Trypanocidal Constituents in Plants 2.¹⁾ Xanthenes from the Stem Bark of *Garcinia subelliptica*

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The constituents of the stem bark of *Garcinia subelliptica* (Guttiferae) were investigated based on its trypanocidal activity against epimastigotes of *Trypanosoma cruzi*, the etiologic agent for Chagas' disease. As the active components, nine xanthenes were isolated including two new ones, 4-hydroxybrasilixanthone B and 1,3,5,6-tetrahydroxy-4,7,8-tri(3-methyl-2-butenyl)xanthone. Their structures were determined by spectroscopic analysis. Trypanocidal activity against trypomastigotes, an infectious form of *T. cruzi*, was also estimated as well as cytotoxic activity. Fukugetin, the major component of the bark, showed no activity.

Key words *Trypanosoma cruzi*; *Garcinia subelliptica*; xanthone; trypanocidal activity; Chagas' disease; cytotoxicity

In the preceding paper in this series,¹⁾ we described the identification of trypanocidal constituents in Guaco (*Aristolochia taliscana*, Aristolochiaceae) against epimastigotes of the rotozoan *Trypanosoma cruzi*, which is the etiologic agent of Chagas' disease (American trypanosomiasis). It is transmitted to humans by triatomine bugs or through blood transfusion. In the course of our search for trypanocidal constituents in plants, we have found that the MeOH extract of the stem bark of *Garcinia subelliptica* MERR. (Guttiferae) showed considerable activity.

G. subelliptica (Guttiferae) is indigenous to the Ryukyu Islands and Taiwan. It is a large tree, growing up to 15–20 m high, and has been planted for use as a windbreak in the Yaeyama Islands of Japan.²⁾ Its bark has been traditionally utilized to dye thread and cloth yellow.

Many prenylated xanthenes, garcini-xanthenes A—E^{3–6)} and subelliptenones A—I,^{7–10)} benzophenone derivatives,^{11,12)} and phloroglucinol derivatives^{13–15)} were isolated from the various parts of this plant and their biological activities, such as antioxidant, antibacterial, and superoxide-scavenging activities, reported.^{3–15)}

The life cycle of *T. cruzi* is divided into three stages: epimastigote in the insect gut; trypomastigote, an infectious form in the mammalian blood stream; and amastigote, a proliferative form in mammalian cells. In the course of the isolation of the trypanocidal constituents, we determined the activity of the MeOH extract and fractions only against epimastigotes, because it is easy to cultivate them and they are not infectious. Schlemper *et al.* mentioned the positive correlation between activity against epimastigotes *in vitro* and activity against trypomastigotes *in vivo*.¹⁶⁾ However, it is necessary to determine the activity against trypomastigotes and amastigotes (stages in the mammalian body) *in vitro* to know whether the trypanocidal constituents against epimastigotes (stage in the insect gut) could become promising new chemotherapeutic agents for the treatment of Chagas' disease. Following the evaluation of trypanocidal activity against epimastigotes, we examined the trypanocidal activity against trypomastigotes and cytotoxicity against human cells using uterine carcinoma (HeLa) cells *in vitro*. In this paper, we describe the structures of two newly isolated xanthenes and the *in vitro* trypanocidal and cytotoxic activity of the iso-

lated compounds from the stem bark of *G. subelliptica*.

MATERIALS AND METHODS

Plant Materials The bark of *G. subelliptica* was collected at Ogimi in Okinawa Prefecture in June 2001. The bark was air-dried and powdered.

Instruments and Reagents The instruments used in this study were a JEOL JNM-A500 for NMR spectra (500 MHz for ¹H-NMR, 125 MHz for ¹³C-NMR), JEOL JMS-HX 110 for MS spectra, and a Shimadzu 200S double-beam spectrometer for UV spectra. The following materials were used for chromatography: Diaion HP-20 (a styrene polymer, Mitsubishi Chemical Industries, Ltd.), Silica gel 60N (Cica), Sephadex LH-20 (Pharmacia Fine Chemicals), YMC gel (ODS, YMC Co. Ltd.). Newborn calf serum (NCS) was purchased from Nacalai Tesque, and Dulbecco's modified Eagle's medium (DMEM) from Sigma. Gentian violet and ketoconazole were purchased from Wako. Other reagents are the same as in the previous paper.¹⁾

Cultivation of *T. cruzi* The strain of *T. cruzi* used in this study and the cultivation method of epimastigotes were the same as those described in the previous paper.¹⁾ The trypomastigotes were harvested from the culture supernatant of mouse kidney carcinoma (LLC-MK2) cells infected with *T. cruzi*.¹⁷⁾ LLC-MK2 cells were maintained in DMEM containing 10% fetal bovine serum (FBS) at 37 °C under 5% CO₂. After inoculation of trypomastigotes into LLC-MK2 cells, the medium was changed to DMEM containing 10% NCS.

Assay of Trypanocidal Activity Against Epimastigotes: Fractions and compounds were first dissolved in dimethyl sulfoxide (DMSO) and then diluted with liver infusion-tryptose (LIT) medium. The final DMSO concentration was maintained at less than 1%. The 1% DMSO solution itself had no effect on the motion of epimastigotes. Fifty microliters of each sample solution prepared by the serial two-fold dilution method and cell suspension (*ca.* 2×10⁶ epimastigotes/ml) was placed in 96-well microplates in duplicate and incubated at 26 °C for 48 h. The control was free from the test samples. The motion of epimastigotes was observed under an inverted light microscope (×100). Each test was performed twice. The activity was expressed as the MC₁₀₀

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value ($\mu\text{g/ml}$ or μM), which was defined as the minimum concentration at which all the epimastigotes become immobilized after 48-h incubation.

Against Trypomastigotes: The sample solutions were prepared in the same way as described above using DMEM instead of LIT medium. Fifty microliters of each sample solution and cell suspension (*ca.* 2×10^6 trypomastigotes/ml) was placed in 96-well microplates in duplicate and incubated at 37°C for 48 h under 5% CO_2 . The activity was expressed as the MC_{100} value as in the case of epimastigotes.

Assay of Cytotoxicity The cytotoxicity of isolated xanthenes against human uterine carcinoma (HeLa) cells was examined using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Mosmann.¹⁸⁾ The detailed procedure was described in a previous paper.¹⁹⁾

Extraction and Isolation of the Active Constituents from the Bark of *G. subelliptica* The dried and powdered stem bark of *G. subelliptica* (500 g) was extracted with MeOH (1000 ml) under reflux for 1 h and filtered. The residue was further extracted for 30 min. Another 1000 g of the sample was extracted in the same manner, and all filtrates were concentrated and dried *in vacuo* to give a dark brown residue (190 g). All epimastigotes were terminated at the concentration of 1 mg/ml of the MeOH extract after 48-h incubation.

The MeOH extract was suspended in 60% MeOH and centrifuged. The precipitates were extracted with MeOH and then with AcOEt. The 60% MeOH solution was passed through a column of Diaion HP-20, and the column was washed with 60% MeOH (fr. 1, 55 g). The MeOH solution was passed through the same column and the column was washed with MeOH (fr. 2, 97 g). The AcOEt solution was treated in the same way (fr. 3, 30 g). Only fraction 2 showed activity at 1 mg/ml. Fraction 2 was separated into six fractions by silica gel column chromatography using CHCl_3 -MeOH (fr. 2-1, 0.7 g; fr. 2-2, 3.6 g; fr. 2-3, 10.4 g; fr.

2-4, 6.3 g; fr. 2-5, 19.8 g; fr. 2-6, 25.0 g). MC_{100} values ($\mu\text{g/ml}$) of frs. 2-1—2-6 were >1000 , 250, 250, >1000 , >1000 , and >1000 , respectively. Fractions 2-2 and 2-3 were chromatographed successively on silica gel (hexane-AcOEt), Sephadex LH-20 (CHCl_3), and YMC gel (MeOH- H_2O) columns to afford garciniaxanthone B³⁾ (**1**, 17 mg), garciniaxanthone A³⁾ (**2**, 18 mg), subelliptenone H¹⁰⁾ (**3**, 88 mg), subelliptenone B⁷⁾ (**4**, 29 mg), compound **5** (22 mg), compound **6** (48 mg), subelliptenone A⁷⁾ (**7**, 548 mg), garciniaxanthone E⁶⁾ (**8**, 90 mg), and 1,4,5-trihydroxy-2-(1,1-dimethyl-2-propenyl)xanthone²⁰⁾ (**9**, 8 mg). Fukugetin²¹⁾ (**10**, 9.0 g) was precipitated from fr. 2-5.

Compound 5: Yellow amorphous powder, mp 185°C (dec.), λ_{max} (MeOH) nm (log ϵ): 209 (4.18), 285 (sh, 4.14), 310 (4.15), 335 (3.91), high-resolution (HR)-FAB-MS: m/z 408.1214 (Calcd for $\text{C}_{23}\text{H}_{20}\text{O}_7$: 408.1209). ^1H - and ^{13}C -NMR data: see Table 1.

Compound 6: Yellow needles, mp 198 – 200°C (dec.), λ_{max} (MeOH) nm (log ϵ): 210 (4.17), 237 (sh, 4.11), 255 (4.25), 329 (3.95), HR-FAB-MS: m/z 487.2106 (Calcd for $\text{C}_{28}\text{H}_{32}\text{O}_6 + \text{Na}$: 487.2097). ^1H - and ^{13}C -NMR data: see Table 1.

MC_{100} values of compounds **1**–**10** against epimastigotes and trypomastigotes are shown in Table 2. GI_{50} values of compounds **1**–**9** are also shown in Table 2.

RESULTS AND DISCUSSION

Trypanocidal activity-guided fractionation of the MeOH extract of the bark of *G. subelliptica* resulted in the isolation of 10 compounds (Chart 1). Fukugetin (**10**) and seven (**1**–**4**, **7**–**9**) of nine xanthenes have already been isolated from the same plant. They were identified by comparison of the spectral data with those reported. Compounds **5** and **6** were new xanthenes.

The molecular formula of **5** was determined to be $\text{C}_{23}\text{H}_{20}\text{O}_7$ on the basis of the molecular ion peak in HR-FAB-

Chart 1. Chemical Structures of Compounds **1**–**10**

Table 1. NMR Spectral Data^{a)} for Compounds **5** and **6**

No.	5			6		
	C	H	HMBC ^{b)}	C	H	HMBC ^{b)}
1	150.7			162.7		
2	104.8			98.5	6.28 (s)	1, 3, 4, 9a
3	148.1			162.9		
4	126.2			106.7		
4a	145.0			154.6		
5	103.6	6.87 (s)	6, 7, 8a, 10a	130.4		
6	154.2 ^{c)}			151.1		
7	139.0			126.1		
8	120.9			135.7		
8a	108.4			111.9		
9	183.6			183.7		
9a	104.8			104.0		
10a	153.8 ^{c)}			147.7		
1'	116.4	6.71 (d, 10)	1, 3, 3''	21.9	3.58 (d, 7)	3, 4, 4a, 2', 3'
2'	128.6	5.72 (d, 10)	2, 4', 5'	124.0	5.33 (brt, 7)	4', 5'
3'	78.8			131.4		
4'	28.3	1.48 (s)	2', 3', 5'	25.8 ^{c)}	1.64 (s)	2', 3', 5'
5'	28.3	1.48 (s)	2', 3', 4'	18.0 ^{d)}	1.82 (s)	2', 3', 4'
1''	121.6	8.05 (d, 10)	8, 3'', 4'', 5''	25.3	3.48 (d, 7)	6, 7, 8, 2'', 3''
2''	133.6	5.94 (d, 10)	8, 3'', 4'', 5''	123.9	5.11 (brt, 7)	4'', 5''
3''	76.8			132.0		
4''	27.2	1.47 (s)	2'', 3'', 5''	25.8 ^{c)}	1.67 (s)	2'', 3'', 5''
5''	27.2	1.47 (s)	2'', 3'', 4''	18.2 ^{d)}	1.79 (s)	2'', 3'', 4''
1'''				29.1	4.13 (d, 7)	7, 8, 8a, 2''', 3'''
2'''				125.5	5.10 (brt, 7)	4''', 5'''
3'''				130.7		
4'''				25.9 ^{c)}	1.68 (s)	2''', 3''', 5'''
5'''				18.3 ^{d)}	1.79 (s)	2''', 3''', 4'''
1-OH		13.16 (s)	1, 2, 9a		13.57 (s)	1, 2, 9a

a) δ ppm in acetone- d_6 at 500 MHz (^1H -NMR) and 125 MHz (^{13}C -NMR). Coupling constants are J in Hz in parentheses. Signal assignments are based on 2-dimensional ^1H - ^1H shift correlation spectroscopy (COSY) and heteronuclear multiple-quantum connectivity spectroscopy (HMQC). b) Correlated carbons. c, d) May be interchangeable within the same column.

MS at m/z 408.1214 $[\text{M}]^+$ (Calcd for $\text{C}_{23}\text{H}_{20}\text{O}_7$, 408.1209). The ^1H -NMR spectrum showed the presence of a hydrogen-bonded hydroxyl group (δ 13.16, s), one isolated aromatic hydrogen (δ 6.87, s), and two 2,2-dimethylpyran rings [δ 1.47 (6H, s), 5.94 (d, $J=10$ Hz), 8.05 (d, $J=10$ Hz), δ 1.48 (6H, s), 5.72 (d, $J=10$ Hz), 6.71 (d, $J=10$ Hz)]. It was deduced from the highly deshielded olefinic hydrogen signal (δ 8.05) that one of the pyran rings was located at C-7 and C-8 in the deshielding region of the carbonyl group, as in brasilixanthone B.²²⁾ In the differential nuclear Overhauser effect (NOE) experiment, the olefinic proton signal at δ 6.71 showed a response to irradiation of the hydroxyl proton signal (δ 13.16) and *vice versa*, indicating another pyran ring located linearly on ring A (Fig. 1). The ^{13}C -NMR chemical shifts of carbons in ring A were in good agreement with those of atroviridin,²³⁾ which has the same substitution pattern at ring A. An aromatic hydrogen was located on C-5 based on the correlation between C-8a and C-7 (three bonds), as well as C-6 and 10a (two bonds) in the heteronuclear multiple-bond connectivity (HMBC) spectrum (Table 1). Therefore **5** was determined to be 4-hydroxybrasilixanthone B.

Compound **6** has the same molecular formula as **8**, which is $\text{C}_{28}\text{H}_{32}\text{O}_6$ on the basis of the quasi-molecular ion peak at m/z 487.2106 $[\text{M}+\text{Na}]^+$ (Calcd for $\text{C}_{28}\text{H}_{32}\text{O}_6+\text{Na}$, 487.2097). ^1H - and ^{13}C -NMR spectra revealed the presence of three 3-methyl-2-butenyl units, a singlet aromatic proton (δ 6.28, s), and a chelated hydroxyl group (δ 13.57, s). An aromatic proton was located on C-2 due to three-bond con-

Fig. 1. NOE Correlations of **5** and **6**, and Structures of Brasilixanthone B and Atroviridin

nectivity from a chelated hydroxyl proton in the HMBC spectra. The C_2 -H signal showed a correlation to C-4 as well as to C-1, -3, and -9a. One of the three 3-methyl-2-butenyl units was located at C-4 because the methylene proton signal (H-1', δ 3.58, 2H, d, $J=7$ Hz) showed cross peaks between C-3, C-4, and C-4a. A second one was placed at C-8 because its methylene proton signal (H-1'', δ 4.13, 2H, d, $J=7$ Hz) was deshielded due to the influence of the *peri*-carbonyl group. A NOE correlation was observed between H-1''' and methylene proton signals (δ 3.48, 2H, d, $J=7$ Hz), suggesting that a third one is attached to C-7. ^{13}C -NMR chemical shifts

Table 2. Trypanocidal and Cytotoxic Activity of **1**–**10**, Gentian Violet, and Ketoconazole *in Vitro* (μM)

	Epi. ^{a)}	Try. ^{b)}	HeLa ^{c)}
1 (Garciniaxanthone B)	66	8	50
2 (Garciniaxanthone A)	158	16	17
3 (Subelliptenone H)	190	114	>253
4 (Subelliptenone B)	51	25	43
5	196	147	17
6	172	54	16
7 (Subelliptenone A)	162	54	77
8 (Garciniaxanthone E)	>430	47	10
9	128	48	14
10 (Fukugetin)	>500	>500	
Gentian violet	24	2	2
Ketoconazole	94	377	>188

a) MC_{100} values against epimastigotes. b) MC_{100} values against trypomastigotes.

c) GI_{50} values against HeLa cells.

of ring B carbons in **6** are in agreement with those in **8**. Assignments of six methyl group signals are based on the NOEs in the NOESY spectrum (Fig. 1). Consequently, the structure of **6** was determined to be 1,3,5,6-tetrahydroxy-4,7,8-tri(3-methyl-2-butenyl)xanthone and it is called isogarciniaxanthone E.

Compounds **1**–**10** were estimated for their trypanocidal activity against epimastigotes and trypomastigotes of *T. cruzi* *in vitro*. Gentian violet²⁴⁾ and ketoconazole²⁵⁾ were also tested for comparison. The results are summarized in Table 2. All xanthenes from the stem bark of *G. subelliptica* showed 1.3 to more than 10 times stronger activity against trypomastigotes than against epimastigotes. The order of potency against epimastigotes was **1**, **4** > **9** > **2**, **6**, **7** > **3**, **5** > **8**. On the other hand, it was **1** > **2**, **4** > **6**, **7**, **8**, **9** > **3** > **5** against trypomastigotes. The tendency of potency is roughly the same between the two stages, except for **8**.

The following are the effects of substitution patterns on the activity against trypomastigotes, although the number of xanthenes examined might not be sufficient to clarify the structure–activity relationships. Among them, **1** has the strongest activity, although it is four-fold weaker than that of gentian violet. Compounds **2**, **6**, and **9** have the same substitution pattern in ring A as **1**. Substitution of one prenyl unit to ring B increases the activity, although two units decrease it. In compounds **6** and **7**, the difference in the substitution pattern in ring A had no influence. Isomers **6**–**8**, which have three isoprenyl units and four hydroxyl groups in their structures, showed almost the same activity. Formation of a pyran ring linear to ring A or B (**5** or **3**) reduced the activity greatly. Considering the highest yield (0.11%) among xanthenes, subelliptenone A (**7**) is responsible for the trypanocidal activity of the MeOH extract in spite of its moderate activity. Many xanthenes show toxicity against HeLa cells, but the structure–trypanocidal activity relationship was slightly correlated with that of cytotoxic activity. Garciniaxanthone B (**1**) is one of the candidates for further investigation *in vivo* because of its high trypanocidal activity and rather low cytotoxic activity. Fukugetin (**10**), the major compound in the

stem bark, showed no activity against both epimastigotes and trypomastigotes.

Acknowledgments The authors are grateful to Prof. K. Kinjo of Ryukyu University for providing the stem bark of *G. subelliptica*. Our thanks are also due to Dr. S. Hamano of Kyushu University for his helpful advice. We thank Ms. Y. Iwase and Mr. H. Hanazono for the NMR and MS measurements. This work was supported in part by a Grant-in-Aid for Scientific Research (C) (No. 14572028) from the Ministry of Education, Culture, Sports, Science and Technology.

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