Isolation and identification of bile salts conjugated with cysteinolic acid from bile of the red seabream, *Pagrosomus major*

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Abstract Bile salts present in gallbladder of wild and cultured red seabream, *Pagrosomus major*, a marine teleost were analyzed. The bile from wild red seabream was found to contain two previously unknown bile salts along with two known bile salts, taurocholate and taurochenodeoxycholate. Isolation of each bile salt was performed by column chromatography. Fast atom bombardment mass spectra of the unknown bile salts showed the molecular ions $(M-H)^-$ of *m/z* 544 and 528 which are shifted 30 mass units upfield compared to those (*m/z* 514 and 498) of taurocholate and taurochenodeoxycholate, respectively; this is consistent with the presence of cysteinolic acid (mol wt 155) instead of taurine (mol wt 125). Enzymatic hydrolysis of the bile salts released cholic acid and chenodeoxycholic acid, respectively, and an amino acid that was identified as D-cysteinolic acid by direct comparison with an authentic sample. From these results, the bile salts in the bile of wild red seabream were identified as the conjugates of cholic acid and chenodeoxycholic acid with cysteinolic acid. ^{1}H- and ^{13}C-magnetic resonance spectra of the bile salts were also consistent with the proposed structure. The cysteinolic acid conjugates were found only in wild and not in cultured red seabream; this distinction seems to result from differences in dietary cysteinolic acid. — **Une, M., T. Goto, K. Kihira, T. Kuramoto, K. Hagiwara, T. Nakajima, and T. Hoshita.** Isolation and identification of bile salts conjugated with cysteinolic acid from bile of the red seabream, *Pagrosomus major*. *J. Lipid Res.* 1991. **32:** 1619–1623.

Supplementary key words conjugated bile acids • cholic acid • chenodeoxycholic acid • D-cysteinolic acid

The red seabream, *Pagrosomus major*, is one of the most valuable and expensive fish in Japan, and the aquaculture of the marine teleost is under development in various districts in the country. However, cultured red seabream is inferior in quality to the wild type, presumably because of differences in diet. In order to improve the quality of cultured red seabream, studies on nutrition especially lipid nutrition, of the cultured fish have been carried out extensively, but no information is available on the comparison of bile salts in wild and cultured fish. Bile salts play a very important role in lipid digestion and absorption.

In this study, we report the chemical structure of the bile salts of wild and cultured red seabream; we found D-cysteinolic acid-conjugated cholic and chenodeoxycholic acids only in wild and not in cultured red seabream.

EXPERIMENTAL

Reference compounds

Taurocholate and taurochenodeoxycholate were purchased from Sigma Chemical Co. (St. Louis, MO). D-Cysteinolic acid (mp 278–279°C, $[\alpha]_D$ + 6.7) was a generous gift from Dr. K. Ito (Faculty of Applied Biological Science, Hiroshima University).

General

Infrared (IR) spectra, proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR), and optical rotations were obtained as described previously (1). Gas-liquid chromatography–mass spectrometry (GLC-MS) and thin-layer chromatography (TLC) were carried out as described previously (2).

Chiral monitor, ACS 750/25 (Applied Chromatography Systems, Ltd., UK) was used for the measurement of the chirality.

Fast atom bombardment (FAB) mass spectra were obtained on a JEOL (Tokyo, Japan) D-300 mass spectrometer. Typical experimental conditions were: xenon atom beam, 10 KV accelerating potential, and 20 mA emission current. Underivatized bile salts were dissolved in glycerol, and the solution was inserted into the ion source.

Abbreviations: TLC, thin-layer chromatography; TC, taurocholate; TCDC, taurochenodeoxycholate; HPLC, high performance liquid chromatography; FAB, fast atom bombardment; GC-MS, gas chromatography–mass spectrometry.

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High performance liquid chromatography (HPLC) was carried out using a Waters (Milford, MA) M-45 solvent-delivery system equipped with a Shimadzu (Kyoto, Japan) SPD-1 UV detector; the wavelength was 205 nm. A TSK-GEL ODS-80TM (4.6 mm i.d. × 15 cm, Tosoh, Tokyo, Japan) column was used. A mixture of methanol–20 mM phosphate buffer (pH 2.5)–water–acetonitrile 15:5:2:1 (by vol) was used as the moving phase and the flow rate was 1 ml/min.

Extraction of bile salts from the gallbladder bile

Gallbladders from 15 wild red seabream were cut under ethanol (50 ml), and the extracts were filtered. Evaporation of the solvent from the filtrated extract gave crude bile salts (5.4 g) as a solid. Similarly, crude bile salts (4.5 g) were obtained from 12 cultured red seabream.

Chromatographic separation of bile salts

The crude bile salts were chromatographed on a column of silica gel using a mixture of chloroform–methanol–acetic acid–water 13:4:2:1 (by vol) as an eluting solvent. Purification of the bile salts was performed by reversed phase column chromatography (Lobar column, Merck, Darmstadt, Germany) using a mixture of methanol–water 3:2 (by vol) as the moving phase. The column effluents were monitored by TLC.

Enzymatic hydrolysis of bile salts

The isolated bile salts were dissolved in 2.0 ml of 0.1 M acetate buffer (pH 5.6) and the solution was incubated at 37°C with cholylglycine hydrolase (EC. 3.5.1.24; Sigma Chemical Co.) in the presence of 0.5 ml of 0.2 M disodium salt of EDTA and 0.5 ml of β-mercaptoethanol. After an 18-h incubation period, the incubation mixture was passed through a Sep-pak C_{18} cartridge. The cartridge was washed with 10 ml of water and then 30 ml of methanol. The water eluate was filtered by Centricut V-10 (Kurabo Industries Co. Ltd., Osaka, Japan). The filtrate was lyophilized thoroughly and the residue was dissolved in a small portion of water and passed through a column of Dowex 50 (16 ml, H^+ form). After washing the column with three column-volumes of water, the eluants and washings were combined, and applied to a column of Dowex 2 (16 ml, OH^- form). Amino acids adsorbed were then eluted with 4% acetic acid (210 ml), and the solvents were evaporated to dryness. The methanol eluates from the Sep-pak C_{18} cartridge were evaporated to dryness.

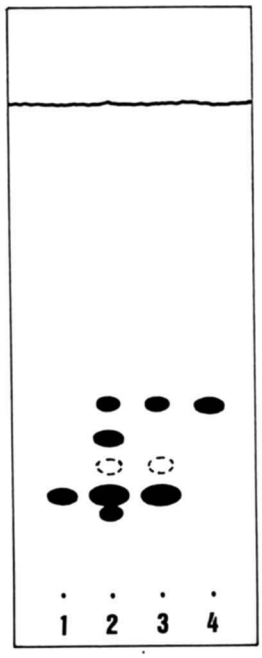
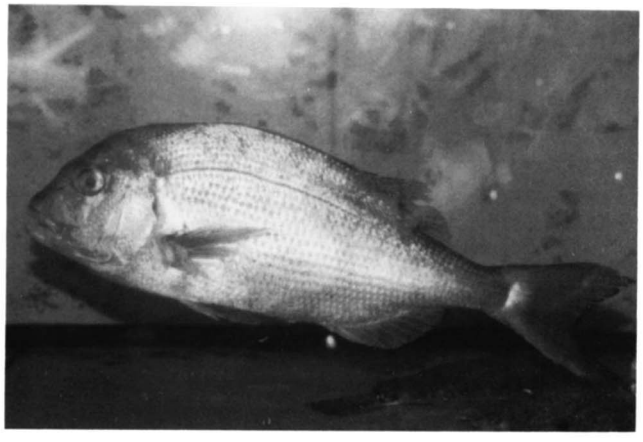


Fig. 1. Representative thin-layer chromatogram of the bile salts from wild and cultured red seabream and standards. 1, Taurocholate; 2, bile salts from wild red seabream; 3, bile salts from cultured red seabream; 4, taurochenodeoxycholate. Solvent system: n-butanol–acetic acid–water 17:2:1 (by vol).



Red seabream, *Pagrosomus major*.

RESULTS

The crude bile salts from red seabream were analyzed by TLC. As shown in **Fig. 1**, lane 2, two compounds (bile salt 1, R_f 0.32, and bile salt 2, R_f 0.17) were observed with taurocholate (TC, R_f 0.20) (lane 1) and taurochenodeoxycholate (TCDC, R_f 0.39) (lane 4) in the bile obtained from wild (lane 2) but not from cultured fish (lane 3). HPLC analysis of the bile salts obtained from wild red seabream also showed two additional peaks (bile salt 2, 14.4% and bile salt 1, 24.5%) that eluted faster than TC (45.8%) and TCDC (15.3%), respectively (**Fig. 2**). The crude bile salts (900 mg) from the native fishes were chromatographed on a column of silica gel (60 g) to get four fractions (**Table 1**). The eluate from fraction II was further purified by reverse phase column chromatography to

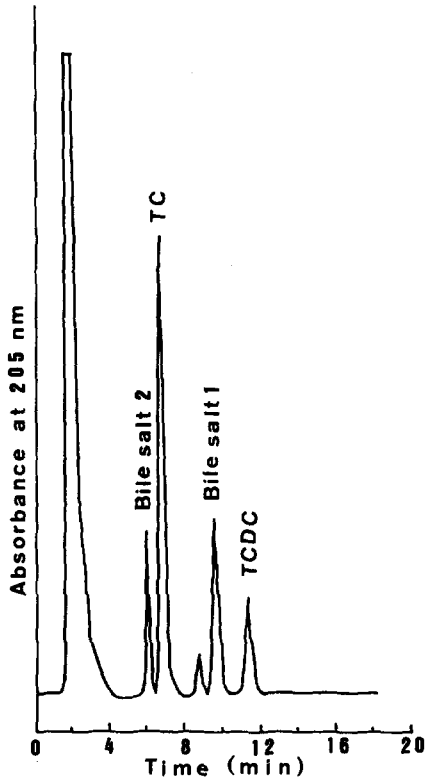


Fig. 2. High performance liquid chromatogram of the bile salts from wild red seabream. The HPLC conditions are described in Experimental.

get bile salt 1 (29 mg) as a powder: $[\alpha]_D$ + 18.5; IR V_{max}: 3400 (hydroxyl), 1645 (amide), 1550 (amide), 1400, 1210 (sulfonic acid), 1050. The eluate from fraction IV was further purified on a column of silica gel (4 g) to give bile salt 2 (11 mg) as a powder: IR V_{max}: 3400 (hydroxyl), 1620 (amide), 1560 (amide), 1430, 1200 (sulfonic acid), 1050.

The FAB mass spectrum of bile salt 1 showed the ion $(M-H)^-$ of *m/z* 528, which was shifted 30 mass units upfield compared to that (*m/z* 498) of TCDC. IR spectrum of bile salt 1 closely resembled that of TCDC except for the presence of the band of 1400 cm^{-1}. The ^{13}C-NMR spectrum (**Table 2**) of bile salt 1 showed excellent agreement with C-1 through C-24 of TCDC. The only difference between both the spectra discerned in the signals was due to the amino acid moieties. The ^1H-NMR spectrum (**Table 3**) of bile salt 1 also showed close agreement with TCDC, except for signals corresponding to the amino acid moiety. On the basis of these spectroscopic data, it was considered that bile salt 1 is chenodeoxycholic acid conjugated with an unusual amino acid. Thus, bile salt 1 (10 mg) was incubated with cholylglycine hydrolase (90 units) in acetate buffer. The incubation mixture was passed through a Sep-pak C_{18} cartridge, which was washed successively with water and methanol. The eluate obtained from the water washings was purified by means of ion exchange chromatography. The chromatographic behavior on TLC plates and ^{13}C-NMR spectrum of the amino acid obtained were completely identical with those of authentic cysteinolic acid (**Table 4**). Since the measurement of the optical rotation could not be performed because of the insufficient amount of the amino acid obtained after hydrolysis of bile salt 1, the chirality was determined by chiral monitoring. On chiral monitoring the amino acid dextrorotatory (+) was authentic D-cysteinolic acid. The eluate obtained from the methanol washings was methylated and trimethylsilylated. The resulting derivative was analyzed by GC-MS. Its retention time was completely identical with that of the corresponding derivative of chenodeoxycholic acid. The identity of the bile acid was established through comparison of mass spectra of the natural and authentic samples. Thus, we

TABLE 1. Silica gel column chromatography of bile salts from the gallbladder bile of wild red seabream

Fraction	Volume	Weight	Eluate
	ml	*mg*	
I	35	66	TCDC
II	82	362	TCDC, bile salt 1, and TC
III	135	271	TC
IV	80	71	TC and bile salt 2

TCDC, taurochenodeoxycholate; TC, taurocholate. Eluant was a mixture of chloroform-methanol-acetic acid-water 13:4:2:1, by volume.

TABLE 2. ^{13}C-NMR data on bile salts

	TCDC	Bile Salt 1	TC	Bile Salt 2
1	36.61	36.60	36.55	36.54
2	31.40	31.40	31.24	31.23
3	72.91	72.90	72.95	72.95
4	40.52	40.52	40.53	40.52
5	43.24	43.23	43.27	43.25
6	35.92	35.93	35.89	35.89
7	69.08	69.09	69.10	69.11
8	40.84	40.83	41.09	41.07
9	34.08	34.09	27.93	27.92
10	36.26	36.25	35.95	35.94
11	21.81	21.82	29.61	29.61
12	41.09	41.09	74.06	74.09
13	43.72	43.73	47.56	47.55
14	51.56	51.57	43.02	43.02
15	24.65	24.66	24.26	24.27
16	29.28	29.30	28.70	28.72
17	57.36	57.36	48.09	48.08
18	12.21	12.22	13.04	13.04
19	23.43	23.43	23.20	23.19
20	36.94	37.01	36.93	37.01
21	18.94	19.00	17.78	17.82
22	33.20	33.11	33.19	33.07
23	34.25	34.35	34.29	34.36
24	176.56	176.62	176.62	176.68
1'[a]	51.52	52.76	51.54	52.78
2'	36.63	50.07	36.64	50.03
3'		64.07		64.08

TCDC, taurochenodeoxycholate; TC, taurocholate.
[a] $^{2'}_{}\text{-NH-}^{1'}\text{CH}_2\text{-CH}_2\text{-SO}_3$, $^{2'}_{}\text{-NH-CH(}^{3'}\text{CH}_2\text{OH)-}^{1'}\text{CH}_2\text{-SO}_3$.

TABLE 3. ^{1}H-NMR data of bile salts

	Bile Acid Moiety						Amino Acid Moiety		
	18-CH_3	19-CH_3	21-CH_3	3β-H	7β-H	12β-H	1'-CH_2SO_3	2'-CH-NH	3'-CH_2OH
TCDC	0.69(s)	0.92(s)	0.97(d)	3.36(m)	3.79(m)		2.95(t)	3.58(t)	
Bile salt 1	0.69(s)	0.92(s)	0.97(d)	3.36(m)	3.79(m)		3.02(m), 3.08(m)	4.31(m)	3.69(m)
TC	0.71(s)	0.91(s)	1.02(d)	3.39(m)	3.79(m)	3.95(m)	2.95(t)	3.58(t)	
Bile salt 2	0.71(s)	0.91(s)	1.03(d)	3.39(m)	3.79(m)	3.96(m)	3.02(m), 3.08(m)	4.33(m)	3.69(m)

Multiplicities are shown in parentheses. TCDC, taurochenodeoxycholate; TC, taurocholate. All samples were measured as CD_3OD solution.

concluded that bile salt 1 is N-chenodeoxycholyl-D-cysteinolic acid (I) (**Fig. 3**).

The structure of bile salt 2 was studied in the same manner. The FAB mass spectrum of bile salt 2 showed the molecular ion $(M-H)^-$ at *m/z* 544, which shifted 30 mass units upfield compared to that (*m/z* 514) of TC. The ^{13}C- and ^{1}H-NMR spectra of bile salt 2 showed excellent agreement with those of TC except for signals corresponding to the amino acid moieties. Further, the enzymatic hydrolysis of bile salt 2 released cholic acid and D-cysteinolic acid, the structures of which were confirmed by the same procedures as described above for the analysis of the hydrolyzates of bile salt 1. On the basis of these results, bile salt 2 was identified as N-cholyl-D-cysteinolic acid (II) (Fig. 3).

DISCUSSION

As far as we are aware, extensive investigations dealing with the bile salts of the vertebrates have indicated that marine teleosts have almost exclusively taurine-conjugated C_{24} bile acids, taurocholate and taurochenodeoxycholate, as their major biliary constituents (3). This is true in the case of red seabream. The major bile salts of both wild and cultured red seabream were also taurocholate and taurochenodeoxycholate. In addition, we found cysteinolic acid conjugates of cholic acid and chenodeoxycholic acid as the fourth and second most abundant bile salts in the bile of wild red seabream. Thus, the present study constitutes the first demonstration of a vertebrate bile that contains considerable amounts of bile salts conjugated with an unusual amino acid rather than taurine and glycine. D-Cysteinolic acid has been found in some species of marine algae (4, 5). It comprises 41% and 21% of total amino acids in *Ulva pertusa* and *Enteromorpha linza*, respectively. Furthermore, the existence of D-cysteinolic acid has been reported in muscle of blue crab, prawn, Pacific mackerel, and sardine (6). Although the origin of D-cysteinolic acid present in fish is still not confirmed, the present finding that cysteinolic acid-conjugated bile acids were detected only in the bile of wild red seabream and not cultured red seabream suggests that cysteinolic acid present in wild fish originates from food. The similarity in chemical structure between cysteinolic acid and taurine suggests that the conjugation of bile acids with D-cysteinolic acid is catalyzed by the enzyme system that catalyzes the conjugation with taurine. In this connection, it would be interesting to study whether the cysteinolic acid-conjugated bile acids could be produced in cultured red seabream as well as mammals whose diets are sup-

TABLE 4. Physico-chemical and chromatographic data of natural and authentic D-cysteinolic acid

Method	Natural Cysteinolic Acid	Authentic D-Cysteinolic Acid
	R_f values	
TLC[a]		
System 1[b]	0.49	0.49
System 2[c]	0.45	0.45
	ppm	
^{13}C-NMR		
1'[d]	50.08	50.08
2'	50.67	50.67
3'	61.58	61.58
Chirality	+	+

[a]Plate: silica gel G (0.25 mm thickness, Merck).
[b]n-Propanol-water, 2:1 by volume.
[c]n-Propanol-acetic acid-water, 15:1:4 by volume.
[d]The number of carbons referred to in Table 2.

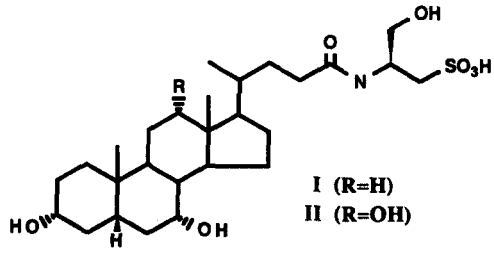


Fig. 3. Structures of D-cysteinolic acid-conjugated bile acids.

plemented with D-cysteinolic acid. It would be interesting to search the occurrence of cysteinolic acid conjugates in other species of fish, especially Pacific mackerel and sardine, whose muscle has been found to contain D-cysteinolic acid.

An extensive literature exists regarding the effect of side-chain modifications on transport and metabolism of bile salts. For example, the side-chain length influences the physiologic properties of bile acids (7, 8), and the amino acid moiety of bile acid conjugate greatly influences bacterial modification and hepatic transport (9–12). Thus it seems unlikely that the cysteinolic acid-conjugated bile acids exhibit the same biological properties as taurine-conjugated bile acids. If the cysteinolate conjugates act as biologically active substances to promote biliary excretion of cholesterol and intestinal absorption of products during lipid digestion better than natural taurine-conjugated bile acids, the difference in quality between wild and cultured red seabream would be explained as a result of the occurrence and absence of cysteinolate conjugates in these fishes. Studies to answer these questions are now in progress.

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