Characterization of sarcosylsarcoursodeoxycholic acid formed during the synthesis of sarcoursodeoxycholic acid

Ashok K. Batta, Gerald Salen, and Sarah Shefer

Department of Medicine and Sammy Davis, Jr. Liver Institute, UMDNJ-NJ Medical School, Newark, NJ 07103, and Veterans Administration Medical Center, East Orange, NJ 07019

Summary This report describes the isolation of sarcosylsarcosine conjugate of ursodeoxycholic acid (UDCA) formed during the synthesis of sarcoUDCA by the mixed anhydride method. The compound was characterized by its chemical ionization mass spectrum. The diamino acid conjugate was formed only when the free amino acid was used for conjugation. This was confirmed by the isolation of glycylglycoUDCA during the conjugation of UDCA with free glycine but not with glycine ethyl ester hydrochloride. Pure sarcoUDCA was prepared by conjugation of UDCA with sarocisine methyl ester hydrochloride while sarcoUDCA on further reaction with the protected sarcosine derivative gave pure sarcosylsarcoUDCA in 52% yield.—**Batta, A. K., G. Salen, and S. Shefer. Characterization of sarcosylsarcoursodeoxycholic acid formed during the synthesis of sarcoursodeoxycholic acid. *J. Lipid Res.* 1989. **30:** 771–774.

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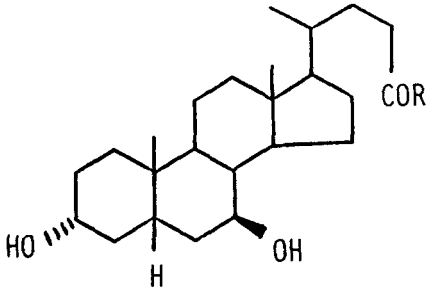
Ursodeoxycholic acid (3α, 7β-dihydroxy-5β-cholan-24-oic acid, UDCA) is increasingly being used as a gallstone-solubilizing agent due to its greater patient tolerance and reduced side effects (1). UDCA is 7β-dehydroxylated to a much smaller extent than its 7α-hydroxy epimer, CDCA, to form the hepatotoxic LCA (2, 3). Humans are believed to be protected from LCA toxicity, due to their capacity to sulfate LCA (4); however, it has recently been shown that certain individuals may not be able to sulfate LCA effectively (5). This emphasizes the need for litholytic agents that are not 7-dehydroxylated to LCA.

Recently, the biliary secretion, intestinal absorption, and metabolism of the sarcosine (N-methylglycine) conjugate of UDCA (sarcoUDCA, **Fig. 1, I.**) was studied in the rat (6). It was found that this unnatural conjugate of UDCA was effectively absorbed from the intestine and secreted into the bile intact (6, 7). When administered orally to the hamster, 77% of the sarcosine conjugate was recovered in the feces in 6 days, whereas 95% of the free UDCA similarly administered was converted into LCA (6). In another study, chronic feeding of sarcoUDCA showed no toxicity in the mice (8). Thus, there is preliminary evidence that sarcoUDCA, like the glycine- and taurine-conjugated bile acids, circulates well in the enterohepatic circulation, but, unlike the glycine- or taurine-conjugated bile acids, resists bacterial 7-dehydroxylation as well as deconjugation (9).

In order to further study the metabolism and toxicity of sarcoUDCA in the rat, we required the synthesis of large quantities of this compound. Kimura et al. (6) have recently reported the synthesis of this compound by the method of Norman (10). However, a TLC examination of the reaction products revealed to us the presence of a more polar compound in approximately 15% yield. This report describes the synthesis of this polar compound characterized as sarcosylsarcoUDCA (Fig. 1, II.) from its chemical ionization mass spectrum. We also report the

Abbreviations: UDCA, ursodeoxycholic acid, 3α, 7β-dihydroxy-5β-cholan-24-oic acid; CDCA, chenodeoxycholic acid, 3α, 7α-dihydroxy-5β-cholan-24-oic acid; LCA, 3α-hydroxy-5β-cholan-24-oic acid; sarcoUDCA, sarcosine conjugate of ursodeoxycholic acid; sarcosylsarcoUDCA, sarcosylsarcosine conjugate of ursodeoxycholic acid; glycoUDCA, glycine conjugate of ursodeoxycholic acid; glycylglycoUDCA, glycylglycine conjugate of ursodeoxycholic acid; EEDQ, 2-ethoxy-1- ethoxycarbonyl-1 2-dihydroquinoline; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; CIMS, chemical ionization mass spectrometry.



I. R = NCH_2COOH (CH_3)

II. R = NCH_2CONCH_2COOH (CH_3 CH_3)

Fig. 1. Structures of sarcoUDCA and sarcosylsarcoUDCA; I: sarcoUDCA; II: sarcosylsarcoUDCA.

synthesis of sarcoUDCA without contamination due to sarcosylsarcoUDCA.

MATERIALS AND METHODS

UDCA was a gift from Tokyo Tanabe, Inc., Japan. All chemicals were A. C. S. grade and were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). The solvents used for HPLC were all HPLC grade and were purchased from Waters Associates Inc. (Milford, MA).

Melting point

Melting points were determined on a Thermolyne apparatus, model MP-12,600, and are uncorrected.

Thin-layer chromatography (TLC)

The TLC of the conjugates of UDCA was performed on silica gel O plates (Analabs, North Haven, CT) in a solvent system of chloroform–methanol–acetic acid 40:6:4 (v/v/v). The spots were visualized by spraying the plate with 10% H_2SO_4 followed by a solution of 3.5% phosphomolybdic acid.

High-performance liquid chromatography (HPLC)

The HPLC of UDCA and its conjugates was performed on a Waters associates ALC 201 system using a Waters Associates model 401 refractive index detector and a Waters Associates Redial-PAK C_{18} reversed-phase column (5 μm particle size). The bile acid conjugate (10–25 μg) dissolved in methanol (10–20 μl) was injected into the column for HPLC analysis. The mobile phase consisted of methanol–water–acetic acid 650:350:16 adjusted to pH 5.1 with 10 N NaOH (11, 12). The solvent flow rate was maintained at 2 ml/min (operating pressure, ca. 2000 p. s. i.). The various conjugates of UDCA were detected by refractive index changes and the detector response was recorded with a Hewlett-Packard (Lexington, MA) model 3380 integrator.

Mass spectrometry

The chemical ionization mass spectra (CIMS) of sarcoUDCA and sarcosylsarcoUDCA were recorded on a Model VG 70-250 mass spectrometer. The sample was deposited as a methanol solution on the direct insertion probe and a 1:3 mixture of nitrous oxide and methane was used as the carrier gas.

Synthesis of sarcoUDCA; isolation of sarcosylsarcoUDCA

(a) To a solution of UDCA (1 g) in dioxane (7.5 ml) containing tri-n-butylamine (0.5 ml) was added 0.25 ml of ethyl chloroformate at 0°C. After 15 min, a solution of 0.35 g sarcosine in 3.4 ml 1 N NaOH was added and the reaction mixture was allowed to stand at room temperature for 3 h. The reaction mixture was then worked up according to Kimura et al. (6) (total yield, 0.9 g). TLC examination of the product showed two major spots, R_f 0.72 and 0.50, in addition to a faint spot due to UDCA, R_f 0.91. The two compounds were found to be in a ratio of 85:15 by HPLC and were isolated in pure form by preparative TLC. Their melting points, TLC, and HPLC characteristics are given in **Table 1** and the major fragments in their chemical ionization mass spectra are given in **Table 2**.

(b) A suspension of ethyl sarcosinate hydrochloride (1.1 g) in 70 ml ethyl acetate containing 1 ml of triethylamine was stirred at room temperature for 30 min. UDCA (2 g) and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (1.73 g) were then added to the reaction mixture. The resulting suspension was refluxed for 30 h and worked up as de-

TABLE 1. TLC and HPLC characteristics of the various conjugates of UDCA

Compound	Melting Point	TLC[a] (R_f)	HPLC[b] (Retention Volume)
			ml
UDCA	202–204°C	0.91	36.1
SarcoUDCA	197–200°C	0.72	15.2
SarcosylsarcoUDCA	212–215°C	0.50	12.8
GlycoUDCA	223–224°C	0.57	12.6
GlycylglycoUDCA	215–217°C	0.35	10.7

[a]Solvent system: chloroform–methanol–acetic acid 40:6:4 (v/v/v).
[b]Solvent system: water–methanol–acetic acid 350:650:16; the pH of the solution was adjusted to 5.1 with 10 N NaOH. Flow rate, 2 ml/min. Pressure ca. 2000 p.s.i.

TABLE 2. Chemical ionization mass spectral fragmentation pattern of sarcoUDCA, sarcosylsarcoUDCA and glycylglycoUDCA

Fragment	SarcoUDCA	SarcosylsarcoUDCA	GlycylglycoUDCA
M^+	462 (32%)[a]		506 (100%)
M^+-1		533 (92%)	
M^+-18		515 (90%)	488 (15%)
$M^+-2 \times 18$		497 (38%)	
M^+-Sarcosine		445 (95%)	
$M^+-(CH_2CONHCH_2COOH)$			392 (16%)
M^+-(side chain + 2 × H_2O)	255 (36%)	255 (25%)	255 (10%)
Sarcosylsarcosine		159 (70%)	
Glycylglycine			131 (20%)
Sarcosine	88 (100%)	88 (100%)	
Glycine			74 (100%)

[a]The numbers in the parentheses denote the relative abundance of the mass-ion fragments.

scribed by Tserng, Hachey and Klein (13). TLC examination of the reaction product showed only one major spot corresponding to sarcoUDCA (yield 1.85 g). The above reaction sequence was repeated using 1.5 g of sarcoUDCA in place of UDCA and the products were isolated in an identical fashion. TLC examination of the reaction product showed one major spot corresponding to sarcossysarcoUDCA (yield 0.9 g).

Synthesis of glycoUDCA; isolation of glycylglycoUDCA

(a) A solution of UDCA (1 g) in dioxane (7.5 ml) containing tri-n-butylamine (0.5 ml) and ethyl chloroformate (0.25 ml) was reacted with glycine (0.31 g) in 1 N NaOH (3.4 ml) exactly as described above for the synthesis of sarcoUDCA, method (a). The reaction product showed a major spot TLC, R_f 0.57 and a minor spot R_f 0.35. The two compounds were found to be in a ratio of 9:1 as seen by HPLC and were obtained in pure form by preparative TLC. The faster moving compound (R_f 0.57) was found to be identical with glycoUDCA by direct comparison with a standard sample. The slower moving compound, R_f 0.35, showed a retention volume of 10.7 ml on HPLC (Table 1) and its structure was confirmed as glycylglycoUDCA by its CIMS fragmentation pattern (M^+ 520 amu, Table 2).

(b) A suspension of ethyl glycinate hydrochloride (0.5 g) in 35 ml ethyl acetate containing 0.5 ml triethylamine was reacted with 1 g UDCA and 0.86 g EEDQ exactly as described above for the synthesis of sarcoUDCA, method (b). GlycoUDCA obtained in this way (1.05 g) was reacted further with ethyl glycinate hydrochloride (0.5 g) when 0.98 g of crude glycylglycoUDCA was obtained (one major spot on TLC, R_f 0.35, and retention volume 10.7 ml on HPLC). The compound was purified by preparative TLC and the pure compound was found to be identical with glycylglycoUDCA obtained by method (a) above.

RESULTS AND DISCUSSION

Glycine-conjugated bile acids can be easily prepared by the mixed anhydride method of Norman (10) in which the bile acid mixed anhydride is treated with the sodium salt of glycine. During the synthesis of glycoCDCA by this method, Hofmann (14) noted the formation of a more polar compound (2–8%) suspected of being a glycylglycine conjugate of CDCA. Still, the method has been widely used and recently, Kimura et al. (6) have noted that the mixed anhydride method gave the best yield of sarcoUDCA. In order to prepare sarcoUDCA for our biological experiments, we used the conditions reported by Kimura et al. and found that almost 15% of a polar compound was consistently formed. Suspecting it to be the sarcosylsarcosine conjugate of UDCA, we isolated the compound in pure form in order to characterize it. The compound was subjected to chemical ionization mass spectrometry and an intense peak at *m/z* 534 was observed. This agreed with the molecular weight of sarcosylsarcoUDCA. The structure was conclusively proved by ion fragments at *m/z* 88, due to the loss of a sarcosine unit [$^+N(CH_3)CH_2COOH$] and *m/z* 159, due to the loss of sarcosylsarcosine unit [$^+N(CH_3)CH_2CON(CH_3)CH_2$ COOH]. Ion fragment at *m/z* 445 was due to the loss of a sarcosine unit from the molecular ion fragment. In an identical fashion, the CIMS of sarcoUDCA showed a molecular ion fragment at *m/z* 462 and a base ion fragment at *m/z* 88 due to the sarosine unit.

Apparently, the excess free sarcosine in the reaction mixture further reacted with sarcoUDCA formed since, in an experiment where ethyl sarcosinate hydrochloride was used and the conjugation was carried out in the presence of EEDQ as described by Tserng et al. (13), no appreciable amount of sarcosylsarcoUDCA was detected. Conjugation of the bile acid by the EEDQ method was found to be rather slow, probably because of the secondary amino group in sarcosine. However, an approxi-

mately 72% yield was obtained when the reaction time was increased from 6 to 30 h. It is thus obvious that the protection of the -COOH group in sarcosine as the ethyl ester yields sarcoUDCA free from contamination due to sarcosylsarco-UDCA. Further conjugation of sarcoUDCA with ethyl sarcosinate hydrochloride yielded sarcosylsarcoUDCA in over 50% yield.

In order to further prove that the free amino group is responsible for the formation of the dipeptide conjugate, glycoUDCA was synthesized by the method of Norman (10). Indeed, almost 10% glycylglycoUDCA was formed in the reaction product. This was in agreement with the observation made by Hofmann during the preparation of glycoCDCA (14). When we used the method of Tserng et al. (13), glycoUDCA was obtained free of glycylglycoUDCA. Further reaction of glycoUDCA with ethyl glycinate hydrochloride then afforded almost pure glycyl glycoUDCA in over 80% yield. It may thus be concluded that the method of Tserng et al. (13) is to be preferred for the synthesis of pure glycine or sarcosine conjugates of bile acids, since the protected acid group in the amino acid prevents its further conjugation with the conjugated bile acid formed.

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