Food Sci. Technol. Res., **6** (3), 216–220, 2000

HIV-1 Reverse Transcriptase-Inhibitory Compound in *Salvia officinalis*

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Received February 28, 2000; Accepted May 11, 2000

Methanol extracts of 98 specimens of the family Labiatae were assayed for their HIV-1 revers transcriptase-inhibitory activity. Potent activity was found in the genera *Salvia* and *Glechoma*. The major inhibitory compound in *S. officinalis* was identified as oleanolic acid, a pentacyclic triterpenoid, by means of ^{1}H-NMR, ^{13}C-NMR, and EI-MS. This compound showed an IC_{50} value of 1.6–2.0 μg/ml, similar to the authentic oleanolic acid preparation. Oleanolic acid was not a common component of the genus *Salvia* and not the sole inhibitory compound in *Salvia*.

Keywords: HIV-1, reverse transcriptase, Labiatae, *Salvia officinalis*, oleanolic acid, pentacyclic triterpenoid

An old Chinese maxim says "Food and medicine have the same origin." Since older days, food has been considered to have three biological functions: nutritional, appetitive, and pharmacological. A number of foods, especially herbs and spices, have been used as traditional and folk medicines (Stuart, 1979; Bown, 1995). In the 1980s, Japanese food scientists divided the above third function into bioregulative, biodefensive, and more detailed subfunctions (Arai, 1995), according to the development of analytical techniques.

In 1983, the AIDS-causing pathogen was found to be HIV-1 (human immunodeficiency virus-1), and its genetic properties and life cycle were rapidly identified. The first anti HIV-1 drug of clinical use was 3′-azido-3′-dideoxy thymidine (AZT) and thereafter many other chemically synthesized nucleosides were in the treatments (Johnston & Hoth, 1993). These compounds are the analogs of the substrate of HIV-1 reverse transcriptase (RT), and inhibit RT competitively. Noncompetitive and non-nucleoside RT-inhibitors have been screened in nature. Inhibitory polyphenols, such as tannin and flavonoid, and terpenoids were obtained from higher plants, and glycan sulfates from seaweeds (De Clercq, 1998).

We have studied the RT-inhibitory activity in the hot water extracts of herbs and spices commercially available and many wild plants collected from deserts and tropical forests. In all cases, the RT-inhibitory compounds obtained from the hot water extracts were polyphenols, which were so large in amount that the other inhibitory compounds could not been detected (Kobayashi *et al.*, 2000).

The present paper describes the RT-inhibitory activity in methanol-extracts of herbs, spices, and related plants and the structural analysis of one of the effective inhibitors.

Materials and Methods

Plant samples Plant samples used for the first screening of potent RT-inhibitors were young whole plants belonging to the family Labiatae, i.e. 59 samples in the subfamily Lanioideae, 5 in Lavanduloideae, 6 in Ocimoideae, 2 in Rosmarinoideae, and 26 in Scutellarioideae. All these plants were the gift of Dr. T. Fukuda, Tokyo Metropolitan Medicinal Plants Garden, and were collected in July, 1996. Data on the RT-inhibitory activity of selected samples of these plants are shown in Table 1. Among 14 spp. of the genus *Salvia* examined in the second screening, *S. japonica*, *S. sclarea*, and *S. miltiorrhiza* were the gift of Prof. S. Yamanouchi, Drug Plant Garden, Coll. Pharmacy, Nihon University, collected in September, 1996, and the others were purchased from Flower shops in Tokyo the same month. Data on the RT-inhibitory activity of these samples are shown in Fig. 5.

Extraction and purification Labiatae samples were dried in the shade and powdered with a mortar to which was added 10 volumes (v/w) of methanol; they were allowed to stand for a week at room temperature, centrifuged, and the supernatants were dried *in vacuo* and used to determine the RT-inhibitory activity. The sample for identification of the RT-inhibitory compound in *Salvia officinalis* was further purified by repeated methanol extraction, chloroform/H_2O partition, methanol/*n*-hexane partition, silica gel column chromatography, and μ-Bondasphere C_{18} HPLC, as shown Fig. 1.

Instrumental analysis Molecular weight was analyzed with a JEOL LMS-SX 102A spectrometer in EI mode and the ^{1}H and ^{13}C NMR spectra in CD_3COCD_3 by a JEOL A600. Oleanolic acid used as standard was purchased from Sigma Aldrich Japan, Tokyo.

Assay of HIV-1 RT activity The inhibitory activity was assayed according to the method of Ono *et al.* (1989). In short, 20 μl of the sample, 10 μl of H_2O, 10 μl of enzyme mixture (2.0 μl of 40 mM Tris-HCl, pH 8.0, 0.4 μl of 8 mM DTT, 1.5 μl of 30 mM KCl, 0.3 μl of 6 mM $MgCl_2$, 0.2 μl of 2 μg/ml poly(A) $p(dT)_{15}$, 0.5 μl of 10 μM dTTP, 1.0 μl of 0.37 μM [^{3}H]-dTTP, 2.5 μl of glycerol, and 6 μl of H_2O), and 10 μl of HIV-RT 0.01U were placed in an Eppendorf tube in this sequence and the enzyme reaction was begun at 37°C for 30 min. The reaction was stopped by addition of 20 μl of 0.2 M EDTA-2Na, and the mixture was filterted through a DE81 nitrocellulose filter, washed, and dried. Radioactivity on the filter was assayed by a Beckman

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Table 1. RT-inhibitory activity in crude methanol extracts of the selected spp. and related ones in the family Labiatae.

Scientific name	Subfamily	Inhibition %[a]
Glechoma hederacea v. *gradis*	Lamioideae	93
Lycopus incidus	Lamioideae	87
L. uniflorus v. *parviflorus*	Lamioideae	78
Mentha arvensis v. *piperascens*	Lamioideae	72
M. longifolia	Lamioideae	84
Monarda didyma cv. *berggamot*	Lamioideae	91
Prunella vulgaris v. *lilacina*	Lamioideae	84
Salvia miltiorrhiza	Lamioideae	89
S. nipponica	Lamioideae	89
S. officinalis	Lamioideae	80
Ocimum basilicum	Ocimoideae	88
Plectranthus effusus	Ocimoideae	86
Rosmarinus officinalis	Rosmarionoideae	75
R. officinalis cv. creeping	Rosmarionoideae	80

[a]Sample concentration is 100 μg crude methanol extract/ml.

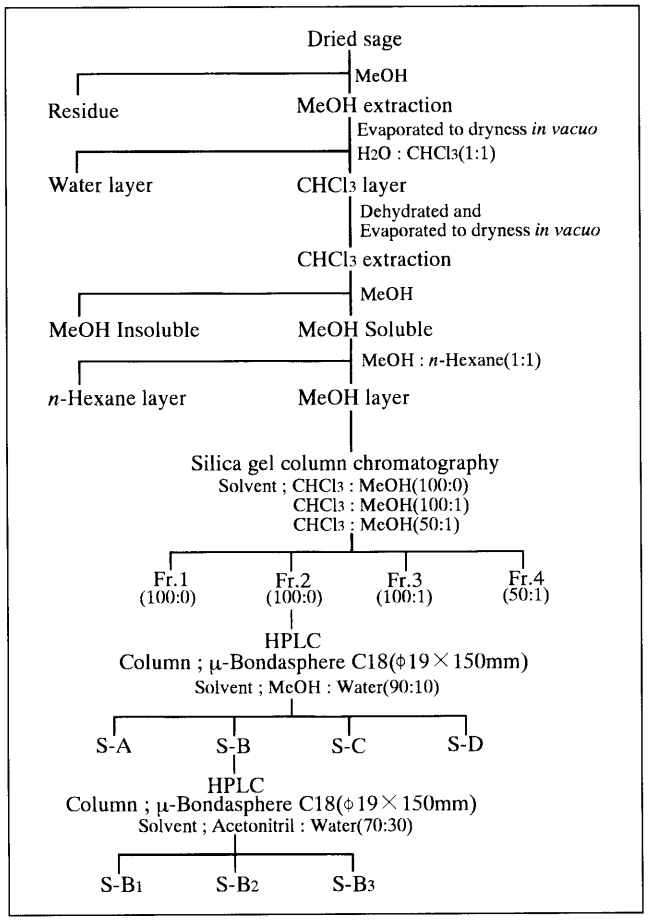

Fig. 1. Procedures for extraction and purification of HIV-1 RT-inhibitory compounds in *Salvia officinalis*.

LS6000TA liquind scintillation counter. HIV-1 RT was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo) and poly(A) $p(dT)_{15}$ was from Boehringer-Manheim-Yamanouchi (Tokyo).

Results

RT-inhibitory activity in family Labiatae The family Labiatae is divided into 9 subfamilies. In this study, 98 plants from 5 subfamilies were examined. RT-inhibitory activity in the crude methanol extracts of these specimens was assayed and found in 8 spp. of Lamioideae, 2 spp. of Ocimoideae, and 2 spp. of Rosmarinoideae. As shown in Table 1, the most potent activity, more than 70% inhibition at 100 μg/ml, was observed in the extracts of *Glechoma hederacea* v. *gradis*, *Monarda digyma* cv. *berggamot*, and 12 other spp. Because of the shortage in sample number, it is not clear whether the activity distributes genus-specific ally or species-specific ally, but it can be said that the genera *Glechoma*, *Monarda*, and *Salvia* are good sources of the HIV-1 RT-inhibitor.

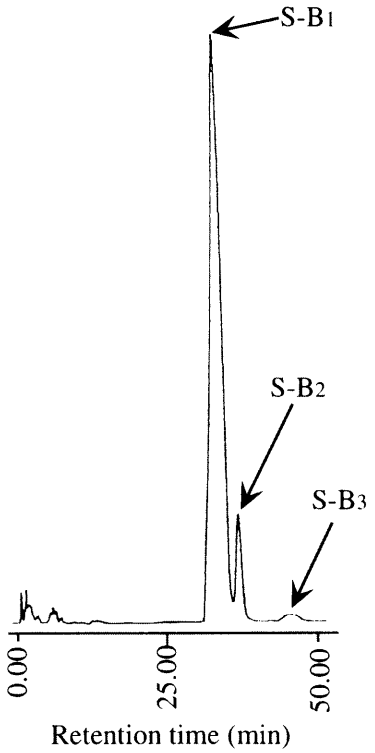

Fig. 2. HPLC profiles of S-B_1, S-B_2, and S-B_3. Column; μ Bondasphere C18 (φ19×150 mm), Solvent; Acetonitrile : Water (70 : 30), Flow rate; 12 ml/min, Detection; UV210 nm.

Table 2. Physical and chemical properties of S-B_1.

Solubility		
Soluble in	*n*-BuOH, CCl_4, $CHCl_3$, DMSO, EtOAc, EtOH, Me_2CO, MeOH, Toluene	
Insoluble in	*n*-Hexane, H_2O	
Color reaction		
Positive	Dichlorophenol indophenol	(dark red)
	2,4-Dinitrophenylhydrazine	(brown)
	Phosphomolybdic acid reagent	(blue)
	Antimony trichloride	(wine red)
	Phosphomolybdec acid	(blue)
Negative	$FeCl_3$	
	Nynhydrin	

Purification and structure analysis of the RT-inhibitory compound in Salvia officinalis Among the above 14 samples showing potent RT-inhibitory activity, *Salvia officinalis* was selected for further study because it showed rather high inhibitory activity and was easily obtained from market. Twice silica gel column chromatography and twice μ-Bondasphere HPLC, showed the RT-inhibitory activity to be concentrated in S-B_1 fraction (Fig. 2). An off-white powdery preparation was obtained

from this fraction and, as shown in Table 2, the results of color reaction indicated that this compound would be steroid or triterpenoid carrying carboxyl group(s).

As shown in Table 3, the ^{1}H-NMR spectrum showed the presence of 48 protons and absence of glycosyl and galoyl groups. The ^{13}C-NMR spectrum exhibited the presence of 29 carbons and signals of the terpenoid ring in the range of δ 30–50 ppm and that of the carboxyl group at δ 178 ppm. The EI-MS spectrum showed a molecular peak of m/z 456 [M], and also fragment peaks of m/z 248 and m/z 203, indicating that S-B$_1$ was composed of an oleanane skeleton and its molecular formula was $C_{30}H_{48}O_3$. (Fig. 3) Confirmation of the presence of the oleanane structure was made by comparing the ^{1}H-NMR spectrum (Fig. 4) of S-B$_1$ with that of oleanolic acid. S-B$_1$ was identified to be ole-

Table 3. ^{13}C-NMR and ^{1}H-NMR data of S-B$_1$ in acetone-d_6.

Position	δ_C	δ_H
1	39.3	1.58 (2H, m)
2	28.1	1.55–1.57 (4H, m)
3	78.8	3.15 (1H, dd, J=11.4, 5.4, 4.2 Hz)
4	39.4	
5	56.2	
6	19.1	
7	33.7	1.31 (2H, td, J=12.6, 4.3, 4.1 Hz)
8	40.2	
9	48.5	1.61 (1H, m)
10	37.8	
11	24.1	1.89 (2H, dd, J=9.0, 3.6 Hz)
12	123.0	5.24 (1H, t, J=7.2, 3.6 Hz)
13	144.9	
14	42.5	
15	28.5	1.80 (2H, m)
16	23.8	2.03 (2H, m)
17	46.9	
18	42.3	2.89 (1H, m)
19	46.8	1.72 (2H, s)
20	31.3	
21	34.5	1.41 (2H, m)
22	30.1	
23	16.3	0.78 (3H, m)
24	28.7	0.99 (3H, s)
25	15.8	0.94 (3H, m)
26	17.6	0.81 (3H, s)
27	26.3	1.17 (3H, s)
28	178.9	
29	23.9	0.95 (3H, m)
30	33.4	0.92 (3H, s)

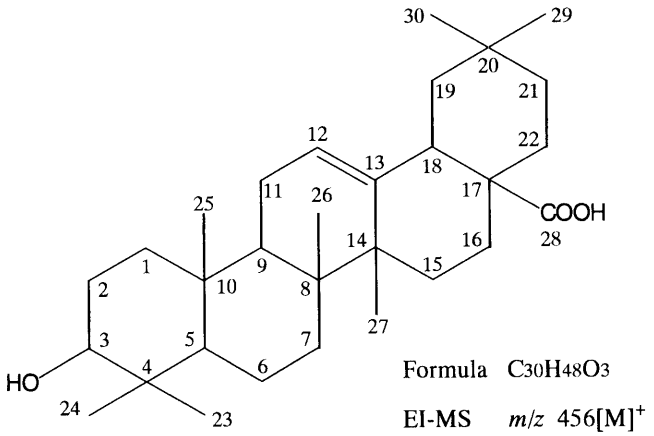

Fig. 3. Structure of RT-inhibitory compound in *S. officinalis* (S-B$_1$).

Table 4. HIV-1 RT-inhibitory activity of S-B$_1$ and oleanolic acid.

	Inhibition (%)			
	Concentration (μg/ml)			
	10	5	2.5	1
S-B$_1$	93	85	55	20
Oleanolic acid	95	91	70	42
S-B$_1$+Oleanolic acid (1 : 1)	97	93	70	22

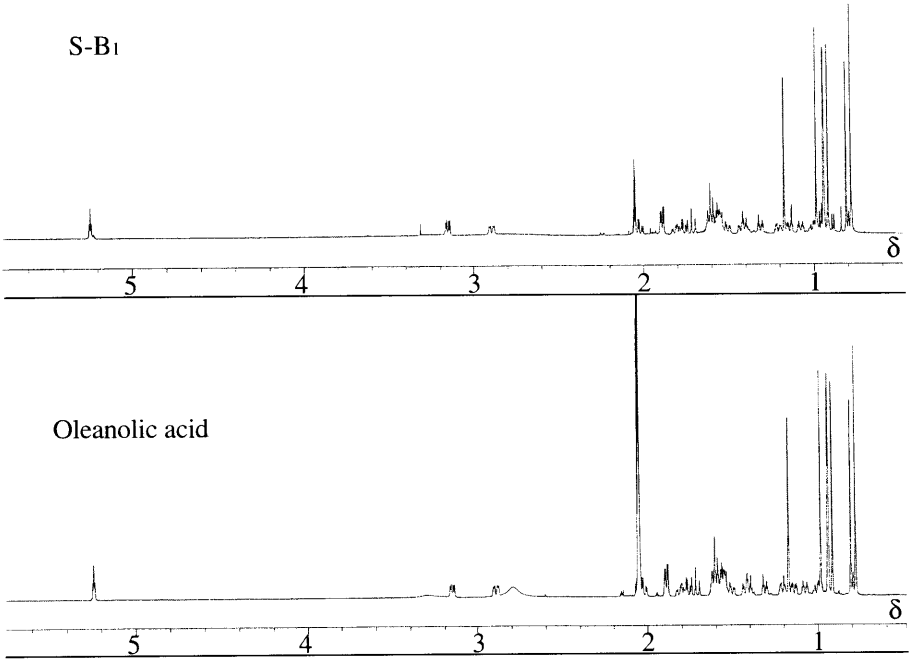

Fig. 4. ^{1}H-NMR spectra of S-B$_1$ and authentic oleanolic acid.

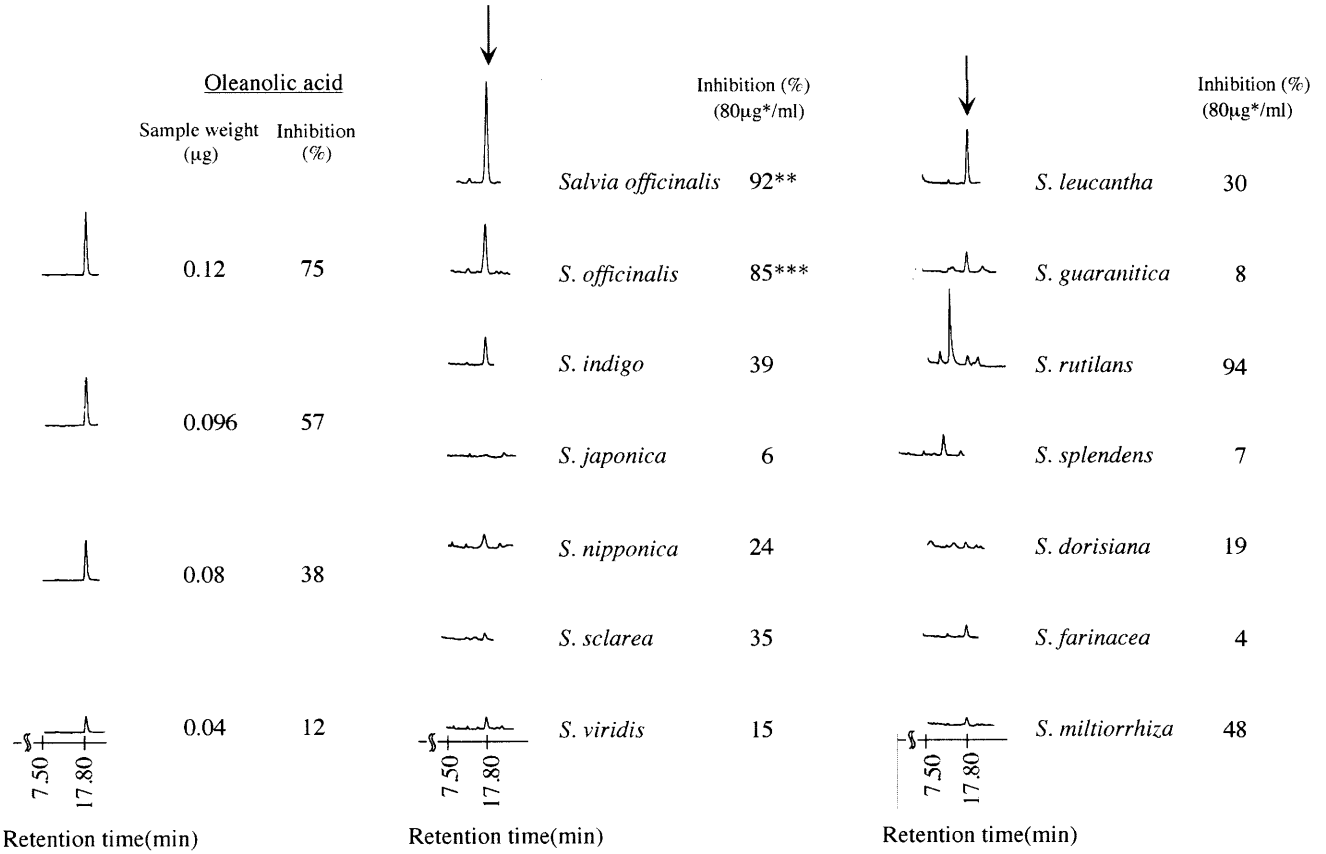

Fig. 5. HPLC profiles and HIV-1 RT-inhibitory activity of methanol extract of *Salvia* spp. *: Crude MeOH extract; **: commercial dry powder; ***: fresh plant from flower shop.

anolic acid. The RT-inhibitory activity of S-B_1 was similar to that of oleanolic acid (Table 4).

Distribution of RT-inhibitory activity and oleanolic acid in genus Salvia Whether oleanolic acid is common in plants of the genus *Salvia* and if its amount is parallel to the RT-inhibitory activity were studied. A peak giving the retention time of 17.80 on μ-Bondasphere C_{18} HPLC (acetonitrile : water=70 : 30, UV 210 nm) was regarded as oleanolic acid. Contrary to expectations, not all the methanol extracts of *Salvia* spp. showed potent inhibitory activity; no or weak activity was observed in a number of them (Fig. 5). In *S. officinalis*, *S. indigo*, and *S. leucantha*, the inhibitory activity was present at the peak corresponding to oleanoic acid and the inhibitory potency was parrarel to the peak level, but this was not the cases of *S. rutilans* and *S. miltiorrhiza*.

These results indicate that the presence of RT-inhibitory activity and of oleanolic acid would not be genus-specific and that the activity in at least a *Salvia* sp. would change according to the specimens used.

Discussion

More than 500 spp. of the genus *Salvia* distribute throughout the world and 18 spp. in Japan (Sakurai, 1984). *Salvia officinalis* is one of the typical herbs used from earliest time for flavoring, improving, preserving, and diversifying everyday foods. It has also been used in the medicinal field as a digestive, hypoglycemic, cholagogic, ammenagogic, anti-asthmatic, carminative, antiseptic, and so on (Stuart, 1979; Bown, 1995).

We clarified in this study that the methanol extract of *S. officinalis* carries potent HIV-1 RT-inhibitory activity and that the major inhibitory compound in this herb is one of the pentacyclic triterpenoics, oleanolic acid. IC_{50} of this compound against HIV-1 RT was calculated to be 1.6–2.0 μg/ml. Oleanolic acid is known to be present in *Olea europaea* (olive), *Betula platyphylla* (birch), and *Eugenia caryophyllata* (clove) rather than in *Salvia* spp. (Connolly & Hill, 1991).

Among various triterpenoids, glycyrrhizin (Ito *et al.*, 1988), betulinic acid and its derivatives (Fujioka *et al.*, 1994; Kashiwada *et al.*, 1996; Evers *et al.*, 1996; Soler *et al.*, 1996), and nigranoic acid (Sun *et al.*, 1996) are famous for their anti-HIV-1 activity. Glycyrrhizin, belonging to the oleanane group, was regarded as a moderate HIV-inhibitor in the early stage of studies on anti-HIV-1, but the major function of this compound may not be RT-inhibition. Betulinic acid and its derivatives, belonging to the lupane group, have attracted the attention of many researchers because they show a strong anti-HIV-1 activity caused by interference with the entry of HIV-1 to the host cells and with its replication in the cells. RT-inhibition was not noteworthy in these compounds. Nigranoic acid was isolated from a Chinese medicinal plant and is the only example evaluated to be a RT-, DNA polymerase-, and RNA polymerase-inhibitor, though the IC_{50} value against HIV-1 RT was as low as 74 μg/ml. Quere *et al.* (1996) reported the presence of HIV-1 protease inhibitory activity in triterpenoids such as oleanolic acid, ursolic acid, betulinic acid and so on.

Numerous reports on the effects of non-nucleoside inhibitors against HIV-1 RT and HIV-1 cytopathicity have been published (De Clercq, 1998). Studies on the quantitative structure-activity relationship were reported between HIV-1-RT and the non-nucleoside inhibitors, i.e. HEPT and carboxanilide derivatives (Kireev *et al.*, 1997; Ren *et al.*, 1998). The relationship between oleanoic acid related-triterpenoids and HIV-1-RT will be introduced in our subsequent research.

Acknowledgments The authors thank Tatsuo Fukuda and Sakae Yamanouchi for their gifts of plant specimens and Katsuhiko Ono for his guidance on RT-inhibition analysis.

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