Tricin from a Malagasy Connaraceous Plant with Potent Antihistaminic Activity

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The bioassay-guided separation of a Malagasy plant, *Agelaea pentagyna*, led to the isolation of a flavonoid, tricin (**1**), with potent inhibitory activity toward exocytosis from antigen-stimulated rat leukemia basophils (RBL-2H3). The structure−activity relationships among structurally related natural and synthetic flavonoids are also discussed.

Since the incidences of atopy dermatitis and pollen allergy are increasing and have been widely serious in Japan in recent years, we have been searching for new antiallergic substances from natural sources and screening compounds with antihistaminic activity in many medicinal and/or toxic plants and have found some active compounds.[1−3] On screening of antihistaminic activity in some Malagasy plants, a MeOH extract of leaves of *Agelaea pentagyna* (Lam.) Baill. (Connaraceae) (Vahimainty in Malagasy) showed strong activity. This species grows in Madagascar and some east African countries and is regarded as a poisonous rather than a medicinal plant. We report here bioassay-guided fractionation of *A. pentagyna* that resulted in the isolation of a flavonoid, tricin (**1**), which exhibited potent antihistamine release activity and accounts for some, but not all, of the observed activity of the methanolic extract. Although Amellal et al. claimed that a catechol moiety (*ortho*-hydroxyl groups) is essential to show antihistaminic activity, tricin without any catechol moiety showed strong activity.[4] They used an assay method similar to ours, except that compound 48/80 and ionophore A23187 were used as inducers. Thus we examined some natural and synthetic flavonoids, including compounds related to tricin and compounds with a catechol moiety, by our method.

A methanol extract of *A. pentagyna* exhibited 100% and 49% inhibitory activity toward exocytosis granules from rat leukemia basophils caused by antigen-induced stimulation at concentrations of 1 and 0.1 mg/mL, respectively. On solvent partitioning, the activity was distributed in all fractions with varied strength. When considering specific activity, a CH_2Cl_2-soluble fraction (93% at 0.1 mg/mL) was the most active, and purification of this fraction gave tricin (**1**) (Figure 1 and Table 1)[5] as a highly promising active compound with an IC_{50} value of 4.38 μM. To examine the structure−activity relationships of natural flavonoids (**2**−**9**), we assayed their inhibitory activity (Table 1). Some unnatural flavonoids (**10**−**18**) were synthesized for the same purpose. As judged from the results among 5,7-dihydroxyflavonoids, C-acetylation of the 3-position (**17**) reduced the activity to 1/44 of that of **1**, although there are the same functional groups on the B-ring. However, compounds **16** and **17**, which have the same substituents on the B-ring as **1**, still retained some activity.

Methylation of 4′-OH of **1**, yielding **10**, reduced the activity to 1/88, while on removal of 4′-OH, yielding **15**, the activity was completely lost. A compound with no substitution on the B-ring, like **11**, showed no activity, whereas baicalein (**2**) still retained some activity, probably due to the three adjacent hydroxyl groups on the A-ring. A 3′,4′-dihydroxyflavone, luteolin (**6**), showed 1/12 the activity of **1**. However, 3′,4′-dimethoxy (**12** and **13**) and 3′,5′-dinitro (**18**) compounds did not exhibit any activity.

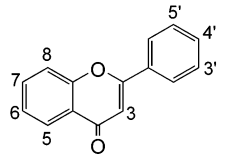

Figure 1. Basic skeleton of flavonoids.

Although with these experimental data we were not able to deduce a general rule for activity, it is tentatively concluded that tricin (**1**) is a 5,7-dihydroxyflavone with a maximal state of activity. Probably, there is another rule for flavonoids with polysubstitutions on the A-ring, such as the baicalein series. It is noteworthy that since tricin (**1**) is widely distributed in Gramineae plants,[6] including cereal plants whose leaves and stalks are generally disposed of or used as feed for cattle, such plants are expected to be new enormous sources of supplemental antiallergic materials, and thus their commercial assessment is in progress.

Experimental Section

General Experimental Procedures. Melting points were measured with a Yanagimoto micro melting point apparatus and are uncorrected. IR spectra were measured on a Horiba FT-710 Fourier transform infrared spectrophotometer. ^{1}H and ^{13}C NMR (DMSO-d_6) spectra were recorded on a JEOL JNM α-400 spectrometer at 400 and 100 MHz, respectively, and MS spectra were recorded on a JEOL JMS SX-102 spectrometer. DNP-specific IgE was purchased from Sigma Co., Ltd. (MO). The highly porous synthetic polymer (MCI gel, CHP-20P) was from Mitsubishi Chemical Co., Ltd. (Tokyo, Japan).

Plant Material. The leaves of *Agelaea pentagyna* were collected at Lokaro Village in Madagascar in November 1998. A voucher specimen (Con-9804) was deposited in the Herbarium of the Botanical and Zoological Institute of Tsimbazaza, Antananaribo, Madagascar.

Measurement of *β*-Hexosaminidase Release. For measurement of the secretion β-hexosaminidase, RBL-2H3 cells were incubated with DNP-specific IgE in complete growth medium in 96-well plates (4×10^4 cells/80 μL of medium/well).[7] Cells were washed twice, and then the medium was replaced with a glucose-saline PIPES-buffered medium containing 1

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Table 1. Inhibitory Activity of Tricin (**1**) and Related Flavonoids on Degranulation of RBL-2H3 Cells

compd	C-3	5	6	7	8	3′	4′	5′	IC_{50} (μM)
tricin (**1**)	H	OH	H	OH	H	OMe	OH	OMe	4.83
baicalein (**2**)	H	OH	OH	OH	H	H	H	H	179
scutellarein (**3**)	H	OH	OH	OH	H	H	OH	H	67.2
cirsimaritin (**4**)	H	OH	OMe	OMe	H	H	OH	H	161
apigenin (**5**)	H	OH	H	OH	H	H	OH	H	124
luteolin (**6**)	H	OH	H	OH	H	OH	OH	H	58.2
tangeretin (**7**)	H	OMe	OMe	OMe	OMe	H	OMe	H	>500
sideritiflavone									
5-methyl ether (**8**)	H	OMe	OMe	OMe	OMe	OH	OH	H	203
sideritiflavone (**9**)	H	OH	OMe	OMe	OMe	OH	OH	H	>500
10	H	OH	H	OH	H	OMe	OMe	OMe	424
11	H	OH	H	OH	H	H	H	H	>500
12	H	H	H	H	H	OMe	OMe	H	>500
13	H	OH	H	OH	H	OMe	OMe	H	>500
14	H	H	H	H	H	OMe	H	OMe	>500
15	H	OH	H	OH	H	OMe	H	OMe	>500
16	H	H	H	H	H	OMe	OH	OMe	97.5
17	Ac	OH	H	OH	H	OMe	OH	OMe	212
18	H	OH	H	OH	H	NO_2	H	NO_2	>500
DSCG									1540

mM Ca^{2+} (Siraganian buffer). Cells were preincubated for 10 min at 37 °C in 40 μL of Siraganian buffer containing a crude extract or pure compounds, which were prepared in DMSO and diluted to give <0.1% DMSO solutions.[8] Cells were then stimulated with DNP−BSA (20 ng/mL) for 15 min. Aliquots (10 μL) of the medium and cell lysate (in 50 μL of 0.1% Triton X-100) were incubated with 10 μL of 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.1 M sodium citrate buffer (pH 4.5) at 37 °C for 1 h. At the end of the incubation, 250 μL of 0.1 M Na_2CO_3−0.1 M $NaHCO_3$ buffer (pH 10) was added. Absorption was measured at 410 nm. Values were calculated as the actual release (percentage of total β-hexosaminidase), after correction for spontaneous release (2−3%), or as a percentage of the maximal response.[2] IC_{50} values were calculated for at least six independent concentrations ranging from 1 to 500 μM due to the limit of solubility. Disodium cromoglycate (DSCG), clinically used as an antiallergic, was used as a positive control, and its IC_{50} value was comparable to the reported value.[4] Cytotoxity of compounds **1**−**18** against RBL-2H3 cells was monitored under an optical microscope. They did not show any cytotoxicity at a concentration of 500 μM.

Bioassay-Guided Extraction and Separation. Dried leaves of *A. pentagyna* (100 g) were extracted three times with hot MeOH to give an extract (10.1 g). The extract was suspended in water and then extracted with hexane (67% inhibition at 1 mg/mL), CH_2Cl_2 (93% inhibition at 0.1 mg/mL), EtOAc (30% inhibition at 0.1 mg/mL), and 1-BuOH (28% inhibition at 0.1 mM), successively (water layer, 70% inhibition at 1 mg/mL). The CH_2Cl_2-soluble fraction was further separated following the activity on an MCI gel CHP-20P column eluted with water, 50% aqueous MeOH, MeOH, and acetone, and a silica gel column eluted with CH_2Cl_2−MeOH (20:1 to 10:1) and CH_2Cl_2−MeOH−H_2O (15:6:1 to 6:4:1), followed by HPLC with ODS (75% MeOH), which yielded tricin (**1**) (6.7 mg) as a pale yellow powder.

Origins of Flavonoids and Synthesis of Tricin and Related Flavonoids. Natural flavonoids (**2**−**9**) were previously isolated from various plants and stored in our laboratory. Tricin (**1**) was further synthesized for assay purpose from acetylsyringic acid and phloroacetophenone by means of the Baker−Venkataraman reaction.

Compounds **10**−**18** were synthesized from the corresponding benzoic acid derivatives and acetophenone derivatives by means of the same reaction. Among them, flavonoids **10**−**13** were known as synthetic compounds, and their physicochemical properties were identical with the reported values.[9−12] Compounds **14**−**18** were synthesized for the first time for assay purposes.

Naturally occurring tricin (1): pale yellow powder; ^{1}H NMR (DMSO-d_6) δ 3.89 (6H, s, CH_3O- × 2), 6.21 (1H, d, J = 2.0 Hz, H-6), 6.56 (1H, d, J = 2.0 Hz, H-8), 6.97 (1H, s, H-3), 7.32 (2H, s, H-2′ and 6′); ^{13}C NMR (DMSO-d_6) δ 56.3 (CH_3O- × 2), 94.1 (C-8), 98.8 (C-6), 103.5 (C-3), 103.6 (C-10), 104.3 (2C, C-2′ and 6′), 120.3 (C-1′), 139.8 (C-4′), 148.1 (2C, C-3′ and 5′), 157.3 (C-5), 163.6 (C-2), 161.3 (C-9), 181.7 (C-4); HRFABMS (negative-ion mode) m/z 329.0663 $[M - H]^-$ (calcd for $C_{17}H_{13}O_7$, 329.0661).[6,14]

Synthesis of Tricin (1). A mixture of 30 g of oxalyl chloride and acetylsyringic acid was refluxed for 3 h. After the oxalyl chloride was removed in vacuo, the residue was dissolved in 50 mL of benzene. The chloride solution was added to a solution of 2′,4′,6′-trihydroxyacetophenone (2.52 g) in 50 mL of pyridine, followed by stirring for 18 h at 25 °C. The reaction mixture was diluted with benzene and washed with 2 N hydrochloric acid, 2 N aqueous NaOH, and then H_2O. The organic layer was evaporated and dried to give 16.0 g of crude ester. A solution of the crude ester in 100 mL of DMSO was added dropwise to a solution of 2.0 g of NaH (50% in mineral oil) in 50 mL of DMSO, which was prepared in advance, with stirring for 30 min at 60 °C under a N_2 stream. After 1 h, the reaction mixture was poured into 2 N aqueous NaOH and washed with CH_2Cl_2. The water layer was acidified with concentrated HCl and then extracted with EtOAc. Evaporation of the organic layer gave 10.8 g of a yellow powder (crude diketone). To a solution of the crude diketone in 30 mL of glacial acetic acid was added 5 mL of concentrated H_2SO_4, and then the solution was heated in a water bath. After cooling in an ice-bath, the solution was neutralized with 10 N aqueous NaOH and then extracted with EtOAc. The residue, obtained on evaporation of the organic solvent, was purified by silica gel column chromatography (CH_2Cl_2−MeOH, 100:1), and then precipitation from EtOH−H_2O afforded 178 mg of tricin (**1**). From the mother liquid, 3-acetyltricin (**17**, 132 mg) was isolated as a byproduct, which was then recrystallized from EtOH−H_2O. The physical properties of **1** were the same as those of naturally occurring tricin within experimental error, and the structure of the byproduct (**17**) was elucidated to be 3-actyltricin from spectroscopic evidence.

3′,5′-Dimethoxyflavone (14): colorless needles (acetone); mp 141 °C; IR (film) ν_{max} 1640, 1606, 1569 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.81 (6H, s, CH_3O- × 2), 6.66 (1H, br s, H-4′), 7.05 (1H, s, H-3), 7.16 (2H, d, J = 1.7 Hz, H-2′ and 6′), 7.46 (1H, br dd, J = 7.8, 6.8 Hz, H-6), 7.75 (1H, br dd, J = 7.8, 6.8 Hz, H-7), 7.77 (1H, br d, J = 7.8 Hz, H-8), 8.00 (1H, br d, J = 7.8 Hz, H-5); ^{13}C NMR (DMSO-d_6) δ 55.7 (CH_3O- × 2), 103.7 (C-4′), 104.4 (2C, C-2′ and 6′), 107.5 (C-3), 118.7 (C-8), 123.3 (C-10), 124.8 (C-5), 125.6 (C-6), 133.1 (C-7), 133.4 (C-1′), 155.7 (C-9), 160.9 (2C, C-3′ and 5′), 162.3 (C-2), 177.3 (C-4); HRFABMS (positive-ion mode) m/z 283.0967 $[M + H]^+$ (calcd for $C_{17}H_{15}O_4$, 283.0970).

5,7-Dihydroxy-3′,5′-dimethoxyflavone (15): pale yellow needles (acetone); mp 284−286 °C; IR (film) ν_{max} 3395, 1649,

1588, 1569, 1503 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.83 (6H, s, CH_3O- × 2), 6.21 (1H, d, J = 2.0 Hz, H-6), 6.54 (1H, d, J = 2.0 Hz, H-8), 6.71 (1H, d, J = 2.0 Hz, H-4′), 7.04 (1H, s, H-3), 7.17 (2H, d, J = 2.0 Hz, H-2′ and 6′), 12.80 (1H, s, 5-OH); ^{13}C NMR (DMSO-d_6) δ 55.7 (CH_3O- × 2), 94.3 (C-8), 99.1 (C-6), 103.9 (2C, C-10 and 4′), 104.5 (2C, C-2′ and 6′), 105.7 (C-3), 132.7 (C-1′), 157.5 (C-9), 160.9 (2C, C-3′ and 5′), 161.4 (C-5), 162.8 (C-2), 164.6 (C-7), 181.9 (C-4); HRFABMS (negative-ion mode) m/z 313.0721 $[M - H]^-$ (calcd for $C_{17}H_{13}O_6$, 313.0712).

4′-Hydroxy-3′,5′-dimethoxyflavone (16): pale yellow needles (EtOH); mp 227−228 °C; IR (film) ν_{max} 3261, 1630, 1604, 1563, 1510 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.89 (6H, s, CH_3O- × 2), 7.05 (1H, s, H-3), 7.36 (2H, s, H-2′ and 6′), 7.47 (1H, ddd, J = 7.8, 4.0, 4.0 Hz, H-6), 7.80 (2H, br d, J = 4.0 Hz, H-7 and 8), 8.02 (1H, br d, J = 7.8 Hz, H-5); ^{13}C NMR (DMSO-d_6) δ 56.3 (CH_3O- × 2), 104.3 (2C, C-2′ and 6′), 105.5 (C-3), 118.5 (C-8), 120.8 (C-1′), 123.3 (C-10), 124.7 (C-5), 125.3 (C-6), 133.9 (C-7), 139.7 (C-4′), 148.2 (2C, C-3′ and 5′), 156.6 (C-9), 163.0 (C-2), 177.0 (C-4); HRFABMS (negative-ion mode) m/z 297.0776 $[M - H]^-$ (calcd for $C_{17}H_{13}O_5$, 297.0763).

3-Acetyltricin (17): colorless needles (EtOH−MeOH); mp 271−272 °C; IR (film) ν_{max} 3349, 1663 1611, 1586, 1513 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.21 (3H, s, CH_3CO-), 3.78 (6H, s, CH_3O- × 2), 6.21 (1H, d, J = 2.0 Hz, H-6), 6.40 (1H, d, J = 2.0 Hz, H-8), 7.19 (2H, s, H-2′ and 6′), 12.39 (1H, s, 5-OH); ^{13}C NMR (DMSO-d_6) δ 18.8 (CH_3CO-), 56.3 (CH_3O- × 2), 94.0 (C-8), 99.1 (C-6), 103.3 (C-10), 107.4 (2C, C-2′ and 6′), 120.5 (C-1′), 127.1 (C-3), 142.9 (C-4′), 147.9 (2C, C-3′ and 5), 157.5 (C-5), 161.5 (C-9), 164.7 (C-2), 164.9 (C-7), 179.5 (C-4), 190.4 (CH_3CO-); HRFABMS (negative-ion mode) m/z 371.0791 $[M - H]^-$ (calcd for $C_{19}H_{15}O_8$, 371.0767).

5,7-Dihydroxy-3′,5′-dinitroflavone (18): yellow needles (acetone); mp >300 °C; IR (film) ν_{max} 3352, 1645, 1610, 1565, 1535, 1345 cm^{-1}; ^1H NMR (DMSO-d_6) δ 6.23 (1H, d, J = 2.0 Hz, H-6), 6.60 (1H, d, J = 2.0 Hz, H-8), 7.42 (1H, s, H-3), 8.96 (1H, br s, H-4′), 9.13 (2H, d, J = 2.0 Hz, H-2′ and 6′), 12.58 (1H, s, 5-OH); ^{13}C NMR (DMSO-d_6) δ 94.6 (C-8), 99.5 (C-6), 104.2 (C-3), 108.3 (C-10), 121.0 (C-4′), 126.6 (2C, C-2′ and 6′), 135.5 (C-1′), 148.9 (2C, C-3′ and 5′), 157.4 (C-5), 161.5 (C-9), 165.0 (C-2), 165.7 (C-7), 181.6 (C-4); HRFABMS (negative-ion mode) m/z 343.0221 $[M - H]^-$ (calcd for $C_{15}H_7O_8N_2$, 343.0202).

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