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Fluorescence Detected Exciton Coupled Circular Dichroism: Development of New Fluorescent Reporter Groups for Structural Studies

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Summary. In collaboration with Jasco Corporation we have recently developed an FDCD (fluorescence detected circular dichroic) instrument J-465, which eliminates the photoselection artifacts and efficiently collects the emitted light from the sample solution based on the ideal ellipsoidal mirror principle. Using the J-465 we have investigated a variety of fluorophores with/without polarization for exciton-coupled FDCD stereochemical analysis. The following three cases extend the FDCD methodology to new challenging areas beyond the limits of conventional CD: (1) substrates containing C=C double bonds, (2) molecules with sterically hindered hydroxyls, and (3) substrates bearing remote stereogenic centers. The pico- to nano-level FDCD analysis described in this paper leads to promising opportunities for the stereochemical analysis of a wide range of natural products with minuscule amounts of sample available.

Keywords. Fluorescence detected exciton coupled CD; Photoselection; Sensitivity enhancement; Cross metathesis; Styrenoid.

Introduction

Circular dichroic spectroscopy (CD) is a general technique for studying chiral molecules [1–3]. In particular, electronic CD, which is associated with transitions between electronic states, is commonly used for structural investigations of organic and inorganic compounds, polymers, and biomacromolecules. The appeal of CD is frequently associated with high structural sensitivity and the relative ease in interpreting or predicting CD spectra, thus making CD the tool of choice for

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complementing NMR and other spectroscopic methods in determining the structures of chiral compounds [1–3].

The key step in any spectroscopic application consists of translating experimental spectra into structural information. A variety of methods, depending on the nature of the molecule, the number and type of chromophores, *etc.*, are available for the interpretation of electronic CD. A most efficient and useful approach, the ''exciton chirality method'', originates in the spatial interaction between two or more strong chromophoric electric dipole transitions that are chirally arranged and close in energy [4, 5]. When the exciton or coupled dipole mechanism of optical activity holds, the CD of a molecule can be quantitatively predicted on the basis of: (1) molecular conformation (relative position and orientation between chromophores) and (2) the spectroscopic properties of the constituent chromophores, *i.e.*, transition energies, dipolar strengths, transition dipole positions, and polarizations. Conversely, the appearance of an exciton coupled CD spectrum offers direct and straightforward information on the absolute molecular structure [4, 5].

In the simple case of two interacting electric dipole allowed transitions, the strength of the exciton coupling, *i.e.*, perturbation and mixing term of the excited states of the two chromophores, is directly proportional to their dipolar strength (and then to ε^2), and inversely proportional to the square of their spatial distance R and to their energy difference ΔE. The coupling is maximal for the degenerate case in which the two transitions are equivalent and the excited states are split into two states separated by the so-called *Davydov* splitting. The exciton coupling also depends on the mutual arrangement of transition dipoles through a simple dipole-dipole coupling term, which is zero for collinear or coplanar dipoles, whereas it reaches a maximum at dihedral angles around 70° for dipoles lying on quasi-parallel planes [4, 5].

A simple example of exciton coupling is when two chirally arranged and spatially close chromophores interact without perturbation from other factors. This results in an exciton-split CD spectrum (a CD couplet) consisting of two *Cotton* effects of opposite signs flanking the absorption band; in degenerate cases when the two chromophores are identical, the couplet crossover point coincides with the absorption maximum. The sign of the CD couplet is defined by the sign of its long wavelength component and reflects the absolute sense of twist between two interacting dipoles. The sense of twist can be inferred by viewing the two dipoles along the direction connecting their centers (the transition dipole positions). If a clockwise rotation is required to superimpose the dipole in the front to that in the rear, the chirality is positive and *vice-versa*. Thus this exciton chirality rule permits one to readily assign absolute configurations from exciton coupled CD spectra; Fig. 1 shows a typical couplet exhibited by a chiral diol bis(4-dimethylaminobenzoate). The couplet amplitude A_{CD}, or the intensity difference between the peak and trough, depends on the coupling strength, and is proportional to ε^2/R^2 and to a geometrical dipolar factor G; the width of the couplet W_{CD}, *Davydov* splitting, is proportional to $G \cdot \varepsilon/R^3$. For non-degenerate cases, A_{CD} is also inversely proportional to the energy difference ΔE between the two isolated transitions.

The exciton chirality approach is widely applicable since the coupled dipole mechanism is usually very strong, and in the presence of suitably arranged chromophores, it dominates the other possible sources of optical activity [3]. In fact,

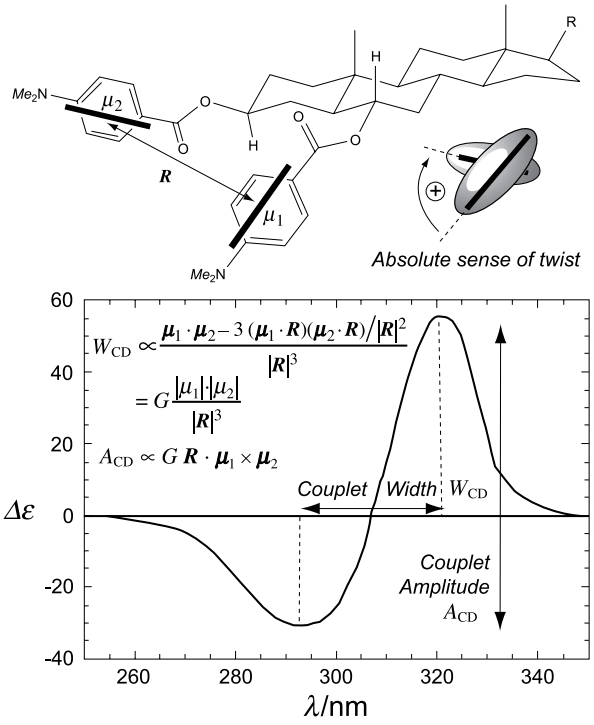


Fig. 1. Exciton-coupled CD spectra of bis(4-dimethylaminobenzoate) of the chiral diol, 5α-cholestan-3β,6α-diol; $\Delta\varepsilon$ is the unit of $mol^{-1}\,dm^3\,cm^{-1}$

when the chiral molecule lacks two proper chromophores, rather than resorting to different tools for interpreting its CD spectrum, it may be convenient to introduce one or two chromophoric groups by chemical derivatization and proceed with the exciton method; in such cases, strong and red-shifted chromophores that do not interact with pre-existing chromophores should be selected [4, 5]. A further recent approach consists of analyzing the CD of supramolecular assemblies formed between the chiral compound acting as a guest and achiral bis-chromophoric hosts, *e.g.*, bis-tetraarylporphyrin tweezers [6–11]. Since the CD signals associated with the exciton mechanism are usually strong, the technique is also very sensitive, and microscale applications are possible: a few microgram or nanogram amounts of sample at micromolar concentration suffices for acquiring a well defined exciton-split CD [12–16]. A second consequence of this enhanced sensitivity is that exciton CD spectra of bis-chromophoric compounds can be significant and diagnostic even when the two chromophores are separated by 40–50 Å [4]. Such features have rendered the exciton chirality method a popular approach for configurational and conformational analyses of chiral molecules, notably in the field of biologically relevant compounds [4, 5].

When three or more chromophores are present, the overall CD can be approximated by summation of all possible pair-wise interacting terms [17]. In more complicated cases, and also when a more quantitative approach is required, the entire exciton coupled CD is calculated. One of the most widely employed calculation is the *DeVoe* coupled oscillator approach [18–20] for structural elucidations

of small molecules [21–26], biopolymers [27–29], and theoretical investigation of exciton interactions [30]. According to this approach each transition dipole is described as a classical damped oscillator with its energy, strength, position, and direction. The optical properties of the oscillator aggregate thus depend only on their dipolar interaction matrix and may be readily calculated.

Despite the advantages of electronic CD, two limitations are relevant.

(i) *Sensitivity.* CD is defined as the differential absorption of left and right circularly polarized radiation (LCP and RCP), and is commonly measured as the differential transmission intensity in the direction of the incident light. The fact that CD is based on *difference* spectroscopy makes it one or two orders of magnitude less sensitive than absorption spectroscopic methods; in ideal cases the *g*-factor ($\Delta A/A$ or $\Delta\varepsilon/\varepsilon$ value) is 10^{-2}. CD is then substantially less sensitive than other spectroscopies such as fluorescence. Even in the most sensitive exciton split CD arising from interaction between two strong chromophores such as tetraarylporphyrins, the concentration limits for CD detection are seldom below $10^{-6\sim7}\,M$ [12–16].

(ii) *Selectivity.* As an example, the CD analysis of a complex consisting of a chiral compound (ligand) attached to a chiral polymeric receptor could be quite challenging since the ligand CD is superimposed on that of the receptor, as is frequently the case encountered in biomacromolecules, where it is especially important to determine the local ligand conformation that often play critical roles in biological processes.

Fluorescence detected circular dichroism (FDCD) combines the structural sensitivity and chiral specificity of CD with the sensitivity and specificity of fluorescence [31–36]. It consists in measuring the differential emission of light from a sample excited with LCP and RCP radiation, and is based on the assumption that the amount of light emitted depends exclusively on the amount absorbed; in other words, the excitation spectrum of the fluorophore parallels the absorption spectrum, and the molecular quantum yield is the same for both circularly polarized components (which may not necessarily be the case) [37]. FDCD is then an indirect way of measuring a differential absorption [31, 32]. It is sensitive to *both* chiral and fluorescent molecules, and therefore is far more specific than standard transmission CD, because in principle the CD associated with a single fluorophoric molecule or moiety may be selectivity extracted in the presence of many non-fluorescent chromophores. Moreover, similar to the higher sensitivity of fluorescence compared to absorption spectroscopy, the direct measurement of emitted radiation against a zero background renders FDCD more sensitive than conventional CD.

The first description of the theoretical basis of FDCD phenomenon and experimental framework date back to 1974 when *Turner*, *Tinoco*, and *Maestre* were able to estimate the selective contribution arising from the chiral fluorophore tryptophan in a mixture with the chiral non-fluorescent chromophore cysteine [31]. The experimental setup used for FDCD detection is as follows: behind a cell placed after the source of CP light (similar to conventional CD instruments), an FDCD photomultiplier (PMT) was placed along the direction orthogonal to the incident radiation (different from CD measurement) to collect the emitted light. A cutoff long-pass filter was inserted between the cell and PMT to prevent scattered light

to be detected. In the simple case of a non-viscous solution of a single chiral and fluorophoric molecule, *e.g.* 10-camphorsulfonic acid, the measured FDCD may be converted into CD by Eq. (1) [31, 32] where A is the sample absorption, $\Delta A = cd\Delta\varepsilon$ (c is the sample molar concentration and d the cell length in cm), F_L and F_R are difference in the emission intensities upon LCP and RCP excitation, and k is a conversion constant specific for each machine. After the FDCD instrument outputs $F_L - F_R$ and $F_L + F_R$ (as AC and DC current components), the absorption A of the same sample affords the desired CD normarized in $\Delta\varepsilon$ units; A must be low to prevent both intermolecular fluorescence quenching and excessive attenuation of the more absorbed CP component [38]. For more complex samples, *i.e.*, in the presence of other non-fluorescent CD-active or fluorescent CD-inactive species, which can give rise to energy transfer with the observed fluorophore, more complicate formulae must be used [32, 38].

$$\Delta A = 2S(1 - 10^{-A})/\ln 10 \cdot 10^{-A}; \quad S = k(F_L - F_R)/(F_L + F_R) \tag{1}$$

Even in most straightforward cases, it soon became clear that FDCD would suffer from a problem not encountered in conventional CD, namely, the anisotropic excitation, known as photoselection, which depends on temporary orientation of the molecules during the irradiation process [39, 40]. In fact, the light absorbed by the system does not equally excite all molecules with various orientations, because the probability of light absorption by a molecule depends on the direction of its electric and magnetic dipoles with respect to the incident light wave vector. This would lead to an emission emerging from a non-isotropic collection of excited molecules, unless unrestricted *Brown*ian movement causes them to rotate rapidly and their orientation between excitation and emission phenomena is randomized leading to an isotropic emission process. The latter is usually the case when the rotational relaxation time is sufficiently shorter than the fluorescence lifetime.

In the presence of photoselection, the observed fluorescence will be polarized, that is, the vertical and horizontal components $F_\parallel$ and $F_\perp$ differ and the ratio $P_F = (F_\parallel - F_\perp)/(F_\parallel + F_\perp)$, known as fluorescence polarization, is non-vanishing. Consequently, the measured FDCD will be a complex function of the isotropic CD and of some particular components of the dipole and rotational strength tensors; in other words, the observed differential emission will not reflect the differential absorption in a straightforward manner. The only exception to this behavior is observed in the uncommon situation when the molecular transition absorption and emission dipoles either coincide or are mutually perpendicular. In all other cases, special instrumental setups have been proposed which would minimize the anisotropic contribution; they consist of placing the PMT along a ''magic'' direction or placing a linear polarizer in front of the PMT with a ''magic'' rotation angle. In practice, however, the sample photoselection will introduce additional severe artifacts in FDCD, due to the linear polarization present in the CP light produced by imperfect optical components in commercial CD instruments. If the LCP/RCP excitation beams contain some residual linear polarization, a difference in the fluorescence signal will be detected not arising from the sample optical activity [41–43]. In practice, the whole measurement will be distorted as with other emission or scattering spectroscopies such as the circular polarization of luminescence and *Raman* spectroscopy [44–46].

The elimination of these artifacts is possible either upon collection of the emitted light along more than one direction, or by specific selection of the unpolarized component of the emitted light. This has been achieved by using a double PMT-system [42, 43], introducing a linear polarizer between the sample and PMT [41], or by conveying the emitted light through an ellipsoidal mirror surrounding the whole cell [47]. Together with Jasco, we have recently developed an ellipsoidal mirror prototype, Jasco FDCD J-465, which can be attached to any modern CD instrument [48]. J-465 not only eliminates artifacts due to fluorescence polarization, but also increases the signal-to-noise ratio S/N by collecting a large fraction of emitted radiation, as discussed in the following [48].

The applications of FDCD reported so far may be divided into three main classes:

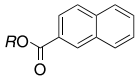
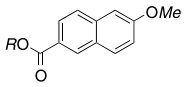
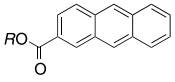
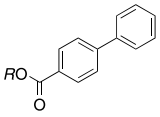
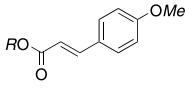
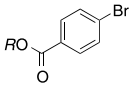
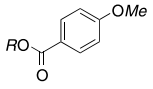
(1) cases when the excited states of chiral fluorescent molecules were investigated and/or their emission properties were exploited;
(2) cases when a selective CD detection of a fluorophore in the presence of other chromophores was required;
(3) cases when FDCD was used as a more sensitive alternative to CD.

To group (1) belong a few interesting applications such as the measurement of lifetime-resolved FDCD [49–51], which helps in separating contributions from multi-component systems, and the quenching detection of chiral molecules also through multidimensional FDCD experiments [52, 53]. To group (2) belong numerous examples of mixtures of a fluorophoric probe and a chromophoric species, *i.e.*, a fluorescent dye associated with a biopolymer) [54–59], or of a fluorophore inserted into a non-fluorescent chiral matrix such as proteins or nucleic acids [60]. In this context, FDCD affords important local chiral information: the FDCD of a single tryptophan within a complex protein may in fact be extracted and changes in its environment accompanying binding or conformational changes can be followed [43, 61, 62]; contribution to CD from the backbone and side chains of poly-tryptophan and poly-tyrosine can be separated [63, 64]; moreover, fluorescent and non-fluorescent aggregation or conformational states can be detected [65, 66]. Despite many interesting reports on FDCD selectivity, this important feature of the method still remains underestimated. Even less exploited is the opportunity for sensitivity enhancement, the second crucial feature of this technique. Apart from the promising employment of FDCD as a detection tool in chromatography [67, 68], electrophoresis [67, 68], and determination of enantiomeric excesses [69], only a few other reports have provided data regarding FDCD sensitivity enhancement, obviously due to the still prevailing photoselection problem [70–75].

FDCD was applied for the first time in 1997 to exciton coupled molecules such as 2-naphthoate, 6-methoxy-2-naphtoate, and 2-anthroate diesters of 1,2-cyclohexanediol and some steroidal di/triesters (Table 1) [74].

It was shown that in these favorable cases, exciton coupled FDCD spectra were in excellent agreement with conventional CD spectra. More importantly, FDCD showed satisfactory S/N ratios at concentrations much lower than those in CD, leading to a 100-fold sensitivity increase; while CD detection limit was of the order of $10^{-6}\,M$, that of FDCD was around $10^{-8}\,M$ [74]. Further investigations using

Table 1. UV and fluorescence properties of previously utilized chromophores[a]

Fluorophore	Solvent	λ_{max}^{abs}/nm(ε)[b]	λ_{max}^{em}/nm	Φ_f	P_f
(a) Strong fluorescence with low polarization					
	*Me*CN	234 (58000)	359	0.32	0.0032
	*Me*CN	237 (48000)	374	0.65	0.0026
	*Me*CN	258 (93000)	434	0.62	0.0023
(b) Strong fluorescence with high polarization					
	*Me*CN	270 (21000)	337	0.66	0.034
(c) Weak or nonfluorescence					
	*Me*CN	306 (23400)	380	0.0005	0.4
	*Me*CN	244 (19500)	n.a.	n.a.	n.a.
	*Me*CN	257 (20400)	n.a.	n.a.	n.a.

[a] Φ_f = fluorescence quantum yield, P_f = fluorescence polarization, R = (1*R*,2*R*)-*trans*-1,2-cyclohexane diol monoester; [b] ε is the unit of $mol^{-1}\,dm^3\,cm^{-1}$

1,2-cyclohexanediol as a chiral scaffold confirmed that similar sensitivity enhancements may be attained for all cases when suitable fluorophores are employed [75]. Such fluorophores should possess, in addition to large ε values, a high fluorescence quantum yield Φ_f (above 0.3) and negligible or low fluorescence polarization P_f (below 0.02). In contrast, the FDCD spectra of 4-phenylbenzoate and especially 4-methoxycinnamate bisesters were not in good agreement with CD, because of artifacts associated with the relatively large P_f. Finally, FDCD spectra of weakly or non-fluorescent 4-methoxycinnamate, 4-bromo, and 4-methoxybenzoate bisesters, as expected, could not be measured. Diesters of 1,2-cyclohexanediol containing two different chromophores demonstrated that the presence of a single favorable fluorophore is sufficient to obtain significant and artifact-free FDCD spectra. Thus the combination of naphthoate-type fluorophores with either 4-methoxybenzoate or 4-phenylbenzoate gave FDCD spectra in which both wings of the exciton couplet were apparent, in full accord with CD [75]. It appears that when the two chromophores are excitonically coupled, the two mixed excited states both become fluorescent and emit a FDCD signal. In other words, a very fast exciton-type energy transfer mechanism presumably occurs in such cases [76–78]. It was also verified

that when a possibility of *Förster*-type energy transfer exists, *e.g.*, from 6-methoxy-2-naphtoate to 2-anthroate fluorophore, a good agreement between FDCD and CD spectra is still attained [75]. On the other hand, a combination between naphthoate-type fluorophores and either totally non-fluorescent chromophores, such as 4-bromobenzoate, or fluorophores with high P_f, such as 4-methoxycinnamate, exhibited FDCD spectra dissimilar to CD due to apparent artifacts. It is important to notice that a Jasco FDCD prototype (J-357) was used for this study analogous to the instrument introduced by *Tinoco* [31]: a single PMT was placed along the direction orthogonal to the incident light, and no polarizer or light-collecting mirrors were introduced. It was thus concluded that [75]:

(1) when suitable fluorophores with high Φ_f and low P_f are already present or introduced in the molecule, exciton coupled FDCD spectra represent a powerful alternative to conventional CD. In fact, FDCD with a sensitivity increase of up to 100-fold disclose the same structural information as that resulting from CD.
(2) Fluorophores with even high Φ_f values but with significant P_f represent a challenge for FDCD if the artifacts due to photoselection are not eliminated by the FDCD device.

It became clear that future applications of FDCD require development not only of new fluorophores with favorable properties but also of improved FDCD detectors which would minimize photoselection effects.

A recently developed ellipsoidal mirror FDCD device (J-465) has shown promising features and is expected to solve the problem of polarized and weak fluorescence [48]. First, by averaging *de facto* radiation emitted all around the sample, it eliminates the artifacts due to photoselection. Second, by collecting a large fraction of emitted radiation, not only along a single direction but multiple directions, it increases the intensity of signal collected by the PMT, thereby leading to sensitive detection of weakly fluorescent samples. A previous device consisting of a linear polarizer, with a proper angle rotation, placed between the sample and PMT (implemented in Jasco FDCD J-405 prototype), was shown to similarly help eliminating FDCD artifacts, but suffers from reduced sensitivity and is therefore of limited applicability [48]. Figure 2 shows the CD and FDCD spectra of (1*R*,2*R*)-*trans*-cyclohexanediol bis(6-methoxy-2-naphthoate) in the viscous solution of methanol/glycerol. In this case fluorescence anisotropy, $P_f = 0.050$, was purposely increased by employing glycerol in addition to methanol. At $10^{-6}\,M$ concentration (Fig. 2a), CD is in good agreement with the artifact-free FDCD obtained by employing either a linear polarizer rotated by 81° with respect to the vertical position (J-405) or the ellipsoidal-mirror light-collecting device (J-465). The FDCD measured with J-357 device where the photoselection is not eliminated differs significantly from the CD. At $10^{-8}\,M$ concentration (Fig. 2b), only FDCD measured with J-465 shows the CD signals with reasonable S/N ratio, whereas at this concentration, both conventional CD and FDCD measured with J-405 are unacceptable.

In the following, three challenging cases so far unexplored by the exciton coupled CD method have been now solved by application of a recently developed cross metathesis/CD chiroptical protocol, and further improved by combination

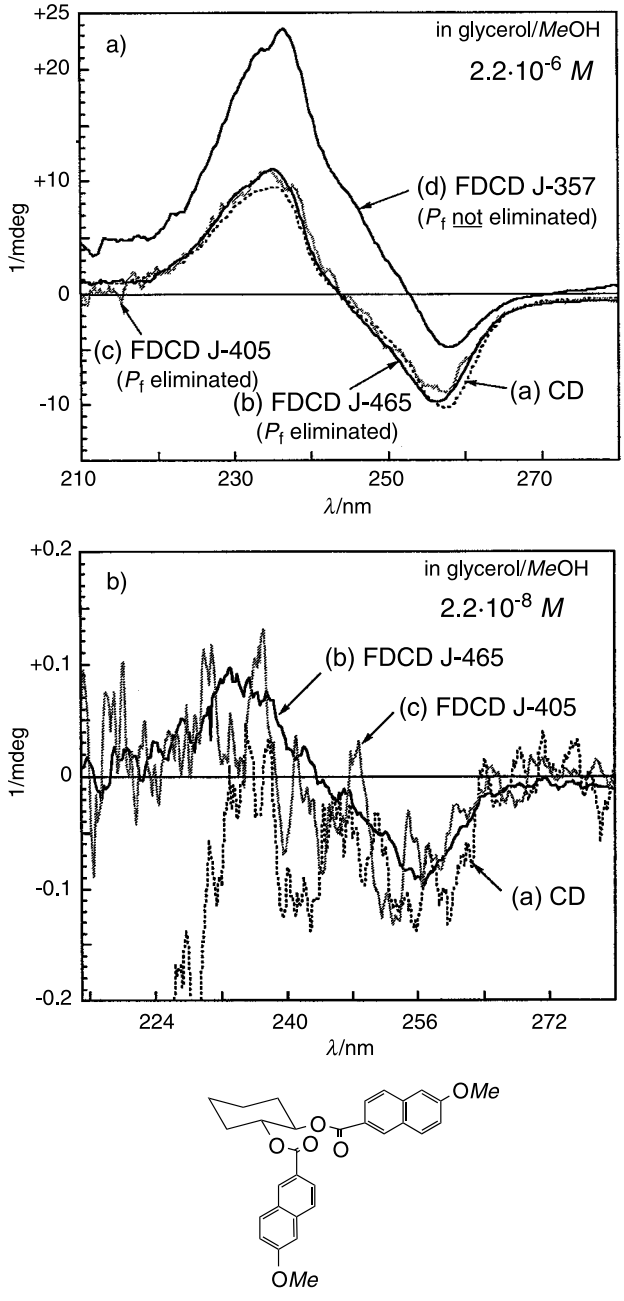


Fig. 2. CD and FDCD of (1*R*,2*R*)-*trans*-cyclohexanediol bis(6-methoxy-2-naphthoate) in glycerol/MeOH ($\Phi_f = 0.37$, $P_f = 0.050$); CD (line a), FDCD by J-465 (line b, ellipsoidal mirror, masks 3.0 mm, light-cut filter, 320 nm), FDCD by J-405 (line c, polarizer 81°), and FDCD by J-357 (line d, P_f not eliminated); $\Delta\varepsilon$ is the unit of $mol^{-1}\,dm^3\,cm^{-1}$

with sensitivity enhanced FDCD detection by employment of the ellipsoidal mirror device J-465. The three cases include: (i) substrates containing C=C double bonds, (ii) compounds bearing sterically hindered hydroxyl groups, and (iii) substrates bearing remote stereogenic centers.

Results and Discussions

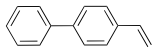
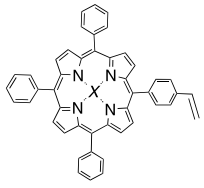
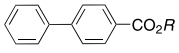
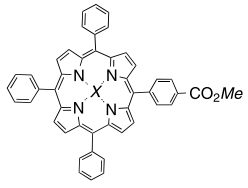
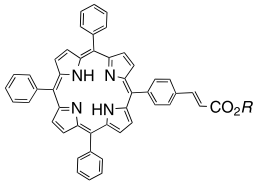
1. *Convenient Fluorophores for Exciton Chirality Studies by FDCD*

The fluorophores used in this study and their UV-Vis and fluorescence properties are listed in Table 2. The fluorophores are categorized into three types 1a–1c according to the methods of introduction into the substrates.

1a. *Styrenoid Fluorophores (**1**, **2**, **3a**, and **3b**) for C=C Double Bond Containing Compounds*

These styrenoid reagents could be introduced into a variety of substituted C=C double bonds by cross metathesis [79, 80] using *Grubbs*' ruthenium catalyst

Table 2. Introduction methods, UV-Vis, and fluorescence properties of **1–6**[a]

Fluorophore		Solvent	λ^{abs}_{max}/nm(ε)[b]	λ^{em}_{max}/nm	Φ_f	P_f
(a) Introduced at C=C Double Bonds by Cross Metathesis						
	**1**	*Me*CN	248 (15000)	304	0.16	0.0020
	**2**	*Me*CN	277 (23700)	331	0.65	0.0029
	**3a** ($X = H_2$)[c]	CH_2Cl_2	418 (420000)	651	0.12	0.0010
	3b ($X = Mg$)[d]	CH_2Cl_2	425 (560000)	661	0.23	0.0029
(b) Introduced at –OH Group by Acylation						
	**4**	*Me*CN	270 (21000)	337	0.66	0.034
	**5a** ($X = H_2$)	CH_2Cl_2	418 (440000)	650	0.11	0.0025
	5b ($X = Mg$)[d]	CH_2Cl_2	425 (540000)	661	0.19	0.0015
(c) Introduced at Sterically Hindered –OH Groups by Acylation and Cross Metathesis						
	**6**[e]	CH_2Cl_2	419 (387000)	651	0.13	0.002

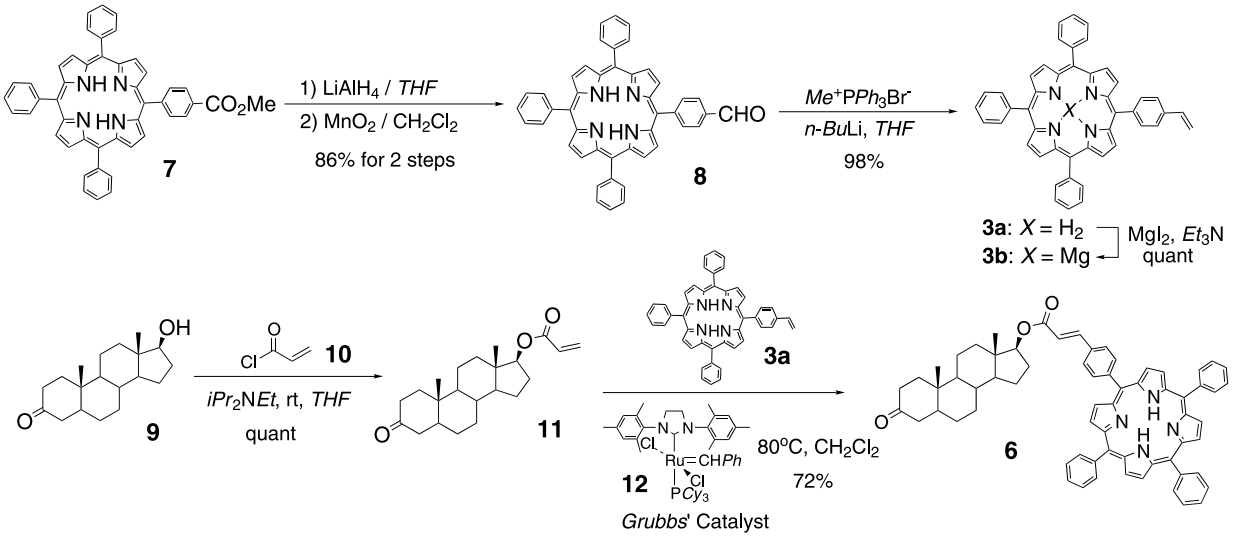
[a] Φ_f = fluorescence quantum yield, P_f = fluorescence polarization; [b] ε is the unit of $mol^{-1}\,dm^3\,cm^{-1}$; [c] preparation is shown in Scheme 1; [d] corresponding magnesium porphyrins **3b** and **5b** were prepared by the reaction of **3a** and **5a** with excess amount of MgI_2 and Et_3N in CH_2Cl_2 at rt in quantitative yields; [e] preparation is shown in Scheme 1, R = 3-oxo-5α-androstan-17β-yl

[81–83]. Whereas styrene (**1**) itself is weakly fluorescent, *p*-vinylbiphenyl (**2**) is a more favorable FDCD fluorophore owing to its red shifted absorbance, higher extinction coefficient, and excellent fluorescent properties (277 nm, $\varepsilon = 23700\,\mathrm{mol^{-1}\,dm^3\,cm^{-1}}$, $\Phi_f = 0.65$, $P_f = 0.0029$).

A fluorophore that possesses a very intense UV-Vis absorption has to be attached when the stereogenic centers are remote and thus expected to give only weak long range exciton coupling [84, 85]. For this purpose, we developed the vinyl-*TPP* (**3a**, $\lambda = 418\,\mathrm{nm}$, $\varepsilon = 420000\,\mathrm{mol^{-1}\,dm^3\,cm^{-1}}$). Although the fluorescence is weaker ($\Phi_f = 0.12$), its high extinction coefficient gives rise to enhanced exciton coupling and thus sensitive FDCD detection. It is noteworthy that the extinction coefficient of corresponding magnesium porphyrin **3b** is even larger ($\lambda = 425\,\mathrm{nm}$, $\varepsilon = 560000\,\mathrm{mol^{-1}\,dm^3\,cm^{-1}}$) and the quantum yield ($\Phi_f = 0.23$) is twice that of **3a** [86]. Since it is known that the radiative lifetime of the porphyrin derivatives is relatively long (*ca.* 10 ns) [86], both vinyl-*TPP* **3a** and its magnesium derivative **3b** show low polarization. The synthesis of **3a** has been reported [87, 88], but this fluorophore can also be readily prepared in high yields from the commercially available tetraphenyl carboxylic acid methyl ester **7** as shown in Scheme 1.

1b. *Fluorescent Carboxylic Acids (**4**, **5a**, and **5b**) for Derivatization of Hydroxyls*

p-Phenylbenzoate **4** shows a high quantum yield ($\Phi_f = 0.66$), but its relatively high polarization ($P_f = 0.034$) [75] has so far restricted its application with previous FDCD techniques. The commercially available *TPP*-carboxylic acid (**5a**, $\lambda = 418\,\mathrm{nm}$, $\varepsilon = 440000\,\mathrm{mol^{-1}\,dm^3\,cm^{-1}}$) employed in observing long range CD exciton coupling [89], has weak fluorescence ($\Phi_f = 0.11$) and bears a low polarization ($P_f = 0.0025$). In accordance with the trend observed for vinyl-*TPP* **3a**, the incorporation of magnesium in **5a** leads to an increase in both extinction coefficient



Scheme 1

(**5b**, $\varepsilon = 540000\,\mathrm{mol^{-1}\,dm^3\,cm^{-1}}$) and quantum yield ($\Phi_f = 0.19$), a two-fold enhancement as compared to **5a**.

1c. *Cinnamate Type Fluorophore (**6**) for Sterically Hindered Hydroxyls*

Cinnamate type fluorophore **6** was particularly developed for derivatizing sterically hindered hydroxyls that are separated over a large distance and therefore are difficult to be analyzed with other chromophores. Fluorophore **6** shows UV-Vis and fluorescent properties compatible to those of **3a** and **5a** ($\varepsilon = 387000\,\mathrm{mol^{-1}\,dm^3\,cm^{-1}}$, $\Phi_f = 0.13$, $P_f = 0.002$). The synthesis of **6** is shown in Scheme 1. Steroid **9** containing a sterically congested 17-hydroxyl was first acylated with acryloyl chloride **10** to give **11** in quantitative yield. The vinyl group in **11** was then subjected to cross metathesis by vinyl-*TPP* **3a** to provide **6** in 72% yield. The detailed chemistry and application to sterically hindered hydroxyls are described in Sections 2c and 2d.

Some of the fluorophores listed in Table 2, such as styrene **1**, have already been used as exciton coupled ''chromophores'' for structural analysis of allylic alcohols, amines, and dihydrofuroangelicins (see below). In this study, these fluorophores are reconsidered as favorable and promising for the sensitivity enhanced FDCD analysis. Successful examples and applications of **1**–**6** toward stereochemical analyses by exciton coupled FDCD are shown in the following Sections. The CD and FDCD results are summarized in Table 3.

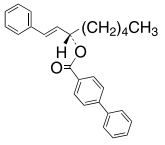
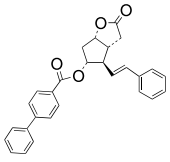
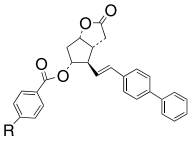
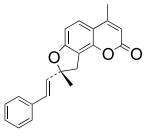
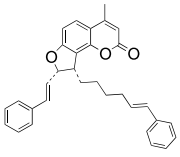
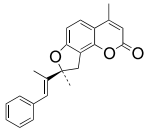
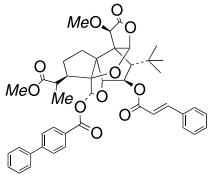
2. *Application of Fluorophores **1**–**6** for Structural Analysis of Selected Chiral Compounds by FDCD. Scope and Limitations*

2a. *Stereochemical Analysis of Allylic and Homoallylic Alcohols*

The configurations of many biologically active natural products with ene moieties are unknown, and moreover, some are available in only minuscule amounts [90–96]. We have recently developed a general microscale CD method where the double bond is converted into a styrene by cross olefin metathesis. The styrenoid chromophore then couples with the allylic acylate or a preexisting chromophore to yield a distinct CD couplet. We have studied ''*Corey*-lactone'' **13** as a model for allylic and homoallylic alcohols (Scheme 2a) [12]. The double bond in **13** (10 μg) was thus reacted with **1** in the presence of catalyst **12** to give styryl alcohols **14a** and **14b**. Acylation with 4-phenylbenzoic acid **4** gave **15a** and **15b**, the conformation being deduced from ^{1}H NMR and conformational analysis as shown in Scheme 2a. The CD of **15a** and **15b** exhibited the expected negative and positive couplets, in agreement with the known chirality of **13** (Fig. 3a, Table 3).

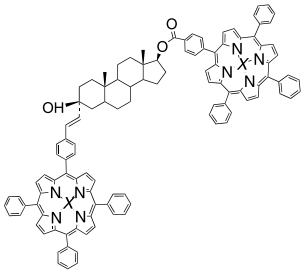
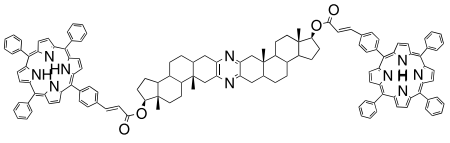
An FDCD exciton analysis utilizing the fluorescence of styrene and 4-phenylbenzoate moieties was performed. A comparison of detection sensitivity of CD and FDCD for **15a** is shown in Fig. 3a (data for **15b** is summarized in Table 3). Whereas the detection limit for CD measurement is at $10^{-6}\,M$, FDCD is still observable at $10^{-8}\,M$, although the fluorescent impurity included in the solvent (excitation at 245 nm) partially interferes with the clear observation of the positive couplet in the lower wavelength region (*vide infra*). It is noteworthy that the

Table 3. Summary of CD and FDCD

Compounds		Solvent	$\Delta\varepsilon/(\lambda/\text{nm})$[a]	A_{CD} amplitude	CD detection limit conc/M	P_f	FDCD detection limit conc/M
Allylic and Homoallylic Alcohols							
	**15b**	*Me*CN	+12 (273) −10 (248)	+21	10^{-6}	0.17	10^{-8}
	**15a**	*Me*CN	−29 (270) +16 (247)	−45	10^{-6}	0.11	10^{-8}
	**17a** ($R = NMe_2$)	*Me*CN	−41 (312) +22 (280)	−63	10^{-6}	0.022	10^{-8}
	18a ($R = Ph$)	*Me*CN	−31 (291) +17 (263)	−45	10^{-6}	0.18	10^{-8}
Dihydrofuroangelicins with Preexisting Coumarin Fluorophore							
	***sty*-19**	*Me*CN	+15 (320) −17 (250)	+32	10^{-6}	0.047	10^{-8}
	***sty*-20**	*Me*CN	−11 (320) +12 (251)	−23	10^{-6}	0.087	10^{-8}
	***sty*-21**	*Me*CN	−14 (32) +14 (243)	−28	10^{-6}	0.030	10^{-8}
Ginkgolide Derivatives with Sterically Hindered –OH Groups							
	**32**	*Me*CN	−33 (291) +18 (263)	−51	10^{-6}	0.18	10^{-8}

(*continued*)

Table 3 (*continued*)

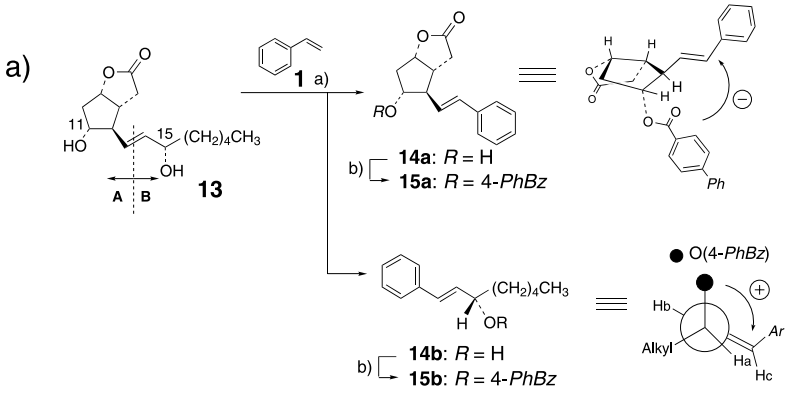
Compounds	Solvent	$\Delta\varepsilon/(\lambda/\text{nm})$[a]	A_{CD} amplitude	CD detection limit conc/M	P_f	FDCD detection limit conc/M
Mono- and Bis-steroids with Remote Stereogenic Centers						
 **35a** ($X=H_2$)	CH_2Cl_2	+83 (424) −44 (414)	+127	10^{-7}	~0	10^{-9}
35b ($X=Mg$)	CH_2Cl_2	+70 (431) −133 (424)	+203	10^{-7}	~0	10^{-10}
 **40**	CH_2Cl_2	−19 (425) +18 (418)	−37	10^{-6}	~0	10^{-8}

[a] $\Delta\varepsilon$ is the unit of $\text{mol}^{-1}\,\text{dm}^3\,\text{cm}^{-1}$

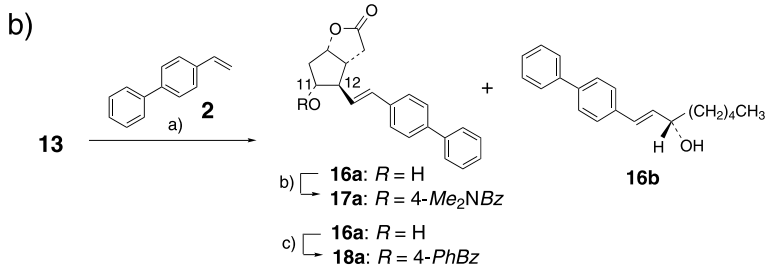
exciton couplet of **15a** can be obtained at $10^{-8}\,M$ using the J-465 instrument even in the presence of extremely high polarization of **15a** ($P_f = 0.11$, Table 3). The results clearly show that the polarization artifacts can be completely eliminated while retaining the highly sensitive CD analysis in emission, namely a 100-fold enhancement in sensitivity compared with CD in transmission.

In order to examine the scope and limitation of the FDCD detection for these allylic type compounds, a more fluorescent 4-phenylstyrene **2** ($\Phi_f = 0.65$) was introduced in **13** (Scheme 2b); the double bond in **13** was replaced with a 4-phenylstyrenoid in the presence of the ''Ru'' catalyst to provide **16a** and **16b**, *ca*. 60% yields. We then attempted to introduce either a non-fluorophore or fluorophore at 11-hydroxyl of **16a** (Scheme 2b); one is the non-fluorescent 4-dimethylaminobenzoate coupled with the fluorophoric 4-phenylstyrenoid [75]. Another is the highly fluorescent 4-phenylbenzoate (**4**, $\Phi_f = 0.66$) [75] aimed at further reducing the FDCD detection limit of **18a** down to $10^{-9}\,M$. The CD and FDCD data of **17a** and **18a** are shown in Figs. 3b and c. In accordance with our previously observed phenomena, the FDCD spectrum of **17a** exhibited a couplet over the entire region with 100-fold enhanced sensitivity over CD; the detection limit for CD was evaluated at $10^{-6}\,M$, whereas for FDCD was at $10^{-8}\,M$. This phenomenon would expand the range of usable chromophores and/or fluorophores pairs for exciton coupled FDCD leading to a wider application of FDCD in structural analyses.

Although two strong fluorophores, 4-phenylstyrenoid and 4-phenylbenzoate ($\Phi_f > 0.6$), were employed in **18a**, the concentration limit could not go below $10^{-8}\,M$; no reliable FDCD couplet signals were observed at $10^{-9}\,M$ (line c in Fig. 3c). In this case also, the fluorescent impurities at 245 nm interfered with



Conditions: a) 10 mol% of *Grubbs*' catalyst **12**, CH_2Cl_2, 40°C, 66% for **14a**, 63% for **14b**; b) 4-phenylbenzoic acid, *EDC*, *DMAP*, CH_2Cl_2, room temperature, quant for **15a**, 80% for **15b**



Conditions: a) 10 mol% of *Grubbs*' catalyst, CH_2Cl_2, 40°C, 60% for **16a**; b) 4-dimethylaminobenzoylimidazole, *DBU*, *Me*CN, room temperature, 84%; c) 4-phenylbenzoic acid, *EDC*, *DMAP*, CH_2Cl_2, room temperature, quant

Scheme 2

the FDCD measurements at this very low concentration. Therefore, it is desirable to use the *TPP* fluorophore, such as **5a**, with much larger extinction coefficients ($\varepsilon \sim 440000\,\mathrm{mol^{-1}\,dm^3\,cm^{-1}}$) for performing highly sensitive analyses. In addition, since porphyrins feature red-shifted UV absorption at *ca.* 420 nm, the FDCD measurement would not be hampered by excitations caused by the solvent impurities in the lower wavelength regions even under increased HT voltage conditions. Successful examples using these porphyrin fluorophores are discussed below.

2b. *Dihydrofuroangelicins – Coumarin as a Preexisting Fluorophore*

Although coumarin derivatives are associated with a variety of pharmacological activities [97–101] and contain chromophores having an experimentally accessible absorbance band above 300 nm, which are also fluorescent, the recently developed cross metathesis protocol so far represents the only general method for a chemical/CD approach for determination of their absolute configurations [24, 26, 102–104]. This has been achieved for a series of dihydrofuroangelicins bearing a

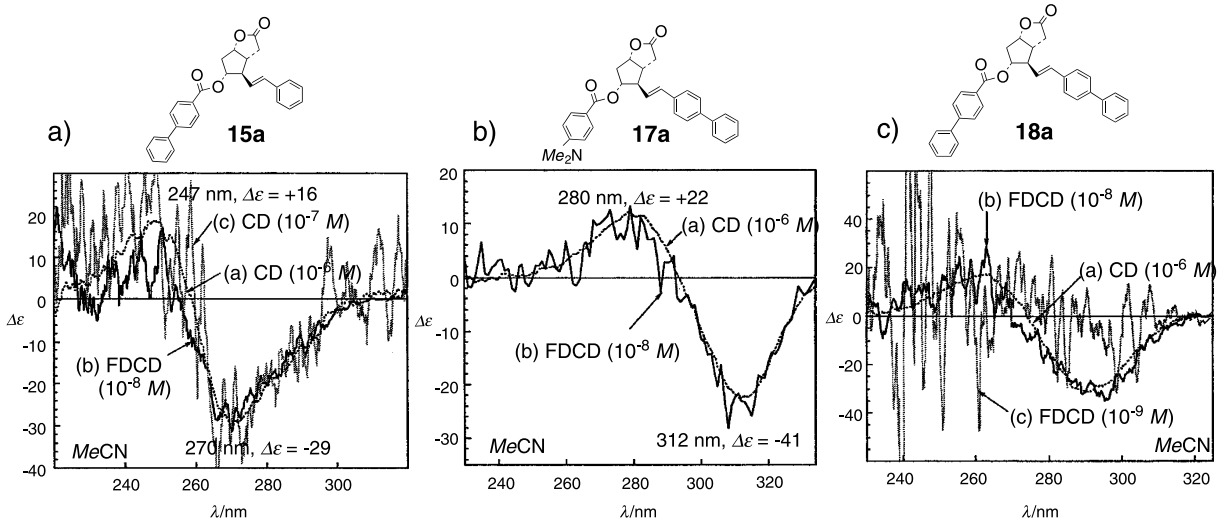
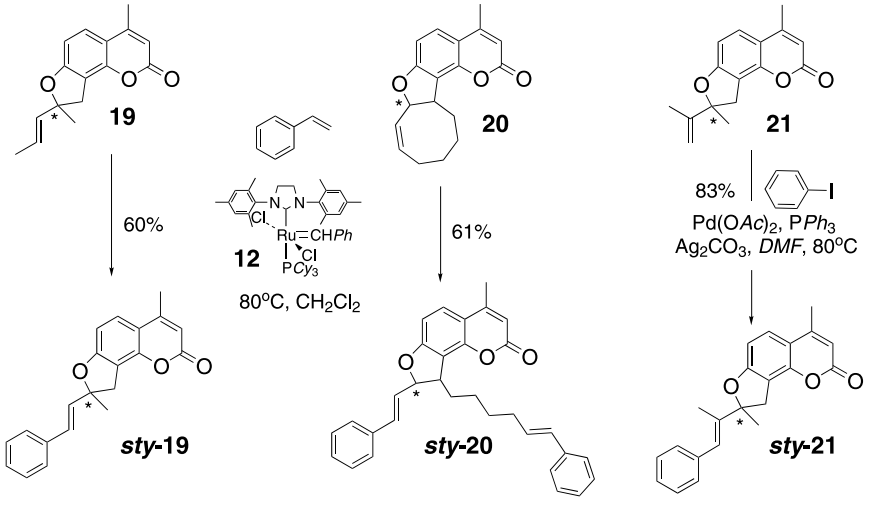


Fig. 3. (a) CD (line a, $c = 1.46 \cdot 10^{-6}\,M$; line c, $c = 1.46 \cdot 10^{-7}\,M$) and FDCD (J-465 device, line b, $c = 1.46 \cdot 10^{-8}\,M$, light-cut filter, 340 nm, masks 4.0 mm, HT voltage, 793 V); (b) CD (line a, $c = 2.33 \cdot 10^{-6}\,M$) and FDCD (J-465 device, line b, $c = 2.33 \cdot 10^{-8}\,M$, light-cut filter, 360 nm, masks 3.5 mm, HT voltage, 777 V), (c) CD (line a, $c = 2.56 \cdot 10^{-6}\,M$) and FDCD (J-465 device, light-cut filter, 340 nm, masks 3.5 mm; line b, $c = 2.56 \cdot 10^{-8}\,M$, HT voltage, 825 V; line c, $c = 2.56 \cdot 10^{-9}\,M$, HT voltage, 900 V); $\Delta\varepsilon$ is the unit of $\mathrm{mol^{-1}\,dm^3\,cm^{-1}}$

variety of C-8 substituted double bonds, namely, 1,2-disubstituted (**19**), endocyclic double bond (**20**), and 1,1-disubstituted (**21**) double bonds (Scheme 3) [24, 26, 102–104].

Upon derivatization of these compounds to the corresponding styrenoids ***sty*-19**-***sty*-21** by cross metathesis and *Heck* reaction [26], moderately intense and clear-cut CD couplets arising from the coupling between the coumarin and the styrene chromophores were observed in the 230–350 nm region. A straightforward



Scheme 3

CD exciton analysis leads to assignment of the absolute configurations of styrenoid derivatives, and therefore the parent compounds (Table 3).

Since both the preexisting coumarin and the styrenoids are fluorescent ($\Phi_f = 0.16$ for styrene and $\Phi_f = 0.050$ for coumarin), these substrates represent interesting models for testing the applicability of FDCD for stereochemical analysis. As summarized in Table 3, ***sty*-19** to ***sty*-21** were analyzed by FDCD with a 100-fold sensitivity enhancement (detection limit $10^{-8}\,M$) with respect to the conventional CD transmission measurements, detection limit at $10^{-6}\,M$. Although the coumarin derivatives ***sty*-19**-***sty*-21** bear large polarization of fluorescence ranging from 0.030 to 0.087 (Table 3), the J-465 instrument completely eliminates the photoselection artifacts and collects much emitted light from the sample solution. Figures 4a and b depict the CD and FDCD curves of **sty-21** ($P_f = 0.030$). As shown in Fig. 4a, the use of the J-465 device (ellipsoidal mirror, line b) and the J-405 device (polarizer at 81°, line c) successfully eliminate the photoselection artifacts at the longer wavelength and lead to excellent agreement between the FDCD and CD spectra at $10^{-5}\,M$. At $10^{-7}\,M$ (Fig. 4b), FDCD measured by J-465 (line b) remains observable, whereas S/N ratios for both the conventional CD (line a) and FDCD obtained by J-405 (line c) are significantly decreased at this concentration. The FDCD detection limit at $10^{-8}\,M$ evaluated for ***sty*-19**-***sty*-21** is the same as that obtained for allylic alcohol derivatives **15a**–**18a**; a similar trend for compounds featuring moderate CD amplitudes is observed (Table 3).

2c. *New Ginkgolide Lactol Derivatives. A Protocol for Sterically Hindered Hydroxyls*

Recently, we have found that the lactone groups in ginkgolide derivatives were smoothly reduced by $NaBH_4$ to give the corresponding lactols creating new stereo-

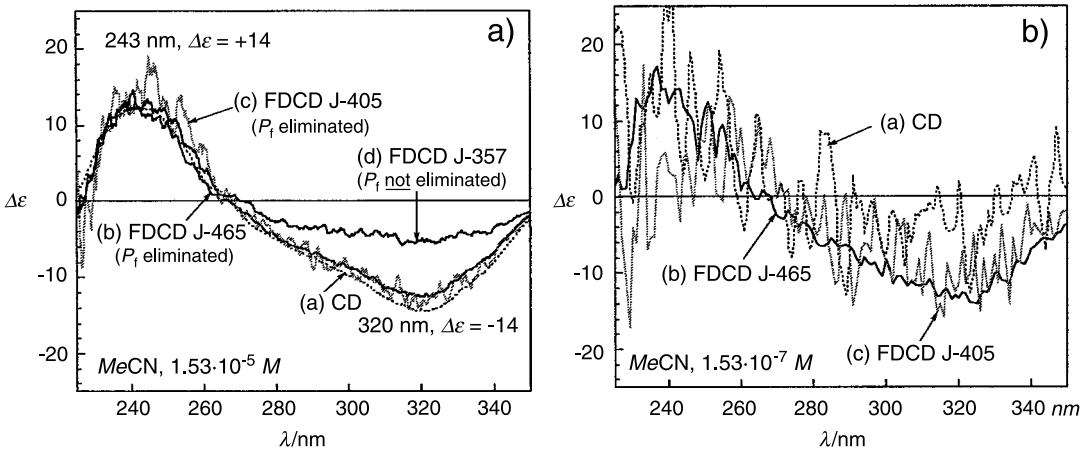
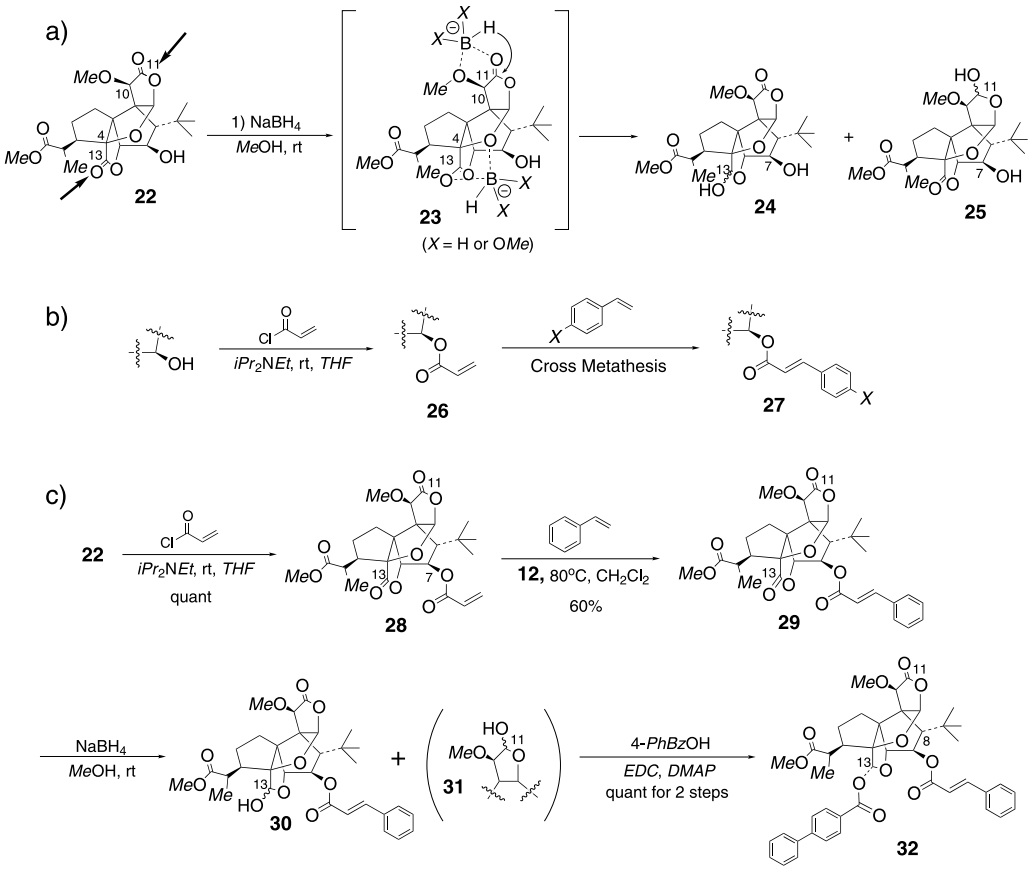


Fig. 4. CD and FDCD of ***sty*-21**; (a) CD (line a) and FDCD by J-465 device (line b, light-cut filter, 380 nm, masks 5.0 mm, HT voltage, 391 V), FDCD by J-405 device (line c, polarizer at 81°, HT voltage, 603 V), FDCD by J-357 device (line d, HT voltage, 448 V); (b) CD (line a), FDCD by J-465 device (line b, HT voltage, 652 V), FDCD by J-405 device (line c, polarizer at 81°, HT voltage, 800 V); $\Delta\varepsilon$ is the unit of $mol^{-1}\,dm^3\,cm^{-1}$



Scheme 4

genic centers at the lactol hydroxyls. An example is shown in Scheme 4a. The $NaBH_4$ treatment of **22**, which was degradated from ginkgolide C [105], quantitatively provided the inseparable mixture of C-13 and C-11 lactol derivatives **24** and **25**, *via* the possible intermediate **23**. The efficient $NaBH_4$ reduction of ginkgolide lactones will expand the ginkgolide-analog library for their biological evaluation [105].

Since both the reduced compounds **24** and **25** bear an additional hydroxy group at C-7 (see Scheme 4a), the stereochemical analysis of newly created stereogenic centers at the lactol hydroxyls could be studied by the exciton coupled method after introduction of suitable chromophores and/or fluorophores at the C-7 hydroxyl and lactol hydroxyls. However, acylation attempts at the ginkgolide 7-hydroxyl with carboxylic acid chromophores, *e.g.*, benzoic acid, were not successful due to the steric hindrance caused by the neighboring *t*-butyl group; the reaction required excess reagents and proceeded in poor yields. For such sterically hindered hydroxyls, a new protocol was developed as shown in Scheme 4b. It utilizes the combined methods of acylation and metathesis; (i) acylation of the sterically hindered hydroxyls with the small and reactive acryloyl chloride [106–110] followed by (ii) cross metathesis with styrenes. According to the method, the ginkgolide derivative **22**

was first reacted with acryloyl chloride in the presence of diisopropylethylamine in *THF* to provide **28** in quantitative yield under mild conditions (Scheme 4c). The introduced vinyl moiety in **28** was then subjected to cross metathesis with styrene, and the cinnamate chromophore was efficiently introduced at the sterically hindered ginkgolide C-7 position in 60% yield. Subsequently, the lactone groups at C-11 and C-13 positions in **29** were smoothly reduced by $NaBH_4$ to give the corresponding lactols **30** and **31** as their four diastereoisomers in quantitative yield. Since these lactol derivatives **30** and **31** could not be separated and existed as its equiblium mixtures, they were continuously acylated by fluorescent 4-phenylbenzoic acid in the presence of *EDC* and *DMAP* to provide **32** as a main diastereoisomer, readily separable from the other isomers by chromatography on silica gel. The 4-phenylbenzoate group was used not only as a ''well-separating acylate agent'' for the hydroxyl-containing compounds [111], but also as one of the exciton coupled fluorophores for FDCD study.

CD and FDCD of the main isomer **32** are shown in Fig. 5a. Compound **32** shows negative exciton coupling between the cinnamate and 4-phenylbenzoate chromophores, with CD amplitude of $A_{CD} = -51$; this is in agreement with that expected from conformational analysis (MMFF and Monte Carlo method) obtained for the 13α-lactone hydroxyl isomer (Fig. 5b). On the other hand, a similar conformational analysis of the diastereomeric 13β-lactone hydroxyl derivative predicted positive CD exciton chirality. Therefore, the main isomer **32** obtained in Scheme 4c was determined to posses the C-13α configuration. The conclusion obtained by the CD/FDCD analysis was also supported by N*O*E between 8-H and 13-H (see Scheme 4c).

Although **32** showed extremely high polarization of fluorescence ($P_f = 0.18$ shown in Table 3), FDCD analysis was performed satisfactorily with J-465; as with the cases of the previously mentioned compounds **15a**–**18a** and ***sty*-19-*sty*-21**, the modest CD amplitude of **32** ($A_{CD} = -51$) enabled the FDCD measurements

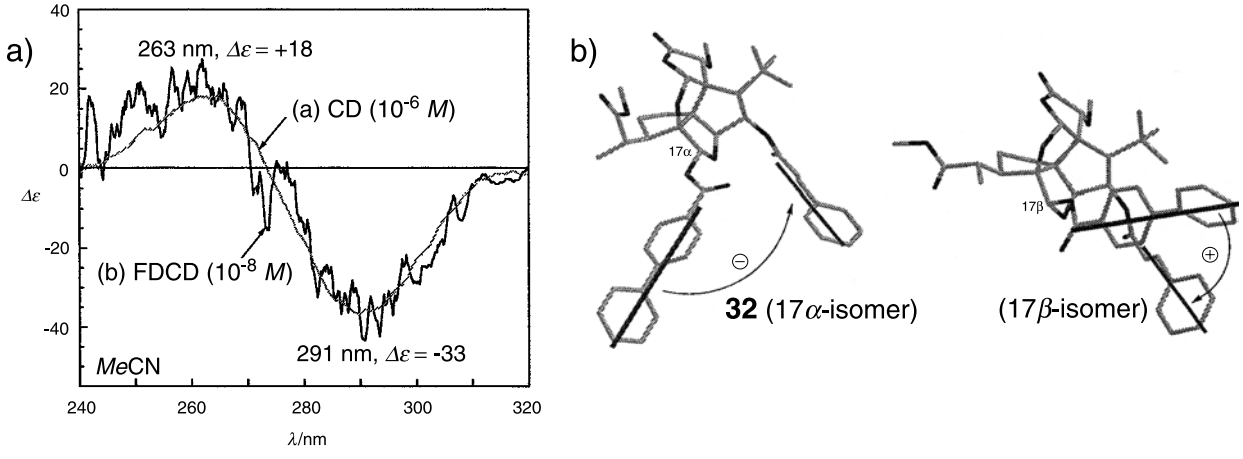


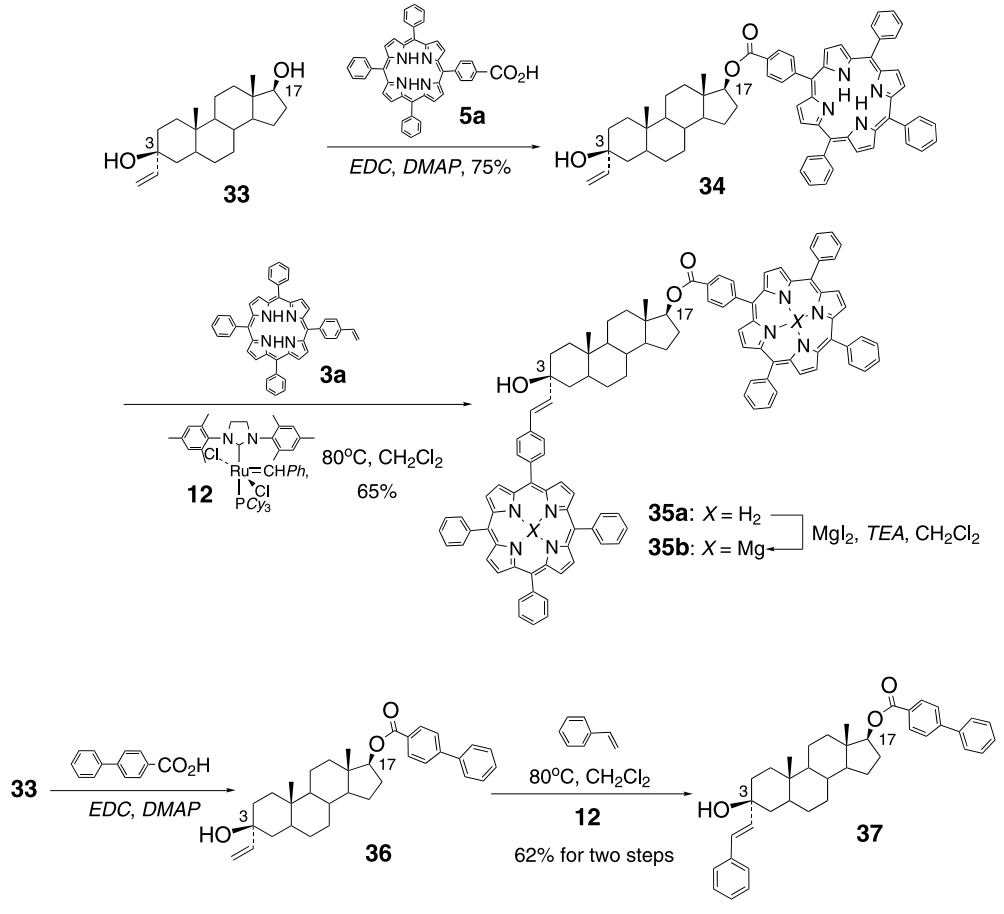
Fig. 5. (a) CD and FDCD of **32**; CD (line a, $c = 2.67 \cdot 10^{-6}\,M$), FDCD (J-465 device, line b, $c = 2.67 \cdot 10^{-8}\,M$, light-cut filter, 340 nm, masks 4.0 mm, HT voltage, 829 V); for the sake of clarity, noisy CD at $10^{-7}\,M$ is not shown; (b) minimized conformation and exciton chirality of **32** and its 17β-isomer; $\Delta\varepsilon$ is the unit of $mol^{-1}\,dm^3\,cm^{-1}$

to be performed down to $10^{-8}\,M$ concentrations, whereas the CD measurement was possible down to $10^{-6}\,M$ (Fig. 5a).

By using a variety of fluorescent 4-substituted cinnamates, *e.g.*, 4-phenylcinnamate or 4-tetraphenylporphyrine (see Section 2d), the application of exciton coupled FDCD will be further extended to a wide range of the sterically congested natural products available only in minuscule amount [16, 112–115], and therefore unsuited for analysis by less sensitive conventional CD analysis.

2d. *Steroid and Dimeric Steroid: Exciton Coupled FDCD Over a Large Distance*

In the case of **33** (Scheme 5) [116], in which the C-17 hydroxyl and C-3 olefin are far apart, we planned to use *TPP*-CO_2H **5a** ($\lambda = 418$ nm, $\varepsilon = 440000\,mol^{-1}\,dm^3\,cm^{-1}$) and vinyl-*TPP* **3a** ($\lambda = 418$ nm, $\varepsilon = 420000\,mol^{-1}\,dm^3\,cm^{-1}$) for both CD and FDCD studies. We have already shown that *TPP*-CO_2H **5a**, which was introduced at the hydroxy or amino group *via* acylates, represents one of the most potent CD reporter groups, especially for the observation of long range exciton coupling [4, 89].



Scheme 5

On the other hand, it is challenging to introduce the vinyl-*TPP* **3a** at the C-3 olefin of **33**; the cross metathesis of the nitrogen-containing porphyrin substrates is rarely reported in the literature since the transition metal catalysts such as *Grubbs*' ruthenium catalyst are believed to be susceptible to nitrogen poisoning. Nevertheless, we explored the vinyl-*TPP* cross metathesis approach keeping in mind that this protocol might be useful for other natural products containing remote OH/C=C or C=C/C=C functionalities, as seen in polyether type marine natural products [90, 91, 117, 118].

We first attached *TPP*-CO_2H **5a** at the C-17 hydroxyl of steroid **33** *via* an ester linkage (Scheme 5). Substrate **33** was then reacted with *TPP*-CO_2H **5a** in the presence of *EDC* and *DMAP* to provide **34** in 75% yield. The cross metathesis of the double bond in **34** with vinyl-TPP **3a** in the presence of *Grubbs*' reagent **12** smoothly provided the corresponding bis-*TPP* derivative **35a** in 65% yield. Since the porphyrin derivatives are easily detectable on the thin layer chromatography (TLC) by their intense deep red color, the reaction monitoring, isolation, and other chemical procedures can be readily performed through microgram-scale handling of the sample. Subsequently, magnesium cations were quantitatively incorporated inside both porphyrins by treatment of **35a** with excess magnesium iodide (10 equivalents) and triethylamine to yield **35b**. In order to compare the CD sensitivity of *TPP*-chromophores in **35b** with that of common aryl chromophores, we also prepared **37** in which styrene (**1**) ($\lambda = 248\,\text{nm}$, $\varepsilon = 15000\,\text{mol}^{-1}\,\text{dm}^3\,\text{cm}^{-1}$) and 4-phenylbenzoate **4** ($\lambda = 270\,\text{nm}$, $\varepsilon = 21000\,\text{mol}^{-1}\,\text{dm}^3\,\text{cm}^{-1}$) are introduced at the C-3 and C-17 positions as shown in Scheme 5.

The CD of **37** (Fig. 6a, dotted line) shows only a positive *Cotton* effect at 269 nm ($\Delta\varepsilon = +11.2\,\text{mol}^{-1}\,\text{dm}^3\,\text{cm}^{-1}$), presumably due to the overlap between the exciton couplet and the individual CD *Cotton* effects resulting from C-17 4-phenylbenzoate and C-3 styrenoid chromophores; since the two stereogenic centers in **37** are separated by a large distance, the exciton coupling between these two chromophores is not sufficiently strong to override the inherent CD *Cotton* effects of each chromophore at the C-3 and C-17, thus failing to give clearcut bisignate couplets. However, when CD of **36** was subtracted from that of **37** (subtraction of the inherent CD *Cotton* effect caused by C-17 4-phenylbenzoate), a small negative counterpart at 247 nm ($\Delta\varepsilon = -2.4\,\text{mol}^{-1}\,\text{dm}^3\,\text{cm}^{-1}$) becomes visible in the resulting difference CD (Fig. 6a).

On the other hand, clear bisignate positive CD couplets with an amplitude of +127 are observed for the bis-porphyrin derivative **35a** as shown in Fig. 6b; the positive exciton chirality of **35a** reflects the clockwise arrangement of porphyrin resulting from the two remote stereogenic centers at C-3 and C-17 separated by 26 Å (see minimized conformation in Fig. 6c).

A conformational analysis with MMFF and Monte Carlo method revealed that **35a** exists as a mixture of several conformers close in energy; 12 minimum energy conformations were isolated within 8.4 kJ/mol. They are due to the variation of at least three degrees of conformational freedom (Fig. 6c): (i) the C3–C=C rotamerism, with three conformers (C=C bond eclipsed to C3–O, C3–C2, and C3–C4 bonds); (ii) the aryl-C=C rotation, with two minima (aryl-C=C dihedral angle about ±30°); (iii) the C17–O rotamerism (C(=O)–O/C17–H17 dihedral angle about ±30°). Although the unrestricted libration of the phenyl-porphyrin bond,

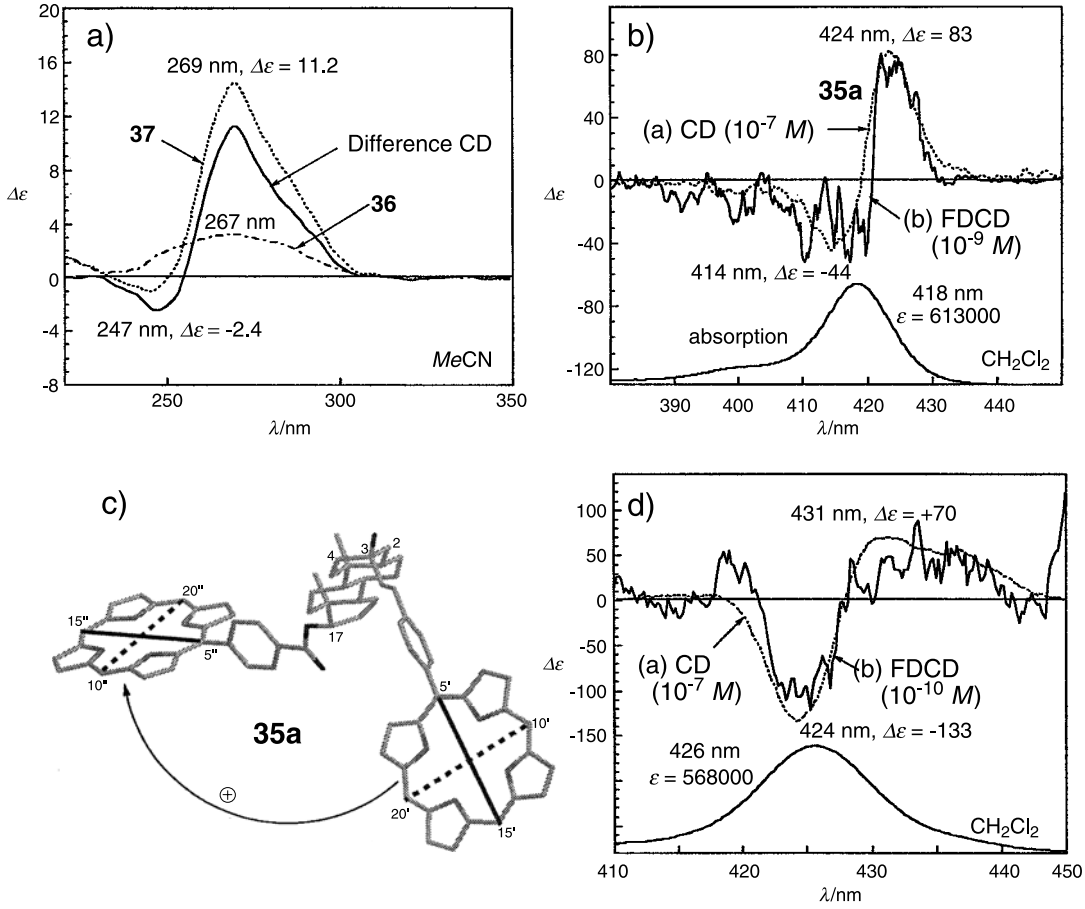


Fig. 6. (a) CD of **36** and **37** and their difference CD; (b) CD (line a, $c = 3.08 \cdot 10^{-7}\,M$) and FDCD of **35a** (J-465 device, line b, $c = 3.08 \cdot 10^{-9}\,M$, light-cut filter, 520 nm, masks 3.0 mm, HT voltage, 778 V); (c) minimized conformation and exciton chirality of **35a**; (d) CD and FDCD of **35b**, CD (line a, $c = 2.91 \cdot 10^{-7}\,M$), FDCD (J-465 device, line b, $c = 2.91 \cdot 10^{-10}\,M$, light-cut filter, 520 nm, masks 3.0 mm, HT voltage, 896 V); ε and $\Delta\varepsilon$ are the unit of $\mathrm{mol^{-1}\,dm^3\,cm^{-1}}$

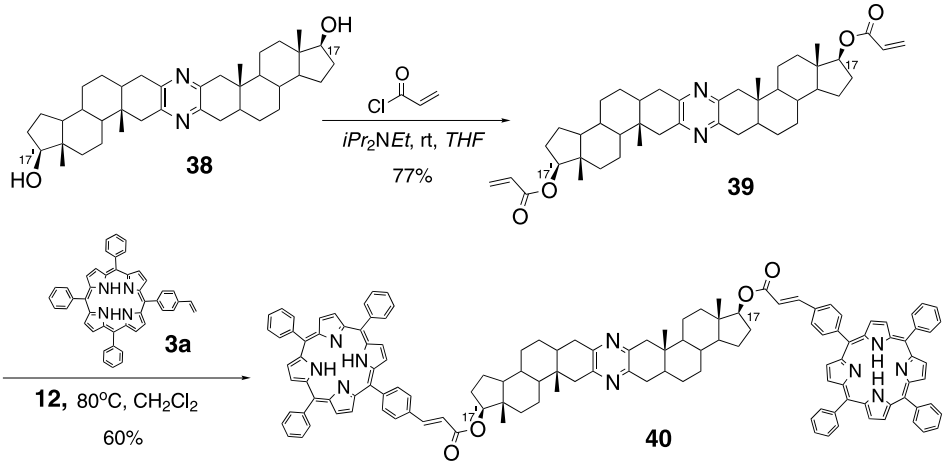
with dihedrals in the range 45 to 135°, should also be considered, this dihedral angle is found to be 90° in all minimum energy conformations. Despite this wide flexibility, it is notable that in all minima the twist defined by 5′–15′ and 5″–15″ porphyrin directions is always positive, with dihedral angles ranging from 32 to 70°. The porphyrin *Soret* band may be described as due to two degenerate transitions with mutually orthogonal transition dipoles [30, 89]. However, due to the libration (largely unrestricted rotation) around the phenyl-porphyrin junction in tetraarylporphyrins a so-called effective transition moment can be considered to exist directed along the 5–15 direction, which is in effect responsible for the sign of exciton coupling in bis-tetraarylporphyrin systems [30, 89]. In conclusion, the observed positive couplet for **35a** is in keeping with a positive chirality defined by the two 5′–15′ and 5″–15″ effective moments.

Fortunately, the FDCD spectrum was also measurable due to the fluorescent property of these *TPP*-derivatives (Fig. 6b). As noted previously, since porphyrin

fluorophores have large extinction coefficients (ε *ca.* 450000 $mol^{-1}\,dm^3\,cm^{-1}$) and the CD amplitude of **35a** exceeded 100, the FDCD measurement was possible even at the highly diluted concentration of $10^{-9}\,M$ despite the long interchromophoric distance. The FDCD detection again achieved a 100-fold enhancement in sensitivity over conventional CD detection as shown in Fig. 6b. Furthermore, introduction of magnesium into porphyrin skeletons (Fig. 6d), highly enhances the FDCD detection, leading to a limit of $10^{-10}\,M$, due to the increase in both the quantum yield and extinction coefficient ($\Phi_f \sim 0.2$, $\varepsilon = 560000\,mol^{-1}\,dm^3\,cm^{-1}$). On the other hand, the concentration limit for CD remains around $10^{-7}\,M$ indicating a 1000-fold enhancement in FDCD sensitivity, the largest increase so far observed.

Encouraged by the highly sensitive FDCD analysis of **35a** and **35b** with a long range coupling distance over 26 Å, the dimeric steroid **40** with the interporphyrin distance increased to 40 Å was measured (Scheme 6). However, the simultaneous bis-acylation of **38** [119] at the two 17-hydroxyls by *TPP*-CO_2H **5a** did not work; the product obtained under several different acylation conditions, *e.g.,* with *EDC* and *DMAP* in dichloromethane, only resulted in a mono-acylated derivative. We therefore utilized the previously established method for sterically hindered hydroxyls employed in Section 2c; bis-steroid **38** was first reacted with the reactive acryloyl chloride, 77% yield, and the vinyl group introduced in **39** was then functionalized by the ''double cross metathesis'' with vinyl-*TPP* **3a** (Scheme 6). By this method, the bis-porphyrin derivative **40** was successfully obtained in ~50% yield from **38**.

The CD spectrum of **40** (Fig. 7a) shows clear negative exciton couplets with an amplitude of −37, reflecting the two remote stereogenic centers at 17 and 17′ positions over a distance of 40 Å (see minimized conformation in Fig. 7b). Ten minimum energy conformations within 9.6 kJ/mol were found for **40** by a MMFF/Monte Carlo conformational analysis. The main degrees of conformational freedom are (Fig. 7b): (i) the C17–O and C17′–O′ rotamerisms, CO–O–C17–H17 dihedral angle about ±30°); (ii) the *s-cis*/*s-trans* C=C–C=O bond isomerism.



Scheme 6

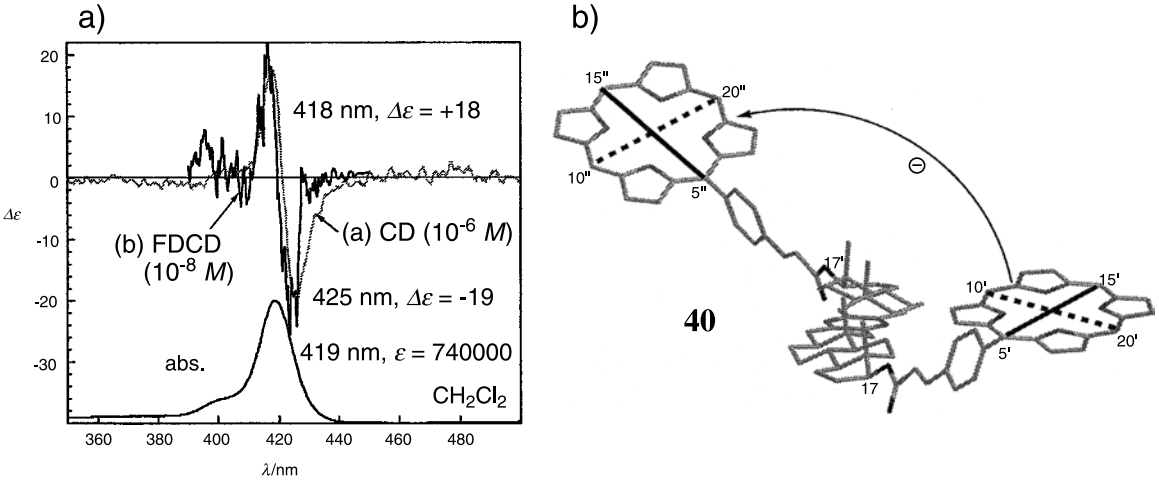


Fig. 7. (a) CD and FDCD of **40**; CD (line a, $c = 1.05 \cdot 10^{-6}\,M$), FDCD (J-465 device, line b, $c = 1.05 \cdot 10^{-8}\,M$, light-cut filter, 520 nm, masks 4.0 mm, HT voltage, 586 V), CD at $10^{-7}\,M$ with low S/N ratio is not shown; (b) minimized conformation and exciton chirality of **40**; ε and $\Delta\varepsilon$ are the unit of $\mathrm{mol}^{-1}\,\mathrm{dm}^3\,\mathrm{cm}^{-1}$

Our attention was then restricted to eight minima with *Boltzmann* population at room temperature above 4%, by considering also entropic factors resulting from the C_2 symmetry system. All show negative twist between 5′–15′ and 5″–15″ directions, with dihedral angles in the −32 to −67° range, in keeping with the observed negative CD couplet. Quantitative CD calculations were also performed on this compound by the *DeVoe* method to investigate the nature of the exciton coupling for such large interchromophoric distances of 39.5–42.5 Å. An hybrid approach was used to describe the *Soret* transition [30, 89], where the 5–15 component was given full dipolar strength (77.4 D^2, extrapolated from the experimental absorption spectrum of 5-(4-(2-methoxycarbonylethenyl)phenyl)-10,15,20-triphenylporphyrin in CH_2Cl_2), and 10–20 component only one half (38.7 D^2). We have previously verified that this approach efficiently describes the exciton interaction in bis-tetraarylporphyrins, by taking into account the libration around the phenyl-porphyrin bonds [30]. All minima gave a negative calculated CD couplet with intensities between −14 and −140; the *Boltzmann*-weighted average was −85, in good agreement with experimental results. Despite the large interchromophoric distance, a moderate exciton coupling is therefore observed for compound **40** owing to the intense extinction coefficient of the *Soret* bands.

Furthermore, as shown in Fig. 7a, FDCD measurement enabled the exciton analysis at even $10^{-8}\,M$ for this extremely long range exciton coupling system. The 100-fold enhancement in sensitivity observed with **40** is in accord with the trend obtained for the exciton-systems with the modest CD amplitudes observed in Sections 2a–2c. The successful microscale analysis performed for both steroid **33** and dimeric steroid **38** provides an intriguing protocol for the stereochemical analysis of many large natural products carrying a variety of functional groups, *e.g.*, sterically hindered hydroxyls, amines, or substituted olefins, which frequently are present in marine toxins [90, 91, 117, 118].

Conclusions

Fluorescence detection of circular dichroism represents an attractive alternative to conventional CD detection whenever specificity toward fluorescent molecules or fluorophoric groups is required, or enhanced sensitivity is desirable because of the restricted amount of compounds, *e.g.,* natural products. Despite these advantages, the application of FDCD has remained for many years restricted due to instrumental inadequacy: namely, residual linear dichroism in circularly polarized light, arising from imperfect optical components, as well as inherent fluorescence polarization typical of many fluorophores, to produce unacceptable artifacts in the FDCD spectra. The recently developed FDCD apparatus (J-465), based on an ellipsoidal mirror device that collects most of the light emitted around the sample, has opened new perspectives to the FDCD technique.

In this paper we described the application of several new FDCD fluorophoric reporter groups, such as styrene, 4-vinylbiphenyl, 4-phenylbenzoate, cinnamate, and porphyrin-based groups which can be conveniently introduced into either olefin moieties by cross-methatesis or at hydroxyls (including sterically hindered ones) by acylation. The paper also provides the examples for efficiency of the ellipsoidal mirror FDCD device, which eliminates photoselection artifacts, augments the intensity of the total fluorescence signal, and overcomes the inconveniencies due to low quantum yields and strong fluorescence polarization. The experimental results highlight the advantage of fluorescence detection in providing FDCD spectra not only in excellent agreement with CD but also at much lower concentrations than conventional CD detection by two-three orders of magnitude reaching $10^{-8}\,M$ in most cases, and down to 10^{-10} M for favorable examples. We believe the structural diversity of the compounds analyzed in this study demonstrates the versatility of the exciton chirality approach, and in particular, the fluorescence based approach as a practical and sensitive technique with a broader applicability.

Experimental

FDCD Measurement

Three different FDCD devices, J-357, J-405, and J-465 attached to Jasco J-810 spectropolarimeter have been used. The scattered light was prevented by employing a long-pass filter in front of the PMT (wavelength used for each measurement is shown in Figure captions). The FDCD signals recorded were converted into the CD spectra based on the equation: $\Delta\varepsilon = \varepsilon_L - \varepsilon_R = 3.032 \cdot 10^{-5} \cdot S \cdot (1 - 10^{-A})/c \cdot d \cdot 10^{-A}$ in which A is UV absorbance, c is molar concentration, d is cell length (cm), and $S = k(F_L - F_R)/(F_L + F_R)$. $(F_L + F_R)$ was measured as DC voltage and k is an instrumental ellipticity constant +28648 (millideg).

Computational Methods

Molecular modeling was performed with Spartan '02 (Wavefunction, Inc., Irvine, CA), using default parameters and convergence criteria. Molecular conformations were calculated with Merck force field (MMFF) using the Monte Carlo conformational search method with 1000 iteration steps. Electronic structure calculations were performed with Gaussian 03 (Gaussian, Inc., Pittsburgh, PA) [120] with ZINDO/S method on input structures minimized with DFT, B3LYP/6-31G(d) level. Coupled oscillator calculations were performed with the *DeVoe* method using a Fortran program developed by

W. Hug [27]. MMFF-optimized structures were used as input geometries; average CD spectra were calculated as *Boltzmann*-weighted at 300 K. Spectral parameters were taken from experimental UV-Vis spectra of the constituent chromophores and ZINDO calculations.

General Procedure of Acryloylation of Sterically Hindered Hydroxyls
Acryloylated Monosteroid (**11**, $C_{22}H_{32}O_3$)

To a solution of 50 mg 17β-hydroxy-5α-androstan-3-one (**9**) (0.172 mmol) and 60.0 mm^3 diisopropylethylamine (0.344 mmol) in 1.0 cm^3 CH_2Cl_2 was slowly added 28.0 mm^3 acryloyl chloride **10** (0.344 mmol) at 0°C. After the mixture was warmed to room temperature and stirred for an additional h, H_2O and saturated aqueous NH_4Cl solution were added, and the resulting mixture was extracted with ethyl acetate. The organic layers were combined, washed with brine, dried (Na_2SO_4), filtered, and concentrated *in vacuo* to give the crude products. Column chromatography on silica gel (gradually from 9 to 25% ethyl acetate in *n*-hexane) gave 60 mg **11** (100%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.73$–1.80 (m, 16H), 0.84 (s, 3H), 1.02 (s, 3H), 1.99–2.04 (m, 1H), 2.07–2.12 (m, 1H), 2.16–2.43 (m, 4H), 4.68 (dd, $J = 8.8$, 8.8 Hz, 1H), 5.81 (dd, $J = 10.4$, 1.3 Hz, 1H), 6.12 (dd, $J = 17.3$, 10.4 Hz, 1H), 6.38 (dd, $J = 17.4$, 1.3 Hz, 1H) ppm; HRFABMS: m/z = calcd for $C_{22}H_{33}O_3$ $[M + H]^+$ 345.2429, found 345.2435.

General Procedure of Cross Metathesis
Porphyrincinnamate (**6**, $C_{66}H_{60}O_3N_4$)

To a solution of 3.0 mg **11** (8.71 μmol) in 2.0 cm^3 CH_2Cl_2 was added 1.7 mg *Grubbs*' second generation ruthenium catalyst **12** (1.99 μmol) and 33.5 mg **3a** (52.3 μmol) at room temperature, and the mixture was stirred at 80°C for 3 h. The reaction mixture was concentrated *in vacuo* to give the crude products which were purified by column chromatography on silica gel (ethyl acetate in *n*-hexane, gradually from 9 to 20%) to afford 6.0 mg **6** (72%) as a deep red colored solid. The reaction could be also performed using 100 μg of **11**. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 0.97$ (s, 3H), 1.05 (s, 3H), 0.82–2.45 (m, 22H), 4.85 (dd, $J = 7.9$, 7.9 Hz, 1H), 6.75 (d, $J = 16.0$ Hz, 1H), 7.72–7.79 (m, 9H), 7.93 (d, $J = 8.1$ Hz, 2H), 8.00 (d, $J = 16.1$ Hz, 1H), 8.20–8.26 (m, 8H), 8.83–8.87 (m, 8H) ppm; HRFABMS: m/z = calcd for $C_{66}H_{61}O_3N_4$ $[M + H]^+$ 957.4743, found 957.4766.

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