Effects of (+)-, (−)- and (±)-Indenestrols A and B on Microtubule Polymerization

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Indenestrol A (IA) is a metabolite of diethylstilbestrol (DES), and indenestrol B (IB) is an analog of IA. IA was simply obtained from *E,E*-dienestrol in the presence of dilute sulfuric acid, and a mixture of IA and IB was formed by thermal cyclization of *E,E*-dienestrol. In order to elucidate the effects of optically active IA and IB on microtubule assembly, the IA and IB enantiomers were separated to >99% purity by high-pressure liquid chromatography using a chiral column. The di(4-bromobenzoate) of (−)-IB was analyzed by X-ray crystallography and its absolute structure was determined as C(3)-*S*. The (+)-, (−)-, and (±)-indenestrols A and B were shown to be inhibitors of microtubule assembly *in vitro* using microtubule proteins from porcine brain. (±)-IB is more active than (±)-IA, and the order of inhibitory activity of the enantiomers on microtubule assembly was (+)-IB>(+)-IA>(−)-IA>(−)-IB.

Keywords optical resolution; indenestrol A; indenestrol B; absolute structure; X-ray crystallography; microtubule assembly; inhibition

The elucidation of the chemical structure of estrogens is important to understand their hormone action, and in 1930 Doisy and his coworkers[1] deduced for the first time the chemical structure of an endogenous estrogenic substance. Subsequent studies have centered on the molecular properties of estrogenic steroids and synthetic estrogens because they are related to receptor ligand binding and hormonal activity.[2]

Diethylstilbestrol (DES), a synthetic estrogen, is a known carcinogen in humans and rodents.[3] The mechanism of carcinogenicity of DES is still unknown, though a number of biological and biochemical effects of DES are dependent on its metabolic activation.[4] DES, which induces neoplastic transformation in cultured cells,[5] is an exceptional carcinogen in that it has no appreciable mutagenic activity.[5-7] Its carcinogenicity can be best explained in terms of aneuploidy[8] due to disturbed assembly of microtubule proteins.[9-12]

Korach *et al.*[13] have investigated the receptor binding and biological activities of a number of DES-related compounds, one of which, indenestrol A, 3-ethyl-2-(4′-hydroxyphenyl)-1-methyl-6-hydroxyindene (IA),[14] is the metabolite of *in vivo* and *in vitro* oxidative metabolism of DES.[15] Indenestrol B, 3-ethyl-2-(4′-hydroxyphenyl)-1-methyl-6-hydroxyindene (IB),[14] is a chemical analog of IA. IA and IB possess a chiral center at the C-1 position and the C-3 position, respectively. Recently, the IA and IB enantiomers were separated on a chiral high-pressure liquid chromatography column, and the absolute structure of (+)-IA[14] has been assigned as C(1)-*R* on the basis of X-ray crystallography (personal communication from Dr. K. S. Korach).[13] The enantiomers of IA show considerable differences in their estrogen-binding affinities,[13] and moreover, the indenestrols have been shown to interact differently with prostaglandin H synthase.[16]

In this study, the enantiomers of IA and IB were separated to >99% purity by high-pressure liquid chromatography (HPLC) using a new chiral column, and the effects of (+)-, (−)- and (±)-IA and (+)-, (−)- and (±)-IB on microtubule polymerization were elucidated by turbidimetric analysis.

Results and Discussion

Synthesis of (+)-, (−)- and (±)-Indenestrol A We found that IA[17] was easily obtained in 97% yield from *E,E*-dienestrol (*E*-DIEN) in the presence of dilute sulfuric acid in methanol (Chart 1). The proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) signals of IA were unequivocally assigned (Table I for ^{13}C-NMR).

Chromatographic separation of the individual IA enantiomers was achieved by HPLC using a Chiralcel OJ column[18] (Daicel Chemical Co.). The chromatographic profile and conditions are shown in Fig. 1, indicating a better separation of the enatiomers than had been obtained in the previous study.[13a] The IA enantiomers were separated on a preparative scale to >99% purity by HPLC. ^{1}H-NMR spectra and mass spectra (MS) of the individual samples were identical with those of (±)-IA, and the melting points (142—143 °C and 140—142 °C, respectively) of (+)- and (−)-IA were lower than that of (±)-IA (mp 175—176 °C). Moreover, the samples collected from the two IA peaks obtained by HPLC gave opposite optical rotations, peak 1 showing $[\alpha]_D^{22.5}$ −281° and peak 2 $[\alpha]_D^{22.5}$ +295°. In the circular dichroism (CD) spectra, (+)-IA has a positive Cotton effect and the (−)-isomer a negative one (Fig. 2). The absolute configuration of the IA enantiomers has been determined by X-ray crystal analysis[13] (Chart 2).

TABLE I. ^{13}C-NMR Spectral Data for IA and IB

Carbon	Chemical shift (ppm)	
	IA	IB
C-1	46.2	133.1
C-2	144.3	146.7
C-3	138.1	51.0
C-4	120.3	123.8
C-5	114.0	112.1
C-6	156.2	157.6
C-7	111.4	106.8
C-8	17.3	11.7
C-9	19.8	24.0
C-10	14.2	8.6
C-3a	137.6	137.8
C-7a	151.3	149.4
C-1′	128.9	128.8
C-2′,6′	130.6	131.0
C-3′,5′	116.1	116.1
C-4′	157.0	157.2

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Synthesis of (+)-, (−)- and (±)-Indenestrol B We demonstrated that a mixture of IA and IB[17] was[19] formed by thermal cyclization when crystals of *E*-DIEN or the *Z*,*Z*-isomer (*Z*-DIEN)[20] were heated at 240 °C for 2 h (Chart 1). Moreover, *Z*-DIEN was obtained by chemical oxidation of DES with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ). All of the ^{1}H- and ^{13}C-NMR signals of IB were assigned (Table I for ^{13}C-NMR). Chromatographic separation of the individual IB enantiomers was performed as described in IA, and the chromatographic profile and conditions are shown in Fig. 1. The IB enantiomers were separated on a preparative scale to >99% purity by using the same HPLC method. The ^{1}H-NMR spectra and MS of the individual samples were identical, but the melting points (94—96 °C and 93—94 °C, respectively) of (+)- and (−)-IB were lower than that of (±)-IB (mp 128—129 °C). Moreover, the samples collected from the two IB peaks gave opposite optical rotations, peak 1 showing $[\alpha]_D^{24}$ −258° and peak 2 $[\alpha]_D^{24}$ +274°. In the CD spectra, (+)-IB has a positive Cotton effect and the (−)-isomer a negative one (Fig. 2).

Since the CD spectral pattern of IB was similar to that of IA, the absolute structures of the IB enantiomers were estimated to be (+)-3*R* and (−)-3*S*, assuming the same asymmetric centers as (+)-1*R* and (−)-1*S* for IA

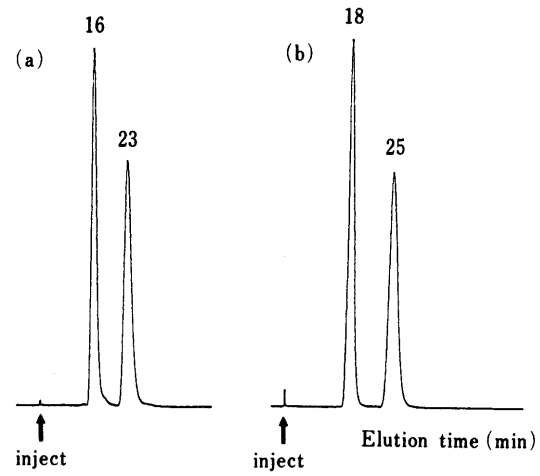

Fig. 1. Chromatograms of IA (a) and IB (b) Enantiomers

A 150-μl sample (5 mg) of each of IA and IB was injected onto a Chiralcel OJ column. The samples were eluted with 20% 2-propanol/*n*-hexane solution at a flow rate of 4 ml/min. Sample detection was done by monitoring UV absorption at 254 nm.

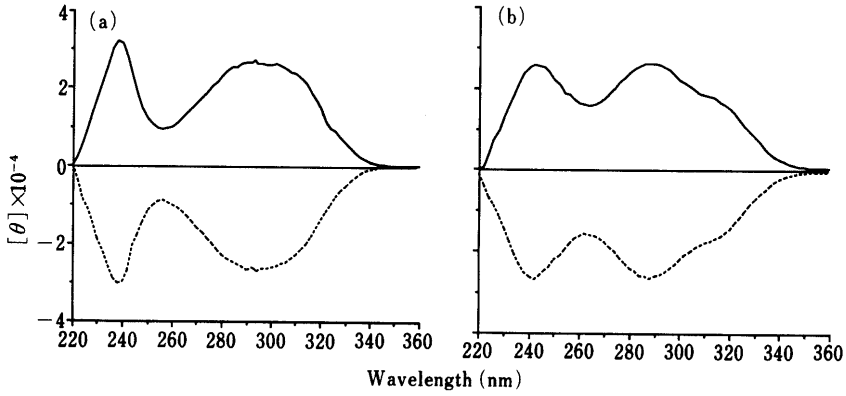

Fig. 2. CD Spectra of (+)- and (−)-IA (a), and (+)- and (−)-IB (b)

(a) (+)-IA (——) and (−)-IA (---). (b) (+)-IB (——) and (−)-IB (---).

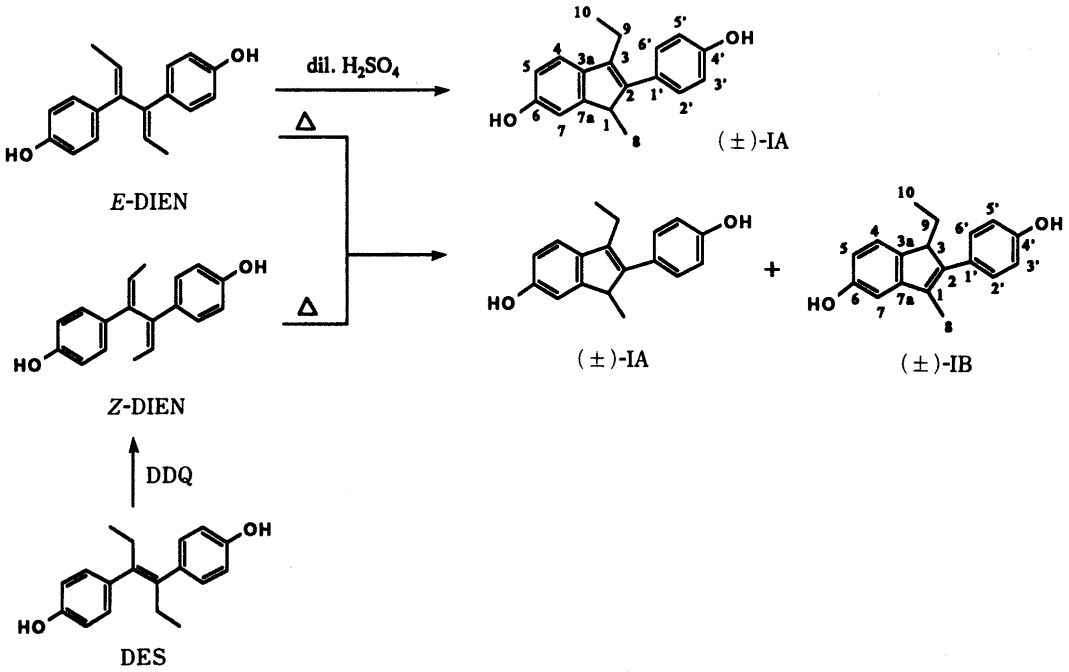

Chart 1

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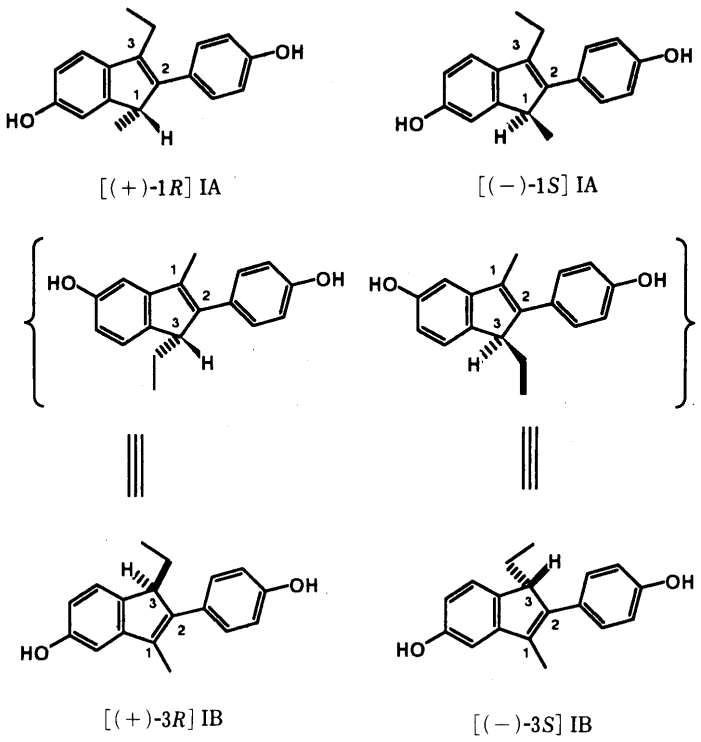

Chart 2

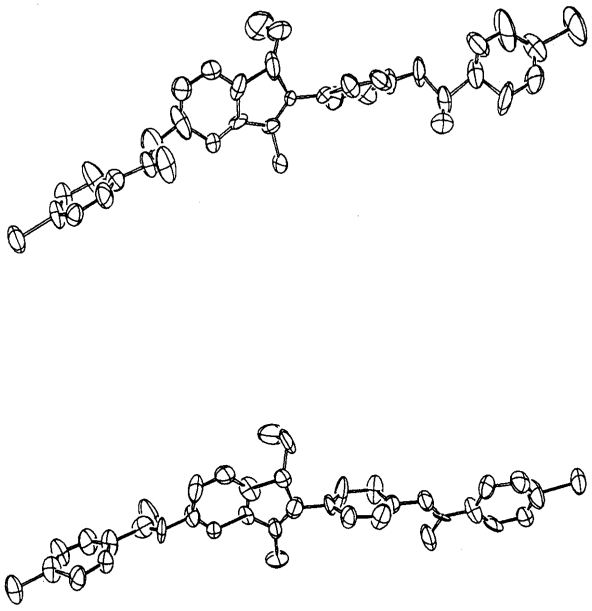

Fig. 3. Molecular Structure of the Two Independent Molecules of the Di(4-bromobenzoate) of (−)-IB

enantiomers, respectively (Chart 2). On the other hand, the absolute configurations of the IB enantiomers were unequivocally determined by X-ray analysis. That is, the highly purified fraction 1 (peak 1) obtained from HPLC was derivatized to form the di(4-bromobenzoate) of (−)-IB for X-ray analysis. The results indicated that the absolute configuration is C(3)-*S* (Fig. 3).

Inhibitory Effects of Indenestrol Stereoisomer on Microtubule Polymerization First, we examined the effects of (±)-IA and (±)-IB on microtubule polymerization. The inhibitory activities of these compounds detected by turbidity measurement depended on the concentrations (50 and 100 μM) used. Figures 4a and 4b show the turbidity values in microtubule polymerization at concentrations of 50 and 100 μM DES, (±)-IA and (±)-IB. These results indicated that the activities decreased in the following order: DES≧(±)-IB>(±)-IA. Moreover, in the presence of 50 and 100 μM DES, (±)-IA and (±)-IB, smaller amounts of microtubules were observed by electron microscopy (data not shown). Secondly, we examined the effects of (+)-, (−)-, and (±)-IA and (+)-, (−)- and (±)-IB on microtubule polymerization. The results indicated that, at 50 and 100 μM, the order of inhibitory activity was: (+)-IB>(+)-IA>(−)-IA>(−)-IB, and that furthermore the activities of (+)- and (−)-IA were weaker than that of (±)-IA (Fig. 4c). Interestingly, however, the activity of (+)-IB was almost the same as that of (±)-IB, and that of (−)-IB was the lowest (Fig. 4d). In order to relate the inhibitory activity to the stereochemistry, the structural formula of each enantiomer of IB was comparatively viewed by rotation through 180° about its long axis as shown in parenthesis in Chart 2. It became clear that the activities of (+)-IA and (+)-IB, which have α-configuration of the 1-methyl or 3-ethyl group (in the structural formula shown in parenthesis), respectively, were stronger than those of (−)-IA and (−)-IB. Moreover, the activity

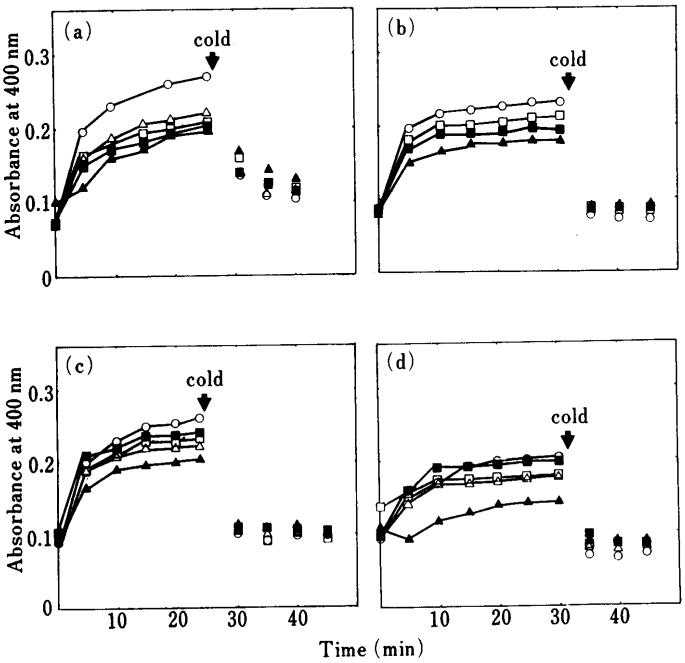

Fig. 4. Turbidimetric Analysis of the Effects of DES, (+)-, (−)- and (±)-IA and (+)-, (−)- and (±)-IB on the Assembly of Microtubules

Test compounds were added at 0 min. (a) ○, control; △, 50 μM DES; ▲, 100 μM DES; □, 100 μM (±)-IA; ■, 100 μM (±)-IB. (b) ○, control; ▲, 50 μM DES; □, 50 μM (±)-IA; ■, 50 μM (±)-IB. (c) ○, control; △, 50 μM (±)-IA; ▲, 100 μM (±)-IA; □, 50 μM (+)-IA; ■, 50 μM (−)-IA. (d) ○, control; △, 50 μM (±)-IB; ▲, 100 μM (±)-IB; □, 50 μM (+)-IB; ■, 50 μM (−)-IB. Arrows indicate the time (25 min) of cold treatment in ice-water.

of (−)-IB, with a 3-ethyl group, was weaker than that of (−)-IA with a 1-methyl group.

Previously, we reported the inhibition of microtubule polymerization by hexestrol isomers [(*R*,*R*)-(+)-hexestrol, (*S*,*S*)-(−)-hexestrol and *meso*-hexestrol] and *dl*-hexestrol.[19] *dl*-Hexestrol showed the highest inhibitory activity among the four isomers. In the present study, equivalent results were obtained. Thus, such a racemic mixture appears to interact differently from the individual isomers with microtubule proteins.

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Experimental

Apparatus for Structural Determination All melting points were obtained on a Shimadzu MM2 micro-melting point apparatus. All ^{1}H-NMR data were recorded in deuterioacetone and are reported as parts per million downfield from Me_4Si ($\delta=0$). ^{13}C-NMR spectra were determined at 67.8 MHz using a JEOL JNM-GX 270 FT NMR spectrometer with 32 k data points for acquisition of free induction decays. For measurement of carbon–proton coupling constants, the coupling information was retained using a gated decoupling facility, which permitted retention of the nuclear Overhauser effect (NOE). Abbreviations used: s = singlet, d = doublet, t = triplet, br = broad, m = multiplet, dd = doublet of doublets, q = quartet. MS were recorded on a JEOL JMS-DX303 mass spectrometer at an ionizing potential of 70 eV. The optical rotations were measured on a JASCO DIP-140 digital polarimeter using a cell with a 10-cm light path, and CD spectra were taken in ethanol using a 0.5-mm cell at room temperature (25 °C) on a JASCO J-20 recording spectropolarimeter. Column chromatography was performed with Kanto Kagaku silica gel (100 mesh). The plates [precoated thin-layer chromatography (TLC) plates, Silica gel 60F-254, Merck] were developed in benzene–acetone (8 : 2, v/v). The compounds were visualized under UV light and/or by spraying with concentrated H_2SO_4 and heating on an electric heater. HPLC was performed on a 10 mm × 25 cm column, using a Waters pump (model 510) and a Waters detector (model 480 spectrophotometer, set at 254 nm). *n*-Hexane–2-propyl alcohol (80 : 20) was used as the eluent.

Materials *E*-DIEN was obtained from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. All other reagents were obtained from Wako Pure Chemical Industries, Ltd.

(±)-IA 1) (±)-IA was prepared by the method of Adler and Hagglund.[17] IA recrystallized from benzene was obtained as colorless needles, mp 175—176 °C. *Anal.* Calcd for $C_{18}H_{18}O_2$: C, 81.17; H, 6.81. Found: C, 81.27; H, 6.75. MS *m/z*: 266 (M^+), 237 (base peak). ^{1}H-NMR δ (ppm): 1.11 (3H, d, $J=7.3$ Hz, 8-CH_3), 1.24 (3H, t, $J=7.3$ Hz, 10-CH_3), 2.61 (2H, q, $J=7.3$ Hz, 9-CH_2), 3.75 (1H, q, $J=7.3$ Hz, 1-H), 6.78 (1H, dd, $J=7.8$, 2.3 Hz, 5-H), 6.91 (2H, br d, $J=8.6$ Hz, 3′,5′-H), 6.97 (1H, d, $J=2.3$ Hz, 7-H), 7.17 (1H, d, $J=7.8$ Hz, 4-H), 7.21 (2H, br d, $J=8.6$ Hz, 2′, 6′-H), 8.10 (1H, br s, 4′- or 6-OH), 8.37 (1H, br s, 4′- or 6-H). ^{13}C-NMR data are shown in Table I.

2) Alternatively, a solution of *E*-DIEN (1 g) in MeOH (30 ml) and 10 N H_2SO_4 (7 ml) was refluxed for 3 h. The reaction mixture was poured into ice-water and extracted with ethyl acetate, and the extract was washed with water, dried (Na_2SO_4), and concentrated *in vacuo* to afford a brown residue (1.2 g), which contained 97% IA and 3% *E*-DIEN, by ^{1}H-NMR. MS and ^{1}H-NMR data for the product recrystallized from benzene were identical with those of an authentic sample (IA).

(+)- and (−)-IA Chromatographic separation of the individual IA enantiomers was achieved by using a HPLC Chiralcel OJ column (Daicel Chemical Co.).[18] The chromatographic profile and conditions are shown in Fig. 1. The first elution product (64 mg) was purified by silica gel (10 g) column chromatography (eluent, benzene–methylene chloride (20 : 80)). Crystallization of the residue from acetone gave (−)-IA as a brown powder, mp 140—142 °C, $[\alpha]_D^{22.5}$ −283° ($c=0.20$, EtOH) (lit. $[\alpha]_D$ −387°).[13a] CD ($c=1.05$ mg/ml, EtOH) [θ] (nm): −30450 (283) (negative maximum). −9120 (256), −27110 (295). MS *m/z*: 266 (M^+), 237 (base peak). ^{1}H-NMR δ (ppm): 1.11 (3H, d, $J=7.3$ Hz, 8-CH_3), 1.24 (3H, t, $J=7.3$ Hz, 10-CH_3), 2.61 (2H, q, $J=7.3$ Hz, 9-CH_2), 3.75 (1H, q, $J=7.3$ Hz, 1-H), 6.78 (1H, dd, $J=7.8$, 2.3 Hz, 5-H), 6.91 (2H, br d, $J=8.6$ Hz, 3′, 5′-H), 6.97 (1H, d, $J=2.3$ Hz, 7-H), 7.17 (1H, d, $J=7.8$ Hz, 4-H), 7.21 (2H, br d, $J=8.6$ Hz, 2′,6′-H), 8.10 (1H, br s, 4′- or 6-OH), 8.36 (1H, br s, 4′- or 6-H). The second elution product (58 mg) was purified by silica gel (10 g) column chromatography (eluent, benzene–methylene chloride (20 : 80)). Elution with benzene–methylene chloride (20 : 80) and crystallization of the residue from acetone gave (+)-IA as a brown powder, mp 142—143 °C, $[\alpha]_D^{22.5}$ +295° ($c=0.21$, EtOH) (lit. $[\alpha]_D$ +365°).[13a] CD ($c=1.01$ mg/ml, EtOH) [θ] (nm): +30870 (238) (positive maximum), +9380 (256), +27070 (293). MS *m/z*: 266 (M^+), 237 (base peak). ^{1}H-NMR δ (ppm): 1.11 (3H, d, $J=7.3$ Hz, 8-CH_3), 1.24 (3H, t, $J=7.3$ Hz, 10-CH_3), 2.61 (2H, q, $J=7.3$ Hz, 9-CH_2), 3.75 (1H, q, $J=7.3$ Hz, 1-H), 6.78 (1H, dd, $J=7.8$, 2.3 Hz, 5-H), 6.91 (2H, br d, $J=8.6$ Hz, 3′,5′-H), 6.97 (1H, d, $J=2.3$ Hz, 7-H), 7.17 (1H, d, $J=7.8$ Hz, 4-H), 7.21 (2H, br d, $J=8.6$ Hz, 2′,6′-H), 8.10 (1H, br s, 4′- or 6-OH), 8.37 (1H, br s, 4′- or 6-OH).

(±)-IB 1) (±)-IB was prepared by the method of Adler and Hagglund.[17] IB recrystallized from benzene was obtained as colorless needles, mp 128—129 °C. *Anal.* Calcd for $C_{18}H_{18}O_2 \cdot C_6H_6$: C, 83.69; H, 7.02. Found: C, 81.86; H, 6.93. MS *m/z*: 266 (M^+), 237 (base peak), 81, 69. ^{1}H-NMR δ (ppm): 0.40 (3H, t, $J=7.3$ Hz, 10-CH_3), 1.58 (1H, m, 9-CH_2), 1.94 (1H, m, 9-CH_2), 2.15 (3H, d, $J=1.7$ Hz, 8-CH_3), 3.84 (1H, br s, 1-H), 6.68 (1H, dd, $J=17.9$, 2.4 Hz, 5-H), 6.81 (1H, d, $J=2.3$ Hz, 7-H), 6.93 (2H, br d, $J=8.6$ Hz, 3′,5′-H), 7.22 (1H, d, $J=7.9$ Hz, 4-H), 7.27 (2H, br d, $J=8.8$ Hz, 2′,6′-H), 7.36 (1.6H out of 6H, s, benzene of solvent), 8.07 (1H, br s, 4′- or 6-OH), 8.41 (1H, br s, 4′- or 6-OH). ^{13}C-NMR data are shown in Table I.

2) Alternatively, *E*-DIEN (503 mg) was heated at 240 °C for 2 h in a glass tube under a nitrogen atmosphere. After cooling, the brown solid was extracted with acetone, and the extract was concentrated *in vacuo*. The ratio (43 : 57) of IA and IB was determined by ^{1}H-NMR analysis and the products were purified by silica gel (100 g) column chromatography. A mixture of the two products was eluted with methylene chloride, and then IB was separated through its acetate by the method of Adler and Hagglund.[17] Its melting point (128—129 °C), MS and ^{1}H-NMR data were identical with those of an authentic sample (IB).

Z-DIEN[20] (100 mg) was heated under the same conditions as described above to give a mixture (102 mg) of IA and IB in a ratio of 43 : 57.

Oxidation of DES with DDQ A solution of DES (1.0 g, 3.7 mmol) and DDQ (1.2 g, 5.5 mmol) in dioxane (40 ml) was refluxed for 5 h. Removal of the solvent under reduced pressure afforded a residue, which was shown to contain a major product (80% yield) and the starting material (5%) by ^{1}H-NMR analysis. The benzene extract of the residue was purified by silica gel (80 g) column chromatography (eluent, benzene–methylene chloride (60 : 40)). Recrystallization from benzene gave *Z*-DIEN as pale yellow needles, mp 193—194 °C. *Anal.* Calcd for $C_{18}H_{18}O_2$: C, 81.17; H, 6.81. Found: C, 81.47; H, 6.87. Its MS and ^{1}H-NMR data were identical with those of an authentic sample.[20]

(+)- and (−)-IB HPLC separation of the individual IB enantiomers was achieved by HPLC using a Chiralcel OJ column (Daichi Chemical Co.),[18] and the chromatographic profile and conditions are shown in Fig. 1. The first elution product (199 mg) was purified by silica gel (10 g) column chromatography (eluent, benzene–methylene chloride (20 : 80)). Crystallization from acetone gave (−)-IB as a brown powder, mp 93—94 °C, $[\alpha]_D^{24}$ −254° ($c=0.21$, EtOH) (lit. $[\alpha]_D$ −246°).[13a] CD ($c=1.15$ mg/ml, EtOH) [θ] (nm): −26920 (242) (negative maximum), −15820 (262), −26550 (288). MS *m/z*: 266 (M^+), 237 (base peak), 81, 69. ^{1}H-NMR δ (ppm): 0.40 (3H, t, $J=7.3$ Hz, 10-CH_3), 1.58 (1H, m, 9-CH_2), 1.94 (1H, m, 9-CH_2), 2.15 (3H, d, $J=1.7$ Hz, 8-CH_3), 3.84 (1H, br s, 1-H), 6.68 (1H, dd, $J=17.9$, 2.4 Hz, 6-H), 6.81 (1H, d, $J=2.3$ Hz, 4-H), 6.93 (2H, br d, $J=8.6$ Hz, 3′,5′-H), 7.22 (1H, d, $J=7.9$ Hz, 7-H), 7.27 (2H, br d, $J=8.8$ Hz, 2′,6′-H), 7.36 (1.6H out of 6H, s, benzene of solvent), 8.07 (1H, br s, 4′- or 5-OH), 8.41 (1H, br s, 4′- or 5-OH). The second elution product (193 mg) was purified by silica gel (10 g) column chromatography (eluent, benzene–methylene chloride (20 : 80)). Crystallization from acetone gave (+)-IB as a brown powder, mp 94—96 °C, $[\alpha]_D^{24}$ +276° ($c=0.20$, EtOH) (lit. $[\alpha]_D$ +261°).[13a] CD ($c=1.05$ mg/ml, EtOH) [θ] (nm): +25,640 (242) (positive maximum), +15830 (264), (+)-26090 (288). MS *m/z*: 266 (M^+), 237 (base peak), 81, 69. ^{1}H-NMR δ (ppm): 0.40 (3H, t, $J=7.3$ Hz, 10-CH_3), 1.58 (1H, m, 9-CH_2), 1.94 (1H, m, 9-CH_2), 2.15 (3H, d, $J=1.7$ Hz, 8-CH_3), 3.84 (1H, br s, 1-H), 6.68 (1H, dd, $J=17.9$, 2.4 z, 6-H), 6.81 (1H, d, $J=2.3$ Hz, 4-H), 6.93 (2H, br d, $J=8.6$ Hz, 3′, 5′-H), 7.22 (1H, d, $J=7.9$ Hz, 7-H), 7.27 (2H, br d, $J=8.8$ Hz, 2′,6′-H), 7.36 (1.6 H out of 6H, s, benzene of solvent), 8.07 (1H, br s, 4′- or 5-OH), 8.41 (1H, br s, 4′- or 5-OH).

Di(4′-bromobenzoate) of (−)-IB (−)-IB (35 mg) was treated with 4-bromobenzoyl chloride (100 mg) in pyridine (0.35 ml) for 2 h at room temperature (26 °C). The reaction product was diluted with ice-water, and extracted with benzene, and the extract was washed with 10% HCl, 5% Na_2CO_3, and water, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent, *n*-hexane–benzene (50 : 50)). Recrystallization from a methylene chloride/*n*-hexane solution gave the di(4-bromobenzoate) derivative of (−)-IB as colorless needles, mp 194—195 °C. ^{1}H-NMR δ (ppm): 0.47 (3H, $J=7.26$ Hz, 10-CH_3), 1.68—1.8 (1H, m, 9-CH_2), 2.0—2.15 (1H, m, 9-CH_2), 2.29 (3H, d, $J=2.0$ Hz, 8-CH_3), 4.12 (1H, br s, 3-H), 7.15 (1H, dd, $J=8.5$, 2.0 Hz, 5-H), 7.33 (1H, d, $J=2.0$ Hz, 7-H), 8.91 (2H, br d, $J=8.9$ Hz, 3′,5′-H), 7.58 (1H, d, $J=8.5$ Hz, 4-H), 7.60 (2H, br d, $J=8.9$ Hz, 2′,6′-H), 7.83 (4H, br d, $J=8.2$ Hz, aromatic H), 8.15 (4H, br d, $J=8.2$ Hz, aromatic H).

Crystal Structure of the Di(4-bromobenzoate) of (−)-IB A colorless prism crystal was obtained from the solution in a mixture of methylene chloride and *n*-hexane and mounted on an automated Rigaku AFC-5 X-ray diffractometer using MoK_α radiation. The unit cell parameters are $a=16.170$ (2), $b=12.046$ (2), $c=7.903$ (1) Å, $\alpha=86.60$ (1), $\beta=76.49$ (1),

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and $\gamma = 69.71\ (3)^\circ$ in space group $P1$ ($Z=2$). Of the 6607 reflections measured with $2\theta \leq 55^\circ$ employing a $2\theta/\omega$ scan mode, 2908 were independently observed at the level of $F > 3\sigma\ (F)$. Three reflections measured every 100 reflections showed no significant variation in intensity. The size of the crystal used was $0.3 \times 0.2 \times 0.2$ mm, but no correction was carried out for absorption. The structure was solved by using MULTAN 78[21] and successive Fourier syntheses, and refined by using the block-diagonal least-squares technique with anisotropic temperature factors for non-hydrogen atoms. Hydrogen atoms except those of the methyl group were included at calculated positions with the equivalent isotropic temperature factors of the bound atoms, but not refined. The refinement was terminated at $R = 0.068$. Calculations were performed with the Direct-Search program system.[22] Twenty Bijvoet pairs which exhibited large effects of anomalous scattering from the bromine atoms were selected and used to determine the absolute configuration. All observed Bijvoet ratios were in agreement with those calculated for the chosen enantiomer, shown in Fig. 3. Some bond lengths and angles differ from typical values because of disorder and/or absorption. Five tables consisting of atomic fractional coordinates, temperature factors, bond lengths, bond angles, and Bijvoet ratios have been deposited as supplementary material.

Preparation of Microtubule Proteins Microtubule proteins were prepared from porcine brain by two cycles of temperature-dependent assembly–disassembly using the methods of Shelanski *et al.*[23] and Ishikawa *et al.*[24] The microtubule proteins were stored at $-80\,^\circ$C for later use.[12]

Microtubule Assembly Assay The effect of the test compounds on microtubule proteins at 37°C was determined by turbidity measurement at 400 nm using a UVIDEC 430B double-beam spectrometer equipped with a thermostatically controlled cell-holder. Microtubule proteins were adjusted to a concentration of 3.0 mg protein/ml in 5 mM 2-(morpholino)ethanesulfonic acid, 0.5 mM $MgSO_4$, 1 mM ethyleneglycol-bis(2-aminoethylether)*N*,*N*,*N*′,*N*′-tetraacetic acid, 50 mM KCl and 1 mM guanosine 5′-triphosphate (pH 6.5), and used as 1-ml aliquots for measurement. Each test compound was dissolved in a 1:1 mixture of dimethyl sulfoxide and *N*,*N*-dimethylformamide[25] and this solution was added to the protein solution at a volume ratio of 2%.

Protein Concentration The concentration of microtubule proteins was determined by the method of Lowry *et al.*[26] using bovine serum albumin as the standard.

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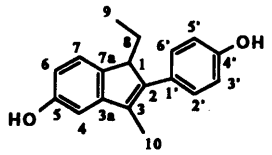

IB

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