 6.8 mmol) and CuI (0.65 g, 3.4 mmol) in 25 mL of THF was added by syringe to a solution of 5-E (0.49 g, 3.6 mmol) in 10 mL of THF at 0 °C. After 15 min at 0 °C and 1 h at room temperature, the reaction mixture was poured into pentane, washed with 2 N HCl and aqueous $NaHCO_3$, dried ($MgSO_4$), and concentrated to give 6-E (0.57 g, 90%) which was shown to be an 8:1 mixture of 6-E/6-Z by gas chromatography (6 ft column, 10% Carbowax 20 M, 80 °C). A pure sample of 6-E was obtained by column chromatography (5% ether–95% pentane, silica gel): ^{1}H NMR (270 MHz, C_6D_6) δ 5.65 (m, 1 H), 3.46 (s, 3 H), 2.22 (d, J = 1.1 Hz, 3 H), 1.41 (d, J = 0.7 Hz, 2 H), −0.14 (s, 9 H); IR (pentane) 1725, 1638 cm^{-1}; mass spectrum, calcd for $C_9H_{18}O_2Si$ m/e 186.1075, found m/e 186.1075, m/e (relative intensity) 186 (0.8), 84 (100), 82 (46), 73 (32), 57 (22), 56 (15), 54 (15).

Reaction of $[(CH_3)_3CH_2]_2CuLi$ (3.5 mmol) with 5-Z (3.5 mmol, 98:2 Z/E) in THF for 16 h at room temperature led to the isolation of 0.45 g of a mixture of starting material (5-Z, 45%) and a 2.3:1 mixture of 6-E and 6-Z (50%).

1-Methoxy-1-(trimethylsiloxy)-3-methyl-1,3-butadiene (7). Methyl 3-methyl-2-butenoate (5.7 g, 50 mmol) in 10 mL of THF was slowly added at 0 °C to a solution of lithium diisopropylamide prepared by addition of a hexane solution of n-BuLi (30.5 mL, 1.64 M, 50 mmol) to $HN[CH(CH_3)_2]_2$ (5.0 g, 50 mmol) in 100 mL of THF. $(CH_3)_3SiCl$ (75 mmol) and 2 mL of triethylamine were added at 0 °C. Hexane (100 mL) was added, and the reaction mixture was filtered, concentrated, and distilled under reduced pressure to give an ~1:1 mixture of 7-Z and 7-E: 5.2 g (66%); bp 40 °C (0.3 mm). When the distillation of 7 was carried out at 65 °C (1 mm) or if the mixture of 7-Z and 7-E was heated to 115 °C for 40 min, isomerization to give a single isomer of 7 was observed. For the thermodynamically favored isomer of 7: ^{1}H NMR (C_6D_6, 270 MHz) δ 4.90 (br d, J = 2.7 Hz, 1 H), 4.65 (m, J_{CH_3} = 1.4 Hz, J_H = 2.7 Hz), 4.23 (s, 1 H), 3.15 (s, 3 H), 2.00 (dd, J = 1.4 Hz, J' = 0.7 Hz, 3 H), 0.14 (s, 9 H); ^{13}C NMR (15 MHz, C_6D_6, off-resonance decoupled) δ 159.4 (s), 141.9 (s), 110.2 (t), 83.7 (d), 56.5 (q), 25.6 (q), 2.2 (q); IR (hexane) 1660 cm^{-1}; mass spectrum, calcd for $C_9H_{18}O_2Si$ m/e 186.1075, found m/e 186.1075, m/e (relative intensity) 149 (38), 146 (100), 131 (20), 89 (16), 75 (14), 73 (40), 59 (14). The only difference observed between the ^{1}H NMR (C_6D_6) of the two isomers of 7 is the CH_3O resonances which appear at δ 3.15 and 3.12.

Methyl (Z)-3-Methyl-4-(trimethylsilyl)-2-butenoate (6-Z). The decomposition of 7 (150 mg, 0.80 mmol) in toluene-d_8 occurred at 117 °C (NMR yield 46%) (k = 9.7 × 10^{-5} s^{-1}, $t_{1/2}$ = 2.0 h) to give a ~1.1 mixture of 6-Z and $(CH_3)_3SiOCH_3$. $(CH_3)_3SiOCH_3$ was isolated in 27% yield by preparative gas chromatography (UCON 50 HB 280X, 64 °C) and identified by comparison of NMR and IR spectra and GC retention times with those of an authentic sample. None of 6-E was detectable by NMR. 6-Z (40% yield) was isolated by preparative thin layer chromatography (3:7 CH_2Cl_2–hexane, silica gel, R_f 0.3): ^{1}H NMR (270 MHz, C_6D_6) δ 5.67 (br s, 1 H), 3.42 (s, 3 H), 2.46 (br s, 2 H), 1.52 (br d, J = 1.1 Hz, 3 H), 0.05 (s, 9 H); ^{13}C NMR (C_6D_6, 15 MHz) δ 169.0, 161.8, 114.4, 52.0, 29.3 (CH_2Si and CH_3C=), 1.0; IR (pentane) 1730, 1640 cm^{-1}; mass spectrum, calcd for $C_9H_{18}O_2Si$ m/e 186.1075, found m/e 186.1075, m/e (relative intensity) 186 (2.3), 89 (29.7), 82 (100), 77 (30.5), 73 (55.3), 59 (17.9).

6-Z was also obtained by photolysis of 6-E. A pentane solution of 6-E in a quartz tube was irradiated in a Srinivasan-Griffin photochemical reactor with mercury lamps (254-nm maximum). The photolysis was followed by gas chromatography which indicated that a photostationary state consisting of a 1.7:1.0 ratio of 6-E/6-Z was established after 16 h. The mixture was separated by thin-layer chromatography (10% ether–pentane, silica gel; R_f 0.5 for 6-E, R_f 0.3 for 6-Z), and the components were identified by ^{1}H NMR.

Registry No. 1, 40988-97-4; 2-E, 49784-51-2; 2-Z, 49784-64-7; 2-E, 76927-58-7; 3-Z, 76915-31-6; 4, 76915-32-7; 5-E, 6372-01-6; 5-Z, 6214-25-1; 6-E, 65263-86-7; 6-Z, 65263-87-8; 7-E, 76927-59-8; 7-Z, 76915-33-8; $(CH_3)_3SiCH_2Li$, 1822-00-0; $(CH_3)_3SiCl$, 75-77-4; $(CH_3)_3SiOCH_3$, 1825-61-2; 4-methyl-3-penten-2-one, 141-79-7; 4-methyl-2-(trimethylsiloxy)-1,3-pentadiene, 6651-46-3; methyl 3-methyl-2-butenoate, 924-50-5.

(20) Jones, D. E.; Morris, R. O.; Vernon, C. A.; White, R. F. M. *J. Chem. Soc.* **1960**, 2349.

(21) For further ^{1}H and ^{13}C NMR see: Brouwer, H.; Stothers, J. B. *Can. J. Chem.* **1972**, *50*, 601.

N-Nitroso Bile Acid Conjugates. 1. Synthesis, Chemical Reactivity, and Mutagenic Activity

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Two *N*-nitroso bile acid conjugates, *N*-nitrosotaurocholic acid (NOTC) and *N*-nitrosoglycocholic acid (NOGC), have been synthesized. Spectral data were consistent with *N*-nitrosamide structures. NOTC and NOGC decomposed in mildly alkaline aqueous solution to give cholic acid and the respective alcohols, 2-hydroxyethanesulfonic acid and glycolic acid. The rates of decomposition for both compounds between pH 7 and 9 indicated a $t_{1/2}$ of ca. 2–4 h at neutral pH. Under simulated gastric conditions, NOTC was formed from taurocholic acid and nitrite. NOTC and NOGC were mutagenic in a bacterial forward mutation assay (*S. typhimurium* TM 677). The results are discussed with respect to the mechanisms of *N*-nitrosamide decomposition.

Cancer of the gastrointestinal tract accounts for a large proportion of the cancers which afflict humans in the United States and other parts of the world.[1] Much attention has been focussed on exogenous sources of carcinogens such as foodstuffs and environmental pollution with comparatively little attention being paid to possible endogenous formation of carcinogens.

One class of chemicals, *N*-nitroso compounds, has been extensively studied in connection with chemical carcino-

(1) R. Peto, *Nature (London)*, 284, 297 (1980).

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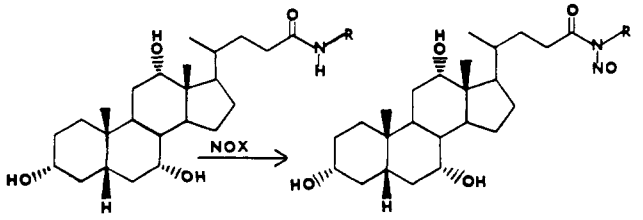
Scheme I. Bile Acid Conjugate N-Nitrosation



genesis, and the *N*-nitrosamines, in particular, have well-established carcinogenicity[2] and have been shown to occur under a wide range of environmental conditions.[3]

In comparison, the *N*-nitrosamides, generally possessing direct acting carcinogenic activity,[2] have attracted comparatively little attention. With the exception of compounds like *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (MNNG) and *N*-methyl-*N*-nitrosourea (MNU), the literature on the physiological occurrence and activity of *N*-nitrosamides is sparse, probably due to their lability and a lack of appropriate analytical methodology. In addition, the fact that amides are more difficult to N-nitrosate than amines[4] may have dissuaded most workers from establishing suitable model systems to study this reaction.[5]

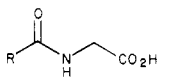
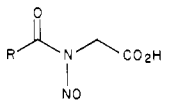
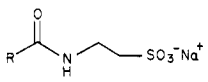
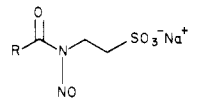
We reasoned that the amide moiety in conjugated bile acids could be N-nitrosated and that these compounds could be used to investigate the possibility of in vivo *N*-nitrosamide formation.

The bile acids are normally present in human bile as amides of glycine of taurine (2-aminoethanesulfonic acid) and enter the upper intestinal tract in this form.[6] Having performed their function of assisting in the absorption of fat they are reabsorbed and circulated in the enterohepatic cycle.[7] However, a fraction (~0.5 g/day) is excreted in the faeces, with the same amount being synthesized to maintain a constant total amount (ca. 2–2.5 g).[8] Since this "pool" circulates two or three times during meals or five to ten times daily then, theoretically, 10–15 g is excreted in the intestine daily. Thus, a large potential source of amides is available for N-nitrosation (Scheme I).

In terms of the mechanism of N-nitrosation the bile acid conjugates possess structural and physical properties which represent those present in a wide range of biological situations. Recent work[9] suggests that the hydroxyl function is more readily nitrosated compared to the amino or amide function. This leads to the formation of nitrite esters capable of further nitrosation, depending on structural and electronic considerations. Although nitrite esters of sterols[10] and bile acids[11] have been prepared, little is known about their ability to nitrosate amines or any other substrates. In addition, bile acids readily form micelles[12] which have a well-documented[13,14] catalytic effect on N-nitrosation.

With these factors in mind we have investigated the properties of N-nitroso bile acid conjugates. The results are described in the following sections.

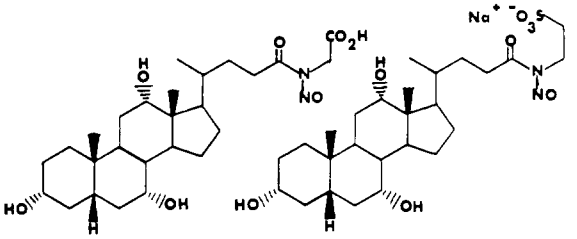
Table I. NMR (250 MHz) Chemical Shifts of *N*-Nitroso Bile Acid Conjugates and Related Compounds

bile acid[b]	shift, δ[a,c]
	13.19 (s, 1, COOH)
	13.20 (s, 1, COOH), 8.19 (t, 1, J = 6 Hz, NH), 3.72 (d, 2, J = 6 Hz, $NHCH_2$)
	13.19 (s, 1, COOH), 4.42 (s, 2, $N(NO)CH_2$)
	7.67 (tr, 1, J_1 = 6.5 Hz, NH), 3.30 (q, 2, J_1 = 6.5 Hz, J_2 = 7.5 Hz, $NHCH_2CH_2$), 2.54 (tr, 2, J_2 = 7.5 Hz, $CH_2CH_2SO_3^-$)
	3.93 (tr, 2, J = 7.5 Hz, $N(NO)CH_2$-CH_2), 2.36 (tr, 2, J = 7.5 Hz, CH_2-CH_2-SO_3^-)

[a] Downfield of Me_4Si in Me_2SO-d_6. [b] R = C_{23} residue of cholic acid. [c] Apart from the steroid "envelope" of absorbances at δ 0–2.20, all the compounds showed resonances at δ 4.35, 4.11, and 4.02 (all s or d, 1 exch 3α-, 7α-, and 12α-OH not assigned) and 3.77, 3.60, and 3.17 (s, 1, C_{12}, C_7, and C_3 CHOH, respectively).[3,2] [d] The center line of both of these triplets was split with an apparent coupling constant of 3 Hz. In order to check whether any other protons (or an induced assymmetry due to a nearby chiral center) caused this effect, we carried out a homonuclear proton decoupling experiment. Irradiation of the resonance at 3.93 ppm caused the 2.36-ppm resonance to collapse to a singlet and vice versa. No other part of the spectrum was affected.

Results

Synthesis and Characterization of *N*-Nitroso Bile Acid Conjugates. The treatment of two bile acid conjugates, taurocholic acid (TC) and glycocholic acid (GC), with either nitrous acid or nitrosyl chloride in acetic acid afforded products whose physical and chemical properties were entirely consistent with the *N*-nitrosamide structures shown.



N-Nitrosoglycocholic acid (NOGC)

N-nitrosotaurocholic acid sodium salt, (NOTC)

The UV–visible spectra in methanol showed characteristic[15] triplets of absorbances between 380 and 430 nm, with extinction coefficients of less than 100, and single

(2) H. Druckrey, R. Preussmann, S. Ivankovic, D. Schmähl, J. Afkham, G. Blum, H. D. Mennel, M. Müller, P. Petropoulos, and H. Schneider, *Z. Krebsforsch.*, **69**, 103 (1967).
(3) I. S. Krull, G. Edwards, M. H. Wolf, T. Y. Fan, and D. H. Fine, *ACS Symp. Ser.*, **No. 101**, 175–194 (1979).
(4) S. S. Mirvish, *Toxicol. Appl. Pharmacol.*, **31**, 325 (1975).
(5) A recent exception is Y. Kakuda and J. I. Gray, *J. Agric. Food Chem.*, **28** 584 (1980).
(6) P. P. Nair and D. Kritchevsky, Eds., "The Bile Acids", Plenum Press, New York, 1973, Vol. 2, pp 55–80.
(7) Reference 6, pp 103–149.
(8) Reference 6, pp 58–61.
(9) B. C. Challis and D. E. G. Shuker, *J. Chem. Soc., Chem. Commun.*, 315 (1975); M. Yamamoto, T. Yamada, and A. Tanimura, *Shokuhin Eiseigaku Zasshi* **20**, 15 (1979).
(10) M. N. Akhtar, *Adv. Photochem.*, **2**, 263 (1964).
(11) I. Tanasescu and M. Terdic, *Acad. Repub. Pop. Rom. Fil. Chuj, Stud. Ceccet. Chim.*, 11, 303 (1963); *Chem. Abstr.*, **58**, 4619*f* (1963).
(12) Reference 6, 1971, Vol. 1, pp 302–332.
(13) Y.-K. Kim, S. R. Tannenbaum, and J. S. Wishnok, Proceedings of the 6th Meeting on Analysis and Formation of *N*-Nitroso Compounds, Budapest; *IARC Sci. Publ.*, **No. 31**, 207–214 (1981).
(14) J. H. Fendler and E. J. Fendler, "Catalysis in Micellar and Macromolecular Systems", Academic Press, New York, 1975, pp 242–247.
(15) R. B. Brundrett, M. Colvin, E. H. White, J. McKee, P. E. Hartman, and D. L. Brown, *Cancer Res.*, **39**, 1329 (1979).

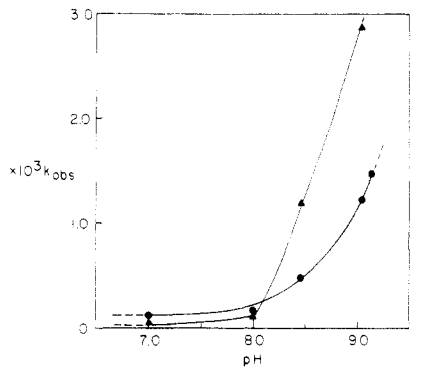


Figure 1. Partial pH–rate profile of the decomposition of NOTC and NOGC in aqueous buffers (see Experimental Section for details): ▲ = NOTC; ● = NOGC.

peaks at ca. 250 nm (ϵ ~4000–5000). Infrared spectra (KBr disks) showed a carbonyl absorption at 1725 cm^{-1} for both compounds and nitroso absorptions at 1500 and 1510 cm^{-1} for NOTC and NOGC, respectively.

The field-desorption (FD) mass spectra of GC, TC, and NOGC all showed major peaks corresponding to M + H + Na (*m/e* 489, 561, and 518 respectively). The TC spectrum, in addition, had a strong peak at *m/e* 291.5, corresponding to $(M + 2Na)^{2+}$; this peak was observed in the FD spectrum of TC.[16] The most prominent peak at higher mass for NOTC was at *m/e* 562, corresponding to loss of 28 mass units from M + H + Na. This fragmentation may be analogous to a thermal rearrangement, followed by loss of N_2, which has been reported for some *N*-nitrosamides.

Detailed analyses of the fragmentation patterns for these compounds, in the absence of appropriate model systems, are not complete. A more extensive survey of the FD mass spectral properties of the *N*-nitroso bile acid conjugates will be described in a subsequent report.

In addition, the 250-MHz ^1H NMR spectra of NOTC, NOGC, GC, TC, and cholic acid were obtained. The chemical shifts of interest are summarized in Table I. In all these cases the major part of the spectrum (δ 0–2.2, corresponding to the steroid backbone) remained essentially unchanged, making the assignment of the side-chain resonances reasonably straightforward.

Satisfactory elemental analyses could not be obtained of chromatographically homogenous, recrystallized samples of either *N*-nitroso bile acid conjugate.

Decomposition in Aqueous Solution. NOTC and NOGC decompose in aqueous solution as determined by the disappearance of the characteristic visible absorbances. This decomposition is markedly catalyzed by increasing hydroxide ion concentration (Figure 1).

Above pH 8 the reactions were carried out in borate buffer which is known not to catalyze *N*-nitrosamide decomposition.[17] Below pH 8 phosphate buffer was used which catalyzed the reaction; therefore, rates of decomposition at zero buffer concentration were obtained by extrapolation of values obtained at several different buffer concentrations. A linear relationship was observed between the rate of decomposition and phosphate buffer concentration.[18]

Thus, the *N*-nitroso bile acid conjugates have half-lives of about 2–4 h at neutral pH; that is, they are reasonably stable under conditions that might be expected to be encountered in the upper intestinal tract.[19]

Table II. Percentage Yields of the Major Products of Decomposition of *N*-Nitroso Bile Acid Conjugates in Borate Buffer (pH 8, 0.05 M) at 37 °C

	cholic acid	HO⟨ ⟩CO_2H	HO⟨ ⟩SO_3H
NOTC	94		61[a]
NOGC	50	85	

[a] In addition, there were two other, as yet unidentified, products observed in the GLC trace.

Scheme II. Decomposition of NOTC and NOGC in Aqueous Alkaline Solution

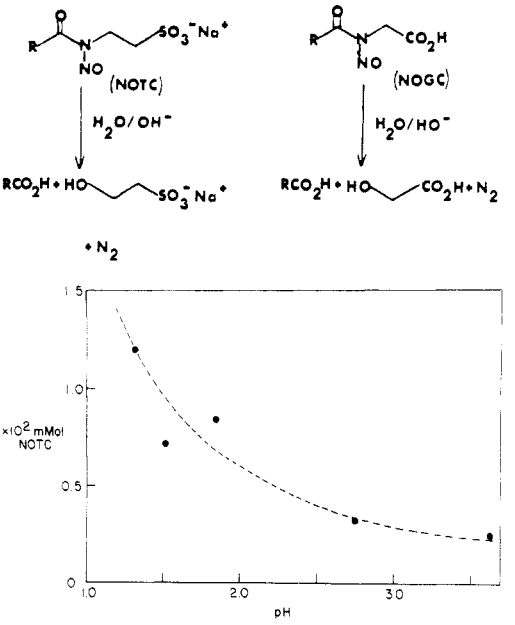


Figure 2. Product yield vs. pH for the N-nitrosation of taurocholic acid in simulated gastric juice: initial [NOTC] = 10 mM; [$NaNO_2$] = 40 mM; overnight reaction.

Cholic acid was the main steroidal product of aqueous decomposition of NOTC and NOGC as determined by TLC and gas–liquid chromatography (Table II). NOTC afforded 2-hydroxyethanesulfonic acid (isethionic acid), and NOGC gave 2-hydroxyacetic acid (glycolic acid) as the major alkyl fragments (Scheme II).

Formation of *N*-Nitroso Bile Acid Conjugates. When taurocholic acid (TC) was nitrosated under simulated gastric conditions (HCl/NaCl buffer at 37 °C), the yield was low even after prolonged reaction times (ca. 12 h). The actual amount formed, however, may be higher because NOTC decomposes at pH ~1.5. A 0.2-mM solution of NOTC in pH 1.5 HCl/NaCl buffer was assayed by high-pressure LC, and the peak due to NOTC was found to decrease with time with a $t_{1/2}$ of ca. 12 h.

Figure 2 shows the yield of *N*-nitrosotaurocholic acid (NOTC) as a function of pH, as determined by high-pressure LC upon termination of the reaction by neutralization to ca. pH 5. When an aliquot of the reaction solution was treated with sodium hydroxide and analyzed, the product disappeared as would be expected from rapid alkaline hydrolysis.[20]

In a manner similar to that for other simple amides, the N-nitrosation of taurocholic acid (TC) is catalyzed by increasing acidity.[4] In contrast to amines, amides do not

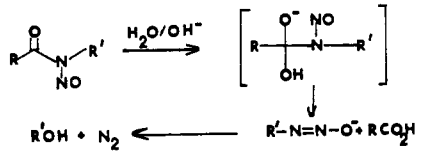
(16) R. Shaw and W. H. Elliott, *Biomed. Mass Spectrom.*, **5**, 433 (1978).
(17) C. N. Berry, Ph.D. Thesis, University of London, 1973.
(18) C. N. Berry, B. C. Challis, and A. D. Gribble, *J. Chem. Soc., Perkin Trans. 2*, in press.
(19) Reference 6, p 121.
(20) Reference 18; B. C. Challis and S. P. Jones, *J. Chem. Soc., Perkin Trans. 2*, 703 (1979); E. H. White and D. J. Woodcook in "Chemistry of the Amino Group", S. Patai, Ed., Wiley, New York, 1968, Chapter 8.

Table III. Mutagenic Activity of Bile Acid Conjugates in TM 677

	control[e]	NOTC[b]	NOGC[c]
10^5(mutant fraction)[a,d]	5 (15[f])	122 ± 21	151 ± 22

[a] Mutant fraction = number of mutant cells observed/total number of surviving cells. [b] 1.12 mmol. [c] 1.07 mmol. [d] No activation. [e] Me_2SO. [f] 99% upper confidence limit of background.

Scheme III. Aqueous Alkaline Decomposition of N-Nitrosamides



show a pH maximum for nitrosation and generally do not react at pH ≥5.

Under similar reaction conditions glycocholic acid is insoluble due to protonation of the conjugate base (pK_a = 3.8).[12]

Mutagenic Activity. N-Nitroso bile acid conjugates were isolated from reaction solutions by preparative thin-layer chromatography. NOTC and NOGC thus isolated were assayed for mutagenic activity in a forward mutation assay using strain TM 677 of *S. typhimurium* which is sensitive to a broad range of known mutagens.[21] Both NOTC and NOGC were positive whereas the nonnitrosated substrates showed no activity (Table III).

Discussion

Two N-nitroso bile acid conjugates have been synthesized and characterized. They belong to the class of N-nitrosamides which are known to be carcinogens[2] and mutagens.[15] Despite having more complex substituents than the simpler carcinogenic N-nitrosamides (for example, N-methyl-N-nitrosoacetamide) they exhibit a closely similar type of chemical reactivity.

Both NOTC and NOGC readily decompose in mildly alkaline aqueous solutions with the evolution of nitrogen. The mechanism of this decomposition has been studied by several workers[20] and is thought to involve an initial nucleophilic attack on the carbonyl function with subsequent rate-limiting decomposition of the tetrahedral intermediate to displace a diazotate moiety (Scheme III).

There is evidence that the diazotate intermediate decomposes further to give a diazonium ion which is either quenched by the aqueous solution or can be trapped out by added nucleophiles. In the case of NOGC and NOTC, base-catalyzed decompositions can give rise to diazoacetic acid (a known carboxymethylating agent)[22] and 2-diazoethane sulfonic acid (Scheme III) which can hydrolyze to 2-hydroxyethanesulfonic acid (isethionic acid, ISA). ISA is a known urinary metabolite in man;[23] however, its source remains unknown despite the efforts of several research groups.[24] Although ISA is nominally the deamination product of taurine, taurine itself has been unequivocally shown *not* to be the source in vivo.[25] Thus, we are actively investigating the metabolism of both N-nitroso bile acids with particular emphasis on NOTC because of its unusual breakdown product.

Not surprisingly, both compounds were mutagenic, in the absence of activation, in both forward and reversion bacterial mutation assays and showed stronger responses in the reversion strains that are most sensitive to base substitution.[26] NOGC and NOTC are currently being tested for carcinogenicity in whole animals.

Experimental Section

Caution! *In view of the hazardous nature of N-nitroso compounds, all work was carried out in efficient fume hoods. N-Nitrosamides were destroyed by using concentrated aqueous alkali.*

Bile acids obtained from Vega Biochemicals or Sigma Chemical Co. were found to be chromatographically homogeneous and were therefore used without further purification. Thin-layer chromatography was carried out on silica gel G with $CHCl_3$–*i*-PrOH–*i*-Pr_2O–acetic acid (3:2:2:2 v/v; used for the separation of complex mixtures of bile acids) or $CHCl_3$–MeOH–Et_2O (1:1:1 v/v; for monitoring reactions of single bile acids). Bile acids were visualized by spraying the plates with phosphomolybdic acid in acetic acid according to Haslewood.[27]

Nitroso compounds were visualized by spraying with Shinn's reagent.[28] After being sprayed, nitrite esters showed up immediately as purple spots, and N-nitrosamides appeared only after irradiation of the plate with either short- or long-wave UV light.

N-Nitroso Taurocholic Acid (NOTC). Sodium taurocholate (5 g), in glacial acetic acid (50 mL) containing anhydrous sodium acetate (8 g, ~10-fold excess), was treated with a slow stream of NOCl gas (Matheson) at 10–15 °C for ca. 30 min until a yellow-green color persisted. Upon completion of the addition and after the mixture was stirred for another 30 min, TLC showed that all the starting material had been consumed and that two major products were formed which are positive to phosphomolybdic acid (PMA) and Shinn reagent (*without* UV irradiation). Treatment of a small aliquot of the reaction mixture with methanol and purging with nitrogen afforded only a single product by TLC. (This procedure converts excess NOCl to methyl nitrite and causes steroidal nitrite esters to transesterify to give methyl nitrite. Methyl nitrite is then removed by purging with N_2.) This compound was PMA positive but Shinn positive only after UV irradiation.

Methanol (ca. 20 mL) was added to the rest of the reaction solution and nitrogen bubbled through until a piece of filter paper spotted with Shinn's reagent held over the mouth of the flask no longer turned purple, indicating that no more methyl nitrite was being produced. The resulting solution was filtered through Celite to remove precipitated salts and freeze-dried (freeze-drying was used to minimize product decomposition). The residue (containing NOTC and inorganic salts) was dissolved in the minimum volume of water saturated with ether. (A concentrated aqueous solution of NOTC was found to become extremely viscous, no doubt due to its surfactant properties. Small amounts of ether were completely miscible with this solution, and only when sufficient ether is added to form a separate layer does the solution become mobile again.)[29] Sodium chloride was added until crystallization began. When the mixture was cooled overnight in the refrigerator, the product crystallized and was filtered off, washed with ice-cold saturated sodium chloride solution saturated with ether, and dried in vacuo over P_2O_5 at room temperature. Extraction of the pale yellow solid with a minimum volume of cold absolute ethanol afforded a pale yellow, clear solution which was treated with ether until a persistent cloudiness resulted. After the mixture was cooled overnight, the crystals were filtered off, washed with ice-cold ether, and dried in vacuo to give sodium N-nitrosotaurocholate: 3.8 g

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(72%); mp 182–184 °C dec (discolors at 110 °C); UV max (MeOH) 241 nm (ε 4095), 390 (52), 405 (74), 426 (73); IR (KBr) 1725 (C=O), 1500 (N=O) cm^{-1}; mass spectrum (field desorption, T_s = 79 °C, voltage 6.7 kV, wire current 15–18 mA), m/e (relative intensity) 564 (29), 562 (57), 518 (39), 510 (57), 508 (57), 506 (32), 505 (57), 498 (68), 496 (32), 490 (75), 489 (39), 460 (21), 448 (32), 438 (86), 437 (93), 420 (100), 411 (36), 410 (64), 409 (39), 391 (36), 390 (57), 389 (57).

***N*-Nitroso Glycocholic Acid (NOGC).** Glycocholic acid (5 g) was treated with NOCl as described above. On completion of the reaction and after the mixture was freeze-dried, the residue was dissolved in ice–water (300 mL) and carefully acidified with concentrated HCl with vigorous stirring until further addition of acid gave no precipitate. The solid was filtered off, washed with water to remove excess acid, and dried over P_2O_5 in vacuo. The residue was dissolved in ethanol to give a clear yellow solution to which water was added until a persistent cloudiness was observed. After the mixture was cooled overnight, the crystals were filtered off, washed with cold water, and dried in vacuo over P_2O_5 to give *N*-nitrosoglycocholic acid: 4.0 g (75%); mp 91–93 °C; UV max (MeOH) 248 nm (ε 4690), 387 (58), 403 (95), 422 (88); IR (KBr) 1725 (C=O), 1510 (N=O) cm^{-1}; mass spectrum (field desorption, T_s = 78 °C, voltage 6.7 kV, wire current 10–15 mA), m/e (relative intensity) 518 (53), 516 (56), 505 (43), 497 (50), 496 (100), 468 (42), 467 (57), 465 (61), 439 (51), 439 (42), 437 (40), 436 (31), 432 (35), 430 (78), 426 (69), 423 (22), 421 (79), 411 (20), 410 (69), 409 (53), 392 (62), 389 (36), 374 (30), 366 (71), 340 (61).

High-Pressure Liquid Chromatography. *N*-Nitroso bile acid conjugates were analyzed by reversed-phase, high-pressure LC on LiChrosorb RP18 (25 cm × 4 mm i.d., stainless-steel column) using an isocratic eluant of ammonium phosphate (30 mM, pH 4.3)/acetonitrile (2:1) at 1.5 mL/min. Both NOGC and NOTC had identical retention times (9 min) with this system. A mixture of the two was resolved by using ammonium phosphate (30 mM, pH 2.3)/acetonitrile (1:1) at 1.5 mL/min, where the retention times were 2.6 and 6.4 min for NOTC and NOGC, respectively. Both compounds were detected by using UV monitoring of the column eluate at 250 or 410 nm.

Mutagen Assays. Forward (TM 677) bacterial mutation assays were carried out as described by Thilly and co-workers.[21]

Decomposition of NOTC and NOGC. The decomposition of the *N*-nitroso bile acids in aqueous solution was followed by monitoring the disappearance of the UV absorbance at 245 nm (NOTC) or 250 nm (NOGC). Above pH 8 the reactions were carried out in borate buffer (0.1 M). Below pH 8 phosphate buffer was used. All reactions were carried out in a Beckman DU-8 double-beam spectrophotometer with the reaction cells thermostated to 37 °C. The reactions were found to give good first- (or pseudo-first) order kinetics over at least 3 half-lives.

Products of Decomposition. NOGC or NOTC (5 mL of 10 mM solution in 0.05 M borate buffer, pH 8) was stirred in the dark at 37 °C until TLC showed that all the *N*-nitrosamide had disappeared. Aliquots (200 μL) were acidified, evaporated to dryness, and extracted with ether/methanol (9:1 v/v). This solution was treated with diazomethane at 0 °C and then analyzed by GLC. On a 3 ft × 2 mm glass column containing 3% SP-2250 on Supelcoport (100–120 mesh), with the injection port at 300 °C, the column at 275 °C, and a helium flow rate of 60 cm^2/min, methyl cholate has a retention time of 6.5 min. The yields were determined by treating standard solutions of cholic acid in a similar manner.

Cholic acid was identified by GC/MS in the following manner. An ether/methanol solution of methyl cholate was prepared as described above. The solvent was removed in a stream of nitrogen and the residue dissolved in dry pyridine (200 μL). Trifluoroacetic anhydride (50 μL) was added, and the mixture was allowed to stand at room temperature for 15 min. Aliquots (2 μL) of this solution were injected onto the column of a Hewlett-Packard 5992A gas chromatograph–mass spectrometer. The retention time of methyl 3α,7α,12α-tris(trifluoroacetoxy)-5β-cholanoate, on a 3 ft × 2 mm glass column containing 3% SP-2250 on Supelcoport (100/120 mesh), with the injection port at 300 °C, the column at 250 °C, and a helium flow rate of 25 cm^2/min, is 4.1 min. Both NOTC and NOGC reaction mixtures and cholic acid gave almost identical ion chromatograms. The mass spectra (70 eV) of the derivatives were identical and displayed characteristic ions at m/e 482 (M^+ – 228, 2 × trifluoroacetic acid [TFA]), 367, (M^+ – 2TFA + side chain), 253 (M^+ – 3TFA + side chain), 211 (M^+ – 3TFA + side chain + D ring). This fragmentation is identical with that described in the literature.[30]

Isethionic acid was determined as its dimethyl derivative after a treatment of an aliquot of the reaction solution with ethereal diazomethane in the presence of a trace of boron trifluoride. Methyl 2-methoxyethanesulfonate was chromatographed on an OV-17 column and its identity confirmed by GC/MS.[31]

Glycolic acid was analyzed as methyl glycolate, after treatment with diazomethane, on a 0.8% KOH on 4% Carbopack plus Carbowax 20M column.

Full details of the latter analyses will be published elsewhere.

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Registry No. NOTC sodium salt, 76757-84-1; NOGC, 76757-85-2; cholic acid, 81-25-4; taurocholic acid Na, 145-42-6; glycocholic acid, 475-31-0; hydroxyacetic acid, 79-14-1; 2-hydroxyethanesulfonic acid, 107-36-8.

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