Pergamon

Tetrahedron, Vol. 53, No. 35, pp. 11961-11970, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(97)00709-5

Configurational Assignment of Brassinosteroid Sidechain by Exciton Coupled Circular Dichroic Spectroscopy

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Abstract: A microscale method was developed to determine the sidechain configuration of brassinosteroids, a class of potent plant growth promoters. Microscale naphthoylation followed by circular dichroic (CD) spectroscopy measurement in acetonitrile unambiguously differentiate between the two possible vicinal *syn*-diol configurations, (22*R*,23*R*) and (22*S*,23*S*). © 1997 Elsevier Science Ltd.

Brassinosteroids are a new group of phytohormones with high growth stimulating and anti-stress activities, and which exist in only minuscule amounts.[1-3] After the isolation and characterization of brassinolide in 1979[4] about 40 members have been discovered in a broad spectrum of monocots and dicots as well as in gymnosperms, thus suggesting their ubiquitous occurrence in plants.[5] Structurally most brassinosteroids are A/B-*trans* fused steroids with 2α,3α-diol functionalities, where ring B is 6a-oxa-6-oxo-, 6-oxo- or 6-deoxo. A characteristic feature for all native members found so far is the (22*R*,23*R*) vicinal diol in the side chain.

Hydroxylation of suitable Δ^{22}-unsaturated sterol precursors with OsO_4 represents a key step in the synthesis of brassinosteroids, which are necessary for structural characterization of new brassinosteroids and for practical applications. Although usage of chiral ligands leads to a higher stereoselectivity of the desired native (22*R*,23*R*)-diol,[6] the (22*S*,23*S*)-isomer with lower bioactivity[7] is also formed in these reactions.[8] However, since there exists no general method for configurational assignments, a versatile and rapid analytical method based on exciton coupled circular dichroism (ECCD) of their pernaphthoates is reported here.

ECCD method is a microscale procedure for determining the absolute configurations and conformations of compounds containing two or more chromophores in solution.[9,10] Hydroxyl and amino groups are converted into para-substituted benzoates, naphthoates, and other chromophores. Provided the chromophores are close-by in space, the electric transition moments will couple and give rise to a bisignate CD. A positive absolute twist between the electron transition moments of the coupled chromophores, i.e., positive chirality, will give rise to a split CD curve with positive first and negative second Cotton effects at longer and shorter wavelength, respectively. The amplitude (A-value) of the split CD, defined as the difference between $\Delta\varepsilon_1$(first Cotton effect, longer wavelength) and $\Delta\varepsilon_2$(second Cotton effect, shorter wavelength), is inversely proportional to the distance between the two chromophores.[9] Importantly, in case of three or more identical[11,12] or different chromophores,[13,14] the exciton split CD curve can be approximated by pair-wise addition of the interacting

chromophores. Thus, in a compound with three chromophores, A/B/C, the CD is represented by three corresponding pair-wise contributions, namely, A/B, B/C, and A/C ("pair-wise additivity rule").[14b]

In the present case, a series of synthetic brassinosteroids with 22*R*,23*R*-(**1a, 3a, 5a, 7a, 9a**) and 22*S*,23*S*-(**2a, 4a, 6a, 8a, 10a**) stereochemistry have been investigated (Figure 1). Compounds **1a-5a** and **7a-9a** were prepared as described in the literature (**1a, 2a**[15]; **3a, 4a**[16]; **5a**[17]; **7a, 8a, 9a, 10a**[18]). Compound **6a** was synthesized via the isopropylidenedioxy derivative **12'** of **12**[6] followed by isomerization of the 3α,5-cyclo-6-ketone to the corresponding Δ^2-6-ketone **6a'** and subsequent deprotection with HCl in MeOH (Scheme 1). The low μg scale naphthoylation of brassinosteroids and reference CD spectra of naphthoates (in acetonitrile) which clearly distinguishes the two configurations will be described. Applicability of the ECCD method to acyclic species is briefly discussed in the end.

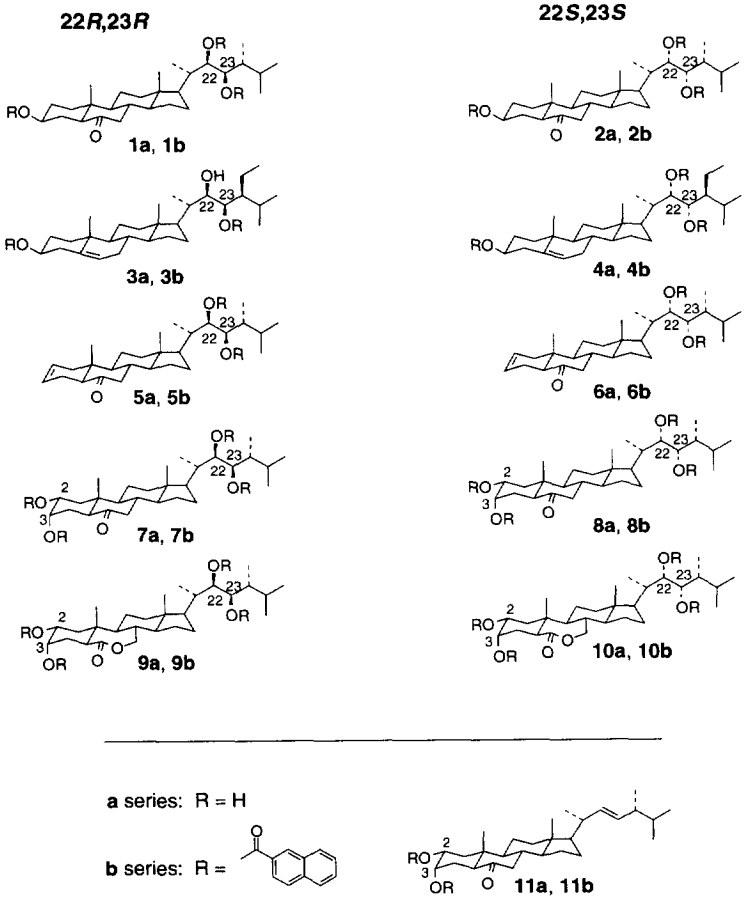
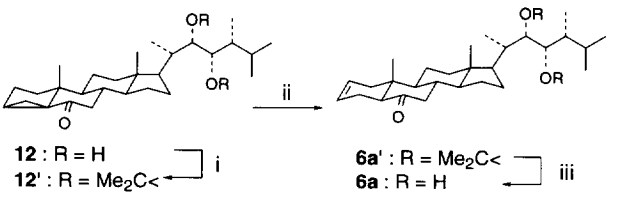


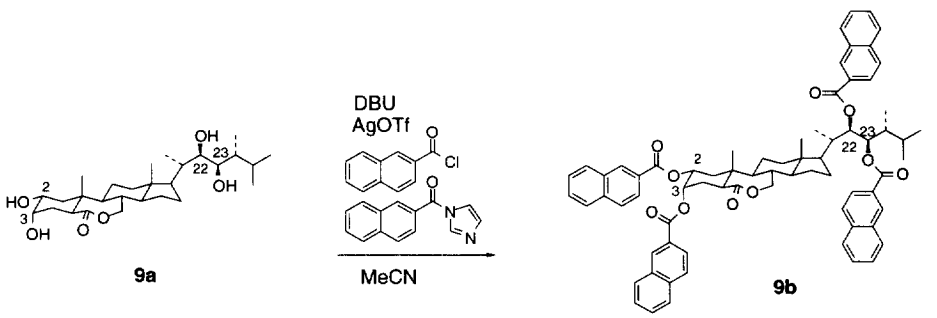
Figure 1. Brassinosteroids and their pernaphthoates



Scheme 1. Synthesis of **6a**

Results and Discussion

Of the numerous possible chromophores, 2-naphthoate was chosen for the current study because its high UV ε-value (59,000 in acetonitrile) makes it suited for lower μg level studies. However, pernaphthoylation of the steroid sidechain did not proceed efficiently under earlier conditions[19] using 1-(2-naphthoyl)imidazole and DBU due to the stringent demand of placing two naphthoate groups on the sidechain. Many other reagents and conditions, including 2-naphthoyl chloride, silver trifluoromethanesulfonate, and DMAP, the same protocol used for perbromobenzoylation and 4-methoxycinnamoylation of oligosaccharides,[20] gave only poor yields. It was found that the pernaphthoates could be obtained in decent yields (upto 60 %) when an acetonitrile solution of the steroid is refluxed with 1-(2-naphthoyl)imidazole overnight. Many of the pernaphthoates, **1b-4b** and **7b-10b**, were prepared by this method, but the yield of **5b** and **6b** were still low, the mononaphthoates being the major products. However, further efforts led to the finding that almost quantitative yields could be attained when 2-naphthoyl chloride and silver trifluoromethanesulfonate were mixed with the 1-(2-naphthoyl)imidazole-DBU solution (Scheme 2). According to this protocol, large scale (ca. 0.5 mg) derivatization of **2a**, **5a**, **6a**, and **11a** proceeded quantitatively, while microscale (10-20 μg) reaction of **1a-10a** gave sufficient pernaphthoates for UV/CD measurements. Preliminary studies suggested that DBU is not working as a catalyst but as a base in this reaction.



Scheme 2. Pernaphthoylation of brassinosteroids.

The UV and CD of pernaphthoates **1b**-**6b** were examined in non-polar (methylcyclohexane) and polar (acetonitrile) solvents. In the case of methylcyclohexane, the (22*R*,23*R*)- and (22*S*,23*S*)-series both exhibited negative couplets but with different amplitudes of -170 and -70, respectively. Although it is possible to differentiate the two series from the amplitudes, this is hardly satisfactory. The differentiation between the two series, however, was clear when acetonitrile was used as solvent (Figs. 2A and 2B). The (22*R*,23*R*)-series, namely, pernaphthoates of 24-epiteasterone (**1b**), (22*R*,23*R*)-22,23-dihydroxystigmasterol (**3b**), and (22*R*,23*R*,24*R*)-22,23-dihydroxy-24-methyl-5α-cholest-2-en-6-one (**5b**) give negative couplets with large amplitudes (A values); the Δε of the first Cotton effect (CE) at 243 nm was -209 to -252, while that of the second CE at 229 nm ranged from +199 to +230. The CD A-values of these compounds thus exceed -400 (Figure 2A). In contrast, the derivatives of the (22*S*,23*S*)-diol series, 22,23,24-trisepiteasterone (**2b**) and (22*S*,23*S*)-22,23-dihydroxystigmasterol (**4b**), (22*S*,23*S*,24*R*)-22,23-dihydroxy-24-methyl-5α-cholest-2-en-6-one (**6b**), show positive CD curves with A-values around +80 (Figure 2B). Since the CD signs of the two series are opposite to each other in acetonitrile, i.e., negative for the (22*R*,23*R*)- and positive for the (22*S*,23*S*)-series, configurational assignment of the sidechain 22,23-*syn*-diol moiety is nonambiguous.

It should be noted that the stereochemistry at C-24 does not affect the CD signs. The configuration is 24*S* in stigmasterol derivatives **3b** and **4b**, while it is 24*R* in compounds **1b**, **2b**, **5b**, and **6b**, yet the CD sign is governed by the configuration of 22,23-diol group as shown on Figure 2.

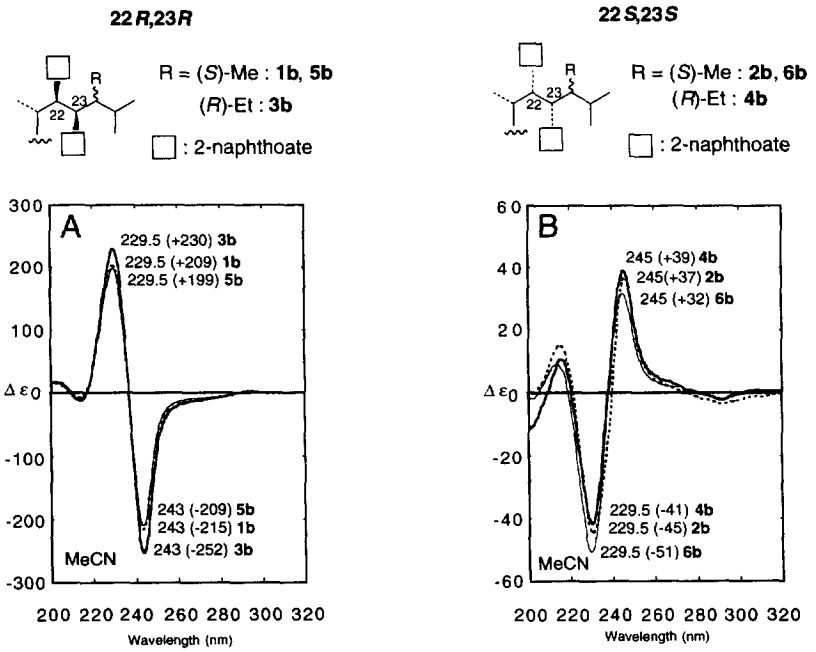


Figure 2. A. CD spectra of **1b** (dotted line), **3b** (bold solid line), and **5b** (thin solid line). B. CD spectra of **2b** (dotted line), **4b** (bold solid line), and **6b** (thin solid line). All spectra were taken in acetonitrile.

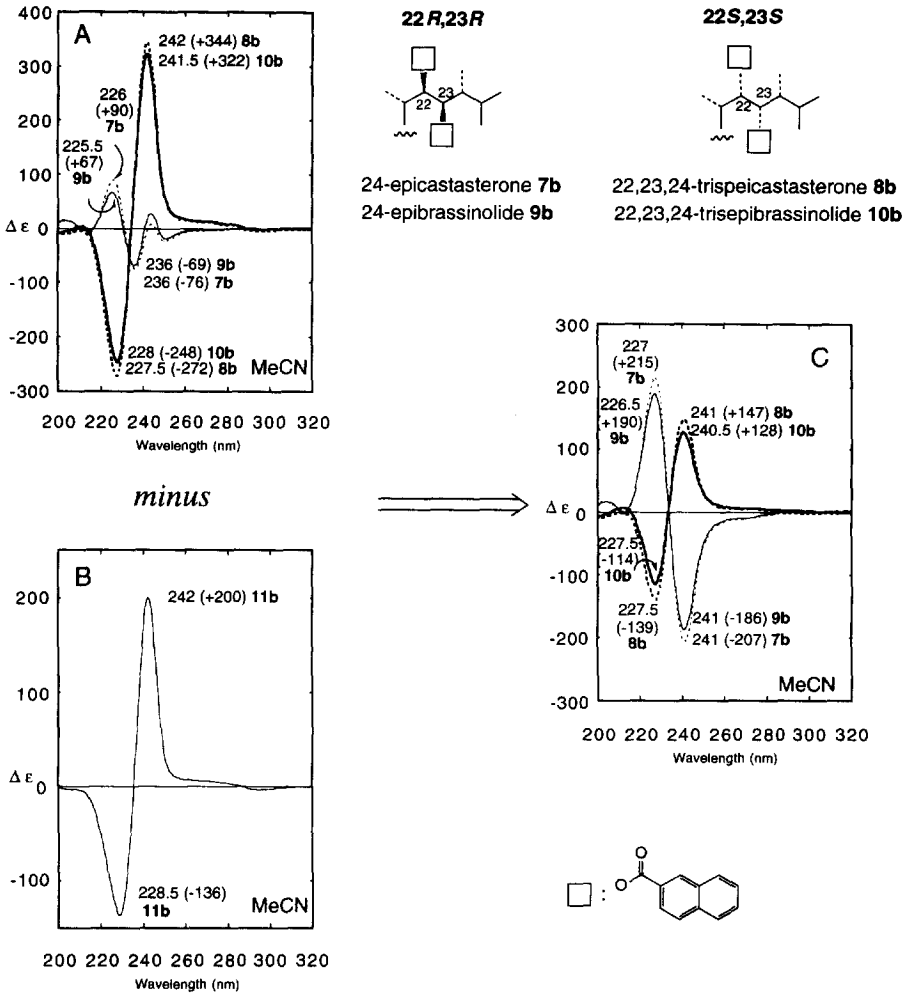


Figure 3. A. CD spectra of **7b** (thin dotted line), **8b** (bold dotted line), **9b** (thin solid line), and **10b** (bold solid line). B. CD of **11b**. C. Difference spectra (spectra in A minus spectrum in B) of **7b** (thin dotted line), **8b** (bold dotted line), **9b** (thin solid line), and **10b** (bold solid line). All spectra were taken in acetonitrile.

Table 1. UV ε-values of bis-, tris-, and tetra-naphthoates in acetonitrile.

	Compounds	ε-value
Bisnaphthoates	**5b**, **6b**,[a] and **11b**	97,000 (232 nm)
Trisnaphthoates	**1b**, **2b**,[a] **3b**, and **4b**[a]	147,500 (233 nm)
Tetranaphthoates	**7b**, **8b**, **9b**, and **10b**	198,500 (232 nm)

[a] UV λ_{max} of these compounds are slightly red-shifted (See Experimental).

Castasterones **7a**, **8a** and brassinolides **9a**, **10a** contain a 2α,3α-diol group, which gives rise to strong positive curve when derivatized. Therefore, the CD spectra of (22*R*,23*R*)- and (22*S*,23*S*)-series were different from the corresponding previous cases shown above (Figure 3A). The (22*S*,23*S*)-series, **8b** and **10b**, showed strong positive curve, while (22*R*,23*R*)-counterparts, **7b** and **9b**, showed much smaller CD with multiple peaks. The two configurations can still be differentiated since the CD curves of the two series were clearly different each other. However, difference in the sidechain configuration becomes much clearer upon subtraction of the CD of (22*E*,24*R*)-2α,3α-dihydroxy-24-methyl-5α-cholest-22-en-6-one bisnaphthoate **11b** (Figure 3B for **11b**; Figure 3C for difference spectra of compounds **7b**-**10b**). These difference spectra are only an approximation of the sidechain CD since some long-range interaction between the skeletal and sidechain vicinal naphthoates clearly exists: the entire CD curves of those tetranaphthoates are made of six pair-wise interactions ("pair-wise additivity rule")[14b] and the subtraction procedure above removes only one of them. However, this qualitative analysis successfully differentiated between (22*R*,23*R*)- and (22*S*,23*S*)-configurations. The difference spectra for the (22*R*,23*R*)-series, **7b** and **9b**, gave strong negative couplets, the sign being the same as that of **1b**, **3b**, and **5b**; the difference spectra for the (22*S*,23*S*)-series, **8b** and **10b**, was a positive couplet, the sign again being the same as that of **2b**, **4b** and **6b**. In the former (22*R*,23*R*)-series the CD amplitudes are both large; however, the amplitudes for the latter (22*S*,23*S*)-series are +286 and +242 for **8b** and **10b**, respectively, which are much larger than those of **2b**, **4b** and **6b**, ca. +80.

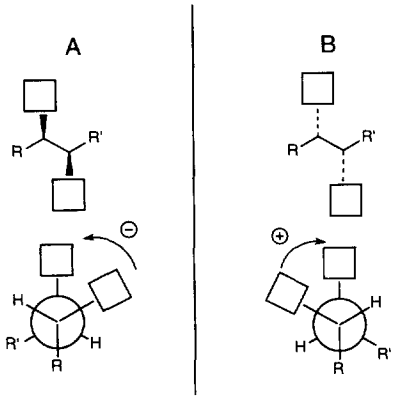


Figure 4. Naphthoates of 1,2-*syn*-diols, and a *gauche* conformation that is presumably most populated in acetonitrile.[21] The squares represent the naphthoate groups.

This amplitude difference in the (22*S*,23*S*)-series is possibly due to the flexible nature of the sidechain moiety. A previous CD study showed that acyclic 1,2-*syn*-diols with the same configuration as in the (22*R*,23*R*)-series mostly adopted a *gauche* conformation giving rise to a *negative* CD curve (Figure 4A).[21] If that is the case, acyclic 1,2-*syn*-diols with the same configuration as in (22*S*,23*S*) should also mainly adopt a *gauche* conformation that gives a *positive* CD curve (Figure 4B). In the case of brassinosteroids, however, the conformational distribution seems different from that of simple model 1,2-vicinal diols because of the presence of many substituents at C-21 and C-24. NMR coupling constants ($^3J_{22H\text{-}23H}$) of (22*R*,23*R*)-series, **7b** and **9b**, in acetonitrile-d_3 were 8.8 and 8.7 Hz, respectively, indicating that the C-22 and C-23 chromophores mainly adopt the *gauche* conformation giving rise to a negative CD as in Figure 4A. On the other hand, *J* values of the (22*S*,23*S*)-series, **8b** and **10b**, were 4.4 and 4.8 Hz, respectively.[22] This result, combined with the weaker amplitude of the positive CD couplet (relative to the negative amplitude of the *R,R* series), suggests that, although the *gauche* conformation as in Figure 4B is still considerably populated, the conformational distribution in (22*S*,23*S*) is more dispersed than in the case of (22*R*,23*R*). In compounds **2b**, **4b** and **6b** (NMR could not be measured in acetonitrile-d_3 because of poor solubility), the *gauche* conformation is even less populated, as

judged from the much weaker CD A-value. In addition to this conformational factor, the above mentioned long-range exciton coupling between the skeletal and sidechain naphthoates also contribute to the differences.

Although it is not easy to predict the CD amplitudes of acyclic compounds, the general trend of CD sign for acyclic vicinal *syn*-diols[21] still holds for the brassinosteroids studied here, and the spectra can be used as references for stereochemical analysis of brassinosteroids with unknown sidechain configuration.

Conclusion

CD spectra of brassinosteroid pernaphthoates were obtained as references for the assignment of the sidechain configuration of unknown brassinosteroids. Although nonempirical analysis of ECCD of acyclic compounds may still be difficult, the method can be used in the microscale structural analyses of a wide variety of acyclic compounds of biological importance.[21,23,24] Once a set of reference spectra or a data bank is established, compounds with unknown stereochemistry can be readily determined without reference compounds. A major advantage of the present CD method is its minuscule scale; this is particularly important in cases such as naturally occurring brassinosteroids where large amounts are not available.

Experimental

General Method. Experimental details for the synthesis of compounds **1a**-**11a** and **12** have been recorded previously.[15-18,25] 2-naphthoyl chloride, silver trifluoromethanesulfonate, 2-naphthoic acid, 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU), 1,1'-carbonyldiimidazole were purchased from Aldrich. 1-(2-naphthoyl)imidazole was prepared as described previously,[19] and purified by silica gel column chromatography: benzene-acetone 2:1(v/v) R_f 0.52. DBU was purified by vacuum distillation before use. All other solvents and reagents were used directly without further purification unless otherwise specified. CD spectra were measured by JASCO J-720 spectropolarimeter. Parameters for CD measurement were as follows, Bandwidth 1.0 nm, Slit width Auto, Sensitivity 10 mdeg, Response 4 sec., Start wave length 400 nm, End wave length 200 nm, Scan speed 100 nm/min., Step resolution 0.5 nm, Accumulation 4. TLC plate used for both analysis and preparation was silica gel 60 F-254, 0.25 mm, E. Merck. NMR spectra were recorded either on Bruker DMX 500 or on Varian VXR 400 instrument and performed in $CDCl_3$ or in acetonitrile-d_3. Chemical shifts (δ) are reported in ppm downfield from internal TMS and coupling constants (J) in Hz. The abbreviations used are as follows: s, singlet; d, doublet; dd, doublet-doublet; t, triplet; m, multiplet; br, broad. Low-resolution and high-resolution FAB mass spectra were measured on a JEOL JMS-DX303 HF mass spectrometer using a glycerol matrix and Xe ionizing gas. CI mass spectra were measured on a NERMAG R10-10 spectrometer with NH_3 as ionizing gas. Bransonic® ultrasonic cleaner 3210R-MT was used for sonication.

Pernaphthoylation with 1-(2-naphthoyl)imidazole (Heating protocol). Steroid (ca. 1 mg) was dissolved in anhydrous acetonitrile (0.5 ml), which was added excess amount (10 equiv.) of 1-(2-naphthoyl)imidazole and refluxed overnight. Product was purified by preparative TLC (20 × 20 cm: solvent: chloroform; R_f values are shown below). Yield depends on substrate: **7b**, **8b**, **9b** and **10b** (60 %); **1b** and **2b** (40%); **3b** and **4b** (20%); Yields of **5b** and **6b** were less than 10%.

Pernaphthoylation with DBU, 2-naphthoyl chloride, silver trifluoromethanesulfonate, and 1-(2-naphthoyl)imidazole. Steroid (10 μg to 0.5 mg) was dissolved in anhydrous acetonitrile (0.1 ml), which was added DBU (1 drop, excess), silver trifluoromethanesulfonate (2.0 mg, 7.8 μmol), 2-naphthoyl chloride (1.5 mg, 7.8 μmol), and 1-(2-naphthoyl)imidazole (2.0 mg, 9.0 μmol). The turbid yellowish solution was stirred overnight at room temperature, quenched by saturated NH_4Cl, extracted with ethyl acetate (5 ml × 2), washed with saturated sodium bicarbonate (5 ml × 2), dried over $MgSO_4$. The crude material was further purified by TLC: 2 × 10 cm plate for microscale (10-20 μg); 20 × 20 cm for large scale (ca. 0.5 mg) derivatization (solvent: chloroform; R_f values are shown below). Large scale derivatization of **2a**, **5a**, **6a**, and **11a** gave quantitative pernaphthoates. Microscale derivatization (10-20 μg) of **1a**-**10a** gave enough products for UV/CD measurements. Isolated products should be pumped overnight before UV/CD measurement, otherwise the CD amplitudes could be smaller than those shown in this paper. Brief sonication (a few seconds) is recommended when **1b**-**6b** are dissolved into acetonitrile.

UV ε-value measurements. Pernaphthoates were lyophilized using benzene and pumped overnight before weighing. Some of the pernaphthoates, **1b-6b**, needed to be briefly sonicated (a few seconds) when dissolved into acetonitrile. The obtained ε-values are shown on Table 1.

24-Epiteasterone trinaphthoate(1b). R_f 0.22 (chloroform). UV λ_{max} 233 nm. ^{1}H-NMR (500MHz, acetonitrile-d_3): δ 0.78 (3H, s), 0.80 (3H, s), 0.97 (3H, d, *J*=7.1), 1.03 (3H, d, *J*=6.2), 1.05(3H, *J*=6.3), 1.28 (3H, d, *J*=6.7), 4.89 (1H, m, H-3), 5.63 (1H, dd, *J*=8.7, 4.0, H-23), 5.78 (1H, d, *J*=8.7, H-22), 7.4-8.10 (18H, m), 8.34 (1H, s), 8.36 (1H, s), 8.60 (1H, s). FABHRMS (*m/z*): calcd. for $C_{61}H_{67}O_7$ $[M+H]^+$ 911.4887, found 911.4901.

22,23,24-Trisepiteasterone trinaphthoate(2b). R_f 0.28 (chloroform). UV λ_{max} 234 nm. ^{1}H-NMR (500MHz, $CDCl_3$): δ 0.73 (3H, s), 0.86 (3H, s), 0.98 (3H, d, *J*=7.6), 1.00 (3H, d, *J*=7.1), 1.01 (3H, d, *J*=6.6), 1.15 (3H, d, *J*=7.0), 5.03 (1H, m, H-3), 5.59 (1H, t, *J*=3.2, H-22), 5.79 (1H, dd, *J*=6.1, 3.2, H-23), 7.60 (6H, m), 7.91 (6H, m), 7.98 (3H, br d, *J*=6.7), 8.08 (1H, dd, *J*=8.6, 1.6), 8.16 (2H, d, *J*=8.5), 8.62 (1H, s), 8.69 (2H, s). FABHRMS (*m/z*): calcd. for $C_{61}H_{67}O_7$ $[M+H]^+$ 911.4887, found 911.4889.

(22*R*,23*R*)-22,23-Dihydroxystigmasterol trinaphthoate(3b). R_f 0.70 (chloroform). UV λ_{max} 233 nm. ^{1}H-NMR (500MHz, $CDCl_3$): δ 0.78 (3H, s), 0.91 (3H, d, *J*=6.8), 1.05 (3H, d, *J*=6.8), 1.08 (3H, s), 1.14 (3H, t, *J*=7.5), 1.32 (3H, d, *J*=6.6), 2.23 (1H, m), 2.49 (2H, d, *J*=7.3), 4.87 (1H, m), 5.40 (1H, d, *J*=4.5), 5.67 (1H, d, *J*=9.6), 5.80 (1H, d, *J*=9.6), 7.35-8.05 (18H, m), 8.22 (1H, s), 8.26 (1H, s), 8.57 (1H, s). FABHRMS (*m/z*): calcd. for $C_{62}H_{68}O_6Na$ $[M+Na]^+$ 931.4913, found 931.4904.

(22*S*,23*S*)-22,23-Dihydroxystigmasterol trinaphthoate(4b). R_f 0.73 (chloroform). UV λ_{max} 234 nm. ^{1}H-NMR (500MHz, $CDCl_3$): δ 0.71 (3H, s), 0.94 (3H, d, *J*=6.8), 0.97 (3H, d, *J*=6.9), 1.03 (3H, t, *J*=7.3), 1.06 (3H, s), 1.10 (3H, d, *J*=7.0), 2.51 (2H, d, *J*=7.6), 4.91 (1H, m), 5.44 (1H, d, *J*=4.0), 5.55 (1H, apparent t, *J*=3.5), 5.80 (1H, br t, *J*=4.5), 7.50-8.15 (18H, m), 8.58 (1H, s), 8.63 (2H, s). FABHRMS (*m/z*): calcd. for $C_{62}H_{69}O_6$ $[M+H]^+$ 909.5094, found 909.5065.

(22*R*,23*R*,24*R*)-22,23-Dihydroxy-24-methyl-5α-cholest-2-en-6-one bisnaphthoate(5b). R_f 0.43 (chloroform). UV λ_{max} 232 nm. ^{1}H-NMR (500MHz, $CDCl_3$): δ 0.72 (3H, s), 0.78 (3H, s), 1.02 (3H, d, *J*=7.1), 1.049 (3H, d, *J*=6.7), 1.054 (3H, d, *J*=6.9), 1.29 (3H, d, *J*=6.4), 5.55 (1H, m), 5.65 (1H, dd, *J*=7.9, 4.7, H-23), 5.70 (1H, m), 5.82 (1H, d, *J*=7.3, H-22), 7.45-7.95 (12H, m), 8.42 (1H, s). FABHRMS (*m/z*): calcd. for $C_{50}H_{59}O_5$ $[M+H]^+$ 739.4363, found 739.4384.

(22*S*,23*S*,24*R*)-22,23-Dihydroxy-24-methyl-5α-cholest-2-en-6-one bisnaphthoate(6b). R_f 0.43 (chloroform). UV λ_{max} 233 nm. ^{1}H-NMR (500MHz, $CDCl_3$): δ 0.72 (6H, s), 0.99 (9H, m), 1.14 (3H, d, *J*=7.0), 5.57 (2H, m), 5.72 (1H, m), 5.70 (1H, m), 5.78 (1H, m, H-22), 7.6 (4H, m), 7.9 (4H, m), 7.98 (2H, dd, *J*=8.0, 3.2), 8.15 (2H, d, *J*=8.6), 8.69 (2H, s). FABHRMS (*m/z*): calcd. for $C_{50}H_{59}O_5$ $[M+H]^+$ 739.4363, found 739.4366.

24-Epicastasterone tetranaphthoate(7b). R_f 0.11 (chloroform), 0.55 (hexane-ethyl acetate, 2:1(v/v)). Note: R_f of **7b** is very close to that of a byproduct: one of trisnaphthoates which gives strong positive CD. The mixture of **7b** and the byproduct can be separated by TLC (developed three times with hexane-ethyl acetate 4:1(v/v), **7b** is the higher spot at R_f 0.28). UV λ_{max} 232 nm. ^{1}H-NMR (500MHz, acetonitrile-d_3): δ 0.82 (3H, s), 0.95 (3H, s), 0.99 (3H, d, *J*=7.12), 1.06 (3H, d, *J*=6.3), 1.07 (3H, d, *J*=6.0), 2.95 (1H, m), 5.37 (1H, m, H-2), 5.65 (1H, dd, *J*=8.8, 4.0), 5.78 (1H, m, H-3), 5.81 (1H, d, *J*=8.6), 7.40-8.10 (24H, m), 8.36 (1H, s), 8.37 (2H, s), 8.39 (1H, s), 8.64 (1H, s). FABHRMS (*m/z*): calcd. for $C_{72}H_{72}O_9Na$ $[M+Na]^+$ 1103.5070, found 1103.5060.

22,23,24-Trisepicastasterone tetranaphthoate(8b). R_f 0.11 (chloroform), 0.55 (hexane-ethyl acetate, 2:1(v/v)). UV λ_{max} 232 nm. ^{1}H-NMR (500MHz, acetonitrile-d_3): δ 0.76 (3H, s), 0.95 (3H, s), 1.00 (3H, d, *J*=6.8), 1.04 (3H, d, *J*=6.9), 1.07 (3H, s), 1.17 (3H, d, *J*=7.0), 3.02 (1H, t, *J*=8.3), 5.38 (1H, m, H-2), 5.57 (1H, apparent t, *J*=4.0*, H-22), 5.81 (1H, m, H-3), 5.83 (1H, m, H-23), 7.45-8.20 (24H, m), 8.38 (1H, s), 8.63 (2H, s), 8.73 (1H, s). *Apparent coupling constant from ^{1}H-NMR measurement: precise coupling constant between H-22 and H-23 ($^3J_{H22,H23}$=4.4 Hz) was obtained by decoupling experiment.[22] FABHRMS (*m/z*): calcd. for $C_{72}H_{72}O_9Na$ $[M+Na]^+$ 1103.5070, found 1103.5040.

24-Epibrassinolide tetranaphthoate(9b). R_f 0.08 (chloroform), 0.49 (hexane-ethyl acetate, 2:1(v/v)). Note: R_f of **9b** is very close to that of a byproduct: one of trisnaphthoates which gives strong positive CD. The

mixture of **9b** and the byproduct can be separated by TLC (developed three times with hexane-ethyl acetate 4:1(v/v), **9b** is the higher spot at R_f 0.20). UV λ_{max} 232 nm. ^{1}H-NMR (500MHz, acetonitrile-d_3): δ 0.85 (3H, s), 0.99 (3H, d, *J*=7.1), 1.07 (9H, m), 1.29 (3H, d, *J*=6.8), 2.46 (1H, m, H-4$_{eq}$), 3.37 (1H, dd, *J*=12.3, 4.6, H-5), 4.10 (1H, d, *J*=12.6, H-7$_a$), 4.33 (1H, m, H-7$_b$), 5.30 (1H, m, H-2), 5.65 (1H, dd, *J*=8.7, 4.0), 5.76 (1H, m, H-3), 5.81 (1H, d, *J*=8.7), 7.15-8.10 (24H, m), 8.36 (1H, s), 8.39 (1H, s), 8.42 (1H, s), 8.65 (1H, s). FABHRMS (*m/z*): calcd. for $C_{72}H_{72}O_{10}Na$ [M+Na]$^+$ 1119.5020, found 1119.5010.

22,23,24-Trisepibrassinolide tetranaphthoate(10b). R_f 0.08 (chloroform), 0.49 (hexane-ethyl acetate, 2:1(v/v)). UV λ_{max} 232 nm. ^{1}H-NMR (500MHz, acetonitrile-d_3): δ 0.78 (3H, s), 0.97 (3H, d, *J*=6.8), 0.99 (3H, d, *J*=6.7), 1.03 (3H, d, *J*=6.9), 1.07 (3H, s), 1.15 (3H, d, *J*=7.0), 2.48 (1H, m, H-4$_{eq}$), 3.44 (1H, dd, *J*=12.4, 4.6, H-5), 4.10 (1H, d, *J*=12.6, H-7$_a$), 4.33 (1H, dd, *J*=12.6, 9.6, H-7$_b$), 5.30 (1H, m, H-2), 5.56 (1H, br t, *J*=4.0*, H-22), 5.78 (1H, m, H-3), 5.81 (1H, br t, *J*=5.1*, H-23), 7.45-8.20 (24H, m), 8.25 (1H, s), 8.62 (2H, s), 8.74 (1H, s). *Apparent coupling constant from ^{1}H-NMR measurement: precise coupling constant between H-22 and H-23 ($^3J_{H22,H23}$=4.8 Hz) was obtained by decoupling experiment.[22] FABHRMS (*m/z*): calcd. for $C_{72}H_{73}O_{10}$ [M+H]$^+$ 1097.5200, found 1097.5210.

(22*E*, 24*R*)-2α,3α-Dihydroxy-24-methyl-5α-cholest-22-en-6-one bisnaphthoate(11b). R_f 0.20 (chloroform). UV λ_{max} 232 nm. ^{1}H-NMR (500MHz, $CDCl_3$): δ 0.75 (3H, s), 0.85 (3H, d, *J*=7.0), 0.87 (3H, d, *J*=7.8), 0.95 (3H, d, *J*=6.8), 1.03 (3H, s), 1.05 (3H, d, *J*=6.6), 5.22 (2H, m, H-22 and H-23), 5.45 (1H, m, H-2), 5.89 (1H, m, H-3), 7.43 (1H, t, *J*=7.0), 7.54 (1H, t, *J*=7.0), 7.62 (2H, m), 7.65 (1H, m), 7.77 (1H, d, *J*=8.6), 7.82 (1H, d, *J*=8.3), 7.93 (1H, dd, *J*=8.6, 1.6), 7.98 (2H, t, *J*=8.9), 8.03 (1H, d, *J*=8.1), 8.13 (1H, dd, J=8.5, 1.5), 8.42 (1H, s), 8.66 (1H, s). FABHRMS (*m/z*): calcd. for $C_{50}H_{59}O_5$ [M+H]$^+$ 738.4363, found 738.4367.

(22*S*,23*S*,24*R*)-22,23-Isopropylidenedioxy-24-methyl-3α,5-cyclo-5α-cholestan-6-one(12'). Keto diol **12**[6] (100mg) in dry ethyl acetate (25 ml) was stirred with 2,2-dimethoxypropane (0.5 ml) and toluene-p-sulfonic acid (5 mg) for 3 h at rt. The solvent was removed under reduced pressure, the residue stirred with aqueous K_2CO_3 (5%, 15 ml) for 10 min., extracted with ethyl acetate, worked up and purified by crystallization: 98 mg (90 %) **12'**; m.p. 143-144 °C (ethyl acetate-hexane); $[\alpha]_D^{28}$ +1.47° (c 1.09, MeOH); IR (nujol): ν_{max} 1716 cm^{-1} (CO); UV (c 1.09, MeOH) λ_{max} 287 nm, ε_M 100; CD (c 12.35, $CHCl_3$) $\Delta\varepsilon_{292}$ -1.78; EI-MS: *m/z* 470 (M$^+$, 1), 455 (M$^+$-15, 18), 399 (M$^+$-71, 18), 370 (M$^+$-100, 4), 355 (455-100, 13), 171 (100); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.74 (3H, s, 18-H$_3$), 0.94 (6H, d, *J*=6.9, 2 methyl), 0.96 (3H, d, *J*=5.2), 1.00 (3H, s, 19-H$_3$), 1.05 (3H, d, *J*=6.6, 21-H$_3$), 1.35 and 1.38 (acetonide-H$_3$), 2.45 (1H, d, 7α-H), 3.83 and 3.99 (22- and 23-H).

(22*S*,23*S*,24*R*)-22,23-Isopropylidenedioxy-24-methyl-5α-cholest-2-en-6-one(6a'). A mixture of 3α,5-cyclo-22,23-acetonide **12'** (100 mg), pyridinium hydrochloride (6 mg), anhydrous LiBr (12 mg) and N,N-dimethylacetamide (2 ml) was heated at 160 °C in an argon atmosphere for 2 h. The reaction mixture was cooled at 0 °C, diluted with ice water and extracted with ethyl acetate. The combined organic extracts were washed with water, dried concentrated and then purified by silica gel chromatography. Elution with hexane-ethyl acetate 8:2 (v/v) afforded 85 mg (85 %) **6a'**; m.p. 182-184 °C ($CHCl_3$); $[\alpha]_D^{30}$ -8.00° (c 1.10, MeOH); IR (nujol): ν_{max} 1712 cm^{-1} (CO); UV (c 1.10, MeOH) λ_{max} 292 nm, ε_M 65; CD (c 13.53, $CHCl_3$) $\Delta\varepsilon_{294}$ -2.04; EI-MS: *m/z* 470 (M$^+$, 3), 455 (M$^+$-15, 33), 399 (M$^+$-71, 12), 370 (M$^+$-100, 8), 355 (455-100, 18), 171 (100); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.70 (3H, s, 18-H$_3$), 0.71 (3H, s, 19-H$_3$), 0.94 (6H, d, *J*=6.6, 2 methyl), 0.96 (3H, d, *J*=6.0), 1.04 (3H, d, *J*=6.6, 21-H$_3$), 1.35 and 1.38 (acetonide-H$_3$), 2.36 (2H, 5α- and 7α-H), 3.82 and 3.98 (2H, 22- and 23-H), 5.63 (2H, m, 2- and 3-H).

(22*S*,23*S*,24*R*)-22,23-Dihydroxy-24-methyl-5α-cholest-2-en-6-one(6a). A solution of Δ^2-acetonide **6a'** (25 mg) was stirred with 4N HCl (0.5 ml) for 6 h at 50 °C. The solvent was removed and the crude product was purified by chromatography. Elution with hexane-ethyl acetate 85:15 (v/v) afforded 20 mg (87%) **6a**; m.p. 153-154 °C (from ethyl acetate-hexane); $[\alpha]_D^{21}$+9.12° (c 1.32, MeOH); IR (nujol): ν_{max} 3507 (OH), 1704 cm^{-1} (CO); UV (c 1.32, MeOH) λ_{max} 281 nm, ε_M 100; CD (c 10.10, $CHCl_3$) $\Delta\varepsilon_{300}$ -2.00; EI-MS: *m/z* 430 (M$^+$, 14), 415 (M$^+$-15, 8), 359 (M$^+$-71, 7), 330 (M$^+$-100, 100); HRMS (*m/z*): calcd. for $C_{28}H_{46}O_3$ [M]$^+$ 430.3475, found 430.3461. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.71 (3H, s, 18-H$_3$), 0.71 (3H, s, 19-H$_3$), 0.89 (3H, d, *J*=6.9, 27-H$_3$), 0.91 (3H, d, *J*=7.2, 28-H$_3$), 0.97 (3H, d, *J*=6.6, 26-H$_3$), 1.03 (3H, d, *J*=6.9, 21-H$_3$), 3.60 (1H, m, 22H), 3.73 (1H, dd, *J*=4.4, 2.5, 23-H), 5.56 and 5.67 (2H, m, 2- and 3-H).

Acknowledgments. This study was supported by NIH GM 34509.

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(*Received* 30 *December* 1996; *accepted* 4 *April* 1997)