Bile acids were analyzed in the bile, small and large intestines, and feces of germ-free rats after a single inoculation with one of six intestinal bacteria that had originally been isolated from human feces. Bacteroides vulgatus and Bifidobacterium longum preferentially deconjugated tauro-\(\Delta^{22}\)-muricholic acid and taurocholic acid, respectively. Clostridium ramosum, Peptostreptococcus productus and Lactobacillus gasseri deconjugated both bile acids, but Escherichia coli did not deconjugate either one. Rats inoculated with bacteria that deconjugated tauro-\(\Delta^{22}\)-muricholic acid produced \(\Delta^{29}\)-muricholic acid in the feces. In contrast, \(\Delta^{22}\)-cholic acid could not be detected in rats inoculated with bacteria that deconjugated taurocholic acid.

In the present study we show that desaturation of the side chain of \(\beta\)-muricholic acid (\(\Delta^{22}\)-\(\beta\)-muricholic acid) occurs upon a single inoculation with predominant bacteria obtained from human feces.

**MATERIALS AND METHODS**

Determination of tauro-\(\Delta^{22}\)-\(\beta\)-muricholic acid. Bile acids in bile obtained from conventional rats and germ-free rats were analyzed by high-performance liquid chromatography (HPLC). For this purpose, a Cosmosil 5C18 column (15 cm \(\times\) 4.6 mm i.d.; Nakarai Kagaku Co., Kyoto, Japan) was used, and 0.3% ammonium phosphate (pH 7.0)/acetoniitrile (20:8, vol/vol) served as mobile phase; the eluent was monitored at 210 nm.

The peak with a relative retention time of 0.36 (peak A) relative to taurocholic acid was detected in bile of conventional rats, while it could not be detected in bile of germ-free rats.

Fraction A was isolated directly from bile of conventional rats by preparative HPLC using a TSK-gel ODS-120T column (25 cm \(\times\) 2 cm i.d.; Tosoh, Tokyo, Japan) and 0.3% ammonium phosphate (pH 7.0)/acetoniitrile (20:8, vol/vol) as mobile phase (monitored at 220 nm) (11). The structure of the compound representing peak A was determined by \(^1\)H and \(^13\)C nuclear magnetic resonance (NMR) spectrometry.

\(^1\)H NMR spectra were recorded on a Varian XL-400 instrument (Varian, Palo Alto, CA) at 399.950 MHz and \(^13\)C NMR spectra on a Varian XL-200 instrument at 50.309 MHz using \[^{2}\]H\(_3\)pyridine as solvent and tetramethylsilane as internal standard (\(\delta\)\(_{\text{H}}\)O and \(\delta\)\(_{\text{C}}\)O). The accuracies of \(\delta\)\(_{\text{H}}\), \(J\) and \(\delta\)\(_{\text{C}}\) were \(\pm 0.002\) ppm, \(\pm 0.3\) Hz and \(\pm 0.03\) ppm, respectively. 2D INADEQUATE (incredible natural abundance double-quantum transfer experiment) \(^13\)C NMR spectra were taken on the 4096 x 2048 matrix with zero-filling in the first domain, and with line-broadening weighting of 40 and 5 Hz, in the first and in the second domain, respectively.

Animals and treatments. Germ-free Wistar male rats (ca. 4 months old) bred at Shionogi Aburahi Laboratories (Shiga, Japan) had been housed under germ-free conditions and were monoassociated orally with six strains of bacteria, i.e., Escherichia coli, Bifidobacterium longum, Bacteroides vulgatus, Clostridium ramosum, Peptostreptococcus productus and Lactobacillus gasseri. These bacteria had originally been isolated from normal human feces, and represented the predominant strain of each group. Each bacterium was cultured for 72 h at 37°C in GAM semifluid medium (Nissui Co., Tokyo, Japan) and then homogenized with a stirrer without access of air. A 1-mL portion of the culture medium containing \(10^8\)–\(10^9\) bacteria/mL was administered to the rats. Conventional rats of the same strain and the same age were also examined.

The rats were fed a commercial diet (Oriental CMF Diet; Oriental Kobo Co., Tokyo, Japan) which had been sterilized by radiation with \(^{60}\)Co (50 KGY). Feces from each rat were collected before inoculation and also each day after inoculation for 6 d. A portion of the feces collected on the last day was utilized to confirm that the
The extracts were combined and dried. A solution in 90% from the dry preparation with absolute ethanol at homogenates was lyophilized. Bile acids were extracted with 20 mL of distilled water, and a portion of the bile with 20 vol of ethanol at 85-90°C for 10 min. After ile- tine and the large intestine, including the cecum, were dominal aorta to sacrifice the animals, the small intesti- and the bile duct was cannulated with a PE-10 poly-ethylene tube to collect bile for 30 min, with the rectal heating plate (15). After removing blood from the ab- dominal aorta to sacrifice the animals, the small intestine and the large intestine, including the cecum, were removed along with their contents.

**Bile acid determination.** Bile acids were extracted from bile with 20 vol of ethanol at 85–90°C for 10 min. After fil- tration, a portion of the extract was dried under a stream of nitrogen and subjected to PHP-LH-20 column chromatography (16) to obtain the fractions of free, glycine-conjugated, taurine-conjugated and sulfated bile acids.

The small and large intestinal contents were homoge- nized with 20 mL of distilled water, and a portion of the homogenates was lyophilized. Bile acids were extracted from the dry preparation with absolute ethanol at 85–90°C for 1 h, and the mixture was filtered. This extraction procedure was repeated three times (17), and the extracts were combined and dried. A solution in 90% ethanol was subjected to PHP-LH-20 column chromatography (16).

The feces were lyophilized and ground in a small mill. Bile acids were extracted from a portion of the feces (usually 0.5 g) by treatment with 15 mL of absolute ethanol at 85–90°C for 1 h, and a filtered solution was obtained (17). The extraction procedures were the same as those used for the intestinal contents.

The fractions obtained after PHP-LH-20 column chromatography were purified on a Sep-Pak C18 cartridge (Water Associates, Milford, MA) and then concentrated under reduced pressure (18). The sulfate fraction was obtained (17). The extraction procedures were the same ethanol at 85-90°C for 1 h, and the mixture was filtered. This extraction procedure was repeated three times (17), and the extracts were combined and dried. A solution in 90% ethanol was subjected to PHP-LH-20 column chromatography (16).

TABLE 1

<table>
<thead>
<tr>
<th>Inoculated bacterial count log N/mL</th>
<th>Bacterial count log N/g wet weight feces</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>9.1</td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em></td>
<td>8.4</td>
</tr>
<tr>
<td><em>Bacteroides vulgatus</em></td>
<td>8.4</td>
</tr>
<tr>
<td><em>Clostridium ramosum</em></td>
<td>8.6</td>
</tr>
<tr>
<td><em>Peptostreptococcus productus</em></td>
<td>8.5</td>
</tr>
<tr>
<td><em>Lactobacillus gasseri</em></td>
<td>8.5</td>
</tr>
</tbody>
</table>

The bacterial counts were more than 10^9/g wet weight of feces (Table 1) using the method of Benno et al. (14), and the remainder was used for bile acid analysis.

Soon after the final collection of feces, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and the bile duct was cannulated with a PE-10 poly-ethylene tube to collect bile for 30 min, with the rectal temperature being kept at 37°C by means of an electrical heating plate (15). After removing blood from the ab- dominal aorta to sacrifice the animals, the small intestine and the large intestine, including the cecum, were removed along with their contents.

**Bile acid determination.** Bile acids were extracted from bile with 20 vol of ethanol at 85–90°C for 10 min. After fil- tration, a portion of the extract was dried under a stream of nitrogen and subjected to PHP-LH-20 column chromatography (16) to obtain the fractions of free, glycine-conjugated, taurine-conjugated and sulfated bile acids.

The fractions obtained after PHP-LH-20 column chromatography were purified on a Sep-Pak C18 cartridge (Water Associates, Milford, MA) and then concentrated under reduced pressure (18). The sulfate fraction was obtained (17). The extraction procedures were the same ethanol at 85-90°C for 1 h, and the mixture was filtered. This extraction procedure was repeated three times (17), and the extracts were combined and dried. A solution in 90% ethanol was subjected to PHP-LH-20 column chromatography (16).

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The fractions obtained after PHP-LH-20 column chromatography were purified on a Sep-Pak C18 cartridge (Water Associates, Milford, MA) and then concentrated under reduced pressure (18). The sulfate fraction was subjected to solvolysis (19). The glycine- and taurine- conjugated bile acids were hydrolyzed using cholyl- glycine hydrolase (EC 3.5.1.24; Sigma, St. Louis, MO) (20), and the deconjugated bile acids were extracted with diethyl ether after acidification with 2 N hydrochloric acid.

The bile acids were methylated with freshly prepared diazomethane and then trifluoroacetylated with trifluoroacetic anhydride (17,21). The bile acid derivatives were quantified by gas–liquid chromatography utilizing a Hewlett Packard (Palo Alto, CA) gas-chromatograph, Model HP5890A, equipped with a hydrogen flame-ion- ization detector and an HP-7673A autoinjector. A capillary column (15 m x 0.25 mm i.d.) coated with DB-17 (J&W Scientific, Folsom, CA) was used. The column was programmed from 200–280°C at a rate of 5°C/min; the injection port was kept at 280°C, and the detector at 300°C.

**RESULTS**

Enzymatic hydrolysis of the compound eluted as peak A (compound A) with cholyglycine hydrolase and methyla- tion with diazomethane produced a methyl ester. The 1H NMR spectrum of the methyl ester derived from com- pound A was quite similar to that of the methyl ester of β-muricholic acid except for two additional signals at δ_1H_ 5.978 (dd, J = 15.5 and 0.6 Hz, 23-H) and δ_1H_ 7.080 (dd, J = 15.5 and 9.0 Hz, 22-H), as is compared in Table 2. The chemical shifts and couplings (J = 15.5 Hz) of these signals indicate the presence of an E-double bond with one hydrogen at the vicinal position. The down-field shifts of the 20-methyl signal (δ_1H_ 1.076, d, J = 6.6 Hz, 21-H) and the ester methyl signal (δ_1H_ 3.731, s, COOCH_3) suggest that the methyl ester of compound A is the methyl ester of β-muricholic acid having an E-double bond at C-22 and C-23. The presence of an α, β-unsaturated carboxy ester system is consistent with the observed UV absorption at 214 nm (22). The 1H NMR spectrum of compound A is essentially the same as that of the methyl ester of compound A except for the presence of three signals at δ_1H_ 5.351 (t, J = 6.7 Hz, CH_2SO_3H), δ_1H_ 4.332 (brq, J = 6.0 Hz, CH_2N), and δ_1H_ 7.070 (t, J = 5.8 Hz, NH) and the absence of the ester methyl signal. These three additional signals of compound A can be assigned to the hydrogens of the taurine moiety. Thus, compound A is proposed to be tauro-A22,β-muricholic acid.

The 13C NMR spectrum of β-muricholic acid obtained in [2H_5]pyridine has been reported by Kuroki et al. (23). We have mostly confirmed the assignments of these signals by 2D INADEQUATE 13C NMR spectroscopy (12,13,24) as shown in Figure 1. In the 2D INADE- QUATE spectrum of β-muricholic acid, all connectivities of the carbons were established except for C-1, C-2, C-3 and C-4, whose signal intensity was too low. However, our assignments were not in agreement with those of Kuroki et al. (23) with respect to C-14 and C-17.

The 13C NMR signals of the methyl ester of β-muricholic acid were assigned by chemical shift comparisons with β-muricholic acid. The chemical shift changes for C-1 through C-21 of the methyl ester vs. the acid are within 0.24 ppm. The changes for C-22 through C-24 are -0.70, -0.38 and -2.22 ppm, respectively, which are greater due to the methyl esterification (see Ref. 25, and references therein). The methyl ester methoxy signal appears at δ_13C_ 51.25.

The 13C NMR spectrum of the methyl ester of com- pound A resembles that of the methyl ester of β-muricholic acid except for the signals originating from the side-chain carbons. The 13C NMR signals were assigned based on data reported for a number of bile acids (23;