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Studies on the Mutagenicity of Swertiae Herba. III.[1] Components Which Become Mutagenic on Nitrite Treatment

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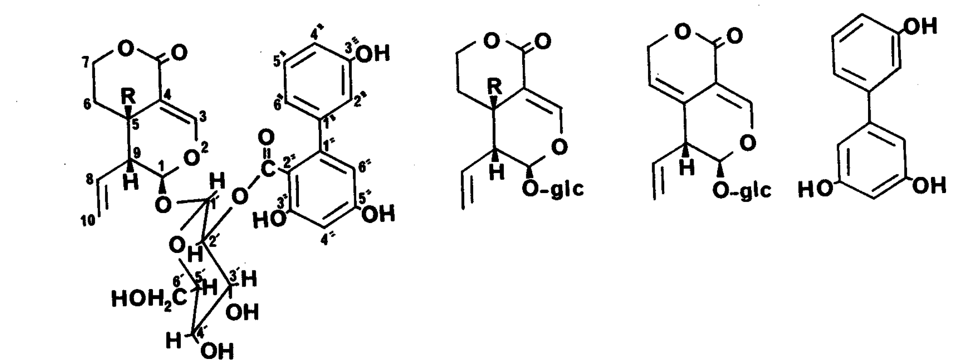
The methanolic extract of Swertiae Herba after treatment with nitrite was mutagenic to *Salmonella typhimurium* TA 98 in the absence of S9 mix. Two active principles were isolated and identified as amarogentin and amaroswerin, and this activity was proved to be associated with their 3,3′,5-trihydroxybiphenylcarboxylic acid moiety. 3,3′,5-Trihydroxybiphenyl obtained from both the glucosides also exhibited mutagenicity after treatment with nitrite. Such activity was not observed for the non-acylated secoiridoid glucosides and xanthone homologues of this drug. Quantitative analysis of the above two active glucosides in the commercial plant was also conducted.

Keywords——Swertiae Herba; *Swertia japonica*; amarogentin; amaroswerin; seco-iridoid glucoside; mutagenicity; *Salmonella typhimurium*; nitrite

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 stomachic. Previously, we reported that the mutagenic principles of this plant are represented by the oxygenated xanthones.[2] A quantitative relation between the content of these xanthones and the mutagenicity of this plant was also reported.[1] Carcinogenic *N*-nitroso compounds are formed from nitrite and secondary amines under acidic conditions.[3] Further, ingested nitrite is mainly formed from nitrate by oral bacteria.[4] Thus one possible cause of human gastric cancer is thought to be direct-acting mutagen-carcinogens produced in the acidic conditions of the stomach. As a continuation of our series of studies on the mutagenicity of this drug, the present paper deals with the identification and quantitative analysis of constituents which show mutagenic activity after treatment with nitrite.

It has already been found that the xanthone derivatives of this plant exhibited mutagenicity only in the presence of S9 mix.[2] It was observed that after treatment with nitrite, the methanolic extract of this plant showed mutagenic activity on *Salmonella typhimurium* TA 98 and TA 100 without S9 mix. However, the xanthone derivatives show no mutagenicity without S9 mix even after treatment with nitrite.

An aqueous suspension of a methanolic extract of this plant was extracted with ether, ethyl acetate and 1-butanol (saturated with water), successively. The nitrite-induced mutagenicity was found to be concentrated in the ethyl acetate extract. Column chromatography of this ethyl acetate extract on Sephadex LH-20 followed by chromatography of the most active fraction (F-4, Fig. 1) on silica gel afforded two active compounds, **1** and **2**. On the basis of a comparison of the ultraviolet (UV), infrared (IR), field-desorption mass (FD-MS) and nuclear magnetic resonance (NMR) spectra as well as other physical constants with those of authentic samples or those given in the literature, **1** and **2** were identified as amarogentin and



1: R=H
2: R=OH

3: R=H
4: R=OH

5

6

Chart 1

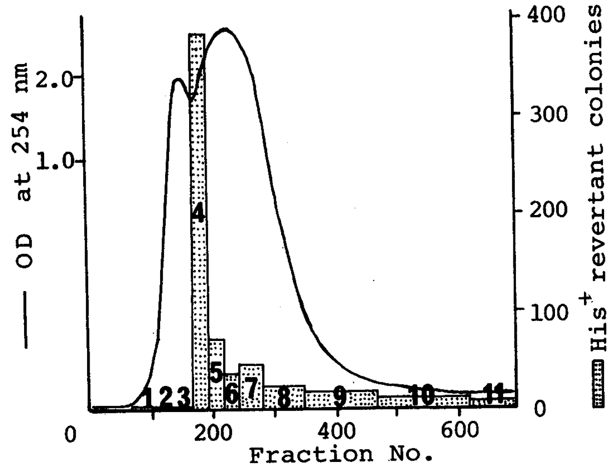


Fig. 1. Sephadex LH-20 Column Chromatogram of Ethyl Acetate Extract

Eluant: ethanol, Ames assay: every fraction (F-1—F-11) was tested on *S. typhimurium* TA 98 without S9 mix after nitrite treatment, and His$^+$ revertant colonies were expressed as the number of revertants generated by each fraction obtained from 1 mg of ethyl acetate extract.

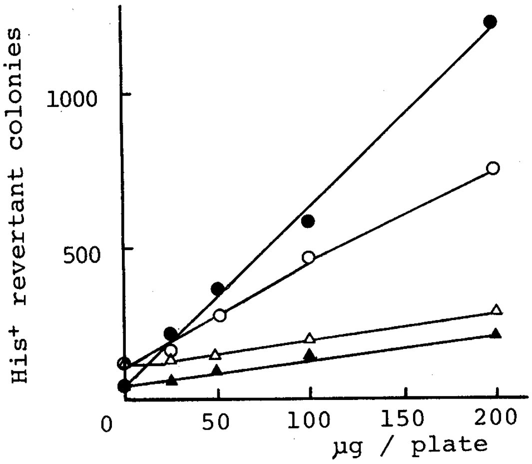


Fig. 2. Dose–Response Curves of Amarogentin (1) and Amaroswerin (2) for Mutagenicity on *S. typhimurium* TA 98 or TA 100 without S9 Mix after Nitrite Treatment

▲—▲, amarogentin (**1**) on TA 98; △—△, amarogentin (**1**) on TA 100; ●—●, amaroswerin (**2**) on TA 98; ○—○, amaroswerin (**2**) on TA 100.

amaroswerin, respectively. These compounds have already been isolated from this plant as bitter principles.[5,6] These seco-iridoid acylglucosides have the characteristic 3,3′,5-trihydroxybiphenylcarboxylic acid group as the acyl moiety. Compounds **1** and **2** themselves were not mutagenic in the presence or absence of S9 mix. However, after treatment with nitrite, both compounds showed remarkable mutagenicity on *S. typhimurium* TA 98 and TA 100 with or without S9 mix. The dose-response curves for each compound after nitrite treatment are shown in Fig. 2. After nitrite treatment, **2** gave 6000 (TA 98) and 3800 (TA 100) net His$^+$ revertant colonies per 1 mg, while **1** gave 1200 (TA 100) and 1100 (TA 98).

Several non-acylated secoiridoid glucosides such as sweroside (**3**), swertiamarin (**4**) and gentiopicroside (**5**) have been isolated from the 1-butanol extract (*vide supra*) and it has been elucidated that **1** and **2** are the biphenylcarboxylic acid esters of **3** and **4**, respectively, as illustrated in Chart 1. Since **3**, **4** and **5** did not show mutagenicity even after treatment with nitrite, the nitrite-induced mutagenicity of **1** and **2** must be attributable to the acyl moiety (the biphenylcarboxylic acid group). Treatment of **1** or **2** with alkali resulted in saponification followed by decarboxylation, affording 3,3′,5-trihydroxybiphenyl (**6**). It was found that after treatment with nitrite, **6** showed mutagenicity similar in intensity to that of **2** (Table I). The nitrite-induced mutagenicity of several synthetic biphenyls and biphenylcarboxylic acids was

TABLE I. Mutagenicities of Biphenyl Derivatives after Nitrite Treatment

Compound	His$^+$ revertant colonies/mg	
	TA 90	TA 100
3,3′,5-Trihydroxybiphenyl (**6**)	6800	13400
2-Hydroxybiphenyl	1200	2800
3-Hydroxybiphenyl	300	1400
4-Hydroxybiphenyl	0	0
2,2′-Dihydroxybiphenyl	1100	1900
4,4′-Dihydroxybiphenyl	0	0
Biphenyl-2-carboxylic acid	0	0
Diphenic acid	0	0

TABLE II. Contents (%) of Seco-iridoid Glucosides

	Samples				
	1	2	3	4	5
1	0.60	0.26	0.50	0.34	0.52
2	0.13	0.55	0.35	0.60	0.23

tested and it was found that the activity depends on the presence and location of hydroxyl groups on the molecule; 2-hydroxybiphenyl, 3-hydroxybiphenyl and 2,2′-dihydroxybiphenyl exhibited weak activity, while no nitrite-induced mutagenicity was observed for 4-hydroxybiphenyl, 4,4′-dihydroxybiphenyl, biphenyl-2-carboxylic acid or diphenic acid (Table I).

N-Nitrosoamines formed from secondary amines have been identified as nitrite-induced mutagens. Recently, mutagen precursors which become mutagenic on nitrite treatment were reported by Osawa *et al.* (sorbic acid,[7] piperine[8]), Wakabayashi *et al.* (β-carboline derivatives,[9] indole-3-acetonitrile[10]), Ochiai *et al.* (tyramine[11]) and Kanamori *et al.* (β-carboline derivatives[12]). The present study is the first to find nitrite-induced mutagenicity of hydroxybiphenyl homologues. Preliminary high-performance liquid chromatographic (HPLC) analysis demonstrated that on treatment with nitrite, **1**, **2** and **6** yielded complex mixtures of unidentified products; separation of these products has not been achieved as yet.

Quantitative analysis of the two active seco-iridoid glucosides in commercial plant materials was conducted by means of HPLC according to Sakamoto *et al.*[13] The contents of **1** and **2** were 0.2—0.6% and 0.1—0.6%, respectively (Table II).

Experimental

General Procedures——^{1}H-NMR and ^{13}C-NMR spectra were taken in CD_3OD 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 **1** and **2** was based on comparison of their UV, IR, ^{1}H-NMR and mp data with the corresponding data in the literature.

Treatment with Nitrite——A sample was diluted or dissolved in 0.3 M acetate buffer (pH 3.2) at appropriate concentrations. The test solution (0.4 ml) was mixed with 0.3 ml of $NaNO_2$ solution (containing 207 mg of $NaNO_2$ in 10 ml of 0.3 M acetate buffer, pH 3.2) and incubated at 37 °C for 60 min. Then, 0.3 ml of ammonium sulfamate solution (containing 343 mg of $H_2NSO_3NH_4$ in 10 ml of 0.3 M acetate buffer, pH 3.2) was added to the solution to stop the reaction with nitrite. After 10 min, 0.1 ml aliquots of the solutions were subjected to mutagenic assay.

Mutagenicity Assay——See the previous paper.[2]

Extraction and Separation of Active Compounds——Commercial Swertiae Herba (500 g) was extracted repeatedly with MeOH and the MeOH solution was concentrated to dryness. A suspension of MeOH extract (110 g) in H_2O was extracted successively with Et_2O, EtOAc and 1-BuOH saturated with H_2O. Potent mutagenicity was found in the EtOAc extract. The EtOAc extract was chromatographed on Sephadex LH-20 (7 cm i.d. × 50 cm) with EtOH and grouped into eleven fractions (F-1—F-11) on the basis of their TLC patterns. After treatment with nitrite, seven fractions (F-4—F-11) showed mutagenicity (Fig. 1).

On silica gel column chromatography with EtOAc–$CHCl_3$–MeOH (30 : 15 : 2), the most active fraction (F-4) gave two active fractions; the former fraction afforded **1** and the latter fraction afforded **2** in yields of 0.05 and 0.1%, respectively. **1**: Colorless needles (from MeOH–EtOAc), mp 229 °C (lit. mp 229—230 °C). FD-MS *m/z*: 587 $(M+H^+)$, 609 $(M+Na^+)$. UV λ_{max}^{MeOH} nm (log ε): 226 (4.44), 260 (4.09), 302 (3.76). IR ν_{max}^{KBr} cm^{-1}: 3400, 1675, 1655, 1610, 1575, 980. ^1H-NMR (CD_3OD) δ: 2.58 (1H, m, C_5-H), 4.29 (1H, d, $J=8$ Hz, $C_{1'}$-H), 4.73 (1H, t, $J=8$ Hz, $C_{2'}$-H), 5.39 (1H, d, $J=1.3$ Hz, C_1-H), 6.16 (1H, d, $J=2.3$ Hz, $C_{6''}$-H), 6.30 (1H, d, $J=2.3$ Hz, $C_{4''}$-H), 7.17 (1H, t, $J=8$ Hz, $C_{5'''}$-H), 7.43 (1H, d, $J=2.6$ Hz, C_3-H). ^{13}C-NMR (CD_3OD) δ: 25.8 (d), 28.7 (t), 43.4 (d), 62.4 (t), 69.5 (t), 71.6 (d), 74.6 (d), 74.8 (d), 78.3 (d), 96.7 (d), 97.1 (d), 103.2 (t), 105.6 (s), 112.8 (d), 114.5 (d), 116.5 (d), 116.9 (d), 121.2 (d), 129.3 (d), 132. 8 (d), 146.5 (s), 148.6 (s), 153.7 (s), 153.7 (d), 157.3 (s), 163.8 (s), 166.0 (s), 167.6 (s), 171.4 (s). **2**: White powder (from MeOH–EtOAc). FD-MS *m/z*: 625 $(M+Na^+)$, 641 $(M+K^+)$. UV λ_{max}^{MeOH} nm (log ε): 225 (4.45), 267 (4.14), 303 (3.82). IR ν_{max}^{KBr} cm^{-1}: 3400, 1680, 1650, 1610, 1580, 990. ^1H-NMR (CD_3OD) δ: 4.27 (1H, d, $J=8$ Hz, $C_{1'}$-H), 4.69 (1H, t, $J=8$ Hz, $C_{2'}$-H), 5.61 (1H, d, $J=1.3$ Hz, C_1-H), 6.18 (1H, d, $J=2.3$ Hz, $C_{6''}$-H), 6.30 (1H, d, $J=2.3$ Hz, $C_{4''}$-H), 7.20 (1H, t, $J=8$ Hz, $C_{5'''}$-H), 7.43 (1H, s, C_3-H). ^{13}C-NMR (CD_3OD) δ: 33.5 (t), 51.9 (d), 62.3 (d), 64.3 (t), 65.8 (s), 71.3 (d), 74.9 (d), 75.0 (d), 78.4 (d), 98.3 (d), 99.0 (d), 103.3 (t), 109.4 (s), 112.7 (d), 114.6 (d), 116.5 (d), 121.0 (d), 121.5 (d), 129.5 (d), 133.3 (d), 146.6 (s), 148.7 (s), 153.7 (s), 153.8 (d), 157.5 (s), 164.0 (s), 166.4 (s), 167.3 (s), 172.0 (s).

3,3′,5-Trihydroxybiphenyl (6)——**1** or **2** (100 mg) was dissolved in 0.2 N NaOH (EtOH) (10 ml) and the solution was refluxed for 30 min. The mixture was acidified with 1 N AcOH and extracted with Et_2O (20 ml × 3). The combined Et_2O extracts were washed with water, dried over Na_2SO_4 and evaporated to dryness. The residue was dissolved in EtOAc and subjected to preparative thin layer chromatography ($CHCl_3$–EtOAc–MeOH 8 : 4 : 1). The band of *Rf* 0.4—0.5 was extracted with EtOAc and recrystallized from benzene–Et_2O giving 7 mg of **6** as white prisms. mp 182—183 °C (lit. mp 182—183 °C). MS *m/z*: 202 (M^+). UV λ_{max}^{MeOH} nm (log ε): 216 (4.19), 252 (3.83), 286 (3.57). IR ν_{max}^{KBr} cm^{-1}: 3300, 1605, 1580, 1240, 995. ^1H-NMR (CD_3OD) δ: 6.24 (1H, t, $J=2.2$ Hz, C_4-H), 6.50 (2H, d, $J=2.2$ Hz, $C_{2,6}$-H), 6.73 (1H, m, $C_{4'}$-H), 6.96 (1H, t, $J=2.2$ Hz, $C_{2'}$-H), 6.99 (1H, m, $C_{6'}$-H), 7.19 (1H, t, $J=8$ Hz, $C_{5'}$-H).

Plant Materials for Analysis——Five specimens of commercial Swertiae Herba (sample Nos. 1—5) were purchased in Hiroshima market in 1983—1984.

Other Materials——Authentic samples of **3**, **4** and **5** were extracted and purified according to the previous paper.[13] Biphenyl-2-carboxylic acid, diphenic acid, 2-hydroxybiphenyl, 3-hydroxybiphenyl, 4-hydroxybiphenyl, 2,2′-dihydroxybihenyl and 4,4′-dihydroxybiphenyl were obtained from Tokyo Kasei Kogyo Co., Ltd., Tokyo.

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