Enantiomeric Excess of a Cruciferous Phytoalexin, Spirobrassinin, and Its Enantiomeric Enrichment in an Achiral HPLC System

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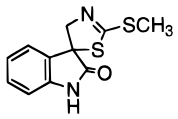
Enantiomeric purity of a cruciferous phytoalexin, spirobrassinin (**1**), was determined by chiral HPLC analysis. The enantiomeric excesses of two natural spirobrassinin fractions separated by nonchiral chromatography were considerably different. A significant enantiomeric enrichment was observed during the nonchiral chromatographic separation of an artificial enantiomeric mixture of **1**.

Plants produce antimicrobial compounds called phytoalexins following exposure to microorganisms. Phytoalexin production is believed to be a part of a plant's chemical and biochemical defense mechanisms.[1] Our previous phytoalexin studies of several economically important cruciferous vegetables revealed that they produce sulfur-containing indole or indole-related compounds.[2] These compounds were established as the first sulfur-containing phytoalexins, and they are biosynthetically related to some characteristic cruciferous constituents, the glucosinolates.[3] In 1987, we reported the isolation of the first oxindole phytoalexin, spirobrassinin (**1**), from *Pseudomonas cichorii*-inoculated Japanese radish (*Rhaphanus sativus* L. var. *hortensis*).[4] Our continuing efforts on the biosynthetic studies of cruciferous phytoalexins revealed that **1** was biosynthesized from brassinin (**2**), which originated from L-tryptophan.[3] However, the absolute configuration of natural (−)-**1** has not yet been determined, while brassinin-related compounds have been investigated by several groups for the past decade.[5,6] Recent interest in compound chirality, as well as successful microbial biotransformation studies[6] of cruciferous phytoalexins, prompted us to determine the absolute configuration of **1**. Preliminary X-ray analysis[7] of natural (−)-**1** indicated that it was not optically pure, and chiral HPLC analysis confirmed this. To determine the enantiomeric purity of natural (−)-**1**, we freshly isolated this substance, avoiding recrystallization. Surprisingly, the enantiomeric excesses (ee) of the two spirobrassinins fractionated by HPLC on an achiral phase were considerably different. Herein, we describe the enantiomeric enrichment of **1** in the nonchiral HPLC system. This is an extremely rare example of enantiomeric enrichment described for a natural product.



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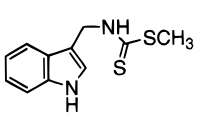

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Racemic **1** was synthesized as reported previously.[3] Chiral analysis and optical resolution were achieved by HPLC with a Sumichiral OA-4700 chiral column (*i*-PrOH-dichloroethane−hexane, 2:8:90) monitored by photodiode array (PDA) and HPLC-CD detectors [Figure 1, (−)-**1**: $[\alpha]_D$ −143.6° (*c* 0.25, CH_2Cl_2), (+)-**1**: $[\alpha]_D$ +142.7° (*c* 0.25, CH_2Cl_2)]. CD curves of the enantiomers were complete mirror images (Figure 2), and they showed moderate Cotton effects at 221 nm [(−)-**1**, $\Delta\epsilon$ 25.9; (+)-**1**, $\Delta\epsilon$ −25.9] and 204 nm [(−)-**1**, $\Delta\epsilon$ -21.0; (+)-**1**, $\Delta\epsilon$ 21.0].

Natural **1** was subjected to chiral HPLC analysis. The major enantiomer, (−)-**1**, eluted later following a small antipodal peak, as evidenced by checking the PDA and CD detectors. Racemization of **1** was not observed after standing in solution for a lengthy period. Recent asymmetric synthesis of natural products and successful development of chiral column technology have clarified that natural products are not always enantiomerically pure.[8] However, reports concerning enantiomeric purity of natural products are limited, except for racemic compounds, since analysis of enantiomeric purity may be quite tedious.

Previously reported **1** was obtained by successive chromatography followed by recrystallization.[4] Occasionally, recrystallization induces enantiomeric enrichment of partially enantio-enriched compounds. To determine more accurate ee values, natural **1** was newly isolated from *P. cichorii*-inoculated turnips (*Brassica campestris* L. ssp. *rapa*) (Brassicaceae) without any recrystallization. A short separation by nonchiral HPLC gave two spirobrassinin fractions, which were identified from their ^{1}H NMR spectra. Surprisingly, enantiomeric excesses of the two spirobrassinin fractions were considerably different (earlier eluted fraction 97.7% ee, later eluted fraction 83.4% ee).

To clarify this unusual phenomenon, partially enantio-enriched **1** [(+)-**1**:(−)-**1** = 73.6:26.4, 47.2% ee, 7.7 mg] was prepared using the resolved (+)-**1** and (−)-**1**, and the mixture was subjected to nonchiral HPLC. A peak (retention time 25−30 min) corresponding to **1** was divided into about 30 fractions. Figure 3 shows the ee of each fraction. As expected, it shows a dramatic change of ee. The first fraction gave almost enantiomeric pure (+)-**1** (99.0% ee), while a later fraction gave almost racemic **1** (5.8% ee). However, virtual elution curves of total (+)- and (−)-**1**, calculated from the area under each enantiomer, were mostly simple. These results suggest that this phenomenon would be caused by a diastereomeric difference between homochiral and heterochiral associations on the surface of the stationary phase. The enantiomeric enrichment phenomenon of nonchiral chromatographic separations has

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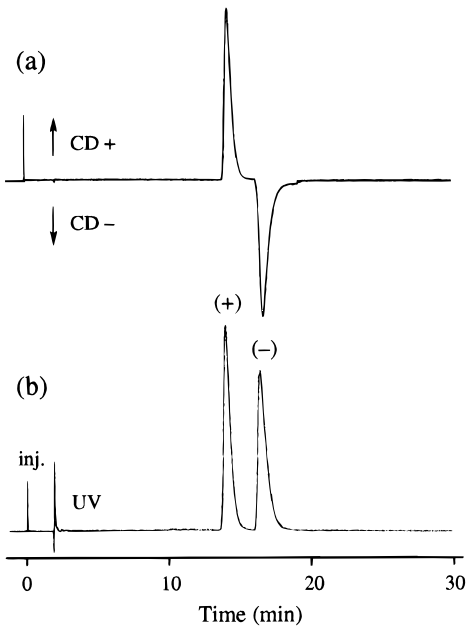

Figure 1. Chiral analysis of (±)-**1** by a Sumichiral OA-4700 chiral column (4 × 250 mm). Conditions: mobile phase, *i*-PrOH−dichloroethane−hexane (2:8:90); flow rate, 1.5 mL/min; detection, (a) on-line CD detection at 308 nm; (b) UV detection at 308 nm. The first eluted peak (R_t 14.0 min) was a (+)-**1**, while a second peak (R_t 16.4 min) was a (−)- **1**.

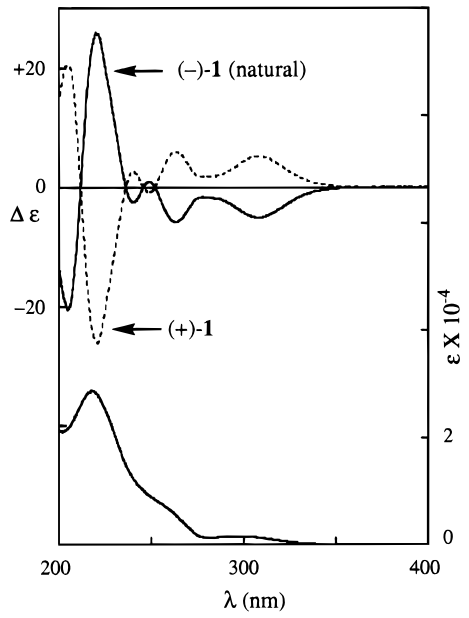

Figure 2. UV and CD spectra of **1** (EtOH). Solid line: CD spectrum of (−)-**1** (major natural enantiomer). Dotted line: CD spectrum of (+)-**1** (minor enantiomer).

been observed in a few cases.[9] However, all compounds reported previously were synthetic compounds except for the case of ^{14}C-labeled nicotine.[9a] This is a novel observation of the enantiomeric enrichment phenomenon during the isolation of a natural product.

The recent interest in and successful application of chiral technology may be expected to more easily reveal the enantiomeric excess of naturally occurring compounds. However, the isolation process usually involves sequential chromatographic methods. These processes may produce enantiomeric enrichment of partially enantiomeric pure natural products. Thus, we must be more careful about enantiomeric excess determinations of natural products, especially compounds possessing proton donor and acceptor

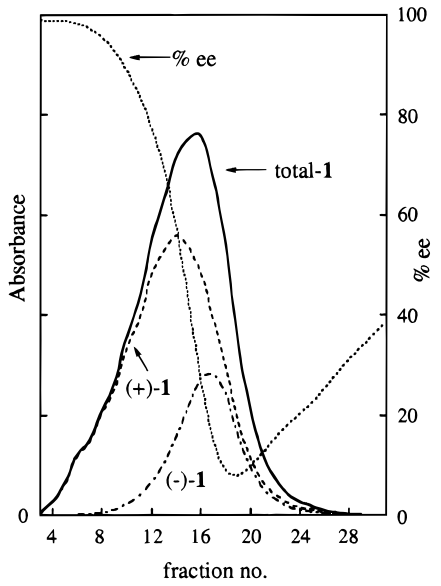

Figure 3. Enantiomeric enrichment of **1** by the HPLC on a nonchiral column (YMC SIL06, 300 × 10 mm). Conditions: mobile phase, 1% *i*-PrOH−$CHCl_3$; flow rate, 1.5 mL/min. Partially enantio-enriched **1** [(+)-**1**:(−)-**1** = 73.6:26.4, 47.2% ee, 7.7 mg] was injected, the large peak of **1** (R_t 25−30 min) was divided into 30 fractions. Enantiomeric excesses of each fraction were analyzed by the chiral HPLC analysis system. Total **1**, (+)-**1**, and (−)-**1** virtual elution curves were calculated from absorption areas of each enantiomer at 254 nm.

moieties. At this stage, we have concluded that the enantiomeric excess of natural (−)-**1** is 95.0% from the average calculation of both fractions. The composite nature of **1** suggests to us a difference in biological activity between the racemate and both of the enantiomers. However, no significant differences were expressed in antifungal bioassay against *Bipolaris leersiae* in vitro between the chemical species.[10] Determination of the absolute configuration of **1** is now under investigation.

Experimental Section

General Experimental Procedures. Melting points were determined using a Mitamura-Riken melting points apparatus and were uncorrected. Optical rotations were determined on a JASCO DIP-1000 digital polarimeter. UV and CD spectra were obtained using a JASCO V-570 and a JASCO J-720 spectrophotometer, respectively. The ^{1}H (400 MHz) NMR spectra were obtained on a JEOL Lambda 400 spectrometer using $CDCl_3$, with tetramethylsilane as an internal standard. HPLC separations were performed on a JASCO 900S instrument equipped with a JASCO MD-1515 multiwavelength detector, a JASCO-Borwin data processing system, and a JASCO CD-1595 on-line CD detector using an YMC-Pack SIL-06 column (10 × 300 mm, YMC Co., Ltd., Kyoto, Japan).

Plant Material. Turnip roots (*B. campestris* L. ssp. *rapa* Tamasato) used were commercial ones. This cultivar is grown widely in Japan.

Extraction and Isolation. Seven turnip roots (*B. campestris* L. ssp. *rapa*) were cut transversely into 2 mm thick disks. The tissues were incubated for 19 h at 20 °C in moist plastic cases covered loosely with polyethylene film. Then they were inoculated with *P. cichorii* (ca. 10^8 cells mL^{-1}). After being incubated at 20 °C for 4 days they were lyophilized. Freeze-dried inoculated turnip tissue (45 g) was extracted with acetone to give an extract (0.67 g), which was triturated with EtOAc. The EtOAc-soluble portion was submitted to Si gel short column chromatography, with the EtOAc-eluted part concentrated in vacuo, to give 199 mg of material, which was chromatographed over Si gel (2% MeOH−CH_2Cl_2) to give a crude spirobrassinin fraction (25 mg). Final purification was performed by HPLC on a YMC-Pack SIL-06 column with 2%

MeOH−CH_2Cl_2 and 0→10% *i*-PrOH−CH_2Cl_2 as eluants to give two natural (−)-spirobrassinin fractions A (9.6 mg, 97.7% ee) and B (2.2 mg, 83.4% ee).

Natural Spirobrassinin (1):[4] colorless needles; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.63 (3H, s, SCH_3), 4.51 (1H, d, J = 15.1 Hz, CH_2), 4.69 (1H, d, J = 15.1 Hz, CH_2), 6.91 (1H, d, J = 7.8 Hz, H-7), 7.09 (1H, ddd, J = 7.8, 7.6, 1.0 Hz, H-5), 7.27 (1H, ddd, J = 7.8, 7.8, 1.3 Hz, H-6), 7.37 (1H, d, J = 7.6 Hz, H-4), 8.19 (1H, br s, NH).

HPLC Chiral Analysis and Separation. HPLC was carried out on a Sumichiral OA-4700 chiral column (4 × 250 mm, Sumika Chemical Analysis Service Ltd., Osaka, Japan) using *i*-PrOH−dichloroethane−hexane (2:8:90) at a flow rate of 1.5 mL/min. The first eluted peak (R_t 14.0 min) was (+)-**1**, while a second peak (R_t 16.4 min) was (−)-**1**.

(+)-Spirobrassinin (1): colorless needles; mp 143−145 °C; $[\alpha]_D$ +142.7° (*c* 0.25, CH_2Cl_2); UV (EtOH) λ_{max} (ϵ) 218.0 (28800), 260.0 (sh) (6480), 295.6 (1550) nm; CD (EtOH) λ_{max} ($\Delta\epsilon$) 204.4 (21.0), 221.0 (−25.9), 240.0 (2.6), 248.8 (−1.0), 263.6 (5.9), 308.2 (5.1) nm.

(−)-Spirobrassinin (1): colorless needles; mp 142−144 °C; $[\alpha]_D$ −143.6° (*c* 0.25, CH_2Cl_2); UV (EtOH) λ_{max} (ϵ) 218.0 (28800), 260.0 (sh) (6480), 295.6 (1550) nm; CD (EtOH) λ_{max} ($\Delta\epsilon$) 204.4 (−21.0), 221.0 (25.9), 240.0 (−2.6), 248.8 (1.0), 263.6 (−5.9), 308.2 (−5.1) nm.

Separation of an Artificial Enantiomeric Mixture of 1. Partially enantio-enriched (+)-**1** was prepared by mixing racemic **1** and (+)-**1**. Enantiomeric excess of the mixture was determined by HPLC chiral analysis as 47.2% ee. The enantiomeric mixture (7.7 mg) was submitted to HPLC on the YMC SIL06 column (300 × 10 mm) using 1% *i*-PrOH−$CHCl_3$ at 3 mL/min. The large peak of **1** (R_t 25−30 min) was divided into 30 fractions (1 fraction volume ca. 500 μL). Enantiomeric excesses of each fraction were analyzed by the chiral HPLC analysis system.

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(7) Crystallization of natural (−)-**1** from CH_2Cl_2−hexane yielded colorless clear crystals. However, the X-ray analysis of **1** was unsuccessful since the crystals were complex mixed ones.

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(10) Inhibition tests were examined under a microscope after 20 h incubation at 25 °C. All chemical species [(±)-**1**, (+)-**1**, (−)-**1**] showed weak inhibition against fungal mycelial of *B. leersiae* at the same concentration (1 mM). The detailed experimental procedure will be published together with antifungal activity of a spirobrassinin analogue.

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