Chem. Pharm. Bull.
32(6)2290—2295(1984)

Studies on the Mutagenicity of Swertiae Herba. I. Identification of the Mutagenic Components

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(Received September 12, 1983)

The mutagenicity of the extract of Swertiae Herba is attributable to its xanthone derivatives. Seven active principles were isolated and six of them were identified as methylbellidifolin, methylswertianin, swertianin, bellidifolin, norswertianin and desmethylbellidifolin, all of which have already been isolated from this plant. The seventh mutagenic compound, a new xanthone derivative formulated as 5,8-dimethylbellidifolin, was also obtained from methylbellidifolin by methylation with diazomethane as a minor product. The structure–mutagenicity relationship of these compounds, together with some other derivatives, is discussed.

Keywords——Swertiae Herba; *Swertia japonica*; xanthone derivative; mutagenicity; *S. typhimurium*; ^{1}H-NMR; 5,8-dimethylbellidifolin

Swertiae Herba (*Swertia japonica* MAKINO Gentianaceae; Japanese name: Senburi or Toh-yaku) is a well-known Japanese folk medicine and has been used as a bitter stomachic. Recently, it has been reported that a crude extract of this plant exhibited mutagenicity in the Ames test.[1)] In connection with a study on the separative analysis of the secoiridoid glucosides,[2)] we have investigated the xanthone glucosides of this plant, reporting the structure of a new glucoside, isoswertianolin, and structure revisions of swertianolin and norswertianolin.[3)] As a continuation of these studies, this paper reports the isolation of mutagenic principles of this plant and the identification of several xanthone derivatives as the active components. The structure–mutagenicity relationship of xanthone derivatives is discussed.

An aqueous suspension of the methanolic extract of this plant was extracted with ether, ethyl acetate and 1-butanol (saturated with water), successively. The mutagenicity in the Ames test with S9 mix was concentrated in the ethereal extract. Column chromatography of this ethereal extract on Sephadex LH-20 afforded four active fractions, F-I—IV as shown in Fig. 1. Column chromatography of F-I on silica gel afforded three yellow pigments, **1**, **2** and **3**. Recrystallization of F-II and -III gave yellow pigments **4** and **5**, respectively, while F-IV was subjected to column chromatography on silica gel followed by preparative thin layer chromatography (TLC), affording yellow pigments **6** and **7** (Fig. 2). All of these compounds, **1**—**7** were mutagenic to TA 100 with S9 mix.

On the basis of ultraviolet (UV), infrared (IR), electron impact mass (EI-MS) and nuclear magnetic resonance (NMR) spectra as well as comparison of other physical constants, **1**, **2**, **4**, **5**, **6** and **7** were identified as methylbellidifolin, methylswertianin, swertianin, bellidifolin, norswertianin and desmethylbellidifolin, respectively, all of which have already been isolated from this plant.[4)]

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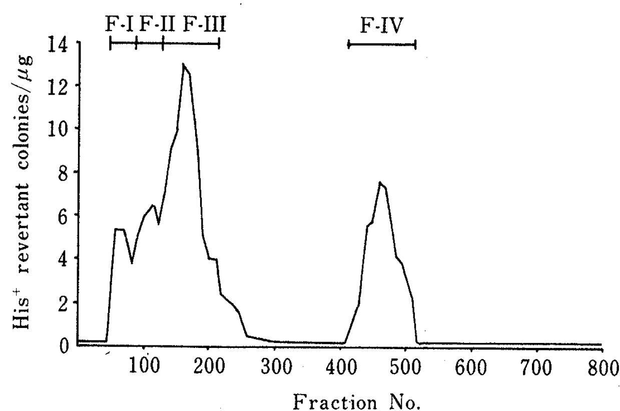


Fig. 1. Sephadex LH-20 Column Chromatogram of Ethereal Extract

Eluant, ethyl acetate; Ames test, every tenth fraction was tested on TA 100 with S9 mix.

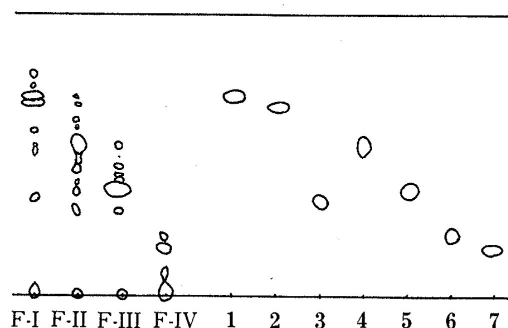
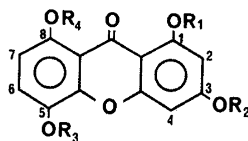


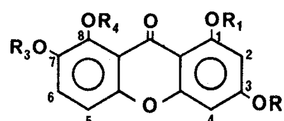
Fig. 2. Thin Layer Chromatograms of the Fractions Separated on a Sephadex LH-20 Column (F-I—IV) and of Purified Xanthones (1—7)

Plate, Kiesel gel 60 F-254; solvent, benzene–ethyl acetate (4 : 1); detection, UV at 254 nm.



	R_1	R_2	R_3	R_4
7	H	H	H	H
5	H	Me	H	H
1	H	Me	Me	H
3	H	Me	Me	Me
9	Ac	Me	Me	Me
8	Me	Me	Me	H
10	Me	Me	Me	Ac
16	H	Me	H	Glc
17	H	Me	Glc	H

Glc: β-glucopyranosyl.
Ac : acetyl.



	R_1	R_2	R_3	R_4
6	H	H	H	H
4	H	Me	H	H
2	H	Me	Me	H
11	H	Me	Me	Me
14	Ac	Me	Me	Me
12	Me	Me	Me	H
15	Me	Me	Me	Ac
13	Me	Me	Me	Me

Chart 1

The UV and proton nuclear magnetic resonance (^{1}H-NMR) (Table I) spectra suggested that a new pigment, **3** might be an isomer of 1,5-dimethylbellidifolin (**8**) which has been obtained from **5** by methylation with diazomethane.[5] In the present study, **1** was subjected to methylation with diazomethane, and **8** and a small quantity of its isomer were obtained. This isomer was identical with **3**. The structure of **3** was finally confirmed by the acetylation shifts of its aromatic proton signals in comparison with those of **8** (Table I). It is known that acetylation of a phenolic hydroxyl group results in remarkable deshielding of the aromatic protons at the *ortho*- and *para*-positions, while protons at the *meta*-position are almost unaffected.[6] On going from **3** to its acetate (**9**), the signals assignable to the *meta*-situated aromatic protons (H-2 and -4) were evidently displaced downfield by 0.21 and 0.37 ppm, respectively, while signals due to the *ortho*-located protons (H-6 and -7) remained almost unshifted. In contrast, the spectra of **8** and its acetate (**10**) indicated that H-7 was deshielded on acetylation by 0.18 ppm, while signals due to other aromatic protons appeared at almost the same positions in the spectra of both compounds, **8** and **10**. It follows that **3** can be

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Table I. ^{1}H-NMR Chemical Shifts in DMSO-d_4

	Aromatic protons H-2 H-4	H-6 H-7	Hydroxyl protons	Methoxyl protons	Acetyl protons
7	6.21, 6.41 (d, J=2.2 Hz)	7.23, 6.61 (d, J=8.8 Hz)	11.9, 11.2, 11.1, 9.6		
5	6.40, 6.62 (d, J=2.2 Hz)	7.26, 6.64 (d, J=8.8 Hz)	11.9, 11.1, 9.7	3.88 (3H)	
1	6.39, 6.66 (d, J=2.2 Hz)	7.44, 6.72 (d, J=8.8 Hz)	11.9, 11.2	3.91, 3.90 (3H, each)	
3[a]	6.33, 6.50 (d, J=2.2 Hz)	7.19, 6.72 (d, J=8.8 Hz)	13.2	3.98, 3.96, 3.88 (3H each)	
9[a]	6.54, 6.87 (d, J=2.2 Hz)	7.14, 6.68 (d, J=9.5 Hz)		3.96, 3.92, 3.90 (3H each)	2.48 (3H)
8[a]	6.35, 6.61 (d, J=2.4 Hz)	7.17, 6.68 (d, J=8.8 Hz)	12.6	3.99, 3.95, 3.91 (3H each)	
10[a]	6.33, 6.58 (d, J=2.2 Hz)	7.12, 6.86 (d, J=8.8 Hz)		4.00, 3.94, 3.90 (3H each)	2.46 (3H)
		H-5			
6	6.19, 6.33 (d, J=2.2 Hz)	7.26, 6.86 (d, J=8.8 Hz)	11.9, 11.7, 11.1, 9.3		
4	6.36, 6.56 (d, J=2.2 Hz)	7.29, 6.87 (d, J=8.8 Hz)	11.9, 11.7, 9.4	3.89 (3H)	
2	6.38, 6.58 (d, J=2.2 Hz)	7.49, 6.97 (d, J=8.8 Hz)	11.9, 11.8	3.90, 3.86 (3H each)	
11[a]	6.31, 6.34 (d, J=2.2 Hz)	7.34, 7.16 (d, J=8.8 Hz)	13.3	4.00, 3.93, 3.88 (3H each)	
14[a]	6.53, 6.71 (d, J=2.9 Hz)	7.27, 7.12 (d, J=8.8 Hz)		3.94, 3.90, 3.90 (3H each)	2.49 (3H)
12[a]	6.33, 6.45 (d, J=2.2 Hz)	7.20, 6.78 (d, J=8.8 Hz)	13.4	4.00, 3.93, 3.93 (3H each)	
15[a]	6.34, 6.42 (d, J=2.2 Hz)	7.31, 7.26 (d, J=9.5 Hz)		3.95, 3.90, 3.88 (3H each)	2.50 (3H)
13	6.45, 6.57 (d, J=2.2 Hz)	7.47, 7.22 (d, J=8.8 Hz)		3.89, 3.86, 3.85, 3.80 (3H each)	

a) In $CDCl_3$.

formulated as 5,8-dimethylbellidifolin.

The mutagenic activities of **1**—**8** were observed in the presence of S9 mix, being negative without S9 mix. The mutagenicities of these compounds were more evident towards *Salmonella typhimurium* TA 100 than TA 98. The dose–response curves of the mutagenicity and the specific activity of each compound are shown in Fig. 3 and Table II. 1,3,5,8-Tetrahydroxyxanthone (**7**), a non-methylated derivative, and its monomethyl ether (**5**) have significantly higher activities than the more methylated homologues (**1**, **3** and **8**). The activities of **5** and **7** are comparable to that of quercetin, which is a highly mutagenic natural flavonol.[7]

It was noted that the activities of 1,3,7,8-tetrahydroxyxanthone (**6**) and its 3-monomethyl ether (**4**) were evidently less than those of the corresponding 1,3,5,8-tetra substituted derivatives (**7** and **5**). For further confirmation of this structure–activity relationship, methylation of **2** was carried out. On methylation with diazomethane, **2** yielded three compounds, **11**, **12** and **13**. Based on the ^{1}H-NMR spectra (Table I), **13** was assigned as a permethyl ether of **6**, while **11** and **12** were concluded to be isomeric trimethyl ethers of **6**. The structures of **11** and **12** were established by comparison of the aromatic proton signals (acetylation shift) with those of the corresponding acetates, **14** and **15**, as in the case of **3** and **8**

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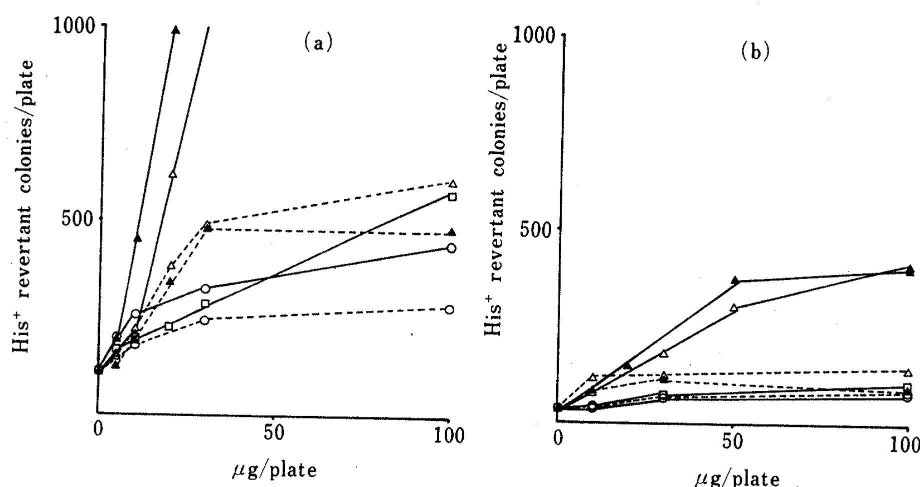

Fig. 3. Dose–Response Curves of Xanthone Derivatives for Mutagenicity to TA 100 with S9 Mix (a) and to TA 98 with S9 Mix (b)

○—○, methylbellidifolin (**1**); ○---○, methylswertianin (**2**); □—□, 5,8-dimethylbellidifolin (**3**); ▲---▲, swertianin (**4**); ▲—▲, bellidifolin (**5**); △---△, norswertianin (**6**); △—△, desmethylbellidifolin (**7**).

Table II. Mutagenicities of Xanthone Derivatives

Compound	His$^+$ revertant[a] colonies/μg	Compound	His$^+$ revertant[a] colonies/μg
7	47.5	**6**	16.5
5	53.3	**4**	16.5
1	7.9	**2**	3.4
3	8.2	**11**	5.8
8	8.2	**12**	2.4
		13	2.0

a) *Salmonella typhimurium* TA 100 with S9 mix.

(*vide supra*). It follows that **11** is identical with decussatin previously isolated from *Swertia decussata* and *S. chirata*,[8] and **12** can be formulated as 1,7-dimethylswertianin.

The mutagenic activities of these methylated compounds (**11**, **12** and **13**) were less than those of **4** and **6** and also than those of **1**, **3** and **8** (Table II), affording additional evidence for the argument that the methylation of the 1-, 5-, 7- and 8-hydroxyl groups in the xanthone series decreases the mutagenicity, and that the activities of the 1,3,7,8-oxygenated series are less than those of the corresponding 1,3,5,8-oxygenated series for the xanthone derivatives.

As already reported in the flavonoid series, glycosylation led to the disappearance of the mutagenicity with or without S9 mix[7c] as in the case of the xanthone derivatives. No mutagenicity was observed with swertianolin (**16**) and isoswertianolin (**17**),[3] isomeric β-D-glucosides of **5**, up to a concentration of 500 μg per plate (both of those compounds were previously isolated from Swertiae Herba). Other constituents of this plant, oleanolic acid, swertisin (a flavone glucoside), amarogentin, amaroswerin, sweroside, swertiamarin and gentiopicroside (bitter secoiridoid glucosides) also showed no mutagenicity under the conditions of the present study.

Quantitative analysis of the xanthone derivatives in this plant and the quantitative relationship between the contents and the mutagenicity will be described in a subsequent paper.[9]

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Experimental

General Procedures——^{1}H-NMR spectra were taken in DMSO-d_6 or $CDCl_3$ on a JEOL JNM GX-270 spectrometer (internal standard: tetramethylsilane (TMS)) at 270 MHz. Melting points were taken on a micro hot stage and are uncorrected. IR, UV and mass spectra were measured with JASCO A 202, Hitachi 557 and JEOL JMS D-300 spectrometers, respectively. Identification of known compounds, **1**, **2**, **4**, **5**, **6**, **7**,[4] **8**[5] and **11**[8] was based on comparison of their UV, IR, ^{1}H-NMR and mp data with the corresponding data in the literature.

Mutagenic Assay——Mutagenicity assay was carried out by the method of Ames *et al.*[10] with some modification.[11] The test samples were dissolved in dimethyl sulfoxide (DMSO). Tester strains were cultured overnight in Difco broth medium, then 100 μl of cell suspension, the sample solution and 500 μl of 100 mM phosphate buffer (pH 7.4) in the presence or absence of S9 mix were mixed and incubated at 37 °C for 15 min. Then, 2 ml of soft agar containing 0.7% Difco agar, 0.6% NaCl, 0.5 mM histidine and 0.5 mM biotin was added to the above mixture and the whole was poured on top of agar plates with Vogel–Bonner essential minimum culture medium containing 2% D-glucose. The plates were incubated at 37 °C for 48 h and the numbers of His$^+$ revertant colonies were counted. 4-NQO was used as the positive reference compound. The numbers of revertant colonies shown in Fig. 3 and Table II are averages of three plates.

Extraction and Separation of Xanthones——Commercial Swertiae Herba (205 g) was extracted repeatedly with hot MeOH and the MeOH solution was concentrated to dryness. A suspension of MeOH extract (49 g) in H_2O was extracted successively with Et_2O, EtOAc and 1-BuOH saturated with H_2O. Potent mutagenicity was found in the Et_2O extract. The Et_2O extract was chromatographed on Sephadex LH-20 (7ϕ × 50 cm) with EtOAc to give four active fractions, F-I, -II, -III and -IV.

On silica gel column chromatography with C_6H_6–Et_2O (20 : 1), F-I afforded **1**, **2** and **3** in yields of 0.02, 0.02 and 0.003%, respectively. **1**: Yellow needles (from MeOH), mp 188 °C (lit. mp 187 °C). $FeCl_3$ (+). MS *m/z*: 288 (M^+). UV λ_{max}^{MeOH} nm (log ε): 203 (4.61), 252 (4.50), 276 (4.30), 330 (4.15). IR ν_{max}^{KBr} cm^{-1}: 3450, 1665, 1640, 1605, 1580. **2**: Yellow needles (from MeOH), mp 191 °C (lit. mp 190 °C). $FeCl_3$ (+). MS *m/z*: 288 (M^+). UV λ_{max}^{MeOH} nm (log ε): 202 (4.48), 236 (4.38), 266 (4.40), 327 (4.11). IR ν_{max}^{KBr} cm^{-1}: 3450, 1670, 1605, 1575. **3**: Pale yellow needles (from EtOH), mp 215 °C. $FeCl_3$ (+). MS *m/z*: 302 (M^+). High-MS: Calcd: $C_{16}H_{14}O_6$: 302.0790. Found: 302.0798. UV λ_{max}^{MeOH} nm (log ε): 203 (4.58), 250 (4.48), 273 (4.15), 327 (4.08). IR ν_{max}^{KBr} cm^{-1}: 3450, 1660, 1615, 1585.

Recrystallization of F-II from EtOAc gave **4** in a yield of 0.04%. **4**: Yellow needles (from EtOAc), mp 221 °C (lit. mp 220 °C) $FeCl_3$ (+). MS *m/z*: 274 (M^+). UV λ_{max}^{MeOH} nm (log ε): 203 (4.44), 237 (4.28), 261 (4.43), 322 (4.10). IR ν_{max}^{KBr} cm^{-1}: 3450, 1660, 1640, 1605, 1580.

Recrystallization of F-III from EtOAc gave **5** in a yield of 0.15%. **5**: Yellow needles (from EtOAc), mp 264 °C (lit. mp 263 °C). $FeCl_3$ (+). MS *m/z*: 274 (M^+). UV λ_{max}^{MeOH} nm (log ε): 202 (4.54), 253 (4.50), 277 (4.32), 332 (4.14). IR ν_{max}^{KBr} cm^{-1}: 3450, 1660, 1635, 1615, 1595.

On silica gel column chromatography with C_6H_6–EtOAc (8 : 1) and subsequent preparative TLC (on a plate of Kiesel gel 60 F_{254} S, 20 × 20 cm, Merck; solvent C_6H_6–EtOAc (4 : 1)), F-IV afforded **6** and **7** in yields of 0.005 and 0.002%, respectively. **6**: Yellow needles (from 50% EtOH), mp 335 °C (lit. mp 335 °C). $FeCl_3$ (+). MS *m/z*: 260 (M^+). UV λ_{max}^{MeOH} nm (log ε): 203 (4.57), 236 (4.36), 264 (4.38), 327 (4.09). IR ν_{max}^{KBr} cm^{-1}: 3340, 1660, 1640, 1610, 1590. **7**: Yellow needles (from 50% EtOH), mp 317 °C (lit. mp 317 °C). $FeCl_3$ (+). MS *m/z*: 260 (M^+). UV λ_{max}^{MeOH} nm (log ε): 202 (4.41), 252 (4.37), 276 (4.16), 333 (4.14). IR ν_{max}^{KBr} cm^{-1}: 3400, 1670, 1645, 1620, 1595.

Methylation of 1——An excess of ethereal CH_2N_2 was added to a solution of **1** (40 mg) in MeOH (50 ml), and the mixture was allowed to stand at 20 °C overnight. After work-up in the usual way, the crude product was recrystallized from EtOAc to give **8** in a yield of 72%. **8**: Yellow needles (from EtOAc), mp 206 °C (lit. mp 205 °C). $FeCl_3$ (+). MS *m/z*: 302 (M^+). UV λ_{max}^{MeOH} nm (log ε): 203 (4.55), 247 (4.41), 275 (4.21), 315 (4.10). IR ν_{max}^{KBr} cm^{-1}: 3500, 1600, 1610, 1590.

The mother liquid of **8** was subjected to high performance liquid chromatography (HPLC) to give pale yellow needles, mp 215 °C (from EtOH), in a yield of 10%. This product was identified as **3** by mixed mp and comparison of UV, IR, mass and ^{1}H-NMR spectra with those of an authentic sample (*vide supra*). Conditions of HPLC; μ-Bondapak C_{18} column, Waters, 7.8 mmϕ × 30 cm; solvent, MeOH–H_2O (25 : 75); flow rate, 4 ml/min. After this methylation, only a trace of trimethylbellidifolin was identified by TLC.

Methylation of 2——**2** (40 mg) was methylated in the same way as above and the crude product was separated by silica gel column chromatography with C_6H_6–EtOAc (8 : 1) to give **11**, **12** and **13** in yields of 53, 19 and 18%, respectively. **11**: Pale yellow needles (from MeOH), mp 162 °C (lit. mp 150 °C). $FeCl_3$ (+). MS *m/z*: 302 (M^+). UV λ_{max}^{MeOH} nm (log ε): 202 (4.36), 238 (4.42), 261 (4.33), 308 (4.07). IR ν_{max}^{KBr} cm^{-1}: 3450, 1660, 1600, 1570. **12**: Yellow needles (from MeOH), mp 178 °C. $FeCl_3$ (+). MS *m/z*: 302 (M^+). High-MS: Calcd: $C_{16}H_{14}O_6$: 302.0790. Found: 302.0797. UV λ_{max}^{MeOH} nm (log ε): 203 (4.44), 239 (4.38), 257 (4.48), 315 (4.13). IR ν_{max}^{KBr} cm^{-1}: 3450, 1640, 1605, 1580. **13**: Colorless needles (from 50% EtOH), mp 115 °C. $FeCl_3$ (−). MS *m/z*: 316 (M^+). High-MS: Calcd: $C_{17}H_{16}O_6$: 316.0944. Found: 316.0936. UV λ_{max}^{MeOH} nm (log ε): 202 (4.42), 239 (4.54), 251 (4.55), 302 (4.16). IR ν_{max}^{KBr} cm^{-1}: 1655, 1645, 1600, 1585.

Acetylation of 3, 8, 11 and 12——Ac_2O was added to a solution of **3**, **8**, **11** and **12** in pyridine, and each mixture

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was heated at 120 °C for 4 h under stirring. After work-up in the usual way, the crude products were recrystallized from 50% EtOH to give **9**, **10**, **14** and **15**, respectively. **9**: Colorless needles, mp 185 °C. MS *m/z*: 344 (M^+), 302 ($M^+ - COCH_3$). High-MS: Calcd: $C_{18}H_{16}O_7$: 344.0896. Found: 344.0895. **10**: Colorless needles, mp 223 °C. MS *m/z*: 344 (M^+), 302 ($M^+ - COCH_3$). High-MS: Calcd: $C_{18}H_{16}O_7$: 344.0896. Found: 344.0879. **14**: Colorless needles, mp 177 °C. MS *m/z*: 344 (M^+), 302 ($M^+ - COCH_3$). High-MS: Calcd: $C_{18}H_{16}O_7$: 344.0896. Found: 344.0894. **15**: Colorless needles, mp 205 °C. MS *m/z*: 344 (M^+), 302 ($M^+ - COCH_3$). High-MS: Calcd: $C_{18}H_{16}O_7$: 344.0896. Found: 344.0879.

Other Materials——Swertisin, amarogentin, amaroswerin, sweroside, swertiamarin and gentiopicroside were obtained according to the previous paper.[3,12] Oleanolic acid was obtained from F-II and -III, and was identical with an authentic sample.

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