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# Studies on the Constituents of Apocynaceae Plants. Gas Chromatography-Mass Spectrometric Determination of New Flavonoid Triglycosides from the Leaves of *Cerbera manghas* L.(2)[1]

Akiyo Sakushima, Sueo Hisada,[2a] Yukio Ogihara,[2b] and Sansei Nishibe[2a]

*Faculty of Pharmaceutical Sciences, Higashi Nippon Gakuen University[2a] and Faculty of Pharmaceutical Sciences, Nagoya City University[2b]*

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Two new flavonol triglycosides, named manghaslin(I) and clitorin(II), were isolated from the leaves of *Cerbera manghas* L. (Apocynaceae). The structures of I and II were elucidated as quercetin-3-O-L-rhamnosyl-(1→2)-O-[L-rhamnosyl-(1→6)] D-glucoside(I) and kaempferol-3-O-L-rhamnosyl-(1→2)-O-[L-rhamnosyl-(1→6)] D-glucoside(II), respectively, by chemical and gas chromatography–mass spectrometric studies.

**Keywords**——*Cerbera manghas* L.; Apocynaceae; flavonol triglycosides; manghaslin; clitorin; gas chromatography–mass spectrometry; photohydrolysis; mass spectrum; methanolysis

In the previous paper,[3] we reported the isolation of nicotiflorin and rutin from the leaves of *Cerbera manghas* L. (Apocynaceae), whose fruits are used as an anesthetic under the name "Niu-xin-gie-zin" in China.[4]

In our continued studies on the constiuents of this plant, two new flavonoids, named manghaslin (I) and clitorin (II),[5] were isolated.

This paper deals with the structure elucidation of I and II by gas chromatography–mass spectrometry (GC–MS).

The extraction was carried out as described in the previous paper.[3] The butanol-soluble fraction of the extract was subjected to column chromatography on silica gel with 25% methanol–chlorofotm to afford I and II.

Manghaslin (I) was recrystallized from ethanol to give pale yellow needles, $C_{33}H_{40}O_{20}\cdot 1^1/_2H_2O$, mp 194—196°, exhibiting positive Mg–HCl and Zn–HCl tests and a positive ferric chloride reaction.

The infrared (IR) spectrum of I suggested the presence of hydroxyl (3350 $cm^{-1}$) and carbonyl (1660 $cm^{-1}$) groups and an aromatic ring (1600 $cm^{-1}$).

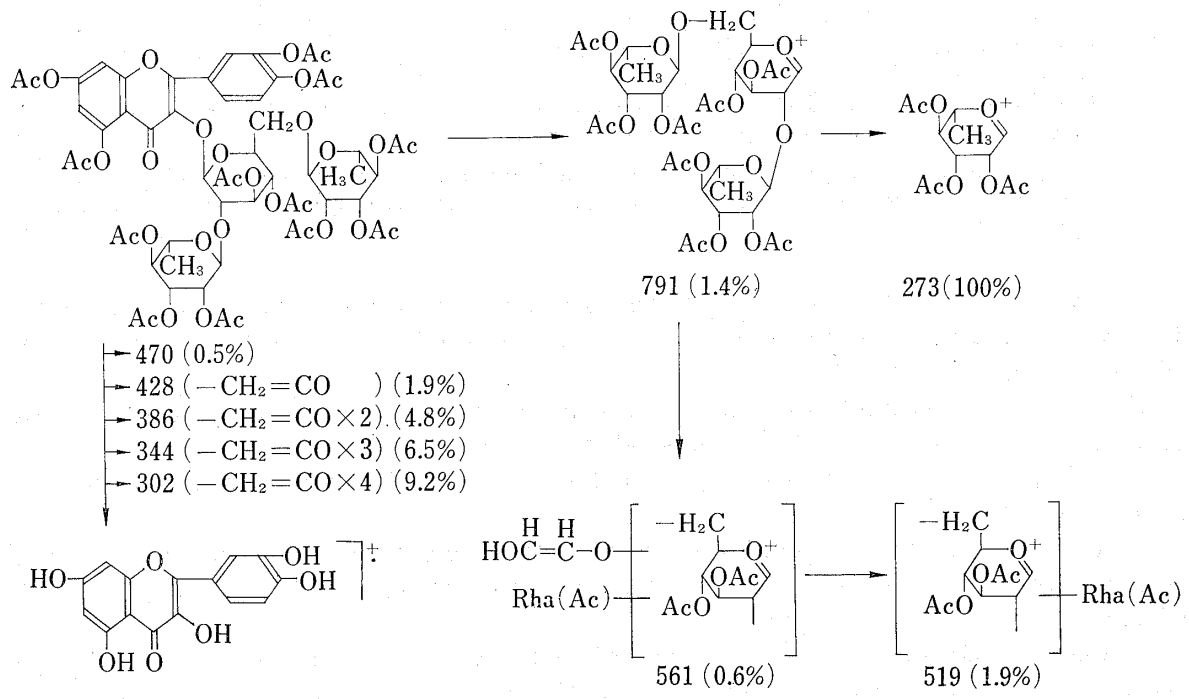
The ultraviolet (UV) spectrum of I showed absorption maxima at 258, 269, and 350 nm, indicating the presence of a flavonol skeleton in I.[6,7] The bathochromic shifts of the absorption maxima on the addition of aluminum chloride and sodium acetate were similar to those of rutin.

1) A. Sakushima, S. Nishibe, and S. Hisada, *Yakugaku Zasshi*, **98**, 1395 (1978).
2) Location: *a) Ishikari-Tobetsu, Hokkaido, 061-02, Japan; b) Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan.*
3) A. Sakushima, S. Nishibe, S. Hisada, Y. Noro, and Y. Hisada, *Yakugaku Zasshi*, **96**, 1046 (1976).
4) Jiag-sa New Medicinal Academy, "Cang-yao-da-ci-dian," Shang-hai Scientific and Technical Press, Hong Kong, 1975.
5) After this work was completed, Morita *et al.* reported the isolation of II from *Clitoria ternatea* L. under the name of clitorin. N. Morita, M. Arisawa, M. Nagase, H. Hsu, and Y. Chen. *Yakugaku Zasshi*, **97**, 649 (1977).
6) T.J. Mabry, K.R. Markham, and M.B. Thomas, "The Systematic Identification of Flavonoids," Spring-Verlag, Berlin, Heidelberg, New York, 1970.
7) J.B. Harborne, T.J. Mabry, and H. Mabry, "The Flavonoids," Chapman and Hall, London, 1975.

The acid hydrolysis of I with 3% sulfuric acid solution gave quercetion, L-rhamnose and D-glucose.

The mass spectrum (MS) of the peracetate of I shoewd fragments due to terminal methylpentose (*m/e* 273), trisaccharide (*m/e* 791, 561, 519), and aglycone (*m/e* 470, 428, 386, 344, 302). The existence of the *m/e* 561 fragment produced from the sugar moiety suggested that the sugar moiety was a branched trisaccharide, as shown in Fig. 1,[8] since the gas chromatogram-peak areas of L-rhamnose and D-glucose were in a ratio of 2: 1.

The methylation of I was carried out by Hakomori's method, and the product was methanolyzed with methanolic 3% hydrochloric acid. The reaction mixture was trimethylsilylated in the usual way. GC–MS analysis of the trimethylsilylated reaction mixture gave fragments of *m/e* 159 (96.7%), 146 (100%), 88 (22.8%) and 75 (66.9%)[9] as shown in Fig. 2, and fragments of *m/e* 101 (31.4%), 88 (100%), and 75 (55.1%).




Ac=$COCH_3$, Rha(Ac)=peracetylated terminal rhamnose residue.

Fig. 1. Scheme for the MS Fragmentation of Peracetylated Manghaslin

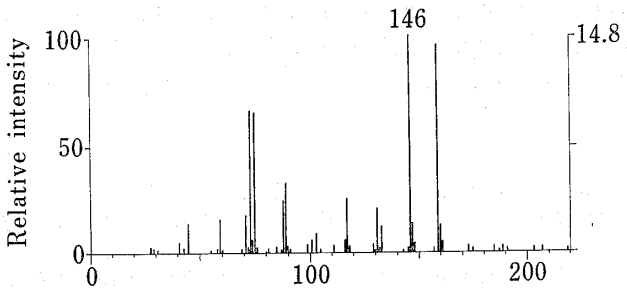



Fig. 2. Mass Spectrum of the TMS Derivative of Methyl 3,4-Di-O-methylglucopyranoside

These fragments suggested the presence of methyl pyranosides of 2,6-di-O-trimethylsilyl-3,4-di-O-methylglucose and 2,3,4-tri-O-methylrhamnose.[9]

On the other hand, GC–MS of permethylmanghaslin after hydrolysis indicated the presence of 5,7,3′,4′-tetramethylquercetin (MS fragments and retention times), and this was confirmed by direct comparison with an authentic sample.

8) T. Komori, Y. Ida, Y. Mutou, K. Miyahara, T. Nohara, and T. Kawasaki, *Biomed. Mass Spectrom.*, **2**, 65 (1975).
9) T. Matsubara and A. Hayashi, *Biomed. Mass Spectrom.*, **1**, 62 (1974).

These results show that two L-rhamnose moieties should be attached at the 2,6-positions of D-glucose, and this view was supported by the following results. The photohydrolysis of I under a UV lamp gave a trisaccharide (MS *m/e*: 791: 561, 519, 331, 273), which was further hydrolyzed to a mixture of rutinose [rhamnosyl-(1→6)-O-glucose] and neohesperidose [rhamnosyl-(1→2)-O-glucose] by 0.5 N sulfuric acid in 50% aqueous ethanol, as shown in Fig. 3.[10]

Therefore, it was considered that the trisaccharide was rhamnosyl-(1→2)-O-[rhamnosyl-(1→6)] glucose.

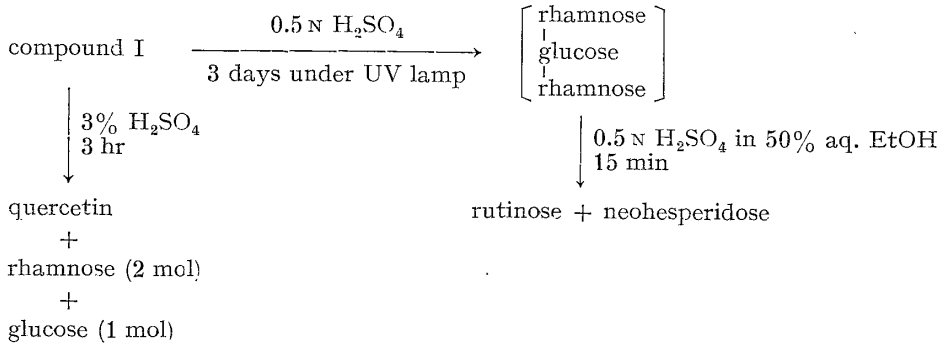



Fig. 3. Photohydrolysis and Hydrolysis of Compound I

Consequently, the structure of I has been established as quercetin-3-O-L-rhamnosyl-(1→2)-O-[L-rhamnosyl-(1→6)] D-glucoside (manghaslin).

Clitorin (II) was recrystallized from ethanol to give pale yellow needles, $C_{33}H_{40}O_{19}\cdot 1\,^1/_2H_2O$, mp 190—194°, exhibiting positive Mg–HCl and Zn–HCl tests and a positive ferric chloride reaction.

The structure of II has been established as kaempferol-3-O-L-rhamnosyl-(1→2)-O-[L-rhamnosyl-(1→6)] D-glucoside (clitorin) on the basis of IR, UV, MS and GC–MS data and hydrolysis in a manner similar to that described for I.

## Experimental

All melting points were determined with a Yanagimoto micro melting point apparatus and are uncorrected. IR spectra were obtained with a Shimadzu IR-400 machine and PMR spectra were taken at 90 MHz with a Hitachi R-40 high resolution PMR spectrometer; chemical shifts are given in the $\delta$(ppm) scale with tetramethylsilane (TMS) as an internal standard. GC was carried out on a Shimadzu GC-6 AM machine equipped with a hydrogen flame ionization detector. Mass spectra were measured on a Shimadzu LKB-9000 (computer, Shimadzu GC-MSPAC-300) with direct inlet of the probe into the ion source. Conditions were as follows: ionizing voltage 20 eV or 70 eV, ionizing current 60 $\mu$A, ion accelerating voltage 3.5 kV. Column chromatography was carried out using silica gel (100—200 mesh, MALLINCKRODT). TLC on Kieselgel $60_{F-254}$ was performed with the following solvent systems, AcOEt: MeCOEt: $H_2O$: HCOOH: $C_6H_6$=4: 3: 1: 1: 2 (upper layer) (TLC-1), AcOEt: MeCOEt: $H_2O$: HCOOH=5: 3: 1: 1 (TLC-2), and the spots were detected with $FeCl_3$ reagent and by spraying with 10% $H_2SO_4$ solution followed by heating.

**Extraction and Isolation of Manghaslin (I) and Clitorin (II)**——Dried leaves of *Cerbera manghas* L. were crushed and treated as described in the previous paper.[3] The BuOH extractives were subjected to column chromatography over silica gel with a $CHCl_3$–MeOH gradient. The fraction eluted with 25% MeOH in $CHCl_3$ was rechromatographed on silica gel with 25% MeOH and 28% MeOH in $CHCl_3$ to afford thirty fractions (frs. 1—30). Frs. 17—22 were rechromatographed on silica gel with 28% MeOH in $CHCl_3$ to give 60 mg of II.

Frs. 23—29 were subjected to column chromatography on silica gel with 30% MeOH in $CHCl_3$ to afford 30 mg of I.

**Properties of Manghaslin (I)**——Pale yellow powder from MeOH–EtOH, mp 194—196°. Dark green to the $FeCl_3$ reaction, deep red to the Mg–HCl test, reddish-pink to the Zn–HCl test. *Rf* 0.07 (TLC-1), *Rf* 0.30 (TLC-2). *Anal.* Calcd for $C_{33}H_{40}O_{20}\cdot 1\,^1/_2H_2O$: C, 50.56; H, 5.53. Found: C, 50.32; H, 5.48. UV $\lambda_{max}^{MeOH}$

10) K. Takaishi, H. Kuwashima, and S. Mashima, Presented at the 75th Annual Meeting of the Pharmaceutical Society of Japan, April 1975. Abstract of Papers, II, p. 209.

nm (log $\varepsilon$): 257 (4.30), 269 (sh), 350 (4.23). UV $\lambda_{max}^{MeOH+NaOAc}$ nm: 272, 320 (sh), 369. UV $\lambda_{max}^{MeOH+AlCl_3}$ nm: 274, 302, 355, 305. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3350 (OH), 2900 (CH), 1660 (C=O), 1600 (C=C), 1060 (C-O).

**Properties of Clitorin(II)**——Pale yellow needles from MeOH–EtOH, mp 190—194°. Greenish-brown to the $FeCl_3$ reaction, deep red to the Mg–HCl test, reddish-pink to the Zn–HCl test. *Rf* 0.12 (TLC-1). *Rf* 0.36 (TLC-2). *Anal.* Calcd for $C_{33}H_{40}O_{19}\cdot 1^1/_2H_2O$: C, 51.61; H, 5.65. Found: C, 51.59; H, 5.68. UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 267 (4.26), 297 (sh), 349 (4.16) UV $\lambda_{max}^{MeOH+NaOAc}$ nm: 272, 300 (sh), 360. UV $\lambda_{max}^{MeOH+AlCl_3}$ nm: 276, 302, 344, 396. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3350 (OH), 2900 (CH), 1665 (C=O), 1605 (C=C), 1050 (C-O).

**Hydrolysis of Manghaslin (I)**——A solution of I (5 mg) in 3% $H_2SO_4$ was heated on a water bath for 3 hr. The precipitate was separated, washed with water, and recrystallized from aq. MeOH to give yellow needles. mp above 300°, UV $\lambda_{max}^{MeOH}$ nm: 258, 269 (sh), 369. IR $\gamma_{max}^{KBr}$ cm$^{-1}$: 3350 (OH), 2900 (CH), 1655 (C=O).

This compound was identified as quercetin by direct comparison with authentic material (IR and UV). The solution was treated with $BaCO_3$ to make it neutral, then the filtrate was evaporated to dryness for sugar examination by GC [glass column (2 m×3 mm), 1.5% OV-1 on Shimalite-W (80—100 mesh)]. The conditions for GC were as follows: column temp., 150—200° (3°/min); injection and detector temp., 300°; carrier gas, $N_2$ (20 ml/min). $t_R$ (min): 5.3, 7.0 (TMS derivative of L-rhamnose, 2 mol). $t_R$: 12.0, 13.1 (TMS derivative of D-glucose, 1 mol).

**Acetylation of Manghaslin (I)**——Compound I (20 mg) was acetylated with acetic anhydride and pyridine in the usual way. The crude acetate was subjected to column chromatography with $CHCl_3$–MeOH to afford peracetylated manghaslin. mp 102—106°, negative to the $FeCl_3$ reaction. IR $\gamma_{max}^{KBr}$ cm$^{-1}$: 2920 (CH), 1760 (C=O), 1660 (C=O). PMR (in $CDCl_3$) $\delta$ (ppm): 0.83—1.26 (6H, m, 2×rhamnosyl C$\underline{H}_3$), 1.86—2.20 (24H, m, 8×aliphatic C$\underline{H}_3$CO), 2.31—2.50 (12H, each s, 4×aromatic C$\underline{H}_3$CO), 3.30—5.75 (17H, m, aliphatic H), 6.90 (1H, d, $J$=3.0 Hz, aromatic 6-H), 7.25—7.47 (2H, m, aromatic 5′,8-H), 7.90—8.20 (2H, m, aromatic 2′, 6′-H). MS *m/e* (%): 791 (1.4) $[C_{34}H_{47}O_{21}]^+$, 561 (0.6) $[C_{24}H_{33}O_{15}]^+$, 519 (1.1) $[C_{22}H_{31}O_{14}]^+$, 470 (0.5) $[C_{23}H_{18}O_{11}]^+$, 428 (1.9) $[C_{21}H_{16}O_{10}]^+$, 386 (4.8) $[C_{19}H_{14}O_9]^+$, 344 (6.5) $[C_{17}H_{12}O_8]^+$, 302 (9.2) $[C_{15}H_{10}O_7]^+$, 273 (100) $[C_{12}H_{17}O_7]^+$.

**Methylation of Manghaslin (I) by Hakomori's Method**——Compound I (5 mg) was methylated in the usual way. The reaction mixture was subjected to chromatography on silica gel with hexane–$CHCl_3$ to afford one spot on TLC. The IR spectrum of this compound showed the absence of hydroxyl groups, and this compound was used for methanolysis and hydrolysis studies without further purification. IR $\gamma_{max}^{CHCl_3}$ cm$^{-1}$: 2920 (CH), 1655 (C=O), 1380 ($CH_3$).

**Methanolysis of Permethylated Manghaslin with Methanolic 3% HCl**——Permethylated manghaslin sealed in a vial was methanolyzed with methanolic 3% HCl under reflux for 10 hr. The reaction mixture was neutralized with $Ag_2CO_3$, filtered and evaporated to dryness *in vacuo*. The residue was examined by GC–MS. GC–MS of the sugar moiety [glass column (2 m×3 mm), 10% DEGS on Shimalite-W (80—100 mesh)] was carried out under the following conditions: column temp., 140° (for TMS derivatives), 170° (for permethyl derivative); injection temp., 200°; separator temp., 280°; carrier gas, He (30 ml/min). $t_R$ (min): 2.6 (methyl per-O-methylrhamnoside). MS *m/e* (%): 101 (31.4) $[C_5H_9O_2]^+$, 88 (100) $[C_4H_8O_2]^+$, 75 (55.1) $[C_3H_7O_2]^+$, 73 (25.9). $t_R$: 16.0 (TMS derivative of methyl 3,4-di-O-methylglucopyranoside). MS *m/e* (%): 159 (96.7) $[C_7H_{15}O_2Si]^+$, 146 (100) $[C_6H_{14}O_2Si]^+$, 133 (12.6) $[C_5H_{13}O_2Si]^+$, 131 (19.6), 117 (23.4), 88 (22.4), 75 (66.9), 45 (13.2).

**Hydrolysis of Permethylated Manghaslin**——Permethylated manghaslin was hydrolyzed with 3% $H_2SO_4$ in the usual way. The reaction mixture was extracted with AcOEt. The AcOEt extract was washed with water, and evaporated to dryness *in vacuo*. The residue was examined by GC–MS. GC–MS of the aglycone moiety [glass column (2 m×3 mm), 2% OV-17 on Chromosorb-W (80—100 mesh)] was carried out under the following conditions: column temp., 260°; injection temp., 300°; separator temp., 320°; carrier gas, He (30 ml/min). $t_R$(min): 38.8 (TMS derivative of 5,7,3′,4′-tetramethylquercetin). MS *m/e* (%): 430 (1.1) $[M]^+$, 415 (100) $[M-CH_3]^+$, 400 (7.7) $[M-30]^+$, 385 (2.6) $[M-45]^+$, 370 (2.1), 357 (3.6) $[M-TMS]^+$, 165 (1.8) $[C_9H_9O_3]^+$.

**Photohydrolysis of Manghaslin (I)**——Compound I (3 mg) was photohydrolyzed with 0.5 N $H_2SO_4$ in 50% aq. EtOH under a UV lamp (15 W, National GL-15) for 3 days then heated on a water bath for 15 min. The reaction mixture was neutralized with $BaCO_3$ and evaporated to dryness *in vacuo*. The residue was examined by GC [glass column (1 m×3 mm), 1.5% OV-1 on Shimalite-W (80—100 mesh)]. The conditions for GC were as follows: column temp., 180—280° (4°/min); carrier gas, $N_2$ (30 ml/min); injection and detector temp., 310°. $t_R$ (min): 21.07, 22.56 (TMS derivative of rutinose). $t_R$: 19.64, 21.41 (TMS derivative of neohesperidose). Trisaccharide (acetate), MS *m/e* (%): 791 (0.9), 561 (2.8), 519 (0.7), 331 (6.1), 273 (100).

**Hydrolysis of Clitorin(II)**——Compound II (3.0 mg) was treated with 3% $H_2SO_4$ in the usual way and the product was recrystallized from aq. MeOH to afford yellow needles. mp above 300°, UV $\lambda_{max}^{MeOH}$ nm: 254 (sh), 268, 368. IR $\gamma_{max}^{KBr}$ cm$^{-1}$: 3350 (OH), 2900 (CH), 1650 (C=O). The filtrate was treated in the usual way, GC: $t_R$ (min): 5.3, 7.0 (TMS derivative of L-rhamnose, 2 mol), $t_R$: 12.0, 13.1 (TMS derivative of D-glucose, 1 mol).

**Acetylation of Clitorin (II)**——Compound II (30 mg) was acetylated with acetic anhydride and pyridine in the usual way. The crude acetate was subjected to column chromatography to afford peracetylated clitorin. mp 134—138°. IR $\gamma_{max}^{KBr}$ cm$^{-1}$: 2920 (CH), 1760 (C=O), 1655 (C=O). PMR (in $CDCl_3$) $\delta$ (ppm): 0.80—1.10 (6H, m, 2×rhamnosyl C$\underline{H}_3$), 1.79—2.20 (24H, m, 8×aliphatic C$\underline{H}_3$CO), 2.31—2.50 (9H, each s,

3×aromatic C$\underline{H}_3$CO), 3.30—5.75 (17H, m, aliphatic H), 6.83 (1H, d, $J$=3.0 Hz, aromatic 6-H), 7.20 (1H, d, $J$=3.0 Hz, aromatic 8-H), 7.30—7.50 (2H, m, aromatic 3′,5′-H), 8.06 (2H, d, $J$=9.0 Hz, aromatic 2′,6′-H). MS *m/e* (%) 791 (1.8) $[C_{34}H_{47}O_{21}]^+$, 561 (0.8) $[C_{24}H_{33}O_{15}]^+$, 519 (0.5) $[C_{22}H_{31}O_{14}]^+$, 412 (6.3) $[C_{21}H_{16}O_9]^+$, 370 (1.2) $[C_{19}H_{14}O_8]^+$, 328 (2.4) $[C_{17}H_{12}O_7]^+$, 286 (3.2) $[C_{15}H_{10}O_6]^+$, 273 (100) $[C_{12}H_{17}O_7]^+$.

**Methylation of Clitorin(II) by Hakomori's Method**——Compound II (5.0 mg) was treated in the usual way, and the reaction mixture was subjected to chromatography on silica gel with hexane–$CHCl_3$ to afford one spot on TLC. The IR spectrum showed the absence of hydroxyl groups, and this compound was used for methanolysis and hydrolysis studies without further purification. IR $\gamma_{max}^{CHCl_3}$ $cm^{-1}$: 2910 (CH), 1660 (C=O), 1380 ($CH_3$).

**Methanolysis of Permethylated Clitorin with Methanolic 3% HCl**——Permethylated clitorin sealed in a vial was methanolyzed with methanolic 3% HCl under reflux for 10 hr. The reaction mixture was treated in the usual way, and evaporated to dryness *in vacuo*. The residue was examined by GC–MS under the conditions described above. $t_R$ (min): 2.6 (methyl per-O-methylrhamnoside). MS *m/e* (%): 101 (31.2) $[C_5H_9O_2]^+$, 88 (100) $[C_4H_8O_2]^+$, 75 (28.8) $[C_3H_7O_2]^+$, 73 (13.4), 45 (14.0). $t_R$: 16.00 (TMS derivative of methyl 3,4-di-O-methylglucopyranoside). MS *m/e* (%): 159 (96.0) $[C_7H_{15}O_2Si]^+$, 146 (100) $[C_6H_{14}O_2Si]^+$, 133 (12.5) $[C_5H_{13}O_2Si]^+$, 131 (19.0), 117 (22.8), 88 (22.4), 75 (68.2), 45 (14.1).

**Hydrolysis of Permethylated Clitorin**——Permethylated clitorin (1.0 mg) was hydrolyzed with 3% $H_2SO_4$ in the usual way. The reaction mixture was extracted with AcOEt. The AcOEt extract was washed with water and evaporated to dryness *in vacuo*. The residue was examined by GC–MS under the conditions described above. $t_R$ (min): 22.4 (TMS derivative of 5,7,4′-trimethyl kaempferol). MS *m/e* (%): 400 (1.2) $[M]^+$, 385 (100) $[M-CH_3]^+$, 370 (9.0) $[M-30]^+$, 355 (2.7) $[M-45]^+$, 327 (5.3) $[M-TMS]^+$, 135 (7.1) $[C_8H_7O_2]^+$.

**Photohydrolysis of Clitorin (II)**——Compound II (3 mg) was photohydrolyzed with 0.5 N $H_2SO_4$ in 50% aq. EtOH under a UV lamp (15 W, National GL-15) for 4 days, then treated in the usual way. The residue was examined by GC under conditions described above. $t_R$(min): 21.07, 22.56 (TMS derivative of rutinose). $t_R$: 19.64, 21.41 (TMS derivative of neohesperidose). Trisaccharide (acetate), MS *m/e* (%): 791 (0.1), 561 (1.0), 519 (0.2), 331 (5.3), 273 (100).