A Collagen Network Formation Effector from Leaves of *Premna subscandens*

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As a part of the search for biologically active plant products, M cells, which form a collagen fiber network *in vitro* after a prolonged culture period, were used. The *n*-BuOH-soluble fraction of a methanol extract of leaves of *Premna subscandens* exhibited promotion of collagen network formation by M cells. Extensive isolation work guided by a bioassay afforded a phenylethanoid, acteoside, as an active compound.

Key words *Premna subscandens*; Verbenaceae; acteoside; M cell; collagen

The collagen protein plays diverse important roles, forming connective tissues, basement membranes and core proteins for bone formation. However, the production of an excess amount of collagen in the liver and lung causes fibrosis and eventually, liver cirrhosis. When undergoing recovery from wounding, the rapid formation of collagen is required in the early phase, and effective resorption of excess collagen in the later phase. In each case, proper turnover of the collagen protein is necessary for the maintenance of homeostasis.

M cells were established by Katsuta *et al*. from Ito cells of rat liver by means of the Nagisa culture.[1)] This is a cell line that forms visible collagen fibers under a phase contrast optical microscope when cultured for a prolonged period with the addition of a proper amount of vitamin C. The functional collagen protein is rich in hydroxyproline, glycine and lysine. Hydroxyproline is biosynthesized through hydroxylation of proline in procollagen, with the aid of molecular oxygen and vitamin C. Since primates can not synthesize vitamin C by themselves, it is a required nutrient, otherwise they suffer from scurvy.

A decoction of *Premna odorata*, an endemic plant in the Philippines, is used as a folk medicine,[2)] and phytochemical investigation has been performed.[3)] For our random screening search for biologically active compounds using M cells, a related plant, *P. subscandens*, collected in Ishigaki Island, has been examined. Of a large number of plant extracts screened, a *n*-BuOH extract of leaves of *P. subscandens* showed a considerable stimulatory effect on the formation of a collagen fiber network. Therefore, we attempted to isolate the active compound from this plant.

In this paper, we report the isolation of an effector of collagen network formation by means of a bioassay-guided procedure.

Experimental

General Fetal bovine serum was purchased from Wittikar (MD, U.S.A.), and L-ascorbic acid phosphate magnesium salt *n*-hydrate (vitamin C), caffeic acid and trypsin were from Wako Pure Chemical Ind. Ltd. (Tokyo). Streptomycin sulfate and benzylpenicillin potassium salt were obtained from Meiji Confectionery Co., Ltd. (Tokyo). Other reagents were of biochemical grade and used without further purification.

^{1}H- and ^{13}C-NMR spectra were measured at 400 and 100 MHz, respectively, on a JEOL JNM α-400 spectrometer in CD_3OD with tetramethylsilane as the internal standard. The martynoside (**2**)[4)] and bioside (**3**)[3)] were from previous experiments.

Cell Culture M cells in culture medium containing 10% dimethylsulfoxide were stored in liquid nitrogen until use. They were subcultured in DM-160 medium supplemented with 10% fetal bovine serum, streptomycin sulfate (100 μg/ml) and benzylpenicillin potassium salt (10 U/ml) in a 60 mm plastic dish at 37 °C under a CO_2 (5%) atmosphere. After the cells had become subconfluent (about 70% confluency), 1.5×10^4 cells in 1 ml of medium were seeded onto circle cover glasses in a 24-well mutiwell plate. When the cells formed a confluent sheet, 10 μl samples in 50% EtOH and 50 μl of 4 mM vitamin C in H_2O were added. Each experiment was performed in triplicate. Every three days, the medium was replaced, and samples and vitamin C were added. After 20 d, the amount of collagen fibers formed, and morphological changes were determined under a phase contrast optical microscope. Cells were not fixed and not stained. The biological activities of additives were assessed as follows: strong inhibition, $--$ (see Fig. 2a for example); weak inhibition, $-$ (intermediate between strong inhibition and no effect); no effect (same as control), $\pm$(see Fig. 2b); weak stimulation, $+$ (intermediate between no effect and strong inhibition); strong stimulation, $++$ (see Fig. 2c).

Mallory–Azan Staining Cover glasses on which cells and collagen fibers were adhered were treated with trypsin at 0 °C under microscopic inspection. When most cells were removed, cover glasses were taken out and dried. Collagen fibers remaining were stained by the Mallory–Azan method.[5)]

Plant Material *Premna subscandens* was collected in Ishigaki Island and identified by Anki Takushi of the Okinawa Prefectural Experimental Station of Forestry, whose help the authors gratefully acknowledge.

Extraction and Isolation Air-dried leaves of *P. subscandens* (840 g) were extracted with MeOH. The precise chromatographic conditions were described elsewhere.[6)]

Acteoside amorphous powder, $[\alpha]_D^{23}$ $-81.9°$ (c=1.67, MeOH), ^{1}H-NMR (CD_3OD): essentially the same as the reported data; ^{13}C-NMR (CD_3OD): δ18.5 (C-6″), 36.5 (C-7), 62.3 (C-6′), 70.4 (C-5″), 70.6 (C-4′), 72.0 (C-3″), 72.2 (C-8), 72.3 (C-2″), 73.8 (C-4″), 76.0 (C-2′), 81.7 (C-3′), 103.0 (C-1″), 104.2 (C-1′), 114.7 (C-2‴), 115.2 (C-8‴), 116.3 (C-5‴), 116.5 (C-2), 117.1 (C-5), 121.3 (C-6), 123.2 (C-6‴), 127.6 (C-1‴), 131.5 (C-1), 144.6 (C-3), 146.1 (C-4), 146.8 (C-4‴), 148.0 (C-7‴), 149.8 (C-3‴), 168.3 (C-9‴).[7)]

Results and Discussion

As the first step, the *n*-BuOH-soluble fraction (56.3 g) of the MeOH extract of the leaves of *P. subscandens* was separated by highly porous synthetic resin (Diaion HP-20) column chromatography. The biological activity of each fraction was assayed at concentrations of 2 μg/ml and 20 μg/ml. Chart 1 shows that marked activity was concentrated in the

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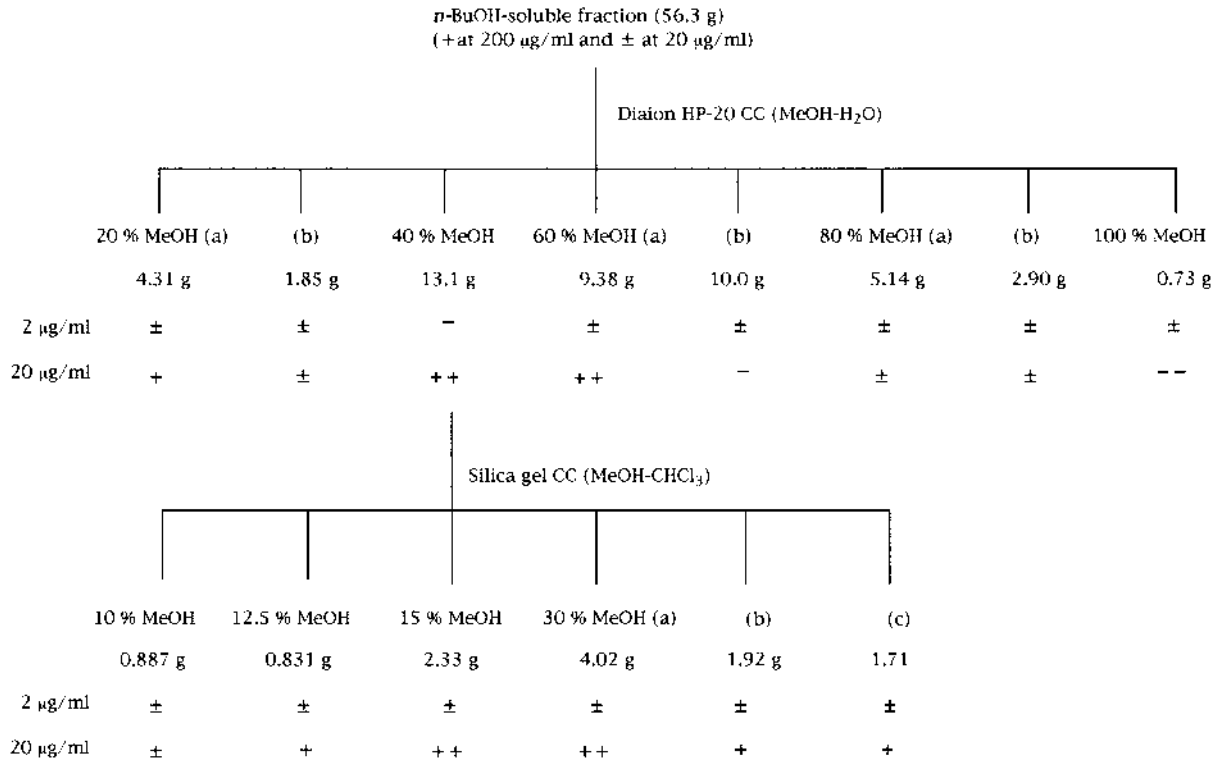

Chart 1. Bioassay-Guided Separation of the *n*-BuOH-Soluble Fraction Obtained from Leaves of *P. subscandens*

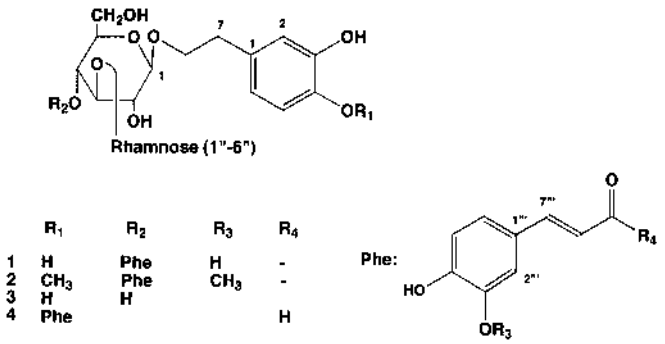

Fig. 1. Structures

40% and 60% (a) MeOH in H_2O fractions (Chart 1). The residue of one (40% MeOH eluate) of the fractions, which showed the strongest activity, was further separated by silica gel column chromatography to give active fractions in the 15% and 30% (a) MeOH in $CHCl_3$ eluates. Since the major components of the two eluates were the same on TLC (silica gel, $CHCl_3$–MeOH–H_2O, 16 : 6 : 1), the residue of the former eluate was finally purified by octadecyl silica gel [Cosmosil 75 C_{18}-OPN, MeOH–H_2O (10% MeOH to 50% MeOH in H_2O as a linear gradient), Nacalai Tesque, Kyoto] gravity column chromatography to give a homogeneous compound (1.26 g) on TLC. The structure of this compound was elucidated to be that of the known phenylethanoid named acteoside (or verbascoside) (**1**) by comparison of the ^{1}H- and ^{13}C-NMR spectroscopic data with those reported. [7]

Table 1 shows that **1** exhibited a stimulatory activity at the minimum concentration of 6 μg/ml (*ca.* 10 μM, final concentration). The stimulatory activity is shown in Fig. 2c, which shows the formation of a more complex network of collagen fibers by the addition of acteoside at a concentration of 20 μg/ml, compared with control (Fig. 2b). The relatively large black masses which can be seen in Fig. 2a—c, are not collagen fibers, but assembled M cells. The effective concentration of around 10—20 μg/ml is nearly the same as the results of other *in vitro* assays, such as the inhibition of the activities of 5-lipoxygenase,[8] protein kinase C,[9] and so on. As a means to clarify the structure–activity relationship, a structurally related compound, martynoside (**2**), and parts of acteoside, bioside (decaffeoyl acteoside) (**3**) and caffeic acid (**4**) were examined, but they did not exhibit any activity. Judging from these results, the catechol moieties in both the aglycone and acyl portions must be required for expression of the biological activity. To confirm that the fibers formed are made of the collagen protein, they were stained by the Mallory–Azan method.[5] The fibers were detected as blue lines as shown in Fig. 2d. The fibers of acteoside-treated cells (Fig. 2c) were densely distributed and thinner, when compared to those of control cells which formed sparse and rather thicker fibers.

In the case of healing from wounds, acteoside may be an effective remedy, since production of minute networks with

Table 1. Dose–Response Activity of **1** and Related Compounds (**2**, **3** and **4**)

Compound	2 3.2	4 6.4	6 9.6	8 13	10 16	15 24	20 μg/ml 32 μM
Acteoside (**1**)	±	±	+	+	++	++	++
Martynoside (**2**)	±	±	±	±	±	±	±
Bioside (**3**)	nd	nd	nd	nd	±	±	±
Caffeic acid (**4**)	nd	nd	nd	nd	±	nd	±

nd: not determined.

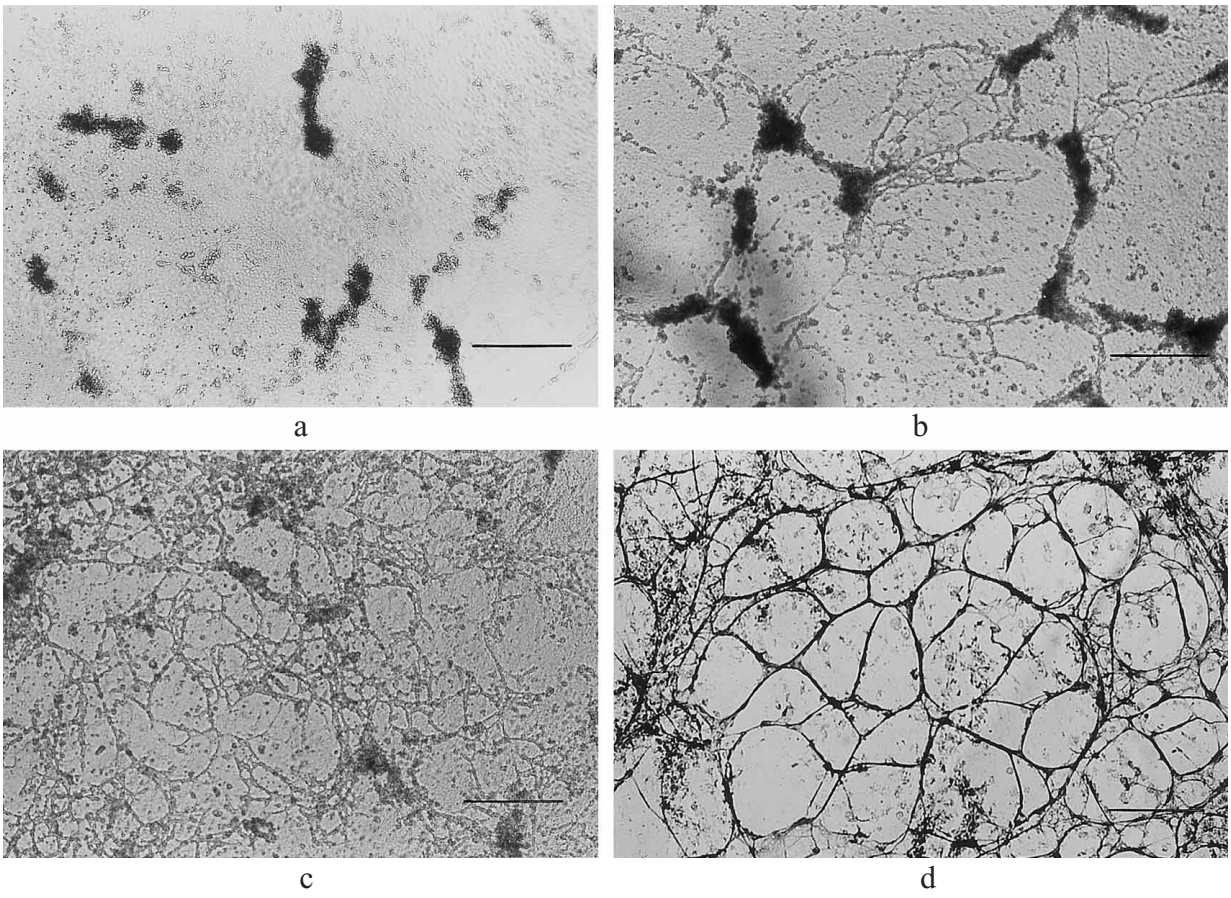

Fig. 2. Micrographs of M Cells

Bars in Fig. 2a—c indicate 250 μm and that in Fig. 2d 150 μm. a) An example of a strongly inhibited state (this micrograph is from a different experiment). b) Control. Vitamin C was added and cultured for 20 d. c) After M cells had been maintained for 20 d in the presence of acteoside at a concentration of 20 μg/ml. d) Stained collagen fibers, obtained under the same conditions of Fig. 2c, by the Mallory–Azan method.

more slender collagen fibers is favorable for rapid granulations and for avoidance of formation of ugly cicatrices which result from thick collagen fibers. Although quantitative analysis of the collagen protein formation was not performed at this time, it is of interest as to whether acteoside stimulated the production of the collagen protein or forced the formation of a complex collagen network without a change in the level of collagen protein production.

Acteoside (**1**) is a phenylethanoid widely distributed in the plant kingdom and is generally abundant in terms of yield.[10] Recently, many studies have revealed that acteoside shows a variety of biological activities.[11] Although this phenylethanoid is a known compound, our bioassay-guided search for biologically active compounds, resulted in the isolation of acteoside as a promising compound.

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