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Chiral cruciferous phytoalexins: Preparation, absolute configuration, and biological activity

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This paper is dedicated to Professor Koji Nakanishi on the occasion of his 80th birthday.

Abstract—Synthesized by an efficient one-pot spirocyclization method, two chiral cruciferous phytoalexins, 1-methoxyspirobrassinin (**2**) and 1-methoxyspirobrassinol methyl ether (**4a**), were prepared through optical resolution using the chiral HPLC method of corresponding racemates. The absolute configuration of natural (+)-**2** was elucidated as *R* by using the direct comparison of ECD and VCD spectra with those of known (*S*)-(−)-spirobrassinin (**1**). Another chiral phytoalexin, (−)-**4a**, had its absolute configuration 2*R*,3*R* elucidated through the comparison of observed and calculated VCD. Interestingly, the absolute configurations of natural (*S*)-(−)-spirobrassinin (**1**) and (*R*)-(+)-1-methoxyspirobrassinin (**2**) were opposite of each other, even though their structures are almost similar, with the exception of an N-methoxy group. A significant difference in the antiproliferative activity between (2*R*,3*R*)-(−) and (2*S*,3*S*)-(+)-**4a** was observed.

1. Introduction

When plants are exposed to microorganisms, they begin to produce antimicrobial secondary metabolites, called phytoalexins, *de novo*.[1–4] More than 30 indole and indole-related phytoalexins were isolated from the plant family cruciferae,[5,6] a family that includes many important crops (e.g., cabbage, turnips, Chinese cabbage, Japanese radish, wasabi, broccoli, rapeseed, arabidopsis, etc.) that are cultivated world wide. These cruciferous phytoalexins show attractive biological activities, such as antitumor activities[7–11] and antimicrobial activities.[5] With respect to the examinations of various biological activities of cruciferous phytoalexins, as well as to the dietary importance of brassicaceous vegetables in cancer chemoprevention,[12] it is quite important to investigate synthetic approaches to indole phytoalexins and their analogs in order to construct phytoalexin libraries.[13]

In 1987, the first spiroindoline phytoalexin, (*S*)-(−)-spirobrassinin (**1**), was isolated from the *Pseudomonas cichorii*-inoculated Japanese radish (*Raphanus sativus*);[14] however, its synthesis and absolute stereochemistry were only recently fully described.[13,15] Absolute configuration of (*S*)-(−)-**1** was determined the exciton, calculated electronic CD methods and X-ray crystallographic analysis.

To date, no studies regarding the stereochemistry of the other three spiroindoline[3,5′]thiazolidine-type phytoalexins, 1-methoxyspirobrassinin (**2**),[16,17] 1-methoxyspirobrassinol (**3**),[18,36] and 1-methoxyspirobrassinol methyl ether (**4a**),[18] have been described. In recent times, Pedras et al. reported a simple determination method for finding enantiomeric purity by means of a chiral NMR solvation agent[19] since some cruciferous phytoalexins may not have complete enantiomeric purities. Moreover, during nonchiral chromatographic separation, the partially enantioenriched **1** showed enantiomeric excesses (ee) varying depending on its chromatographic

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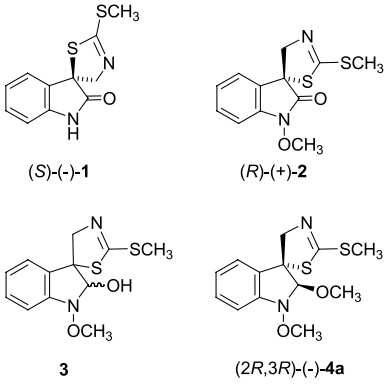
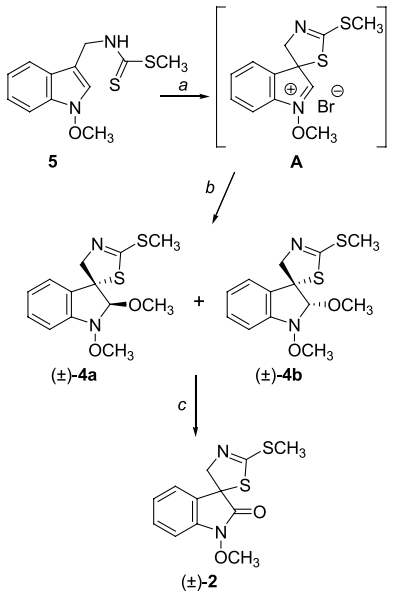
fractions, called enantiomeric enrichment phenomenon.[13,20] These interesting stereochemical phenomena, as well as interest in the biological activities of chiral cruciferous phytoalexins, prompted us to continue our stereochemical studies in this area.

In this paper, we describe the preparation of two chiral cruciferous phytoalexins and the determinations of these absolute configurations as (*R*)-(+)-**2** and (2*R*,3*R*)-(−)-**4a** through electronic circular dichroism (ECD),[21,22] vibrational circular dichroism (VCD),[23–26] and a chemical correlation. Interestingly, the structurally related natural spirobrassinin (**1**) and 1-methoxyspirobrassinin (**2**) show different absolute configurations, suggesting that N–OCH_3 has an effect on their biosynthetic pathways. We also examined the antiproliferative activities of each enantiomer.





Scheme 1. Reagents and conditions: (a) Br_2, dioxane or CH_2Cl_2; (b) see Table 1; (c) PCC, CH_2Cl_2 (37%).

Table 1. Influence of reagents on diastereoselectivity

Reagent/solvent	**4a:4b**[a]	Yield[b] (%)
CH_3OH/dioxane	36:64	74
CH_3OH /CH_2Cl_2	52:48	62
CH_3ONa,15-crown-5/CH_2Cl_2	69:31	71

[a] Determined by ^1H NMR spectroscopy in crude product.
[b] Crude product.

2. Results and discussion

2.1. Synthesis

In order to compare chiroptical properties and to submit all enantiomers for biological assay, optical actives **1**, **2**, and **4a** were prepared by the enantioselective chromatography from the corresponding racemic compounds. These racemic compounds were prepared with the modified spirocyclization strategy, which was stated in the preliminarily reported procedure.[27] This biomimetic procedure was considerably effective in constructing the spiroindoline[3,5′]thiazolidine ring system via the simple one pot reaction from the corresponding indole phytoalexins. In the earlier report, we described the synthesis of **2** through the spirocyclization of 1-methoxybrassinin (**5**).[28–30] This was done with dioxane dibromide in dioxane with the presence of methanol[27] yielding a mixture of natural (**4a**) and unnatural (**4b**) diastereoisomers in a ratio of 36:64 (Scheme 1, Table 1). When treatment with bromine in dry dichloromethane was used in the reaction, the ratio of **4a**:**4b** increased to 52:48. This result indicates the influence of dioxane as a solvent on diastereoselectivity. Presumably, the dioxane attacks the intermediate methoxyiminium ion **A** from the less hindered thiazoline CH_2 side with the formation of an unstable oxonium ion. This further reacts with the methanol that approaches from the sulfur side, which results in the formation of the unnatural diastereoisomer **4b**. However, the diastereoisomer **4a** is preferable because the methoxy group can be found in this species in a higher volume. Finally, the best diastereoselectivity (69:31) was achieved by the cyclization reaction with bromine in dry dichloromethane, which delivered the methoxy group in the form of a sodium methoxide with 15-crown-5-ether complex.

2.2. Optical resolution

The chiral auxiliary method had been previously performed to enantioresolve racemic **1**;[13,15] however, the chiral HPLC technique was applied in the present work because of its practicality as a semi-preparative scale enantiomeric separator. Optical resolutions were done by using chiral HPLC on the CHIRALCEL® OD column (1 cm $\phi \times 25$ cm) with hexane : 2-propanol = 3:1 at a speed of 5 mL/min. In each case, a couple of the UV peaks were in good separation, especially the separation factor α of **4**, which was amazingly high. The elution time and the separation factors α are as follows (t_0 = 3.2 min was used to calculate α): For **1**, $t(-)$ = 12.0 min; $t(+)$ = 14.2 min; α = 1.3. For **2**, $t(+)$ = 9.0 min; $t(-)$ = 10.6 min; α = 1.3. For **4a**, $t(+)$ = 4.8 min; $t(-)$ = 14.3 min; α = 6.9. The first-eluted enantiomers of **1** and **2** were identified as natural enantiomers through comparison of their optical rotations (Table 2), while the second-eluted enantiomer of **4a** was also a natural one. Reported optical rotation values of naturally occurring compounds were generally

Table 2. Optical rotation values of each enantiomers

	Reported value[a]	1st-eluted enantiomer	2nd-eluted enantiomer
1	$[\alpha]_D$ −69.5° ($CHCl_3$; *c* 1.14) $[\alpha]_D$ −53° ($CHCl_3$; *c* 0.30)	$[\alpha]_D$ −101.4° ($CHCl_3$; *c* 1.0) *S*	$[\alpha]_D$ +99.6° ($CHCl_3$; *c* 1.0) *R*
2	$[\alpha]_D$ +19. 05° (CH_3OH[b]) $[\alpha]_D$ +40.8 ° (CH_3OH; *c* 0.09)	$[\alpha]_D$ +61. 1° (CH_3OH; *c* 1.0) *R*	$[\alpha]_D$ −62.7° (CH_3OH; *c* 1.0) *S*
4a	$[\alpha]_D$ −1.9° ($CHCl_3$; *c* 1.57)	$[\alpha]_D$ +24.2° ($CHCl_3$; *c* 0.4) 2*S*,3*S*	$[\alpha]_D$ −18.6° ($CHCl_3$; *c* 0.4) 2*R*,3*R*

[a] $[\alpha]_D$ values of naturally isolated compounds were cited from Refs. 14,19 (**1**), Refs. 16,17 (**2**), and Ref. 18 (**4a**). For **1** and **2**, two publications have each reported their $[\alpha]_D$ values, which are similar.
[b] *c* was not mentioned.

smaller than ones of optical pure compounds, especially for **4a**, suggesting poor stereoselectivity of their biosyntheses. Interestingly, for the chiral HPLC of **1**, each enantiomer's elution order was opposite that of **2**, even though the only difference between the two molecules was a simple methoxy group attached to the N nucleus of the indole ring. It is suggested that a hydrogen bond within the NH group in **1** could be critical for chiral recognition between the chiral substrates and the chiral column surface.

2.3. Absolute configuration

In order to determine their absolute configurations, we examined the electronic and vibrational CD studies of **1**, **2**, and **4a**. The vibrational CD (VCD), an extension of the CD from electronic to vibrational transitions in molecules,[23–26] is a powerful new tool that can be used to determine the absolute configurations in a chiral molecule. Figure 1 shows the VCD, IR, CD, and UV spectra of natural (*S*)-(−)-(**1**), (+)-**2**, and (−)-**4a**. Because most major VCD signals show opposing signs, the direct comparison of the VCD curves of (*S*)-(−)-(**1**) and (+)-**2** suggests that the absolute configuration of (+)-**2** is *R*. Their IR spectra are also almost identical, as are the ECD spectra of (*S*)-(−)-(**1**) and (+)-**2**, which are mirror images of each other. The split Cotton effect, which could be found around 210 nm of (+)-**2**, is consistent with a previous interpretation that was attributed to two transition dipole moments interacting in **1**.[13,15] Again, this supports the idea that the absolute configuration of (+)-**2** is *R*.

On the other hand, the VCD and ECD spectra of (−)-**4a** show different patterns, since their chromophores were considerably different toward the IR and UV spectra. We examined theoretical VCD studies in order to elucidate the absolute configuration of **4a**. VCD has a significant advantage in non-empirically elucidating the absolute configuration to compare the measured VCD spectra with the simulated VCD spectra issued from the quantum mechanical ab initio methods with density functional theory (DFT) calculations.

Using molecular mechanics with an MMFF94S force field from the CONFLEX program,[31] conformational analyses were conducted. The 11 lower conformers were selected so that the cumulative Boltzmann weighted population sum was over 95%. To obtain accurate energies, and IR and VCD spectra, geometry optimizations and a harmonic frequency analysis were performed using DFT calculations at the B3PW91/6-31G(d,p) level. Four conformer spectra, whose cumulative Boltzmann weighted population sums within the 11 conformers was 97%, were superposed with Boltzmann weights.[32]

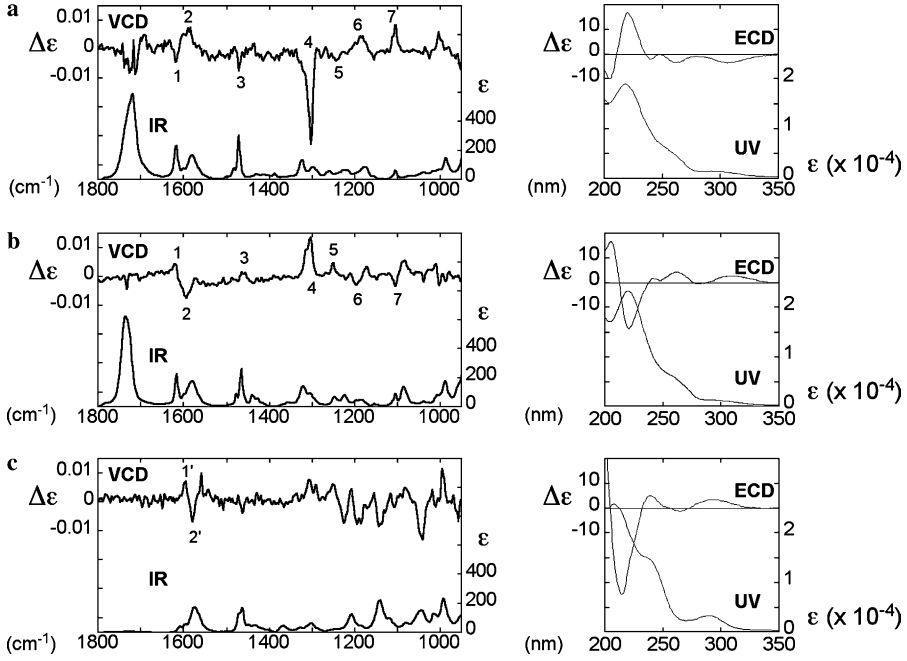


Figure 1. VCD and ECD spectra of (a) (*S*)-(−)-**1**, (b) (+)-**2**, and (c) (−)-**4a**. VCD and IR spectra are in the left column, and ECD and UV spectra are in the right column. Numbered peaks in the VCD spectrum of (+)-**2** showed signs opposite those of the corresponding VCD peaks in (*S*)-(−)-**1**. For (−)-**4a**, VCD patterns in the well-isolated 1600 cm^{-1} region were similar to that of (+)-**2**.

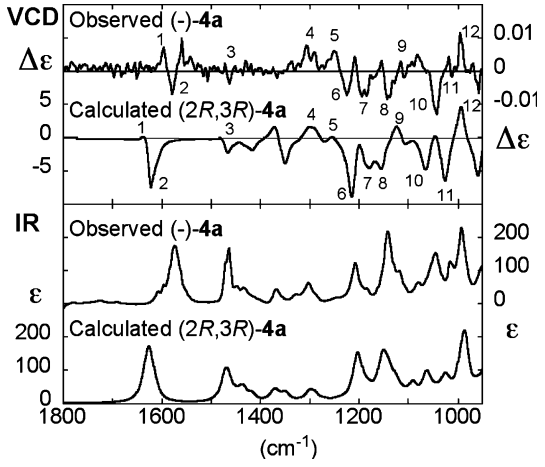


Figure 2. Comparison of IR (lower frame) and VCD (upper frame) spectra observed for (−)-**4a** with those calculated for (2*R*,3*R*)-**4a**.

Between the observed and calculated VCD spectra, the signs of the major VCD bands showed reasonable agreement with only a slight difference in their wave number frequencies (Fig. 2). The comparison suggests that the absolute configuration of the naturally occurring (−)-**4a** is 2*R*,3*R*. In order to confirm this result, a chemical transformation was performed. A naturally occurring (−)-**4a** (99% ee) was transformed to 1-methoxyspirobrassinin (**2**) using oxidation reaction of PCC in dichloromethane with 52% yield. Its ECD spectrum was identical with that of (*R*)-(+)-**2**. The chiral HPLC study also supported this result. Alternatively, unnatural type (+)-**4a** (99% ee) was oxidized to (*S*)-(−)-**2**, whose absolute configuration was also determined by the ECD and the chiral HPLC study. Therefore, absolute configuration of a naturally occurring (−)-**4a** was concluded as 2*R*,3*R*, thus supporting the result of the VCD study.

2.4. Biological activity

Antiproliferative activity was examined by an MTT (thiazolyl blue) test[33] using selected human cancer cell lines (Jurkat cells, MCF-7, and HeLa). The highest antiproliferative activity was found with the natural 2*R*,3*R* enantiomer of 1-methoxyspirobrassinol methyl ether (**4a**), which, at a concentration of 10^{-4} mol L^{-1}, significantly inhibited Jurkat cell growth (human leukemia) to 36.9% of the solvent control after a 72-h incubation. However, the 2*S*,3*S* enantiomer in the same concentration only slightly influenced cell survival (79.8% of the control value), while the concentration of racemic **4a** moved to 72.3%. On the other hand, there was weak activity and no differences were found in the antiproliferative effects between enantiomers and the racemate of 1-methoxyspirobrassinin (**2**) on Jurkat cells. Both phytoalexins exhibited weak activity on the proliferation of the MCF-7 and HeLa cells.

3. Conclusion

In summary, two chiral phytoalexins, 1-methoxyspirobrassinin (**2**) and 1-methoxyspirobrassinol methyl ether (**4a**), were prepared using the chiral column through the optical resolution of their corresponding racemates, which were synthesized by the efficient one pot spirocyclization strategy. Using direct comparison of their ECD and VCD spectra, the absolute configuration of natural (+)-**2** was elucidated as *R*. It was also established through the comparison of observed and calculated VCD that the absolute configuration of (−)-**4a** was 2*R*,3*R*. This VCD application to stereochemical studies of sulfur-containing natural biological active compounds, which was successful, indicates the potential power of VCD for other sulfur-containing biological active products, such as antibiotics. Interestingly, the absolute configurations of natural (−)-spirobrassinin (**1**) and (+)-1-methoxyspirobrassinin (**2**) were opposite of each other, even though their structures were very similar, with the exception of an N-methoxy group. This surprising result could prompt further metabolic studies of 1-methoxyindole alkaloids[34] and biosynthetic studies of the 1-methoxybrassinin (**5**) series.[35] A significant difference in antiproliferative activity between (2*R*,3*R*)-(−)-**4a** and (2*S*,3*S*)-(+)-**4a** was observed, even though (*R*)-(+)-**2** and (*S*)-(−)-**2** showed the same weak activities on Jurkat cells.

4. Experimental

Melting points were determined on a Koffler hot-stage apparatus and remained uncorrected. In $CDCl_3$ solutions, ^1H and ^{13}C NMR spectra were measured on a Bruker Avance spectrometer (600 MHz for ^1H, 150 MHz for ^{13}C). Chemical shifts (δ) are reported in ppm downfield from TMS, and the coupling constants (J) are given in Hz. The mass spectra and HRMS were recorded on a JEOL JMS-FABmate spectrometer at an ionization energy of 70 eV. TLC with Macherey-Nagel Alugram®Sil G/UV254 plates monitored the reaction course. Preparative column chromatography (flash chromatography) was performed over the Kieselgel Merck Type 9385 at 230–400 mesh. A Shimadzu LC-6A liquid chromatograph instrument equipped with a Shimadzu SPD-6AV UV–Vis spectrometric detector was used to perform enantio resolutions. A Hitachi D-2520 GPC-Integrator was used to depict elution curves. Optical rotations were measured at room temperature in a 1 cm cell with a Perkin-Elmer Polarimeter 343 at the sodium D-line. UV and CD spectra were obtained in a 1 mm quartz cell on a JASCO J-810 spectrometer. IR and VCD spectra of each enantiomer were measured using Bomem/BioTools Chiral*ir* and JASCO JV-2001 spectrometers. The samples were dissolved in $CDCl_3$ (concentration: 0.15 M for **1** and **4a** and 0.25 M for **2**) and then placed in a BaF_2 cell (pathlength: 71 μm for **1** and **4a**, and 50 μm for **2**), and the VCD spectra were recorded for 3 h at a 4 cm^{-1} resolution. A solvent IR spectrum was utilized to correct sample IR absorption, and VCD spectra were corrected by enantiomer VCD spectra. Spectroscopic grades solvent were purchased from Wako Pure Chemical Industries, Ltd., and $CDCl_3$ was obtained from Cambridge Isotope Laboratories.

4.1. (±)-1-Methoxyspirobrassinol methyl ether (4a)

Dry dichloromethane (4.20 mL) and powdered molecular sieve (3 Å) were added to a stirred mixture

of 1-methoxybrassinin (**5**)[29,30] (210 mg, 0.788 mmol). Powdered anhydrous K_2CO_3 (218 mg, 1.576 mmol) and a freshly prepared solution of bromine (139 mg, 0.045 mL, 0.867 mmol) in 2 mL of dry dichloromethane was then added. After stirring for 1 min, a freshly prepared solution of CH_3ONa–15-crown-5-ether complex in dry dichloromethane (1.91 mL, 0.867 mmol) was added. The stock solution was prepared by dissolving 54 mg (1 mmol) of CH_3ONa in 2 mL of dry CH_3OH with a subsequent addition of 220 mg (0.20 mL, 1 mmol) of 15-crown-5-ether. Methanol was thoroughly evaporated and the residue was dissolved in 2 mL of dry dichloromethane with molecular sieve (3 Å). Stirring was continued for another 10 min, and the reaction mixture was diluted with dichloromethane (20 mL) and washed with brine (2 × 25 mL). The residue obtained after the solvent's evaporation was subjected to chromatography on 25 g of silica gel (hexane/diethyl ether, 3:1), affording 86 mg (37%) of natural diastereoisomer **4a** as a colorless oil and R_f (hexane/diethyl ether, 3:1) 0.35, which was fully identical with the described natural product.[18] Unnatural diastereoisomer **4b**, 26 mg (19%), R_f (hexane/diethyl ether, 3:1) 0.16 was obtained as colorless crystals, mp 77–79 °C (hexane). HRMS (EI) Calcd for $C_{13}H_{16}N_2O_2S_2$: 296.0653, Found: 296.0649; MS (EI) *m/z* (%) 296 (19, M^+), 265 (100), 233 (59), 218 (24), 192 (54); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.26 (1H, dd, J = 7.7, 7.5 Hz, H-7), 7.23 (1H, d, J = 7.6 Hz, H-5), 7.02 (1H, dd, J = 7.4, 7.5 Hz, H-6), 6.95 (1H, d, J = 7.9 Hz, H-8), 4.62 (1H. s, CH), 4.51 (1H, d, J = 15.3 Hz, H_a), 4.33 (1H, d, J = 15.3 Hz, H_b), 3.95 (3H, s, N-OCH_3), 3.71 (C-OCH_3), 2.56 (3H, s, SCH_3); ^{13}C NMR (150 MHz, $CDCl_3$) δ: 166.4 (>C=N), 147.5 (C-7a), 129.9 (C-6), 129.1 (C-4a), 124.0 (C-5), 123.1 (C-4), 112.7 (C-7), 105.5 (C-2), 73.3 (CH_2), 70.2 (C-3), 63.8 (N-OCH_3), 59.6 (C-O*C*H_3), 15.1 (SCH_3).

In order to give two optical pure enantiomers, an optical resolution by (±)-**4a** chiral HPLC was perfomed. (2*S*,3*S*)-(+)-**4a** (first-eluted enantiomer): $[\alpha]_D$ + 24.2° (CH_3OH; *c* 0.4); UV (CH_3OH, nm) λ_{max} (ε): 208 (25800), 238 (sh, 14800), 290 (3300); CD (CH_3OH) λ_{ext} ($\Delta\varepsilon$): 214 (+34.7), 239 (−5.3), 265 (+1.1), 291 (−3.5). Naturally occurring (***2R,3R***)-(−)-**4a** (second-eluted enantiomer): $[\alpha]_D$ −18.6 ° (CH_3OH; *c* 0.4); UV (CH_3OH, nm) λ_{max} (ε): 208 (25800), 238 (sh, 14800), 290 (3300); CD (CH_3OH, nm) λ_{ext} ($\Delta\varepsilon$): 214 (−34.7), 239 (+5.3), 265 (−1.1), 291 (+3.5). IR ($CDCl_3$ cm^{-1}) ν_{max}: 1575, 1463, 1209.

4.2. (±)-1-Methoxyspirobrassinin (2)

After spirobrassinol (**3**) was prepared by the spirocyclization method of 1-methoxybrassinin (**5**), CrO_3 oxidation of spirobrassinol (**3**) was used to prepare racemic **2**.[27] In order to create a stereochemical relay between chiral **2** and **4a**, a direct microscale transformation method, which consists of PCC oxidation from **4a**,**b** to **2**, was developed. A solution of a distereoisomeric mixture of 1-methoxyspirobrassinol methyl ether (**4a** and **4b**, 20 mg, 0.068 mmol) in dry dichloromethane (260 μL) was added to a vigorously stirred slurry of pyridinium chlorochromate (44 mg, 0.20 mmol) and anhydrous magnesium sulfate (37 mg, 0.30 mmol) in dry dichloromethane (100 μL). The resulting mixture was stirred overnight at room temperature (28 h, reaction was monitored by TLC, eluent hexane-diethyl ether 3:1) and then diluted with diethyl ether (1.5 mL). The brown slurry was filtered through a 0.5-cm column (diameter 0.5 cm) of silica gel, and the column was washed with diethyl ether (3 × 2 mL). The filtrate was then dried over anhydrous sodium sulfate and filtered again. A small amount of silica gel was added to the filtrate, and the solvent was evaporated. The obtained sample preabsorbed on the silica gel was chromatographed on 3 g of silica gel (Merck 60, 40–63 μm, eluent hexane-diethyl ether 3:1), affording 7 mg (37%) of **2**. The spectral data was fully identical to the previously described natural product.[16,17] As described in the text, optical resolution was performed by chiral HPLC of (±)-**2** in order to give two optically pure enantiomers. Naturally occurring (***R***)-(+)-**2** (first-eluted enantiomer): $[\alpha]_D$ + 61.1° (CH_3OH; *c* 1.0); UV (CH_3OH, nm) λ_{max} (ε): 220 (23400), 257 (sh, 6300), 290 (1300); CD (CH_3OH, nm) λ_{ext} ($\Delta\varepsilon$): 204 (+16.8), 220 (−18.5), 241 (+1.7), 258 (+4.1), 307 (+2.8). (***S***)-(−)-**2** (second-eluted enantiomer): $[\alpha]_D$ −62.7° (CH_3OH; *c* 1.0); UV (CH_3OH, nm) λ_{max} (ε): 220 (23400), 257 (sh, 6300), 290 (1300); CD (CH_3OH, nm) λ_{ext} ($\Delta\varepsilon$): 204 (−16.8), 220 (+18.5), 241 (−1.7), 258 (−4.1), 307 (−2.8) nm. IR ($CDCl_3$ cm^{-1}) ν_{max}: 1736, 1618, 1467, 1322.

4.3. Oxidation of chiral (+)-4a and (−)-4a with PCC

Oxidation reactions were performed using a KONTES Microscale glassware kit under a nitrogen atmosphere. A solution of (+)-**4a** (7.8 mg, 0.026 mmol) or (−)-**4a** (6.9 mg, 0.023 mmol) in dry dichloromethane (0.1 mL) was added to a vigorously stirred slurry of pyridinium chlorochromate (16.5 mg, 0.077 mmol for (+)-**4a**, 14.3 mg, 0.066 mmol for (−)-**4a**) and anhydrous magnesium sulfate in dry dichloromethane (0.05 mL). The resulting mixture was stirred overnight at room temperature (20 h) and then diluted with diethyl ether (0.75 ml). After work-up, silica gel chromatographic separation gave 3.0 mg (42%) of (*S*)-(−)-**2** from (+)-**4a**, and 3.4 mg (52%) of (*R*)-(+)-2 from naturally occurring (−)-**4a**. Optical rotation values of enantiomers were almost identical with the ones shown in Table 2. Due to small amount of sample, direct comparisons of ECD spectra were examined.

(***S***)-(−)-**2 from** (+)-**4a:** CD (CH_3OH, nm) λ_{ext} ($\Delta\varepsilon$): 204 (−16.8), 220 (+18.5), 241 (−1.7), 258 (−4.1), 307 (−2.8).

(***R***)-(+)-**2 from** (−)-**4a:** CD (CH_3OH, nm) λ_{ext} ($\Delta\varepsilon$): 204 (+16.8), 220 (−18.5), 241 (+1.7), 258 (+4.1), 307 (+2.8).

4.4. HPLC analyses of chiral 2 derived from optically pure 4

An analytical chiral HPLC was performed on a CHIRALCEL® OD column (0.46 cm ϕ × 25 cm) with hexane : 2-propanol = 3:1 at a speed of 1 mL/min. An HPLC analysis of racemic **2** was performed in order to find the time of elution (t_0 = 4.2 min, t(+) = 9.4 min; t(−) = 10.7 min; α = 1.3). The compound **2** converted

from each chiral **4** showed a single UV peak, which indicated that the oxidation reactions were conducted without racemization. The elution times of **2** from (+)-**4** (10.7 min) and (−)-**4** (9.4 min) corresponded to (*S*)-(−)-**2** and (*R*)-(+)-**2**, respectively.

4.5. Cell culture

The Jurkat cells (human acute T-lymphoblastic leukemia cells), MCF-7 cells (breast cancer), and HeLa cells (cervix cancer) were kindly provided by Dr. M. Hajdúch (Olomouc, Czech Republic). The cells were maintained in RPMI 1640 medium (Jurkat and HeLa cells) or Dulbeco's medium (MCF-7 cells) with Glutamax-I supplemented with 10% fetal calf serum, penicillin (100 IU mL^{-1}), and streptomycin (100 μg mL^{-1}) (all from Invitrogen, UK) in an atmosphere of 5% CO_2 in humidified air at 37 °C. Cell viability, estimated by trypan blue exclusion, was greater than 95% before each experiment.

4.6. Assessment of antiproliferative activity by MTT assay

The antiproliferative activities of the synthesized compounds were examined by an MTT (thiazolyl blue) test[33] using selected human cancer cell lines (Jurkat cells, MCF-7 and HeLa). A total of 8×10^4 cells were briefly plated per well in 96-well polystyrene microplates (Sarstedt, Germany) in a culture medium containing the tested chemicals at final concentrations of 10^{-4}–10^{-6} mol L^{-1}. Ten microliters of MTT (5 mg mL^{-1}) were added to each well after a 72-h incubation. After an additional 4 h, during which insoluble formazan was produced, 100 μL of 10% sodium dodecyl sulfate were added to each well, and another 12 h allowed the formazan to dissolve. At 540 nm, absorbance was measured with an automated MRX microplate reader (Dynatech laboratories, UK). The absorbance of the control wells was taken as 100%, and the results were expressed as a percent of the control.

4.7. Statistical analysis

For all experiments, the mean values and standard deviations (from three experiments) were calculated with the ArcusQuickstat software package. In order to evaluate the statistical significance observed between groups, Student's *t* test was employed. A statistical significance was considered to be present if $P < 0.05$.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.06.001.

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32. The density functional theory at the B3LYP/6-31G(d,p) level was used throughout for calculations carried out with Gaussian03. In order to create the final spectra, frequencies were scaled by a factor of 0.97 and intensities were convoluted with Lorentzian bandshapes, whose half-widths at the half maximum was $10\,cm^{-1}$. The sum of the Boltzmann conformer weights used in the last step covered more than 97% of those of all conformers for which the DFT calculations were carried out.
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36. Compound **3** was isolated as an optical inactive and exists as an inseparable diastereomeric mixture due to the equlibrium of its hemiaminal group. We therefore did not investigate this during this study.