Neuritogenesis of Herbal (+)- and (–)-Syringaresinol Separated by Chiral HPLC in PC12h and Neuro2a Cells

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Syringaresinol isolated from *Epimedium koreanum* NAKAI and *Magnolia officinalis* REHD. et WILS. was subjected to optical resolution by chiral HPLC to give (+)- and (–)-enantiomers. The two syringaresinol enantiomers, as well as a mixture of their glucosides, showed dose-dependent neuritogenesis in a concentration range from 0.24 to 24 μ M in PC12h cells.

Key words syringaresinol; optical isomer; neuritogenesis; PC12h cell; *Epimedium koreanum*

We have carried out a continuing study of the neuron-stimulative substance in plants and found many lipophilic constituents that promote the differentiation (neurite outgrowth) of paraneurons, such as PC12h cells.^{1,2)} The lipophilic extracts of the aerial parts of *Epimedium koreanum* NAKAI or *Epimedium macranthum* MORR. et DECNE. have been demonstrated to have neuritogenetic activity in PC12h cells and stimulate these cells excitedly to respond to carbachol.²⁾ We have preliminarily found an active component of the Et₂O extract of *E. koreanum* syringaresinol, which was found as itself or its glucosides in many of traditional Chinese drugs, including *Acanthopanax senticosus* HARMS³⁾ and the bark of *Eucommia ulmoides* OLIV.⁴⁾ It was previously shown to have a prominent effect on PC12h cells.⁵⁾ But the syringaresinol samples we isolated from the extract of *E. koreanum* seemed to be peculiarly racemic mixtures on the data of HPLC analysis with a chiral stationary phase. We report here the separation of optical isomers of syringaresinol from each of two species of plant, and the comparison of the biological activity of individual enantiomers and a glucoside mixture.

MATERIALS AND METHODS

The concentrated Et₂O extract (35.2 g) of *E. koreanum* collected in northern China in July, 1998 was prepared at the Tianjin Institute of Medical and Pharmaceutical Science and chromatographed on a silica gel (Kieselgel 60) column by a successive elution with CHCl₃, CHCl₃–MeOH (3 : 1) and MeOH (each 2 l). The evaporation residue (17.5 g) of the CHCl₃ fraction was chromatographed on a silica gel column by a successive elution with *n*-hexane, *n*-hexane–EtOAc (5 : 1), benzene–EtOAc (1 : 1), benzene–EtOAc–MeOH (1 : 1 : 0.1) and benzene–EtOAc–MeOH (1 : 1 : 1). The most bioactive fraction from the benzene–EtOAc–MeOH (1 : 1 : 0.1) eluate was chromatographed on TSK HW 40 (Tosoh, Japan) with an eluent, MeCN–H₂O (1 : 1). The main fraction of the eluate was purified by Sephadex-LH20 chromatography with MeOH–H₂O (1 : 1) to give syringaresinol (3 mg), which was identified by comparison with UV, MS and NMR spectral data of an authentic sample.

Magnolia officinalis REHD. et WILS., collected in China, was purchased from Matsuura Yakugyo Co., Ltd. (Nagoya, Japan). The voucher specimen (No 228110) was deposited in the natural product laboratory of the Research Laboratory, Zenyaku Kogyo Co., Ltd. Syringaresinol-4'-*O*- β -D-glucoside (100 mg) isolated from the bark of *M. officinalis* according to the method of Yahara *et al.*⁶⁾ was hydrolyzed by β -glucosidase (Sigma, U.S.A.) in 0.1 M acetate buffer (pH 5.0) at 37 °C overnight. The reaction mixture was extracted three times with EtOAc to afford syringaresinol (31 mg). Each of the two racemic syringaresinol samples obtained from *E. koreanum* and *M. officinalis* was subjected to HPLC using a Chiralcel OD column (Daicel Chemical, Japan) and EtOH–*n*-hexane (8 : 2) as a mobile phase to give optically pure isomers (colorless needles).

Optical rotations were measured on a Perkin-Elmer 247 polarimeter. High resolution mass spectra were recorded on a JMS-SX102 MS instrument. NMR spectra were recorded on a JEOL EX270 NMR spectrometer. HPLC was performed on a Chiralcel OD (Daicel Chemical) column (0.46 $\times$ 25 cm) using a JASCO TRI ROTAR-V PUMP with a UVIDEC-100-V UV detector (eluent, EtOH–*n*-hexane (8 : 2) (flow rate, 0.5 ml/min; detection, UV at 254 nm).

PC12h cells were generously donated by Dr. Hatanaka, Osaka University, Japan, and cultured in Dulbecco's modified Eagle medium supplemented with precolostrum calf serum and heat-inactivated horse serum for growth, as described previously.⁷⁾ Neuro2a cells were purchased from the Institute for Fermentation, Osaka, Japan, and cultured in Eagle's minimum essential medium supplemented with non-essential amino acids and fetal bovine serum for growth. After 24 h of culture, the culture medium was replaced by serum-free medium to treat these cell lines with test compounds as specified in the results. Syringaresinol-4'-*O*- β -D-glucoside was dissolved in distilled water and (+)- and (–)-syringaresinol in 75% dimethylsulfoxide before use. The neurite outgrowth of these cells was evaluated by measuring the length of the longest neurites of individual cells following 48 h of treatment with the test compounds as described previously.⁷⁾ One hundred cells in ten random fields in two culture dishes were

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averaged for each treatment.

RESULTS AND DISCUSSION

Optical Resolution by HPLC of Syringaresinol Syringaresinol samples isolated from *E. koreanum* and *M. officinalis* were identified by comparison with UV, MS and NMR spectral data of an authentic sample. Their optical rotation was estimated at almost naught, but varied from sample to sample. So they were then subjected to chiral chromatography (Fig. 1). On the basis of the results, the syringaresinol samples were supposed to be racemic mixtures.

Actually, identification of (+)- and (−)-syringaresinol on the chromatograms was done by comparison with those of syringaresinol samples isolated from *M. officinalis*, because the amount was not enough to determine the optical rotation of separate enantiomers of samples from *E. koreanum*. The physical data of the enantiomers (in preceding and subsequent eluates, respectively) from *M. officinalis* are (+)-syringaresinol ($[\alpha]_D^{20} +53.3^\circ$, $c=0.36$, CHCl_3 ; mp $187.5\text{--}189.5^\circ\text{C}$) and (−)-syringaresinol ($[\alpha]_D^{20} -50.6^\circ$, $c=0.32$, CHCl_3 ; mp $188.5\text{--}190.5^\circ\text{C}$), occurring in the ratio of *ca.* 2 : 1 according

Fig. 1. Optical Resolution of Syringaresinol Samples by HPLC

The syringaresinol samples were applied to a Chiralcel OD column ($0.46 \times 25\text{ cm}$) and eluted isocratically with EtOH-n-hexane (8 : 2) at a flow rate of 0.5 ml/min, with absorption monitored at 254 nm. The elution profiles (A and B) represent syringaresinol samples isolated from *E. koreanum* and *M. officinalis*, respectively. I and II indicate (+)- and (−)-syringaresinol, respectively.

Fig. 2. The Neuritogenesis of Syringaresinol-4'-O- β -D-glucoside and (+)- and (−)-Syringaresinol in PC12h and Neuro2a Cells

Cells were cultured with or without (control) concentrations of test compounds as indicated for 2 d. The neurite outgrowth was measured as described in Materials and Methods for 100 cells in each group and compared with the control, or between treated groups. The values are shown by the means $\pm$ S.E.M. All of the means of treated groups are significantly ($p < 0.01$) large compared with the control. Statistical analysis was performed by ANOVA and Bonferroni's test.

to the elution profile on chiral chromatography.

The Neuritogenic Activity of Optical Isomers of Syringaresinol in PC12h and Neuro2a Cells The neuritogenicity of (+)-syringaresinol obtained from *S. baicalensis* and (−)-syringaresinol from *Scutellaria scandens* BUCH.-HAM. ex D. DON was already reported with PC12h cells.⁵⁾ Here, we elaborate upon the effect of isolated enantiomers of syringaresinol as well as their glucoside mixture from the bark of *M. officinalis* on PC12h and Neuro2a cells. Consistent with our previous results, all of the glucosides and the two enantiomers were individually effective on the neurite outgrowth in both PC12h and Neuro2a cells (Fig. 2). At about $0.2\text{ }\mu\text{M}$ of these compounds the effect was comparable between them in either cell line. But at higher doses (about 2 and $20\text{ }\mu\text{M}$) of each, two enantiomers showed a generally stronger effect than the glucoside, especially, the (−)-isomer was discernibly more effective than any of the other compounds at a concentration of $24\text{ }\mu\text{M}$ in either cell line. The neurite outgrowth activity of the mixture containing two enantiomers of syringaresinol was about similar in extension to the additive activity of each isomer (data not shown).

All of these results indicate that both (+)- and (−)-syringaresinol, as well as a mixture of optical isomers of syringaresinol-4'-O- β -D-glucoside, are effective on neuritogenicity in cultured paraneurons, and that a racemic mixture of (+)- and (−)-syringaresinol would be responsible, at least partly, for the neurite outgrowth, *i.e.* cell differentiation or activation, of the paraneurons with the Et_2O extract of *E. koreanum*. It may be interesting to investigate the effect of these isomers on such activities of the central nervous system of animals as memory and behaviors, because the Chinese raw drug "Japanese name; Inyokaku" made of the dried herb of *Epimedium* species is an important element in many tonics, and is used in the treatment of aging-related amnesia and impotence.⁸⁾

Syringaresinol and its related compounds have several pharmacological activities including an inhibitory effect on cyclic AMP phosphodiesterase (PDE),⁹⁾ a prolonging effect on the exercise time to exhaustion in chronic swimming stressed rat,¹⁰⁾ and an inhibitory effect on gastric ulcer.¹¹⁾ The inhibitory activity on PDE of (+)-syringaresinol-di- β -glucoside and the enzymatic hydrolysate (syringaresinol) was considerable (IC_{50} : $12.5 \times 10^{-5}\text{ M}$ and $17.5 \times 10^{-5}\text{ M}$, respectively), and the activity of (−)-syringaresinol-di- β -glucoside was

less (IC_{50} : $>50 \times 10^{-5}$ M), with specificity in the chiral isomers. Neuritogenesis was induced by cAMP in both PC12 and Neuro 2a cells used in the present study.^{12,13)} Therefore syringaresinol may be able to induce cAMP-dependent neuritogenesis through PDE inhibition. However, in contrast to the inhibitory activity on PDE mentioned above, the neurogenic activity of the (–) isomer was more effective than the (+) isomer. Additional studies will be required to show the mechanism of syringaresinol.

Dual formation by a single enzyme of optically different bonds of specific groups is generally implausible in biology. It could be thought that the racemic formation of the respective bonds in the syringaresinol structure is performed non-enzymatically in those plants reported here. Such occurrence in both enantiomers of syringaresinol is suspected also in *Acanthopanax senticosus* (RUPR. et MAXIM.) HARMS (data not shown), which is useful as a source of tonic raw drug in Chinese traditional medicine. Syringaresinol in either form, even its glucosides, could be considered a key material in the search for neurotrophic and tonic drugs.

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