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HIV Integrase Inhibitory Activity of *Agastache rugosa*

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We have been screening anti-HIV integrase compounds from Korean medicinal plants by using an *in vitro* assay system which is mainly composed of recombinant human immunodeficency virus type 1 integrase and radiolabeled oligonucleotides. From the above screening, the aqueous methanolic extract of the roots of *Agastache rugosa* exhibited a significant activity. Bioactivity-guided chromatographic fractionation of the methanolic extract resulted in the isolation of rosmarinic acid. The structure of the compound was determined by spectroscopic data and by the comparison with the reported values. The IC_{50} of the rosmarinic acid was approximately 10 μg/ml against HIV integrase.

Key words: HIV integrase, *Agastache rugosa*, Labiatae, Rosmarinic acid

INTRODUCTION

Human immunodeficiency virus (HIV) integrase is an enzyme that incorporates the double-stranded DNA product resulting from the reverse transcription of viral RNA into a host genome, and is essential for viral replication and viron production. This enzyme is a potential target for selective antiviral therapy because there is no known functional analog in human cells (Fesen *et al*, 1993). Development of inhibitors targeted for HIV integrase requires an enzyme-specific *in vitro* test system. For *in vitro* assay of HIV integrase, short duplex oligonucleotides corresponding to the ends of linear viral DNA have been used as substrates. Endonucleolytic cleavage of two nucleotides from the 3' end and strand transfer between oligonucleotides, which serve both as mimic of the viral DNA ends and as integration target, were observed. Both cleavage and joining can be detected by using radiolabelled substrate with subsequent analysis of reaction products on autoradiograph of denaturing polyacrylamide gels (Oh and Shin, 1996; Sherman and Fyfe, 1990). We have been screening HIV integrase inhibitory compounds from Korean medicinal plants by using the above *in vitro* assay system and isolated an active compound from the roots of *Agastache rugosa*. *A. rugosa* which belongs to Labiatae has been used for the treatment of cholera, vomiting, and miasma (noxious air) (Lee *et al*, 1976). Monoterpenes (α-pinene, β-pinene and *p*-cymene), sesquiterpenes (α-ylangene, caryophyllene and calamenene) and flavonoids (agastachoside, agastachin, acacetin and tilianin) have been isolated from the aerial parts of this plant (Lee *et al*, 1995). From the roots, triterpenes (erythrodiol-3-*O*-acetate, oleanolic acid, 3-*O*-acetyl oleanolic acid, 3-*O*-acetyl oleanolic aldehyde and maslinic acid) and diterpenes (dehydroagastol, agastanol) have been also isolated (Han, 1987; Han *et al*, 1987). The present report describes the extraction and purification of the compound that shows inhibitory activity against HIV integrase from this plant.

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MATERALS AND METHODS

General experimental procedures

^{1}H-NMR and ^{13}C-NMR spectra were run on a JEOL JNM-LA 300 spectrometer in CD_3OD at 300 MHz and 75 MHz, respectively, with TMS as an internal standard. TLC was carried out on silica gel precoated plates (Art. No. 5715, Merck).

Plant materials

The roots of *Agastache rugosa* were collected in Yangsan, Korea. After dried in the shade, they were chopped finely for extraction. The voucher specimen is deposited in Korea Research Institute of Bioscience and Biotechnology (KRIBB), Korea.

Extraction and isolation

Dried roots (4.5 kg) were extracted with 90% methanol at room temperature and the extract was concentrated under reduced pressure. The concentrated aqueous residues were partitioned with *n*-hexane, ethylacetate and *n*-butanol, successively. Activity-guided separation of the ethylacetate fraction, which showed strong activity in preliminary screening, was carried out with a series of column chromatography (silica gel : chloroform-methanol mixture gradient : 15:1→1 : 1, v/v, RP-18 : methanol-water mixture : 7 : 3, v/v, and Sephadex LH-20 : chloroform-methanol mixture 2 : 1→100% methanol). Compound **1** (415 mg) was isolated and purified using HPLC [RP-18, 10 × 250 mm, acetonitrile-phosphate buffer (0.1M, pH 3.5) 35:65].

Compound 1 (Fig. 1)

^{1}H-NMR (300 MHz, CD_3OD) δ; 7.56 (1H, d, *J*=15.9, H-7), 7.05 (1H, d, *J*=2.1, H-2), 6.95 (1H, dd, *J*=2.1, 8.1, H-6), 6.78 (1H, d, *J*=8.1, H-5), 6.76 (1H, d, *J*=1.8, H-2'), 6.71 (1H, d, *J*=8.1, H-5'), 6.62 (1H, dd, *J*=1.8, 8.1, H-6'), 6.27 (1H, d, *J*=15.9, H-8), 5.20 (1H, dd, *J*=4.5, 8.1, H-8'), 3.11 (1H, dd, *J*=4.5, 14.1, H-7'), 3.01 (1H, dd, *J*=8.1, 14.1, H-7') ^{13}C-NMR (75 MHz, CD_3OD) ; 127.7 (C-1), 115.2 (C-2), 146.8 (C-3), 149.7 (C-4), 116.5 (C-5), 123.2 (C-6), 147.8 (C-7), 114.4 (C-8), 168.5 (C-9), 129.3 (C-1'), 117.6 (C-2'), 145.3 (C-3'), 145.2 (C-4'), 116.3 (C-5'), 128.8 (C-6'), 37.9 (C-7'), 74.6 (C-8'), 173.5 (C-9')

Bioassays

Recombinant HIV-1 integrase was expressed in *Escherichia coli* and purified using a nickel-chelated column in a one-step manner, as described previously (Oh and Shin, 1996). Aliquots of HIV-1 integrase of 0.5 mg/mL as stock solutions were stored at -70°C until used. Two 20-mer oligonucleotides whose sequences resemble the end of U5-LTR were obtained from Korea Biotech., Inc. (Seoul, Korea), namely K16 (U5-LTR, +strand), 5'-TGTGGAAAATCTCTAGCAGT-3'. The oligonucleotides were purified using 20% polyacrylamide gel before use. To construct the oligonucleotide substrate, oligonucleotide K16 (15 pmol) was labeled at the 5' end, using [γ-^{32}P]-ATP of 250 mCi (3,000 Ci/ mmol; 1 Ci=37 GBq; Amersham Life Science, Arlington Heights, IL, USA) and T4 polynucleotide kinase (T4 PNK, New England Biolabs, Beverly, MA, USA) of 10 units in 40 mL of reaction buffer (70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT) for 15 min at 37°C . The labeling reaction was subjected to 10 mM EDTA and heated to 85°C for 15 min to inactivate T4 PNK. After the addition of complementary oligonucleotide K17 (30 pmol), the reaction mixture was boiled for 3 min and cooled slowly. Labeled substrate was separated from unincorporated nucleotide by passage through a Biospin 6 instrument (Bio-Rad, Hercules, CA, USA). A standard reaction assay of endonucleolytic activity was carried out in the presence of potential inhibitor containing 0.1 pmol of duplex oligonucleotide substrate and 15 p mol of HIV-1 integrase in 15 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM $MnCl_2$, 2 mM 2-mercaptoethanol, 2.5 mM CHAPS, 0.1 mM EDTA, 0.1 mM PMSF, 1% glycerol, and 10 mM imidazole in a total volume of 10 mL. Inhibitors or plant extracts were dissolved in 100% DMSO and added to the reaction mixture. Reaction mixtures were incubated at 33°C for 90 min and stopped by the addition of 4 mL of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF. The reactions were heated to 90°C for 3 min and subjected to electrophoresis on a 20% denaturing polyacrylamide gel. Reaction products were visualized by autoradiography of the wet gel. The IC_{50} values were calculated by scanning bands on a phosphoimage analyzer (GS525, BioRad) (Oh and Shin, 1999).

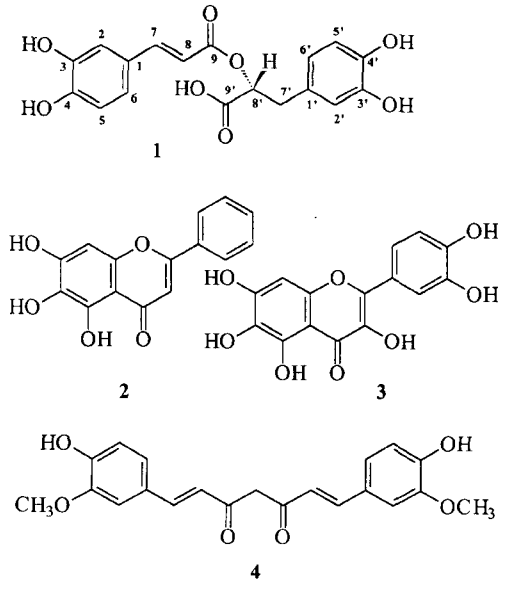

Fig. 1. Structures of HIV-1 integrase inhibitors from plants.

RESULTS AND DISCUSSION

The methanol extract of *A. rugosa* which showed significant inhibitory activity against HIV integrase was subjected to successive purification and isolation. The silica gel column chromatography of the EtOAc fraction with a stepwise gradient eluent from chloroform-methanol 15:1 to 1:1 gave several fractions. After successive column chromatography on RP-18 and Sephadex LH-20, compound **1** (415 mg) was isolated and finally purified using HPLC [RP-18, 10 × 250 mm, acetonitrile-phosphate buffer (0.1 M, pH 3.5) 35:65] (see MATERIALS and METHODS). The ^{1}H-NMR spectrum contained the signals for six aromatic protons at δ 7.05 (1H, d, *J*=2.1 Hz), 6.95

(1H, dd, J=2.1, 8.1 Hz), 6.78 (1H, d, J=8.1 Hz), 6.76 (1H, d, J=1.8 Hz), 6.71 (1H, d, J=8.1 Hz), 6.62 (1H, dd, J=1.8, 8.1 Hz), corresponding to two 1,2,4-trisubstituted phenolic groups. In addition, ^{1}H-NMR spectrum also showed the signals for two trans olefinic bond protons at δ 7.56 (1H, d, J=15.9 Hz), 6.27 (1H, d, J=15.9 Hz), suggesting it has a trans-caffeoyl moiety. This was supported by the ^{13}C-NMR spectrum, which in the low field showed the signals for a caffeoyl moiety at δ 168.5, 149.7, 147.8, 146.8, 127.7, 123.2, 116.5, 115.2, 114.4. The ^{13}C-NMR also showed another carbonyl car-bons at 173.5 and one methylene carbons at δ 37.9. From the above evidence and responding literature data (Kelly *et al,* 1975; Kelly *et al,* 1976), compound **1** was concluded to be a rosmarinic acid (RA). And it was confirmed by co-chromatography with an authentic sample. RA has been reported to occur in several species of the family belonging to Labiatae and Boraginaceae. RA has an antimicrobial, antiviral and antiphlogistic effects, which makes it a valuable product for the pharmaceutical and cosmetic industries (Kuhnt *et al,* 1994; Pearson *et al,* 1997). We examined the HIV inhibitory effects of RA. At the concentration of 10 μg/ml, it inhibited the activity of integrase by 50%, and completely inhibited at 25 μg/ml. Recent SAR studies of HIV-1 integrase inhibitors (Fesen *et al,* 1994; LaFemina *et al,* 1995) showed that the major requirement for potent inhibition of HIV-1 integrase was two vicinal hydroxyl groups on an aromatic ring. Replacement of one or both adjacent hydroxyls with a methoxy group severely reduces potency or renders the compounds inactive, while addi-tion of a third adjacent hydroxyls enhances potency as shown in baicalein (**2**), quercetagetin (**3**: Fesen *et al,* 1994) and gallic acid derivatives (Kim *et al,* 1998). The anti-HIV-1 integrase activity of curcumin (**4**) and its struc-tural analogues were also reported previously (Mazu-mder *et al,* 1995; Mazumder *et al,* 1997). Among these analogues, the structures which had at least one catechol substructures were found to be very potent. Therefore, potent inhibition may require at least two adjacent hydroxyls in space but not necessarily *ortho* to each other on the same ring. Taken together with the results accumulated so far, for possible searching of anti-HIV-1 integrase lead, the chemicals possessing two vicinal hydroxyl groups on an aromatic ring or aromatic rings linked with an appropriate linker with at least a hydroxyl group on each aromatic ring should be considered first. However, since these compounds are reported to be cytotoxic or inactive in cellular assay (Mazumder *et al,* 1997), completely new pharmacophore should be sought from the natural sources along with the structural modification studies of these phenolic compounds isolated so far.

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