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SYNTHESIS OF N-ε-LITHOCHOLYL-L-LYSINE, A COMPONENT OF TISSUE-BOUND LITHOCHOLIC ACID, VIA LITHOCHOLYL-N-HYDROXYSUCCINIMIDE*

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Summary—N-ε-lithocholyl-*l*-lysine is a lithocholic acid modified lysine residue found in proteins from tissues. This paper describes the synthesis of this compound as well as that of the isomeric N-α-lithocholyl-*l*-lysine and the di-substituted N-α-ε-*bis*-lithocholyl-*l*-lysine. The procedure involves the initial coupling of lithocholyl-N-hydroxysuccinimide to the appropriate N-α- or N-ε-CBZ (carbobenzloxy) protected lysine derivative followed by hydrogenolysis with palladium black of the CBZ group using formic acid as the hydrogen donor.

INTRODUCTION

Lithocholic acid is a secondary bile acid formed in the gut by bacterial 7-α-dehydroxylation of chenodeoxycholic acid [1]. A small amount of this bile acid is normally absorbed from the gut and transported to the liver where it is converted to the corresponding sulphate, and the glycine and taurine conjugates. We have recently reported that in addition to these soluble products, lithocholic acid can also appear immobilized in tissue-bound form (tissue-bound lithocholic acid) in which the bile acid is conjugated to the ε-NH_2 groups of lysine residues [2–6]. The isolation of the lysine conjugate from protein hydrolysates and its identification by comparison with synthetic N-ε-lithocholyl-*l*-lysine (**II**) established the nature of the protein-bound residue [2].

The original synthesis of N-α-lithocholyl-*l*-lysine (**I**), N-ε-lithocholyl-*l*-lysine (**II**) and of the disubstituted N-α-ε-*bis*-lithocholyl-*l*-lysine (**III**) were accomplished earlier via the classical mixed anhydride procedure [7] for coupling lithocholic acid with the appropriate CBZ–lysyl benzyl ester followed by catalytic hydrogenation of the CBZ group [2]. This paper describes a simpler synthesis of the lithocholyl lysines (**I–III**) via the N-hydroxysuccinimide ester of lithocholic acid.

EXPERIMENTAL

Materials

N-α-CBZ-*l*-lysine (**IX**) and N-ε-CBZ-*l*-lysine (**VII**) and ninhydrin were purchased from Sigma Chemical Co. Dicyclohexylcarbodiimide, N-hydroxysuccinimide and 8-hydroxy-1,3,6-pyrenetrisulfonic acid and palladium black were obtained from Aldrich Chemical Co.

General methods

Melting points were determined on a Fisher–Johns melting point apparatus and are uncorrected. Elemental analysis were obtained through Robertson Microanalytical Laboratory, Florham Park, New Jersey. Infrared spectra on Nujol mull were recorded on a Pye–Unicam SP-1000 Spectrophotometer. Optical rotations were measured in a Perkin–Elmer Model 141 automatic polarimeter at 589 nm. Thin-layer chromatography was performed on silica gel G plates obtained from Analtech, Newark, DEL. Bile acid positive zones were visualized under ultraviolet after spraying with 8-hydroxy, 1,3,6-pyrenetrisulfonic acid (50 mg/dl in methanol). Ninhydrin spray was used to detect the lysine conjugates in which the α-NH_2 group was free. Catalytic hydrogenolysis of the CBZ derivatives was carried out in Regis Chemical Co. acylation tubes equipped with a Teflon-lined metal screw cap and a locking sleeve to ensure a tight seal.

Synthetic procedures

The following three lysine derivatives were synthesised: N-α-lithocholyl-*l*-lysine (**I**), N-ε-lithocholyl-*l*-lysine (**II**) and N-α-ε-*bis*-lithocholyl-*l*-lysine (**III**) [Fig. 1]. The synthetic approach to these three compounds are shown in Fig. 2.

*Lithocholyl-*N*-hydroxysuccinimide (VI)*

N-Hydroxysuccinimide (**V**), 1.15 g (10 mmol) was dissolved in 40 ml of a 1:1 mixture of the THF–ethyl acetate and added to a solution of lithocholic acid (**IV**), 3.76 g (10 mmol) in 25 ml of THF. A solution

*The systematic nomenclature of bile acids referred to in this paper by trivial names are as follows: Lithocholic acid, 3α-hydroxy-5β-cholan-24-oic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid. The abbreviations used in this paper are: CBZ, carbobenzyloxy-; THF, tetrahydrofuran.

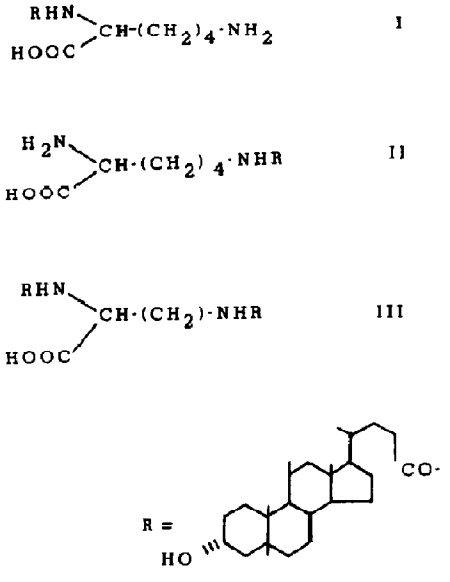

Fig. 1

of dicyclohexylcarbodiimide, 2.06 g (10 mmol) in 10 ml of ethyl acetate, was added slowly while stirring and the reaction was allowed to proceed overnight at room temperature. The precipitated dicyclohexylurea (1.99 g) was filtered and the filtrate was concentrated to dryness in a rotary evaporator. The residue was dissolved in THF and recrystallized from THF–water. The product, dried in a vacuum dessicator, weighed 4.106 g and was obtained in 86.7% yield. For $C_{28}H_{43}NO_5$, calculated: C 71.0%, H 9.15%, N 2.95%; found: C 71.18%, H 9.48%, N 2.94% m.p. 151–153°C; $\{\alpha\}26/D + 28.96°$ (C = 0.145, ethanol), i.r. (nujol) cm^{-1}, 3320 (OH), 1750 (C = 0).

N-α-*lithocholyl*-ε-*CBZ*-l-*lysine* (*VIII*)

N-ε-CBZ-*l*-lysine (**VII**) 140.2 mg (0.5 mmol) was suspended in 10 ml of THF–water (1:1) and gently warmed under running hot water to dissolve the lysine derivative. Sodium bicarbonate, 42 mg was then added, followed by lithocholyl-*N*-hydroxysuccinimide (**VI**), 236.8 mg (0.5 mmol) dissolved in 6.0 ml of THF. The mixture, after having been kept stirred overnight at room temperature, was acidified to pH 2.0 with 1 M HCl and then evaporated to dryness in a rotary evaporator. The residue was extracted with a small volume (about 5.0 ml) of water. The water insoluble residue was dissolved in a mixture of THF–ethyl acetate (1:1), centrifuged at low speed to remove insoluble particles and then evaporated to dryness under partial vacuum in a rotary evaporator. The product (**VIII**) was obtained as a crystalline white power (236 mg) in 73.7% yield. For $C_{38}H_{59}N_2O_6$, calculated: C 71.33%, H 9.29%, N 4.4%; found C 70.47%, H 9.74%, N 4.5%; m.p. 51–53°C; $\{\alpha\}26/D, +17.9°$ (C = 0.43, ethanol); i.r. (nujol) cm^{-1}, 3300 (OH), 1700 (C = 0), 1640 (CONH).

N-α-*lithocholyl*-l-*lysine* (*formate salt*) (*I*)

N-α-lithocholyl-ε-CBZ-*l*-lysine (**VIII**), (125 mg dissolved in 10 ml of glacial acetic acid) was added to 3.0 ml of 90% formic acid containing a small amount of palladium black catalyst. The catalytic hydrogenolysis was carried out in a sealed tube (Regis Chemical Co. acylation tubes) overnight. After the catalyst was removed by successive filtration and centrifugation, the solution was evaporated to dryness in a rotary evaporator. The product was isolated by triturating the oily residue with ether and then recrystallizing from ethanol–ether. The product weighing 33 mg was obtained in 33.4% yield. For $C_{31}H_{54}N_2O_6$, calculated: C 65.45%, H 9.92%, N 4.93%; found: C 64.76%, H 8.98%, N 5.9%; m.p. 116–119°C. i.r. (nujol) cm^{-1} 3300 (OH), 1720 (C = 0) and 1640 (CONH).

N-α-*CBZ*-ε-*lithocholyl*-l-*lysine* (*X*)

N-α-CBZ-*l*-lysine (**IX**), 280.4 mg (1.0 mmol) was dissolved in 20 ml of THF–water (1:1) and 84 mg of solid sodium bicarbonate was added and the mixture was kept stirred. A solution of lithocholyl-*N*-hydroxysuccinimide (**VI**), 473.6 mg (1.0 mmol) in 12 ml of THF was added slowly to the reaction mixture. The reaction was allowed to proceed overnight, after which the mixture was acidified to pH 2.0 with 1 M HCl and then evaporated to dryness in a rotary evaporator. The residue was triturated with about 5.0 ml of water and the aqueous extract was discarded. The precipitate was dissolved in methanol and the undissolved material filtered. The filtrate, upon evaporation to dryness in a rotary evaporator gave a fine iridescent crystalline product weighing 446.4 mg (69.7% yield). For $C_{38}H_{59}N_2O_6$, calculated: C 71.33%, H 9.29%, N 4.38%; found: C 71.92%, H 9.81%, N 4.0%; m.p. 72–74°C; $\{\alpha\}26/D + 13.9°$ (c = 0.115, ethanol); i.r. (nujol) cm^{-1}, 3320 (OH), 1700 (C = 0), 1640 (CONH).

N-ε-*lithocholyl*-l-*lysine* (*II*)

N-α-CBZ-ε-lithocholyl-*l*-lysine (**X**), 100 mg was suspended in 2.0 ml of 90% formic acid and glacial acetic acid was added dropwise with stirring until solution was complete. This solution was added to a suspension of palladium black in 90% formic acid in an acylation tube (Regis Chemical Co). The tube was sealed with the Teflon-lined metal screw cap and catalytic hydrogenolysis was carried out for 2 h. After removal of the catalyst by successive filtration and centrifugation, the acidic mixture was evaporated to dryness in a rotary evaporator under reduced pressure and the product was crystallized by trituration with anhydrous diethyl ether. The product weighing 37 mg was obtained in 46.8% yield. For the formate salt, $C_{31}H_{54}N_2O_6$, calculated: C 67.59%, H 9.88%, N 5.08%; found: C 67.19%, H 10.1%, N 5.1%; m.p. 125–126°C; $\{\alpha\}26/D + 17.5°$ (c = 0.08, ethanol), i.r. (nujol) 3300 (OH), 1720 (C = 0), 1640 (CONH).

Nα-ε-bis-*lithocholyl*-l-*lysine* (*III*)

Lysine monohydrochloride (**XI**), 45.66 mg (0.25 mmol) was dissolved in 5.0 ml of water and the solution was made up to 10.0 ml with THF. Sodium bicarbonate, 63 mg (0.75 mmol) was added to the solution with stirring. A solution of lithocholyl-*N*-hydroxysuccinimide (**VI**), 236.8 mg (0.5 mmol) in 6.0 ml of THF was added slowly and the reaction was allowed to proceed overnight. The reaction mixture was acidified to pH 2.0 with 1 M HCl and then reduced to a small volume in a rotary evaporator. This aqueous suspension was shaken with a mixture

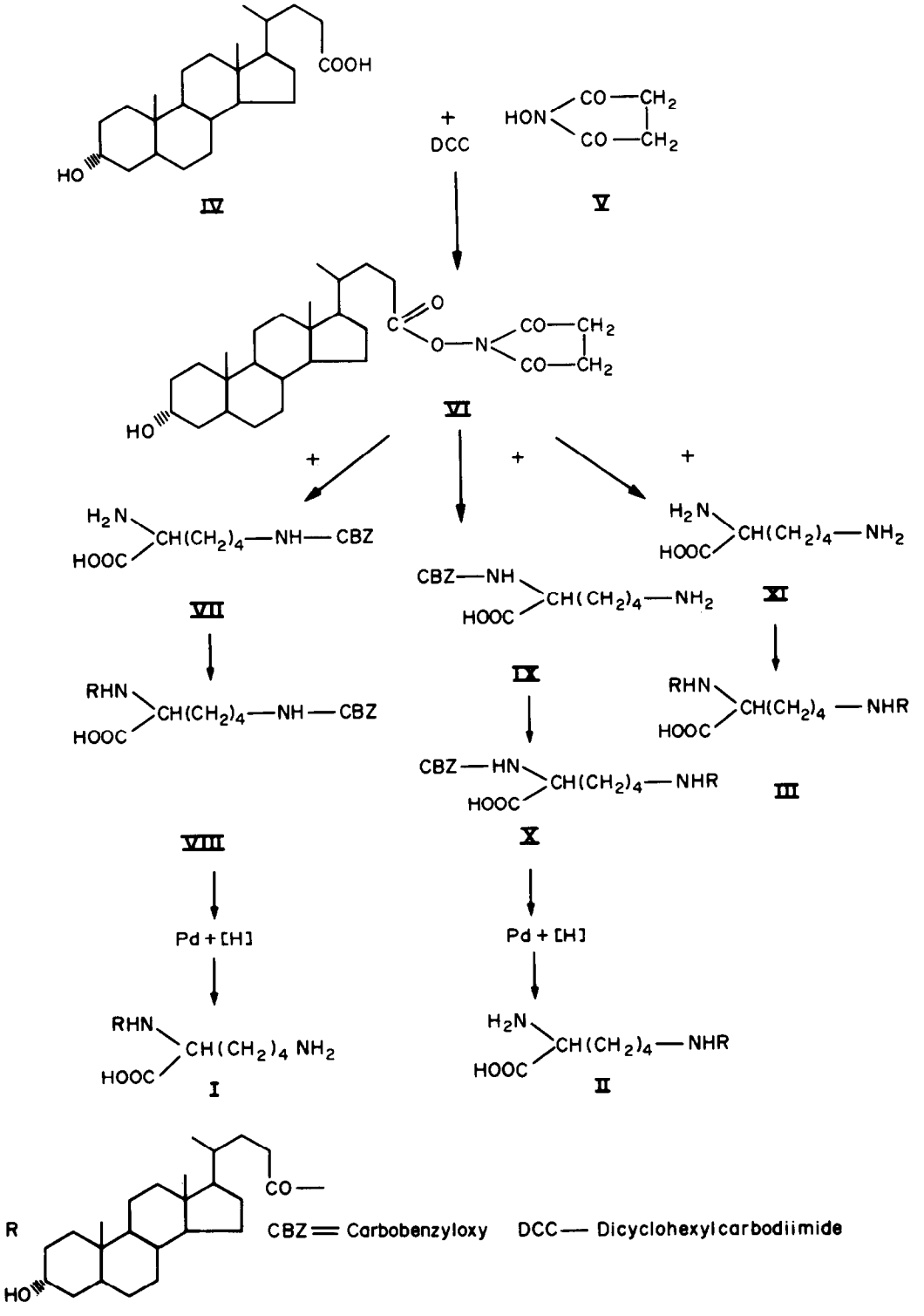

Fig. 2

Table 1. Thin-layer chromatography of lithocholic acid derivatives

Compound	R_f values Solvent A	Solvent B	Solvent C
Lithocholyl-*N*-hydroxy succinimide	0.79	0.82	—
N-α-CBZ-ε-lithocholyl lysine	0.82	0.92	0.04
N-ε-lithocholyl lysine	0.30	0.74	—
N-α-lithocholyl-ε-CBZ lysine	0.80	0.90	0.07
N-α lithocholyl lysine	0.22	0.66	—
N-α-ε-*bis*-lithocholyl lysine	0.84	0.93	0.07
Lithocholic acid			0.61

Solvent systems:
A: Isooctane–isopropanol–acetic acid (2:2:1);
B: Ethanol–ammonia–water (6:1:2);
C: Chloroform–*t*-amyl alcohol (7:3).

of 5 ml of ethyl acetate and 2.0 ml of THF in a separatory funnel. The aqueous layer that separated out was discarded and the organic layer was evaporated to dryness. The residue upon trituration with acetone gave the product as a white precipitate weighing 161 mg with an overall 74.6% yield. For $C_{54}H_{90}N_2O_6$, calculated: C 75.1%, H 10.5%, N 3.3%; found: C 74.22%, H 10.9%, N 3.4%; m.p. 151–153°C; $\{\alpha\}26/D + 30.83°$ (C = 0.227, methanol); i.r. (nujol) 3400 (OH), 1640 (CONH). A mixed m.p. determination between this sample and that prepared earlier gave no depression.

RESULTS AND DISCUSSION

Table 1 presents the thin-layer chromatographic (TLC) data on all of the compounds, using three different solvent systems. In addition to establishing the purity of these compounds, TLC was used to follow the hydrogenolysis of the CBZ derivatives. The products of hydrogenolysis had R_f values lower than the corresponding parent compounds. Furthermore, the positive reaction toward ninhydrin was a distinguishing feature of *N*-ε-lithocholyl-*l*-lysine. The presence of the bile acid moiety was readily established by its characteristic fluorescence upon spraying the plates with 8-hydroxy-1,3,6,-pyrenetrisulfonic acid.

The syntheses described in this paper are relatively simpler than those involving the use of the mixed anhydride procedure which requires the blocking of both the $-NH_2$ and the –COOH functional groups on lysine [2, 7]. *N*-Hydroxysuccinimide esters are crystalline, highly reactive compounds that have been used for the synthesis of peptides [8], *N*-acylaminoacids [9] and fatty acyl CoA and other thiol esters [10, 11]. Since these esters exhibit a high degree of specificity toward amino groups, the carbonyl function on the lysine derivatives could be left unprotected during the coupling reaction.

The final step in these syntheses involves the removal of the CBZ protecting group. This is generally carried out by catalytic hydrogenolysis with hydrogen under pressure, overnight. We have replaced this step by a rapid nonpyroforic procedure where formic acid serves as the solvent as well as the hydrogen donor [12]. This reaction which is completed within 2 h can be readily monitored by following the disappearance of the CBZ derivative on TLC. Furthermore, infrared spectral data showed hydrogenolysis in formic acid did not give rise to the formoxy derivative of the 3-α OH function of lithocholic acid.

REFERENCES

1. Danielsson H.: Mechanisms of bile acid biosynthesis. In *The Bile Acids* (Edited by P. P. Nair and D. Kritchevsky). Plenum Press, New York, Vol. II (1973) pp. 1–32.
2. Nair P. P., Mendeloff A. I., Vocci M., Bankoski J., Gorelik M., Herman G. and Plapinger R.: Lithocholic acid in human liver: Identification of ε-lithocholyl lysine in tissue protein. *Lipids* **12** (1977) 922–927.
3. Nair P. P., Solomon R., Bankoski J. and Plapinger R.: Bile acids in tissues. binding of lithocholic acid to protein. *Lipids* **13** (1978) 966–970.
4. Turjman N. and Nair P. P.: Nature of tissue-bound lithocholic acid and its implications in the role of bile acids in carcinogenesis. *Cancer Res.* **41** (1981) 3761–3763.
5. Turjman N., Mendeloff A. I., Jacob C. and Nair P. P.: Isolation of tissue-bound lithocholic acid from livers of rats treated with methylazoxymethanol. *J. steroid Biochem.* **14** (1981) 1237–1240.
6. Gelb A. M., McSherry C. K., Sadowsky J. R. and Mosbach E. H.: Tissue bile acids in patients with colon cancer and colonic polyps. *Am. J. Gastroenterol.* **77** (1982) 314–317.
7. Vaughan J. R. and Osato R. L.: The preparation of peptides using mixed carbonic carboxylic acid anhydrides. *J. Am. chem. Soc.* **74** (1952) 676–678.
8. Anderson G. W., Zimmerman J. E. and Callahan F. M.: Esters of *N*-hydroxysuccinimide in peptide synthesis. *J. Am. chem. Soc.* **86** (1964) 1839–1842.
9. Lapidot U., Rappaport S. and Wolman Y.: Use of esters of *N*-hydroxysuccinimide in the synthesis of *N*-acylamino acids. *J. Lipid Res.* **8** (1967) 142–145.
10. Al-Arif A. and Blecher M.: Synthesis of fatty acyl CoA and other thiol esters using *N*-hydroxysuccinimide esters of fatty acids. *J. Lipid Res.* **10** (1969) 344–345.
11. Polokoff M. A. and Bell R. M.: Characterization of liver cholic acid coenzyme A ligase activity. *J. biol. Chem.* **252** (1977) 1167–1171.
12. El Amin B., Anantharamaiah G. M., Royer G. P. and Means G. E.: Removal of benzyl-type protecting groups from peptides by catalytic transfer hydrogenation with formic acid. *J. org. Chem.* **44** (1977) 3442–3444.