Study of the Pharmacological Effect of the Bile Salt, Sodium Scymnol Sulfate, from *Rhizoprionodon acutus*. IV. Effects of Naturally Occurring Bile Alcohols, Bile Acids and Their Conjugates on Lesion Development and Vascular Endothelial Cell Injury in a Rat Peripheral Arterial Occlusion Model¹⁾

Hitoshi ISHIDA,* Hidetoshi NAKAYASU, and Kunirou TSUJI

School of Pharmaceutical Science, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan.

Received February 12, 1999; accepted May 12, 1999

A series of naturally occurring bile alcohols, bile acids and their conjugates has been investigated as part of our studies to develop unique anticoagulants with a potent prophylactic effect against vascular endothelial cell injury induced by lactic acidosis *in vivo* and *in vitro*.

In an *in vivo* rat peripheral arterial occlusion model induced by lactic acid injection, oral administration of a single dose of 3 mg/kg scymnol significantly inhibited edematous swelling and development of lower limb lesions, including gangrene, and reduced changes in clotting system functions and serum lactate dehydrogenase activity. It had no effect on clotting system functions in sham-operated rats. The structure–activity relationship suggests that the [24*R*-(+)-5 β -cholestane-3 α ,7 α ,24,26-pentol] or [3 α ,7 α -dihydroxy-5 β -cholan-20-one] structure is important for a potent prophylactic effect following oral administration. Intravenous administration of a single dose of 0.3 mg/kg sodium (25*S*)-scymnol sulfate or scymnol prevented lesion progression as effectively as oral administration of scymnol. Sodium (25*S*)-scymnol sulfate and ursodeoxycholic acid showed clear protective effects against cultured vascular endothelial cell damage due to lactic acidosis which were dose-dependent.

The above results suggest that bile steroids such as scymnol, sodium (25*S*)-scymnol sulfate, ursodeoxycholic acid, and chenodeoxycholic acid may play a role in protecting endothelial cells against injury caused by lactic acidosis. These compounds are candidates for novel anti-ischemic drugs that act by specifically protecting vascular endothelial cells.

Key words scymnol; sodium (25*S*)-scymnol sulfate; chenodeoxycholic acid; ursodeoxycholic acid; argatroban; arterial occlusion

In traditional Chinese medicine,²⁾ the gall bladders of some mammals, especially bear and cow, and cow gallstones, are important crude drugs in a variety of ways such as used as a sedative for biliary colic, as an antiphlogistic for liver disease, as a remedy for jaundice and as a digestive. Bile from various fish, such as shark, carp, mackerel and crucian, has also been used as a crude drug to treat dyspnea due to disorders of the throat and pharynx and as an antiphlogistic for eye diseases.

There have been numerous studies of the structures and pharmacological effects of bile and gallstone components, especially the bile of bears and cows, of which C-24 bile acids such as ursodeoxycholic acid (UDCA),³⁾ chenodeoxycholic acid (CDCA)⁴⁾ and deoxycholic acid (DCA)⁵⁾ are the principal components. There has been less work on bile alcohols and sodium bile alcohol sulfates, such as the C-27 bile alcohols scymnol,⁶⁾ 5 β -chimaerol,⁶⁾ and 5 β -cyprinol⁷⁾ and the C-24 bile alcohol 5 α -petromyzonol,⁸⁾ and their sodium sulfates,^{6–8)} which are the major components of fish bile and little is known about the pharmacological effects of these compounds. In humans, pharmacological studies of bile acids, especially UDCA and CDCA have focused mostly on their biological roles and significance in the liver and digestive tract. UDCA exhibits a choleric effect,⁹⁾ increases liver blood flow¹⁰⁾ and pancreatic juice excretion,¹¹⁾ dissolves cholesterol gallstones,¹²⁾ prevents biliary protein excretion,¹³⁾ increases lipase,¹⁴⁾ activates cholesterol 7 α -hydroxylase and suppresses HMG-CoA reductase,¹⁵⁾ while that CDCA is effective in dissolving gallstones¹⁶⁾ and improving bile secre-

tion in a different manner from that of UDCA.¹⁷⁾ It is also cytotoxic to hepatocytes, erythrocytes, the digestive canal, gall bladder, biliary tract and cell membranes.¹⁸⁾ Based on these findings, UDCA and CDCA have been used clinically as cholagogues to improve bile secretion¹⁹⁾ and resolve gallstones,²⁰⁾ while UDCA has been used to treat chronic liver diseases, such as primary biliary cirrhosis and chronic hepatitis C (in combination with or without human recombinant interferon- α),²¹⁾ and as drug to treat digestive problems due to inflammatory disease of the small intestine.²²⁾ However, to our knowledge, there has been no study on the effects of bile acids on the blood-vascular system.

Recently, we reported that scymnol, 5 β -chimaerol and sodium (25*S*)-scymnol sulfate exhibited protective effects in various experimental models of cerebral anoxia or hypoxia in mice, elevating the partial oxygen pressure of the arterial blood.²³⁾ We also found that CDCA and DCA conjugates had protective effects in a KCN-induced anoxia model. Essel *et al.* reported that pretreatment with UDCA effectively reduced the incidence of hepatic veno-occlusive disease in cancer patients²⁴⁾ undergoing stem cell transplantation, as did heparin, pentoxifylline and prostaglandin E₁.²⁵⁾ An early event in the development of veno-occlusive disease is thought to be endothelial cell damage, which could stimulate local thrombotic mechanisms, leading to microvascular flow insufficiency, production of cytotoxic substances and, ultimately, hepatocellular necrosis.²⁶⁾ It has been suggested that UDCA has a direct protective effect on hepatocytes under hepatic veno-occlusive conditions. However, the mechanisms

* To whom correspondence should be addressed.

involved remain unclear. Very recently, we demonstrated that scymnol effectively prevents lesion development in the rat peripheral arterial occlusion model, induced by lactic acid injection into the femoral artery, by protecting vascular endothelial cells against injury by lactic acidosis.^{1,27)}

The vascular endothelial cells lining blood vessels are multifunctional cells playing a key role in the local control of vascular tone.²⁸⁾ They produce vasoconstrictor endothelin, as well as vasodilators such as endothelial-derived relaxing factor, prostacyclin and C-type natriuretic peptide. Endothelial cells play an important role in the regulation of coagulation, and dysfunction of these cells is important in thrombotic disorders, such as anoxia, advanced atherosclerosis, hypertension, hypercholesterolemia, diabetes and rheumatism due to metabolic abnormalities such as lactic acidosis.^{29,30)} The prophylactic effect of scymnol on lesion development and vascular endothelial cell injury in a rat peripheral arterial occlusion model, and that of UDCA on hepatic veno-occlusive disease in cancer, led us to hypothesize that bile steroids, including scymnol and UDCA, might be effective in the treatment of thrombotic diseases involving arterial tissue damage due to ischemia and hypoxia. The aim of this study is to examine the prophylactic effect of naturally occurring bile alcohols, sodium bile alcohol sulfates, bile acids, and bile acid taurine conjugates, as well as the antithrombotic drug argatroban, on disease progression and endothelial cell damage in a rat peripheral arterial occlusion model. In addition, their effects on damage to endothelial cells induced by lactic acidosis *in vivo* and *in vitro* were examined.

MATERIALS AND METHODS

Materials Scymnol (24R-(+)-5 β -cholestane-3 α ,7 α ,12 α ,24,26,27-hexol), 5 β -chimaerol ((24R,25R)-(+)-5 β -cholestane-3 α ,7 α ,12 α ,24,27-pentol), sodium (25S)-scymnol sulfate ((24R,25S)-(+)-3 α ,7 α ,12 α ,24,26-pentahydroxy-5 β -cholestan-27-yl sodium sulfate) and sodium 5 β -chimaerol sulfate ((24R,25R)-(+)-3 α ,7 α ,12 α ,24-tetrahydroxy-5 β -cholestan-27-yl sodium sulfate) were prepared from the bile of *Rhizoprionodon acutus* according to the procedures reported previously.^{31,32)} 7-Deoxyscymnol (24R-(+)-5 β -cholestane-3 α ,12 α ,24,26,27-pentol) was prepared from megamouth shark and 5 β -cyprinol (5 β -cholestane-3 α ,7 α ,12 α ,26,27-pentol) and 5 β -molanol (5 β -cholestane-3 α ,7 α ,11 α ,26,27-pentol) were prepared from the bile of *Mola mola* L., as reported previously.^{32,33)} 5 α -Cyprinol (5 α -cholestane-3 α ,7 α ,12 α ,26,27-pentol) was prepared from the bile of carp, *Cyprinus caprio*, based on the reported procedures.³⁴⁾ 5 β -Petromyzonol (5 β -cholan-3 α ,7 α ,12 α ,24-tetrol) was prepared by reduction of cholic acid with LiAlH₄ in dry tetrahydrofuran (THF) and its physicochemical data were confirmed to be consistent with reported values.³⁵⁾ Commercial bile acids, cholic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-yl) (Wako Pure Chemical Ind., Ltd.), UDCA (3 α ,7 β -dihydroxy-5 β -cholan-24-yl) (Tokyo Tanabe Pharmaceutical Industries, Ltd.), taurocholic acid (2-[[3 α ,7 α ,12 α -trihydroxy-24-oxo-5 β -cholan-24-yl]amino]ethanesulfonic acid), taurochenodeoxycholic acid (2-[[3 α ,7 α -dihydroxy-24-oxo-5 β -cholan-24-yl]amino]ethanesulfonic acid) and taurooursodeoxycholic acid (2-[[3 α ,7 β -dihydroxy-24-oxo-5 β -cholan-24-yl]amino]ethanesulfonic acid) (Sigma) were used.

Lactic acid was purchased from Nacalai Tesque (Kyoto). Argatroban ((2R,4R)-4-methyl-1-[N₂-((RS)-3-methyl-1,2,3,4-tetrahydro-8-quinoline-sulfonyl)-L-arginyl]-2-piperidine-carboxylic acid monohydrate) was kindly provided by Daiichi Pharmaceutical Co., Ltd. (Tokyo).

Method of Administration Each bile alcohol, sodium bile alcohol sulfate, bile acid or bile acid taurine conjugate was administered as a single oral dose after having been suspended or dissolved in 0.5% methylcellulose-physiological saline before operation or before blood collection. A single dose of scymnol was administered intraperitoneally as a solution in propylene glycol-ethanol-water (2:3:5), while sodium (25S)-scymnol sulfate was dissolved in physiological saline, and argatroban in physiological saline containing 0.3 M D-sorbitol.

Animals Male Wistar rats (13 weeks old) weighing about 290 g, purchased from Japan SLC (Hamamatsu), were used after acclimatization for 4 d.

Administration and Operating Procedure Operations were carried out as described previously.^{1,27,36)} Rats were anesthetized with sodium pentobarbital, 50 mg/kg (i.p.). The femoral artery in the right upper leg was exposed surgically, 2 h after a single oral administration of bile alcohol, bile acid or its conjugate, or 15 min after a single intravenous administration of scymnol or sodium (25S)-scymnol sulfate, or 30 min after a single administration of argatroban, and then 5% lactic acid-saline solution (1:19 v/v, pH 1.67) (0.15 ml) was injected into this femoral artery. Hemostasis was achieved by the application of a drop of surgical sealant to the puncture site of the artery, and the incision closed with surgical binding. Some animals were injected with saline alone in place of lactic acid solution; these are referred to as sham-operated rats.

Macroscopic Observation Development of the lesions was observed daily for 2 weeks, as reported previously.^{1,27,36)} The lesions were graded 0 to IV for gangrene, mummification or loss of digits at 3, 7 and 14 d after the operation as follows: normal appearance, 0; affected region limited to the nails, I; to fingertips, II; necrosis of the fingers, III; gangrene or loss of the fingers, IV. The lesion point score was expressed as the average of the sum of the five finger grades, adding 5 points if gangrene extended to the lower leg, of each animal in the group.

Edematous Swelling Examination As reported previously,^{1,27)} the right and left paws of each animal were amputated 3 h after lactic acid injection and then immediately fixed with neutral 10% formalin. After measuring the thickness of each paw by a digital thickness meter, the difference between the two was calculated and taken as a measure of edematous swelling for each animal.

Tissue for LDH Isozyme Determinations Following previous reports,^{1,37)} a heart tissue sample was excised, weighed, and kept in an ice bath. Tissue was diluted in 10 volumes of phosphate-buffered saline (PBS) and homogenized with a tissue homogenizer. The homogenate was spun at 1000 g for 30 min. The clear supernatant was used to determine LDH activity and LDH isozyme pattern.

Rat Endothelial Cells for Enzyme Assay According to previous reports,^{1,38)} rat endothelial cells (RECs) were isolated from the thoracic aorta by treatment with collagenase in Dulbecco's modified Eagle's medium (DMEM) and plated

into 12-well tissue culture clusters in M199 containing 20% fetal calf serum (FCS). They were then allowed to proliferate until the monolayer reached confluence at 37 °C under a humidified atmosphere containing 5% CO₂, using the collagen prepared from the rat's tail. The cells were confirmed as endothelial cells by reported methods.^{1,38)} The monolayer was washed with PBS three times, lysed with collagenase in DMEM in PBS containing 2% bovine serum albumin, homogenized on an ice bath with a Polytron tissue homogenizer, and then centrifuged at 1000 *g* for 30 min. The clear supernatant was used for the enzyme assays.

Total Serum Lactate Dehydrogenase Activity and Isozyme Pattern in the Rat Model As reported previously,¹⁾ blood samples withdrawn from the jugular vein into a plastic syringe under Nembutal anesthesia at 3 h after operation in some animals were centrifuged at 2000 *g* for 15 min to obtain serum, after standing for 30 min at 37 °C. Total LDH activity was measured with a kit, lactate dehydrogenase C II-TEST Wako (Wako Pure Chemical Industries, Ltd., Osaka). Isozyme electrophoresis was carried out according to previous reports.^{1,37)} Prior to electrophoresis, samples were diluted to approximately 400–600 IU/l with PBS. Isozymes in each sample were separated on agarose gel (Lactate Dehydrogenase Isozyme Test Wako Kit (Wako Pure Chemical Industries, Ltd., Osaka)) by electrophoresis for 20 min at 100 V (Power Station AE-8450, ATTO Corp., Tokyo). After electrophoresis, the isozymes were visualized by a specific colorimetric reaction with the Lactate Dehydrogenase Isozyme Test Wako Kit. Gels were fixed by immersion in EtOH–H₂O–AcOH (14 : 5 : 1) and scanned using a densitometer (Densitograph AE 6905, ATTO Corp., Tokyo). Control LDH isozymes, LDH₁, LDH₂, LDH₃, and LDH₄ prepared from heart and LDH₄ and LDH₅ from culturing rat endothelial cells, were used, as reported previously.¹⁾ Total LDH activity of each isozyme was determined by multiplying the total LDH activity of each sample by the relative fraction of each isozyme determined by densitometry.

Rat Vascular Endothelial Cell Injury Induced by Lactic Acid Acidosis A monolayer of the RECs cultured in M199 containing 20% FCS in each well of a Petri dish was

washed with PBS three times after it had reached the stationary phase, then incubated in M199–saline (1 : 1) containing 0, 0.25, 0.5 1.0 or 1.5% lactic acid (pH 8.09, 2.76, 2.51, 2.31 and 2.16, respectively). After incubation, the cells were washed with PBS three times and stained with 0.02% erythrosine B in PBS for 10 min at 37 °C, according to Watanabe *et al.*³⁹⁾ Photographs were taken and scanned with a film scanner (Coolscan, Nikon Corp., Tokyo, Japan). The number of cells clearly stained with erythrosine B was counted visually and the area stained with erythrosine B was measured by an imaging technique using the Photoshop system (Adobe Systems Inc.). Then, monolayers were treated with 0.1 N NaOH for 12 h at 37 °C, and the clear solution in each well was subjected to spectrophotometry to determine the content of erythrosine B.

Protection of Cells Against Injury Induced by Lactic Acidosis Monolayers of cultured endothelial cells at the stationary phase in the wells of a Petri dish were washed with PBS, and incubated for 2 h in M199–saline (1 : 1) containing 1.0% lactic acid plus the test bile steroid. Vehicle alone was added to the control cultures. After incubation, the cells were washed with PBS three times and stained with 0.02% erythrosine B in PBS for 10 min at 37 °C, and then the area stained with erythrosine B and the content of erythrosine B in cells were measured, as described above.

Analysis of Data Each result is expressed as the arithmetic mean ± S.E. Statistical analysis was conducted using Student's *t*-test. Data from the animal experiments were compared using analysis of variance and Student's *t*-test or Dunnett's multiple range test.

RESULTS

Macroscopic Findings In agreement with previous reports,^{1,27)} the following lesions developed in the rat peripheral arterial occlusion model. From 1 to 2 h, edema of the affected paw gradually developed, reaching a maximum at about 3 h. The whole paw became pale and then dusky within about 5 h after injection. On the next day, the affected area of the nails and/or fingers of the affected paw became dark vio-

Table 1. Effect of Scymnol on Lesion Progression in the Rat Peripheral Arterial Occlusive Disease Model

Group (mg/kg) ^{a)}	Days ^{b)}	Grade of disease and lesion point																			
		3						7						14							
		0	I	II	III	IV	Average	0	I	II	III	IV	Average	0	I	II	III	IV	Average		
Sham-operated	50 ^{c)}	0	0	0	0	0	0.0±0.0** ^{d)}	50	0	0	0	0	0	0.0±0.0**	50	0	0	0	0	0	0.0±0.0***
Control	—	16	5	19	7	3	7.6±1.7	14	8	2	25	1	9.1±1.8	9	5	4	24	8	11.7±1.7		
Scymnol (0.3)	20	8	2	25	1	0	4.9±1.3	14	10	2	16	8	9.9±2.4	12	7	4	17	10	11.2±2.3		
Scymnol (1)	28	5	16	1	0	0	4.0±1.0	21	10	5	12	2	6.4±1.3	13	12	6	12	7	8.8±1.7		
Scymnol (3)	37	7	6	0	0	0	1.3±0.5**	31	8	2	8	1	4.0±1.5*	22	7	6	12	3	6.7±1.7*		
Scymnol (10)	33	9	6	2	0	0	2.7±0.8**	30	8	7	4	1	3.8±0.8*	23	14	1	1	11	5.3±1.0*		
Scymnol (30)	27	6	2	0	0	0	1.4±0.4**	27	5	2	1	0	1.7±0.5**	24	6	2	2	1	2.9±0.8**		
Scymnol (100)	37	3	0	0	0	0	0.0±0.0**	36	4	0	0	0	0.0±0.0**	29	5	5	1	0	2.3±1.1***		
Scymnol (200)	38	2	0	0	0	0	0.0±0.0***	38	2	0	0	0	0.0±0.0***	36	3	1	0	0	0.0±0.0***		
S.S.S. ^{e)} (235)	25	4	4	0	0	0	1.0±0.3***	24	5	1	0	0	1.2±0.3***	25	1	2	2	0	1.8±0.7***		
Argatroban (1)	48	1	1	0	0	0	0.3±0.2**	46	2	1	1	0	0.7±0.4**	41	1	3	2	3	2.5±1.8***		

a) Scymnol or sodium (25S)-scymnol sulfate was administered as a single dose by stomach tube 2 h before lactic acid injection. Argatroban was administered as a single dose by intravenous injection 30 min before lactic acid injection. Control rats received the vehicle alone. Sham-operated rats were injected with saline in place of the lactic acid solution. b) Days after operation. c) Finger number. d) Lesion point scores are represented as means ± S.E. (*n* = 6–11). *, ** and *** indicate significant differences (*p* < 0.05, 0.01 and 0.001, respectively) from the control. e) Sodium (25S)-scymnol sulfate.

let and the dark area gradually extended to the whole paw. From 3 to 14 d after injection, the fingers became gangrenous and then mummified in most animals. The marginal area of the affected part of the paw showed exudation and the gangrenous part of the paw fell off in some cases. Tables 1 and 3 show the grade of disease and average lesion point score for the control rats 3, 7 and 14 d after injection. Paw swelling 3 h after injection is shown in Tables 2 and 4.

Changes in Patterns and Activity of Serum LDH Isozymes Serum LDH activity and LDH isozyme pattern of the control rats are shown in Tables 2, 4 and 5. As in the preceding report,¹⁾ the total LDH activity was markedly increased 3 h after lactic acid injection. The increase in total LDH activity was wholly accounted for by LDH₄ and LDH₅ and the ratio of LDH₄ and LDH₅ increments (0.266) was similar to the reported value for rat endothelial cells in cul-

ture (0.275) (Table 5).

Effect of Argatroban As reported previously,^{1,27,36)} argatroban at 1 mg/kg significantly prevented disease progression and edematous swelling (Tables 1 and 2), and suppressed changes in serum total LDH activity and ratio of LDH₄/LDH₅ in the model (Tables 2 and 5).

Effect of Scymnol When scymnol was given orally as a single dose of 3 to 200 mg/kg before lactic acid injection, the treated rat paws retained a nearly normal appearance for about 10 min after lactic acid injection and then became pale red and flushed, with light edematous swelling, which was inhibited in a dose-dependent manner, lasting for 5 h to 5 d after injection (Tables 1 and 2). In the dose-effect experiments, gangrene was limited to nails or fingertips and only one gangrenous fingertip was lost in two rats at a dose of 3 mg/kg. There was a significant suppression of the increase in total serum LDH activity at 3 mg/kg or more, and of the change in LDH₄/LDH₅ ratio at 10 mg/kg in the model (Tables 2, 4 and 5).

In this model, when scymnol was administered intravenously 20 min before lactic acid injection at a single dose of 1, 3 or 10 mg/kg, it markedly prevented the increase in LDH activity and lesion development.

Effect of Bile Alcohols and Sodium Bile Alcohol Sulfates All the bile alcohols and sodium bile alcohol sulfates examined significantly prevented disease progression, edematous swelling and the increase in the total serum LDH activity following a single oral administration at a dose of 20.5 μ mol/kg, as scymnol did (Tables 3 and 4). The degree of prophylactic effect on lesion development was in the following order, scymnol>sodium 5 β -chimaerol sulfate>5 β -chimaerol>sodium (25S)-scymnol sulfate>7-deoxyscymnol>5 β -molanol>5 α -cyprinol>5 β -cyprinol and 5 β -petromyzonol.

In this model, intravenous administration of sodium (25S)-scymnol sulfate markedly inhibited the increase in LDH ac-

Table 2. Effect of Scymnol on Paw Swelling and Serum Lactate Dehydrogenase Activity in the Rat Peripheral Arterial Occlusive Disease Model

Group (mg/kg) ^{a)}	Paw swelling (mm)	LDH (IU/l)
Sham-operated	—	306.6 $\pm$ 39.2***
Control	4.27 $\pm$ 0.16	1082.0 $\pm$ 77.8
Scymnol (0.3)	3.99 $\pm$ 0.20	1023.0 $\pm$ 149.4
Scymnol (1)	3.71 $\pm$ 0.20*	876.2 $\pm$ 118.0
Scymnol (3)	3.66 $\pm$ 0.22*	816.4 $\pm$ 93.2**
Scymnol (10)	3.41 $\pm$ 0.13**	657.0 $\pm$ 104.8**
Scymnol (30)	2.62 $\pm$ 0.31***	571.0 $\pm$ 22.8***
Scymnol (100)	2.51 $\pm$ 0.43***	556.1 $\pm$ 80.1**
Scymnol (200)	1.39 $\pm$ 0.31***	307.9 $\pm$ 26.7***
S.S.S. (235)	2.22 $\pm$ 0.19***	500.5 $\pm$ 120.2***
Argatroban (1)	3.22 $\pm$ 0.26**	730.0 $\pm$ 70.9*

a) Scymnol or sodium (25S)-scymnol sulfate was administered as a single dose by stomach tube 2 h before lactic acid injection. Argatroban was administered as a single dose by intravenous injection 30 min before lactic acid injection. Control rats received the vehicle alone. Sham-operated rats were injected with saline in place of the lactic acid solution. Values represent means $\pm$ S.E. (n=8–11). *, ** and *** indicate significant differences ($p<0.05$, 0.01 and 0.001, respectively) from the control.

Table 3. Effect of Bile Alcohols, Bile Acids and Their Conjugates on Lesion Progression in the Rat Peripheral Arterial Occlusive Disease Model

Group (mg/kg) ^{a)}	Days ^{b)}	Grade of disease and lesion point																			
		3						7						14							
		0	I	II	III	IV	Average	0	I	II	III	IV	Average	0	I	II	III	IV	Average		
Sham-operated	50 ^{c)}	0	0	0	0	0	0.0±0.0*** ^{d)}	50	0	0	0	0	0	0.0±0.0***	50	0	0	0	0	0	0.0±0.0***
Control	11	8	20	1	0	0	6.4±1.2	5	9	8	13	5	10.5±1.7	3	3	1	16	17	15.8±1.9		
Scymnol	39	1	0	0	0	0	0.1±0.1**	35	5	0	0	0	0.6±0.3***	30	10	0	0	0	1.3±0.3***		
7-Deoxyscymnol	33	5	2	0	0	0	1.1±0.4**	29	8	1	1	1	2.1±0.5**	25	9	2	3	1	3.8±0.7***		
5β-Chimaerol	36	3	1	0	0	0	0.6±0.4**	31	8	1	0	0	1.3±0.5**	26	10	4	0	0	2.5±0.8***		
5β-Cyprinol	31	4	5	0	0	0	1.8±0.7**	19	15	3	3	0	3.8±0.9**	15	15	5	5	0	5.1±1.1**		
5α-Cyprinol	36	7	0	0	0	0	0.9±0.4**	30	9	4	0	0	2.1±0.7**	27	8	3	2	0	4.9±1.1***		
5β-Molanol	34	5	1	0	0	0	0.9±0.4**	26	12	2	0	0	2.0±0.6**	18	15	4	2	1	4.8±0.9***		
5β-Petromyzol	22	6	2	0	0	0	1.7±0.6**	19	8	3	0	0	2.3±0.8**	10	10	8	2	0	5.3±1.5**		
S.S.S.	36	3	1	0	0	0	0.6±0.3**	29	10	1	0	0	1.5±0.5**	24	12	2	1	1	2.3±0.7***		
S.C.S. ^{e)}	37	0	2	1	0	0	0.9±0.6**	35	2	2	1	0	1.1±0.5**	32	3	2	2	1	2.6±0.8***		
UDCA ^{f)}	30	8	1	0	0	0	1.3±0.5**	22	11	3	1	0	2.5±0.8**	17	14	3	5	1	3.8±1.2***		
CDCA ^{g)}	35	5	0	0	0	0	0.6±0.4**	31	9	0	0	0	1.1±0.5**	28	11	1	0	0	1.6±0.5***		
CA ^{h)}	24	4	2	0	0	0	1.3±0.4**	22	5	3	0	0	1.8±0.7**	21	5	1	3	0	2.8±1.0***		
TUDCA ⁱ⁾	33	5	1	1	0	0	1.3±0.6**	29	7	3	1	0	2.0±0.8**	26	7	3	2	2	4.4±1.3**		
TCDC ^{j)}	33	5	2	0	0	0	1.1±0.6**	29	7	2	1	1	2.3±0.9**	22	12	2	3	1	4.6±1.4**		
TCA ^{k)}	19	5	6	0	0	0	4.7±1.8	15	9	2	2	2	6.8±2.2	11	11	2	3	3	8.8±2.2		

a) Bile alcohols and bile acids (20.5 μ mol/kg) were administered as single doses by stomach tube 2 h before lactic acid injection. Control rats received the vehicle alone. Sham-operated rats were injected with saline in place of the lactic acid solution. b) Days after operation. c) Finger number. d) Lesion point scores are represented as means $\pm$ S.E. (n=6–8). ** and *** indicate significant differences ($p<0.01$ and 0.001, respectively) from the control. e) Sodium 5 β -chimaerol sulfate. f) Ursodeoxycholic acid. g) Chenodeoxycholic acid. h) Cholic acid. i) Tauroursodeoxycholic acid. j) Taurochenodeoxycholic acid. k) Taurocholic acid.

tivity and lesion development, with almost similar effects at single doses of 1, 3 and 10 mg/kg, as was the case with scymnol (Tables 6 and 7).

Effect of Bile Acids and Bile Acid Taurine Conjugates

The examined bile acids and bile acid taurine conjugates except taurocholic acid (TCA), all significantly inhibited disease progression and edematous swelling and suppressed the change in activity of serum LDH at a single dose of 20.5 $\mu\text{mol/kg}$ *p.o.*, just as did the bile alcohols. TCA was ineffective (Tables 3 and 4). The degree of prophylactic effect on lesion development was in the following order, CDCA > cholic acid > tauroursodeoxycholic acid (TUDCA) > taurochenodeoxycholic acid (TCDCA) and UDCA.

Effect of Sodium (25S)-Scymnol Sulfate and UDCA *in Vitro*

When rat endothelial cells were incubated in culture

medium containing more than 0.25% lactic acid, many cells were damaged and stained dark brown after treatment with erythrosine B. Lactic acid killed about 24 to 40% of the endothelial cell population over the range 0.5 to 2.5% in medium (Table 8). There was a good correlation between the proportion of cell deaths and the total amount of erythrosine B incorporated in to dead cells.

Sodium (25S)-scymnol sulfate and UDCA showed a clear protective effect against this cell damage due to lactic acidosis, even at a concentration of 25×10^{-6} M. Their protective effect was dose-dependent.

DISCUSSION

This study was conducted to investigate whether naturally occurring bile steroids exhibit a prophylactic effect like that of scymnol on lesion progression and vascular endothelial cell injury in a rat peripheral arterial occlusive model induced by lactic acid injection, and, if so, how they affect endothelial cell damage caused by lactic acidosis *in vivo* and *in vitro*. Argatroban and scymnol served as positive controls, because their prophylactic effect in this model is well established.^{1,27,36)}

The macroscopic and hematological changes that occur in the rat peripheral arterial occlusion model are also well established,^{1,27,36)} and it is considered that the changes in serum LDH activity and isozyme pattern could be almost entirely accounted for by the release of LDH₄ and LDH₅ from injured endothelial cells. The injury to the endothelial cells is thought to result in activation of thrombin *via* a cascade of enzymatic amplification reactions, leading to the formation of occlusive thrombi in microvessels; these fibrin-rich thrombi, in turn, further injure the vascular epithelium in their vicinity.¹⁾ This scenario is supported by the histological findings of both Iwamoto *et al.*³⁶⁾ and Tomikawa *et al.*⁴⁰⁾ who showed that endothelial cells were partially destroyed and the microarterial vessels were occluded by thrombi after injection of 5% lactic acid into rat femoral artery.^{36,40)} Thrombin is a potent agonist of not only for platelet adhesion and aggregation, but also of constriction of vascular smooth muscle.⁴¹⁾ The effectiveness of argatroban against lesion development in this

Table 4. Effect of Bile Alcohols, Bile Acids and Their Conjugates on Paw Swelling and Serum Lactate Dehydrogenase Activity in the Rat Peripheral Arterial Occlusive Disease Model

Group	Paw swelling (mm)	LDH activity (IU/l)
Sham-operated	—	385±56***
Control	4.90±0.06	1054±87
Scymnol	2.80±0.45**	538±65***
7-Deoxyscymnol	3.00±0.45**	638±68***
5 β -Chimaerol	3.12±0.34**	630±74**
5 β -Cyprinol	4.18±0.04***	635±25***
5 α -Cyprinol	4.12±0.14**	636±76**
Molanol	3.64±0.51*	597±52***
5 β -Petromyzol	3.47±0.39*	737±93*
S.S.S.	3.82±0.23**	530±21***
S.C.S.	3.37±0.48*	573±35***
UDCA	3.88±0.14***	631±45***
CDCA	4.09±0.12***	609±56***
CA	3.77±0.39**	706±63**
TUDCA	3.52±0.41**	692±57**
TCDCA	3.52±0.41**	805±71*
TCA	4.30±0.23	828±67

Bile alcohols and bile acids (20.5 $\mu\text{mol/kg}$) were administered as single doses by stomach tube 2 h before lactic acid injection. Control rats received the vehicle alone. Sham-operated rats were injected with saline in place of the lactic acid solution. Values represented as mean±S.E. (n=6–8). *, ** and *** indicate significant differences ($p < 0.05$, 0.01 and 0.001, respectively) from the control.

Table 5. Effect of Scymnol, Ursodeoxycholic Acid and Argatroban on Serum Lactate Dehydrogenase Activity and Isoenzyme Pattern in the Rat Peripheral Arterial Occlusive Disease Model

Group (mg/kg) ^{a)}	Total LDH activity (IU/l)	LDH isoenzyme activity (IU/l) (relative)				
		LDH ₁	LDH ₂	LDH ₃	LDH ₄	LDH ₅
Sham-operated — (<i>p.o.</i>)	491.5±36.9***	13.8±1.0 (2.8%)	2.5±0.2 (0.5%)	2.9±0.2 (0.6%)	38.3±2.9*** (7.8%)	434.0±32.6*** (88.3%)
Control — (<i>p.o.</i>)	1086.0±107.8	21.7±2.2 (2.0%)	4.3±0.4 (0.4%)	6.5±0.6 (0.6%)	162.9±16.2 (15.0%)	890.5±88.4 (82.0%)
Scymnol (1) (<i>p.o.</i>)	875.3±108.1	16.6±2.1 (1.9%)	5.3±0.6 (0.6%)	6.1±0.8 (0.7%)	117.3±14.5 (13.4%)	729.8±90.1 (83.4%)
Scymnol (10) (<i>p.o.</i>)	650.5±63.0**	9.1±3.8 (1.4%)	3.9±0.4 (0.6%)	5.2±0.5 (0.8%)	70.9±6.9** (10.9%)	561.4±54.4** (86.3%)
UDCA (10) (<i>p.o.</i>)	703.2±53.3**	14.8±1.1 (2.1%)	3.6±0.3 (0.5%)	4.2±0.3 (0.6%)	78.8±6.0** (11.2%)	601.8±45.6** (85.6%)
Argatroban (1) (<i>i.v.</i>)	737.4±64.2**	14.7±1.3 (2.0%)	3.7±0.3 (0.5%)	2.9±0.3 (0.4%)	92.2±8.0** (12.5%)	623.8±54.3** (84.6%)

^{a)} Scymnol or ursodeoxycholic acid (20.5 $\mu\text{mol/kg}$) was administered as a single dose by a stomach tube 2 h before lactic acid injection. Argatroban was administered as a single dose by intravenous injection 30 min before lactic acid injection. Control rats received the vehicle alone. Sham-operated rats were injected with saline in place of the lactic acid solution. Values represent means±S.E. (n=10–11). ** and *** indicate significant differences ($p < 0.01$ and 0.001, respectively) from the control.

Table 6. Effect of Scymnol and Sodium Scymnol Sulfate on Lesion Progression in the Rat Peripheral Arterial Occlusive Disease Model

Group (mg/kg) ^{a)}	Days ^{b)}	Grade of disease and lesion point																	
		3					7						14						
		0	I	II	III	IV	Average	0	I	II	III	IV	Average	0	I	II	III	IV	Average
Sham-operated	50 ^{c)}	0	0	0	0	0	0.0±0.0*** ^{d)}	50	0	0	0	0	0.0±0.0***	50	0	0	0	0	0.0±0.0***
Control-1	—	16	9	15	0	0	4.9±1.0	10	1	15	8	6	9.9±1.2	2	2	2	21	13	15.8±1.6
Scymnol (0.3)	19	5	3	3	0	0	1.6±0.5*	16	7	1	4	2	2.6±1.0**	17	5	0	3	5	3.0±1.5***
Scymnol (1)	39	1	0	0	0	0	0.1±0.1***	30	10	0	0	0	1.3±0.6***	27	10	3	0	0	2.0±0.9***
Scymnol (3)	37	3	0	0	0	0	0.4±0.2***	33	6	1	0	0	1.0±0.3***	27	6	0	0	0	1.1±0.4***
Scymnol (10)	31	8	1	0	0	0	1.3±0.5***	33	7	0	0	0	0.9±0.5***	28	11	1	0	0	1.6±0.8***
Control-2	—	11	8	20	1	0	6.4±0.7	3	9	8	13	5	10.5±1.7	3	3	1	16	17	15.8±1.9
S.S.S. (0.35)	19	6	3	2	0	0	3.0±1.4*	13	10	4	2	1	4.7±1.3**	16	4	3	5	2	5.5±2.1***
S.S.S. (1.2)	39	1	0	0	0	0	0.1±0.1***	39	1	0	0	0	0.1±0.1***	35	4	0	0	0	0.4±0.2***
S.S.S. (3.5)	37	3	0	0	0	0	0.4±0.2***	35	2	2	1	0	1.1±0.3***	29	3	2	2	0	1.6±0.7***
S.S.S. (11.7)	36	4	0	0	0	0	0.5±0.5***	33	7	0	0	0	0.9±0.5***	28	7	1	0	0	1.1±0.8***

a) Scymnol or sodium (25S)-scymnol sulfate was administered as a single dose by intravenous injection 20 min before lactic acid injection. Control rats received the vehicle alone (control-1: propylene glycol-EtOH-H₂O=2:3:5, control-2: saline). Sham-operated rats were injected with saline in place of the lactic acid solution. b) Days after operation. c) Finger number. d) Lesion point scores represented as means±S.E. (n=8). ** and *** indicate significant differences (p<0.01 and 0.001, respectively) from the control.

Table 7. Effect of Scymnol and Sodium Scymnol Sulfate on Paw Swelling and Serum Lactate Dehydrogenase Activity in the Rat Peripheral Arterial Occlusive Disease Model

Group (mg/kg) ^{a)}	Paw swelling (mm)	LDH (IU/l)
Sham-operated	—	385±56***
Control-1	—	4.23±0.12
Scymnol (0.3)	3.66±0.14*	931±62
Scymnol (1)	3.46±0.24*	692±58**
Scymnol (3)	2.39±0.48**	618±77**
Scymnol (10)	2.46±0.38**	609±64**
Control-2	—	596±74**
S.S.S. (0.35)	4.20±0.32	983±77
S.S.S. (1.2)	3.54±0.24*	707±38**
S.S.S. (3.5)	2.94±0.47*	566±45***
S.S.S. (11.7)	2.53±0.44**	543±61***
	2.64±0.32**	480±98***

a) Scymnol or sodium (25S)-scymnol sulfate was administered as a single dose by intravenous injection 20 min before lactic acid injection. Control rats received the vehicle alone (control-1: propylene glycol-EtOH-H₂O=2:3:5, control-2: saline). Sham-operated rats were injected with saline in place of the lactic acid solution. Values represent means±S.E. (n=10). *, ** and *** indicate significant differences (p<0.05, 0.01 and 0.001, respectively) from the control.

model appears to be due to a reduction in vascular endothelium damage as a result of its potent thrombin-inhibitory activity in the early phase, because it significantly reduces paw swelling and affects the coagulation system by prolonging activated partial prothrombin time (APPT) and prothrombin time (PT).^{1,27,42)} In contrast, scymnol acts mainly by protecting the endothelial cells, because it significantly reduces paw swelling and tends to restore all values of the coagulation system, platelet functions and LDH activity to those in sham-operated rats, while it has no effect on the clotting system and serum LDH in sham-operated rats.^{1,27)}

The naturally occurring bile alcohols and sodium bile alcohol sulfates examined in this study were isolated from fish biles and are structurally related to scymnol.^{31–35)} All the compounds tested showed a significant preventive effect on disease progression, lesion development, foot swelling and the increase in LDH activity in the model. 5β-Chimaerol, sodium (25S)-scymnol sulfate and sodium 5β-chimaerol sulfate had similar potency, but were slightly less effective than scymnol. 7-Deoxyscymnol exhibited about 1/3 the potency

of scymnol, while 5β-cyprinol, 5α-cyprinol, 5β-molanol and 5β-petromyzonol showed about 1/4 the potency of scymnol. These results indicate that a hydroxyl group at C-7 in the B-ring, hydroxyl groups at C-24 and at terminal C-27 and a 1,3-dihydroxypropyl moiety in the side-chain contribute to the increased potency of C-27 bile alcohols. The hydroxyl group at C-11 or -12, the sulfate ester function at C-26, and the stereo-configuration at C-5 had little effect on the activity after oral administration.

The effects of mammalian bile acids and their taurine conjugates on lesion progression in the model were also examined. All of them, except TCA, exhibited a significant preventive effect after oral administration (Table 3). Their anti-ischemic profile and degree of preventive effect on disease progression at the examined dose were similar to those of bile alcohols and their sodium sulfates. The prophylactic efficacy of CDCA on lesion development in the model was very similar to that of scymnol. Cholic acid showed about half the potency of scymnol, while 5β-petromyzonol was one-fourth as potent as scymnol. UDCA, TUDCA or TCDCA showed about 1/3 to 1/4 the potency of scymnol. These results indicate that potent oral activity requires the carboxyl group at C-24 in the C-24 bile steroid, a hydroxyl group at C-12 slightly increases the activity, and the stereoconfiguration at C-7 affects the activity of the free bile acid but not that of its taurine conjugate. The taurine moiety in TCDCA and TCA reduced the activity, but that in TUDCA did not. These structure-activity relationships suggest that the [3α,7α-dihydroxy-5β-cholanic acid] structure contributes to good oral absorption.

Sodium (25S)-scymnol sulfate and ursodeoxycholic acid significantly prevented edematous swelling of the intima and lesion development and tended to restore the level of serum LDH₄ and LDH₅ activity to that in sham-operated rats, while they had no effect on coagulo-fibrinolytic parameters or platelet function in sham-operated rats (data not shown), as was also found for scymnol.^{1,27)} This finding suggests that the effects of these compounds are due to protecting endothelial cells against damage caused by lactic acidosis.

The prophylactic effects of scymnol and sodium (25S)-scymnol sulfate on lesion development after a single oral

Table 8. Protective Effect of Sodium Scymnol Sulfate and Ursodeoxycholic Acid on Membrane Damage Induced by Lactic Acid in Cultured Rat Endothelial Cells

a) Damage to Endothelial Cells Induced by Lactic Acid

	Lactic acid (%) in medium-1 ^{a)}				
	0.0	0.5	1.0	1.5	2.5
Level (%) of stained cells in total RECs ^{b)} in well	0±0	23.8±0.7***	27.2±1.3***	34.2±0.7***	40.6±1.1***
Total amount (μg) of erythrosine B in RECs in well	6.4±0.5	21.2±0.5***	25.5±0.8***	32.5±0.5***	37.8±0.6***

b) Effect of Bile Steroids on Damage to Endothelial Cells Induced by Lactic Acid

		Concentration (μmol/ml) of test compound in medium-2 ^{c)}			
		0	0.025	0.25	2.5
Total amount (μg) of Erythrosine B in RECs in well	SSS	31.1±0.4	26.6±0.2 ^{^^}	21.0±0.4 ^{^^}	16.4±0.6 ^{^^}
	UDCA	31.3±0.8	26.4±0.5 ^{^^}	23.1±1.3 ^{^^}	— ^{d)}

a) Medium 199-saline (1:1). b) Rat endothelial cells. c) 1.5% Lactic acid in medium 199-saline (1:1). d) Not examined because of poor solubility of ursodeoxycholic acid in the medium. Values represent the means±S.E. (n=3–6). *** and ^^ indicate significant differences ($p<0.001$) from the controls in medium-1 and medium-2, respectively.

dose were *ca.* 1/100 of those after intravenous administration. Based on the reported results, the effect of repeated oral administration of scymnol appears to be *ca.* 1/10 that of intravenous administration.^{1,27)} Accordingly, a higher dose of scymnol or sodium (25S)-scymnol sulfate was required to prevent lesion progression in the model when the compounds were given orally rather than intravenously or when a single oral dose was given rather than a repeated dose.

In humans, oral administration of UDCA, as a single dose of 500 mg/kg, increased the total plasma bile acids by *ca.* 9 times at 1 and 2 h after administration.⁴³⁾ Furthermore, administration of UDCA *p.o.* and intravenously to rats gave similar distribution patterns with half-lives of about 2.5 and 3.75 d, respectively.⁴⁴⁾ These results indicate that substantial amounts of orally administered UDCA are absorbed and enter the enterohepatic circulation. We suggest that an orally administered bile sterol is absorbed as a bile steroid, enter the closed enterohepatic circulation system, and is then slowly transferred to the peripheral blood, perhaps after being converted to active form(s) in the liver

As noted above, scymnol was more potent than its less hydroxylated relatives, 5 β -chimaerol, 5 β -cyprinol and 7-deoxyscymnol, and also its conjugate, sodium (25S)-scymnol sulfate. Furthermore, CDCA was more potent than UDCA or its taurine conjugate, TCDCA. It is well known that UDCA is less hydrophobic than CDCA.⁴⁵⁾ Interestingly, following intravenous administration of the same dose, sodium (25S)-scymnol sulfate was as effective as scymnol in this model, although oral administration the former was less effective than the latter. These findings suggest that the hydrophobic/lyophobic balance of each bile steroid contributes to the efficiency of its oral absorption.

Interestingly, it has been reported that UDCA virtually produces no change in hepatic morphology and function, contrasted with the severe damage induced by CDCA treatment at a dose of *ca.* 100 to 250 μmol/kg.¹⁸⁾ Poupon *et al.* reported that UDCA improved liver function in patients with primary biliary cirrhosis.²¹⁾ However, the mechanisms involved an unknown. We have shown here that UDCA and

CDCA prevent lesion progression in the peripheral arterial occlusion model induced by lactic acidosis and vascular endothelial cell injury caused by lactic acidosis. We, therefore, propose that the efficacy of UDCA in chronic liver disease and hepatic veno-occlusion disease can be explained by its direct protective effect on vascular endothelial cells. However, we cannot yet explain why CDCA has different effects on vascular endothelial cells and hepatocytes. Further studies are necessary to establish the medical applications of naturally occurring bile steroids, including UDCA and CDCA.

To our knowledge, this is the first report that naturally occurring bile steroids and related compounds prevent the endothelial cell damage induced by lactic acidosis, although UDCA has been shown to protect against the membrane-damaging effects associated with hydrophobic bile acids in both hepatocytes and nonliver cells.^{18,46)} UDCA also has a protective effect against mitochondrial membrane permeability transitions promoted by various compounds, including hydrophobic bile salts.⁴⁷⁾ It is also thought to increase the glutathione content of hepatocytes, thereby protecting against the development and progression of cytokine-induced damage.⁴⁸⁾ A study of the mechanism by which bile steroids protect endothelial cells from injury caused by lactic acidosis *in vivo* and *in vitro* is underway.

In summary, orally administered bile steroids, 5 β -chimaerol, sodium (25S)-scymnol sulfate, sodium 5 β -chimaerol sulfate, 7-deoxyscymnol, 5 β -cyprinol, 5 β -molanol, 5 α -cyprinol, 5 β -petromyzonol, cholic acid, UDCA, CDCA, TCDCA and TUDCA, had a significant protective effect against the development of lesions and endothelial cell injury induced by the injection of lactic acid into the femoral artery in the rat peripheral arterial occlusion model. Scymnol and sodium (25S)-scymnol sulfate had a more potent effect following intravenous administration than after oral administration. Sodium (25S)-scymnol sulfate and UDCA also protected rat endothelial cells against damage caused by lactic acidosis *in vitro*. Some naturally occurring bile steroids, such as scymnol, sodium (25S)-scymnol sulfate, UDCA and CDCA, may be clinically useful in preventing thrombotic peripheral arter-

ial occlusive disorders following their oral administration. Their prophylactic action appears to be mainly due to a potent protective effect against the endothelial cell damage produced by lactic acidosis.

REFERENCES

- Ishida H., Nakayasu H., Nukaya H., Tsuji K., *Biol. Pharm. Bull.*, in submitted.
- Akamatsu K., "Wakanyaku," Ishiyaku Press, Tokyo, 1970, pp. 767, 784, 869, 877—879.
- Higuchi K., Kamitori S., Hirotsu K., Takeda H., *Yakugaku Zasshi*, **105**, 1115—1121 (1985).
- Lindley P. E., Mahmoud M. M., Watson F. E., *Acta Cryst.*, **B36**, 1893—1897 (1980).
- Candeloro De Sanctis S., Giglio E., Petri F., Quagliata C., *Acta Cryst.*, **B35**, 226—228 (1979).
- Hammarsten O., *Z. Physiol. Chem.*, **24**, 322—345 (1898); Bridgwater R. J., Briggs T., Haslewood G. A. D., *Biochem. J.*, **82**, 285—290 (1962); Bridgwater R. J., Haslewood G. A. D., Watt J. R., *Biochem. J.*, **87**, 27—31 (1963).
- Haslewood G. A. D., Tammar A. R., *Biochem. J.*, **108**, 263—268 (1968).
- Haslewood G. A. D., Tokes L., *Biochem. J.*, **114**, 179—184 (1977).
- Kitani K., Kanai S., *Life Sci.*, **31**, 1973—1985 (1982).
- Miyaji K., Akiyama T., Ito M., Urakawa T., Shimaji Y., *Jpn. J. Clin. Invest.*, **53**, 1395—1403 (1976).
- Miyasaka K., Kitani K., *Pancreas*, **1**, 264—269 (1986).
- Nakagawa S., Makino I., Ishizaki T., Dohi I., *Lancet*, **2**, 367—369 (1977).
- Kitani K., Kanai S., *Life Sci.*, **30**, 515—523 (1982).
- Ohkawa H., Saeki M., Iwasaki M., *Clinical Report*, **10**, 57—62 (1976).
- Hirabayashi N., Osuga T., *Pharmacometrics*, **15**, 125—132 (1978).
- Roda E., Bazzoli F., Morselli Labate A. M., Mazzella G., Roda A., Sama C., Festi D., Aldini R., Tarroni F., Barbara L., *Hepatology*, **2**, 804—810 (1981).
- Leiss O., Bergmann K., *Scand. J. Gastroenterol.*, **17**, 587—592 (1982).
- Federowski T., Salen G., Zaki G., Shefer S., Mosbach E. H., *Gastroenterology*, **74**, 75—81 (1978); Kitani K., Kanai S., *Jpn. J. Physiol.*, **35**, 443—462 (1985); Hosono J., Iwasaki M., *Clinical Report*, **9**, 3159—3166 (1975).
- Yoshioka M., Hotta S., Yamaguchi K., Tohi K., Miki M., Shiota A., *Diagnosis Treatment*, **64**, 1953—1963 (1976).
- Salvioli G. F., Iigimi H., Carey M. C., *J. Lipid Res.*, **24**, 701—720 (1983); Sugata F., Yamanaka M., Matsumoto T., Katsuta N., *Jpn. J. Gastroenterol.*, **75**, 492—498 (1976).
- Poupon R., Cheien Y., Poupon R. E., Ballet F., Calmus Y., Darnis F., *Lancet*, **1**, 834—836 (1987); Kiso S., Kawata S., Imai Y., Tamura S., Inui Y., Ito N., Matsuzawa Y., *J. Gastroenterol.*, **31**, 75—80 (1996).
- Masuda M., Hosoda S., Baba T., Kashima T., Aki H., Nakagawa M., *Clinical Report*, **10**, 85—102 (1976).
- Ishida H., Kinoshita S., Yamamoto N., Nukaya H., Tsuji K., Kosuge T., *Chem. Pharm. Bull.*, **42**, 2532—2535 (1994).
- Essel J. H., Thompson J. M., Harman G. S., Halvorson R. D., Snyder M. J., Callander N. S., Clement D. J., *Bone Marrow Transplant.*, **10**, 367—372 (1992); Essel J. H., Schroeder M. T., Harman G. S., Halvorson R., Lew V., Callander N., Snyder M., Lewis S. K., Allerton J. P., Thompson J. M., *Ann. Intern. Med.*, **128**, 975—981 (1998).
- Or R., Nagler A., Shpilberg O., Elad S., Naparstek E., Kapelushnik J., Cass Y., Gills S., Chetrit A., Slavin S., Eldor A., *Transplantation*, **61**, 1067—1071 (1996); Bianco J. A., Appelbaum F. R., Nemunaitis J., Almgren J., Andrews F., Kettner P., Shields A., Singer J. W., *Blood*, **78**, 1205—1211 (1991); Bearman S. J., Shen D. D., Hinds M. S., Hill H. A., McDonald G. B., *Br. J. Haematol.*, **84**, 724—730 (1993).
- Catani L., Gugliotta L., Vianelli N., Nocentini F., Baravelli S., Bandini G., Cirio T. M., Tura S., *Bone Marrow Transplant.*, **17**, 277—280 (1996).
- Ishida H., Nakayasu H., Nukaya H., Tsuji K., *Biol. Pharm. Bull.*, **21**, 240—244 (1998).
- Furchgott R. F., Vanhoutte P. M., *FASEB J.*, **3**, 2007—2017 (1989); Vane J. R., Anggard M. D., Botting R. M., *New Engl. J. Med.*, **323**, 27—36 (1990); Yanagisawa M., Kurihara N., Kimura S., Tomobe Y., Kobayashi M., Mitsui Y., Yazaki Y., Goto K., Masaki T., *Nature* (London), **332**, 411—415 (1992); Suga S., Nakao K., Itoh H., Komatsu Y., Ogawa Y., Hama N., Imura H., *J. Clin. Invest.*, **90**, 1145—1149 (1992).
- Moncada S., Higgs A., *New Engl. J. Med.*, **329**, 2002—2012 (1993); Drexler H., *Prog. Cardiovasc. Disease*, **34**, 287—324 (1997).
- Camerer E., Kolsto A. B., Pyrdz H., *Thromb. Res.*, **81**, 1—41 (1996).
- Ishida H., Yamaguchi T., Natsuyama R., Tsuji K., Kosuge T., *Chem. Pharm. Bull.*, **36**, 4408—4413 (1988); Ishida H., Yamamoto N., Nukaya H., Tsuji K., Kosuge T., *Chem. Pharm. Bull.*, **42**, 2514—2517 (1994).
- Ishida H., Miyamoto H., Kajino T., Nakayasu H., Nukaya H., Tsuji K., *Chem. Pharm. Bull.*, **44**, 1289—1292 (1996).
- Ishida H., Nakayasu H., Miyamoto H., Nukaya H., Tsuji K., *Chem. Pharm. Bull.*, **46**, 12—16 (1998).
- Hoshita T., Nagayoshi S., Kazuno T., *J. Biochem. (Tokyo)*, **54**, 369—374 (1963).
- Haslewood G. A. D., Tokes L., *Biochem. J. (Tokyo)*, **108**, 236—238 (1968).
- Iwamoto M., Hara T., Ogawa H., Tomikawa M., *Jpn. Pharmacol Ther.*, **14**, 903—911 (1986).
- Schultze A. E., Gunaga K. P., Wagner J. G., Hoorn C. M., Moorehead W. R., Roth R. A., *Toxicol. Appl. Pharmacol.*, **126**, 301—310 (1994).
- Sugo S., Minamino N., Kangawa K., Miyamoto K., Kitamura K., Sakata J., Eto T., Matsuo H., *Biochem. Biophys. Res. Comm.*, **201**, 1160—1166 (1994).
- Watanabe T., Morita I., Nishi H., Murota S., *PG LT Essential Fatty Acids*, **33**, 81—87 (1988).
- Tomikawa M., Ogawa H., Abiko Y., *Thromb. Diathes. Haemorrh.*, **31**, 86—102 (1974).
- Harver V., Namm D., *Blood Vessels*, **21**, 53—63 (1984); Walz D., Anderson G., Fenton J., *Ann. N.Y. Acad. Sci.*, **485**, 323—332 (1986).
- Kumada T., Abiko Y., *Thromb. Res.*, **24**, 285—298 (1981).
- Matern S., Tietjen K. G., Fackler O., Hinger K., Herz R., Gerok W., *Biol. Effects of Bile Acids*, **25**, 109—118 (1979).
- Federowski T., Salen G., Colalillo A., Tint G. S., Mosbach E. H., Hall J. C., *Gastroenterology*, **73**, 1131—1137 (1977).
- Hofmann A., "Physiology of the Gastrointestinal Tract," Raven Press, New York, 1994, pp. 1555—1576.
- Rodrigues C. M., Fan G., Ma X., Kren B. T., Steer C. J., *J. Clin. Invest.*, **101**, 2790—2799 (1998).
- Rodrigues C. M., Fan G., Wong P. Y., Kren B. T., Steer C. J., *Mol. Med.*, **4**, 165—178 (1998).
- Neuman M. G., Shear N. H., Bellentani S., Tribelli C., *Gastroenterology*, **115**, 157—166 (1998).