A Synthetic Receptor for β,β-Carotene: Towards an Enzyme Mimic for Central Cleavage

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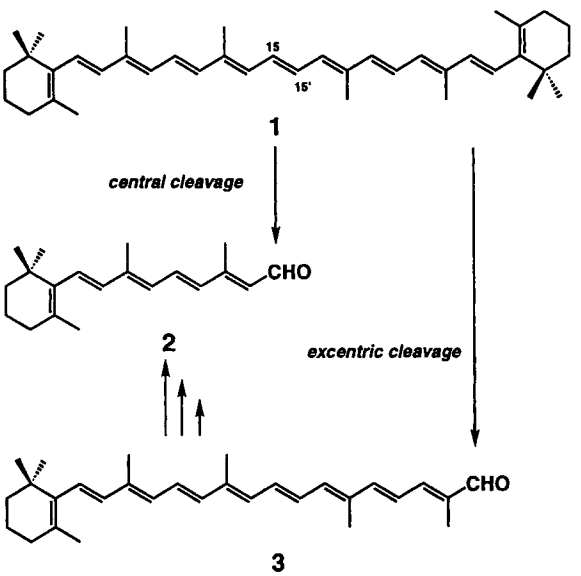
We report on the synthesis of a receptor **4** for β,β-carotene (**1**), and on the binding interaction between the two which yields inclusion complex **5**. The cyclodextrin 'dimer' **4** was obtained *via* condensation of the corresponding 4,4'-(porphyrin-5,15-diyl)bis phenol **8** with 6^A-deoxy-6^A-iodo-β-cyclodextrin (**6**) in the presence of Cs_2CO_3. Fluorescence studies of the binding interaction between 'dimer' **4** and β,β-carotene (**1**) gave a binding constant K_a of $(2.4 \pm 0.1) \cdot 10^6$ M^{-1}

Introduction. – The enzymes (carotene dioxygenases, CDOs) which cleave β,β-carotene (**1**) to provide retinal (**2**) as a precursor for vitamin A are of significance to animal and human nutrition [1]. To date, two modes of cleavage of **1** have been proposed: *i*) the central cleavage of **1** which gives retinal (**2**) directly, and *ii*) the more recently discovered excentric cleavage which yields first apocarotenals, such as **3**, which may be degraded to **2** by β-oxidation (*Scheme 1*) [2][3]. The enzymes catalyzing these reactions have been neither purified nor are their respective mechanisms and cofactors known. Results concerning central cleavage suggest that the enzyme involved places its active

Scheme 1. *Metabolism of β,β-Carotene* (**1**)

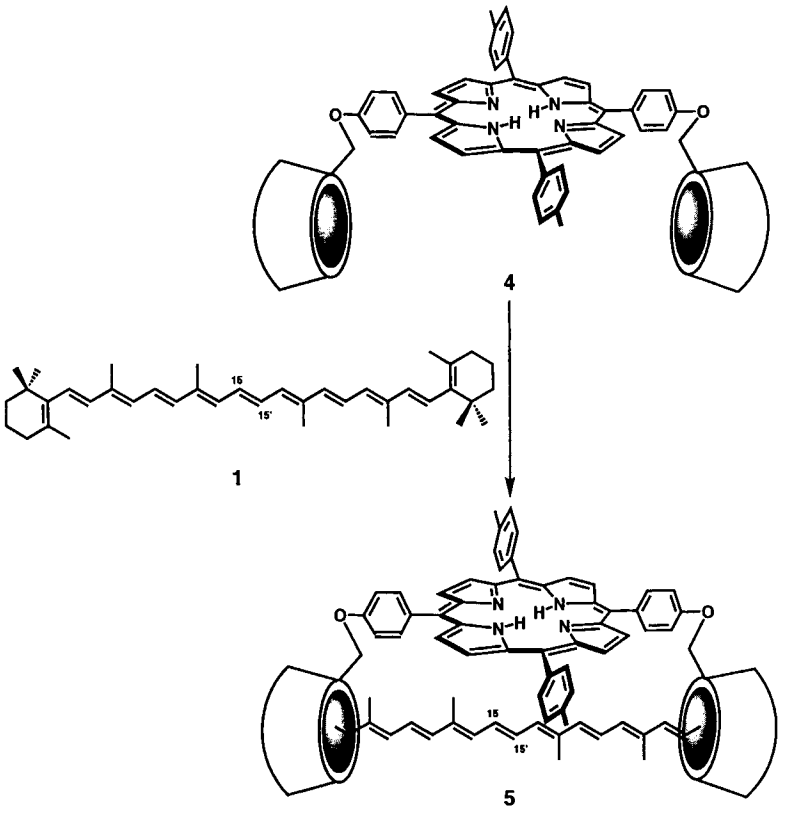


site's metal complex precisely above the C(15)=C(15′) bond to be cleaved [4][5]. The observation that this CDO controls the regiospecific cleavage of one C=C bond out of eleven of the substrate is an intriguing and challenging one. To mimic such a system, we wish to report here on the synthesis of a β,β-carotene receptor as a first step towards an active-site analogue for the dioxygenase.

Results and Discussion. – The design of the supramolecular construct **4** (*Scheme 2*) was found, using the MOLOC program [6], to be an ideal candidate for the binding of β,β-carotene (**1**). Each of the cyclodextrin moieties was shown to be capable of binding one of the cyclohexene end groups of **1**, leaving the porphyrin part of **4** to span the polyene chain (see **5**). Optimization of the complex showed that approximately half of **1** would be included in the cyclodextrin cavities, and that the C(15)=(15′) bond would be located directly under any metal that can be inserted into the porphyrin moiety. In the absence of **1**, several unproductive conformations of **4** are possible due to rotation about the ether linkages; in the presence of **1**, however, an induced fit should be observed yielding the inclusion complex **5**.

As well as having the role of a spacer and potential metal ligand, the porphyrin moiety in **4** was also chosen for its physico-chemical properties. Porphyrins display a

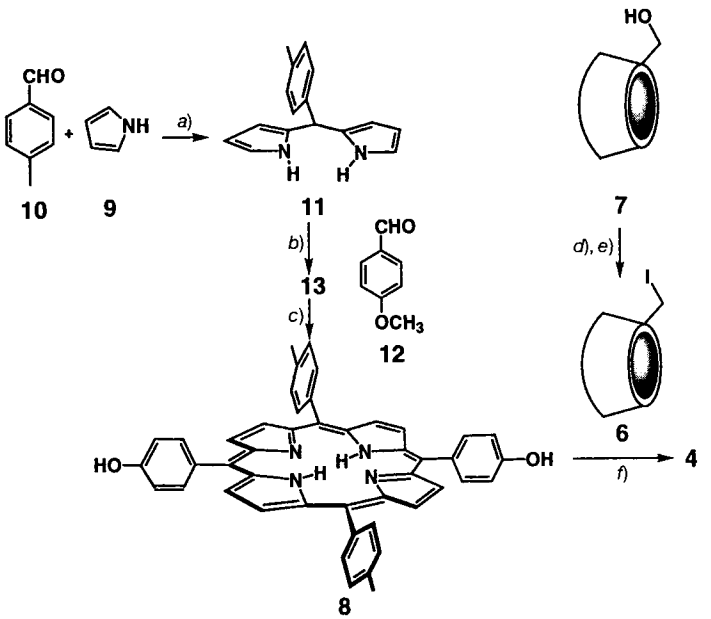
Scheme 2. *Formation of the 1:1 Inclusion Complex* **5** *from β,β-Carotene* (**1**) *and the Cyclodextrin 'Dimer'* **4**



characteristic fluorescence at around 650 nm, and the ability of carotenoids to quench this fluorescence was envisaged as a sensitive probe for the binding interaction of the two entities in an aqueous medium [7]. It could be reasonably assumed from earlier work that a cyclodextrin 'dimer' such as **4** should be capable of providing a binding constant, K_a, for **1** in the region of 10^5-10^7 M^{-1} [8].

The 6^A-deoxy-6^A-iodo-β-cyclodextrin (**6**) was prepared according to established procedures in two steps from β-cyclodextrin (**7**) in 30% overall yield [9] (*Scheme 3*). Both **6** and the intermediate product in the synthesis, 6^A-*O*-(*p*-tosyl)-β-cyclodextrin, were identified unambiguously from their ^{1}H-NMR spectra in (D_6)DMSO and by their MALDI-TOF-MS, the data being in agreement with those published [9]. (Porphyrin-5,15-diyl)-bisphenol **8** was prepred according to a three-step protocol. Firstly, the condensation of 1*H*-pyrrole (**9**) with *p*-tolualdehyde (**10**) in the presence of a catalytic amount of CF_3COOH [10] gave the *meso*-(*p*-tolyl)dipyrromethane **11** in 77% yield, with spectroscopic data identical to published data [11]. The subsequent condensation of **11** with *p*-anisaldehyde (**12**) gave a mixture of five porphyrins as described previously [11]. Porphyrin **13** was the major component, as determined by MALDI-TOF-MS, and could be separated from the mixture in 15% yield based on **11**[1]). The target 4,4′-(por-

Scheme 3



a) 0.1 equiv. of CF_3COOH, r.t. 12 h; 77%. *b*) 0.1 equiv. of CF_3COOH, CH_2Cl_2, r.t., 15 min; 15%. *c*) Excess $AlCl_3$, CH_2Cl_2/EtSH, r.t., 12 h; 70%. *d*) TsCl, pyridine, 5° to r.t. 24 h; 34%. *e*) NaI, DMF, 90°, 5 h; 90%. *f*) 1) Cs_2CO_3, DMF, r.t., 2 h; 2) excess **6**, 80°, 3 days; 66%.

[1]) The total porphyrin yield was 27%. The other porphyrin fractions were identified by ^{1}H-NMR and MALDI-TOF-MS, and corresponded to the 5,10,15,20-tetra(*p*-tolyl)-, 5-(4-methoxyphenyl)-10,15,20-tri(*p*-tolyl)-, 5,10,15-tris(4-methoxyphenyl)-20-(*p*-tolyl)-, and 5,10,15,20-tetrakis(4-methoxyphenyl)porphyrins.

phyrin-5,15-diyl)bisphenol **8** was obtained in 70% from **13** by treatment with $AlCl_3$ in the presence of ethanethiol in CH_2Cl_2 [12]. The *O*-alkylation of the phenol moieties of **8** with the cyclodextrin **6** was achieved *via* the diphenolate, generated by treatment with caesium carbonate, followed by the portionwise addition of **6** under heating over three days. The target cyclodextrin 'dimer' **4** was isolated in 66% yield (based on **8**) by reversed-phase chromatographic separation from unreacted bisphenol **8** and the intermediate mono-cyclodextrinyl-porphyrin derivative. Further purification by HPLC afforded compound **4** of $\geq 99\%$ purity. The compound was characterized by UV/VIS, MALDI-TOF- and ESI-MS, and 1D and 2D NMR.

Determination of K_a. – Carotene is a well-known quencher of porphyrin fluorescence, and electron transfer from carotene to excited-singlet porphyrins has been identified as a quenching mechanism in polar solvents [7]. Thus, we expected that the fluorescence yield of complex **5** should be strongly reduced relative to that of **4**, and sought to determine the binding constant by fluorescence spectroscopy.

The fluorescence of $2.5 \cdot 10^{-7}$ M aqueous solutions of **4** was indeed reduced substantially at low concentrations of added carotene, $[\mathbf{1}]_0 = 1 \cdot 10^{-6}$ M, as shown in the *Figure*. The shape of the fluorescence emission spectra did not change noticeably. This observation clearly indicates association of **1** and **4** in the ground state, because diffusional quenching of porphyrin fluorescence is negligible at these concentrations: the maximum quenching rate $k_q[\mathbf{1}] \leq k_{diff}[\mathbf{1}] \leq 1 \cdot 10^4\ s^{-1}$ cannot compete with the intrinsic decay rate of singlet-excited **4**. The association equilibrium appeared to be established within minutes, the time between preparation of the mixtures and measurement, as the fluorescence yield of the mixtures did not undergo further changes on prolonged standing.

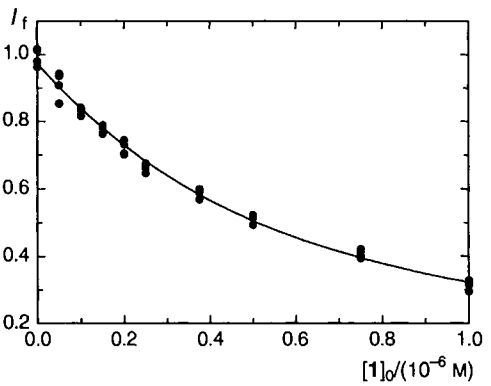


Figure. *Relative fluorescence intensities of aqueous solutions of* **4** *as a function of added β,β-carotene concentration* $[\mathbf{1}]_0$ ($[\mathbf{4}]_0 = 2.5 \cdot 10^{-7}$ M, λ_{exc} 420 nm, λ_{em} 654 nm). The determination of the fitted function (*Eqn. 2a*) is described in the text.

For a quantitative analysis of these data, we consider formation of a 1:1 complex between **1** and **4**, which is defined by the binding constant K_a (see *Eqn. 1*). Dissociation of the excited complex during its lifetime is unlikely and, in any event, would not affect the analysis which is sensitive only to the difference between the fluorescence yields of

4 and **5**. Similarly, a conceivable difference between the absorption spectra of **1** + **4** and **5** would affect only the apparent fluorescence yields of **4** and **5**. Small corrections were applied to the observed fluorescence intensities, I_f, to account for the inner-filter effect arising from absorption by carotene at the excitation wavelength λ_{exc} 420 nm; optically dilute solutions (A 0.05 at λ_{exc}) were used to keep these corrections at 8%. The total observed fluorescence intensity is then given by *Eqn. 2*. The parameters a and b are the apparent fluorescence intensities of **4** and **5**, respectively, at a given concentration, which depend on the quantum yields of fluorescence, the extinction coefficients, and instrumental parameters.

$$\mathbf{1} + \mathbf{4} \rightleftharpoons \mathbf{5},\ K_a = \frac{[\mathbf{5}]}{[\mathbf{1}][\mathbf{4}]} \tag{1}$$

$$I_f = a[\mathbf{4}] + b[\mathbf{5}] \tag{2}$$

Replacement of the variables [**1**] and [**5**] using the mass-balance equations $[\mathbf{1}]_0 = [\mathbf{1}] + [\mathbf{5}]$ and $[\mathbf{4}]_0 = [\mathbf{4}] + [\mathbf{5}]$ yields *Eqns. 1a* and *2a*, respectively, which depend only on the known concentrations of added **1** and **4**, *i.e.*, $[\mathbf{1}]_0$ and $[\mathbf{4}]_0$, respectively, and on the equilibrium concentration of **4**, *i.e.*, [**4**].

$$K_a = \frac{[\mathbf{4}]_0 - [\mathbf{4}]}{[\mathbf{4}]([\mathbf{1}]_0 - [\mathbf{4}]_0 + [\mathbf{4}])} \tag{1a}$$

$$I_f = a[\mathbf{4}] + b([\mathbf{4}]_0 - [\mathbf{4}]) \tag{2a}$$

Eqn. 1a defines the equilibrium concentration of **4**, given the initial concentrations of **1** and **4** and the equilibrium constant K_a. To avoid the necessity of nonlinear fitting, a large excess of one of the components is commonly used that its actual concentration does not differ substantially from the added concentration (*Benesi-Hildebrand* plots) [13]. Such a procedure was impracticable in the present case, and is, in fact, not required. A subroutine for the iterative determination of [**4**] from *Eqn. 1a* was incorporated into a nonlinear least-squares fitting program to find the best values of the parameters a, b, and K_a from *Eqn. 2a*. This obviates the need for introducing any further approximations. Consideration of the error sources suggests that relative errors in I_f should be constant, and this is borne out by the dispersion of the samples. Hence, the logarithmic form of *Eqn. 2a* was used to optimize the parameters, which gave $a = (3.88 \pm 0.04) \cdot 10^6$ counts $s^{-1}\,M^{-1}$, $b = (-0.5 \pm 1.5) \cdot 10^5$ counts $s^{-1}\,M^{-1}$, and $K_a = (2.3 \pm 0.3) \cdot 10^6\ M^{-1}$. The parameter b, the apparent fluorescence yield of the complex **5**, is not significantly different from zero, and its upper limit is *ca.* 3% of the fluorescence yield of the free porphyrin **4**. The quality of the fit was not significantly reduced by fixing the parameter b to zero. This gave the final value of $K_a = (2.4 \pm 0.1) \cdot 10^6\ M^{-1}$ for the binding constant.

An ESI-MS measurement of the H_2O-soluble complex **5** showed the complex as its doubly charged ion at m/z 1722. Subsequent MS/MS of the 1722 peak showed decomposition of the inclusion complex to its two components, **1** and **4**. The UV/VIS spectrum of complex **5** in 2% THF/H_2O showed a 4-nm bathochromic shift to 424 nm in the *Soret* band of the porphyrin with small hypsochromic changes observed in the Q bands.

Sufficiently resolved NMR spectra of complex **5** in D_2O could not be obtained due to extensive line broadening.

Accordingly, there is good evidence for the structures of **4** and **5**, and receptor **4** shows an appreciably high binding constant for substrate **1**. In a later stage of the work, the cleavage of **1** will be studied *via* replacement of the porphyrin moiety of **5** by a metal complex, preferably a porphyrinato ruthenium [14]. Porphyrinato ruthenium complexes are known to cleave C=C bonds to give aldehydes [15]. It is important to note that C=C bond cleavage of β,β-carotene (**1**) to two molecules of retinal (**2**) is essential to prevent product inhibition and obtain catalysis. It can be estimated that the binding constant of retinal (**2**) to 'dimer' **4**, or the corresponding metal complex, will be smaller by three to four orders of magnitude compared to substrate **1**. Indeed, the K_a value of retinal (**2**) to β-cyclodextrin has been reported to be $3.64 \cdot 10^3\ M^{-1}$ [16].

The above described approach has to be distingushed from other porphyrin-containing bis-cyclodextrin systems [8] with regard to two important aspects. Firstly, O-ethers are not prone to oxidation during catalytic turnovers, as may be the case for S-ethers, and secondly, the receptor presented here is designed for an enzyme's natural substrate rather than the substrate being designed to fit, with added tailor-made terminal goups, into the cyclodextrin cavities.

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Experimental Part

General. All reactions were carried out under an inert Ar atmosphere using flame-dried glassware. Solvents were dried under standard conditions and freshly distilled prior to use. Reagents were used as received from *Fluka AG* (Buchs, Switzerland). β-Cyclodextrin was dried at 90° under high vacuum for 24 h prior to use. TLC: precoated glass plates (silica gel *60* F_{254}). Normal-phase column chromatography (CC): silica gel, 0.040–0.063 mm, 230–400 mesh. Reversed-phase column chromatography (RP CC): *LiChroprep*® *RP-8* silica gel with 'nanopure' H_2O and freshly distilled EtOH. HPLC: *Hewlett-Packard-1050* apparatus with a *LiChrospher*®*-100-RP-18e* (5 µm; *250-4)* column; flow rate 1.5 ml/min, temp. 50°; UV detection at 420 and 254 nm. All chromatographic materials were obtained from *Merck AG* (Darmstadt, Germany). Fluorescence spectra: *Jobin-Yvon-Spex-FluoroMax-2* spectrometer, fitted with a temp.-controlled cell housing and integrated magnetic stirrer; solns. in 1-cm quartz cuvettes at 25°; both excitation and emission slits were set at 3 nm. UV/VIS Spectra: λ_{max} in nm. ^{1}H-NMR Spectra: *Varian-Gemini-300* (300 MHz) or *Bruker-DRX-600* (600 MHz) spectrometer; δ in ppm rel. to $SiMe_4$ and coupling constants J in Hz.

5,15-Bis(4-methoxyphenyl)-10,20-bis(4-methylphenyl)porphyrin (**13**). To a soln. of dihydro-*meso*-tolyldipyrromethane (= *N*,5-dihydro-5-(*p*-tolyl)dipyrrin; **11**; 2.5 g, 10.57 mmol, 1 equiv.) and *p*-anisaldehyde (**12**; 1.28 ml, 10.57 mmol, 1 equiv.) in CH_2Cl_2 (170 ml), CF_3COOH (0.81 ml, 10.57 mmol, 1 equiv.) was added. The mixture was stirred at r.t. for 15 min. The reaction was then quenched by the addition of sat. aq. $NaHCO_3$ soln. (170 ml). Extraction of the aq. phase with two portions of CH_2Cl_2, followed by evaporation of the combined org. phase, gave the crude condensation product as a dark solid. This was taken up in CH_2Cl_2 (170 ml), and DDQ (= 4,5-dichloro-3,6-dioxocyclohexa-1,4-diene-1,2-dicarbonitrile; 6 g, 26.44 mmol, 5 equiv. rel. to porphyrin) was added as a soln. in THF. The dark soln. was stirred at r.t. for 15 min after which volatiles were removed *in vacuo*. The crude product was purified by CC (silica gel; hexane/CH_2Cl_2/MeOH/Et_3N 66:33:1:0.5): 565 mg (15%) of **13**. Purple solid. R_f (hexane/CH_2Cl_2/MeOH/Et_3N 50:50:1:0.5) 0.65. UV/VIS (CH_2Cl_2): 420. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.84 (*s*, 8 H, pyr.); 8.1 (*d*, J = 8.73, 4 H_o, (MeO*Ar*)); 8.08 (*d*, J = 8.21, 4 H_o, (Me*Ar*)); 7.54 (*d*, J = 7.84, 4 H_m, (Me*Ar*)); 7.27 (*d*, J = 8.73, 4 H_m, (MeO*Ar*)); 4.09 (*s*, 6 H, O*Me*Ar); 2.69 (*s*, 6 H, *Me*Ar). MALDI-TOF-MS: 704.2 (M^+).

4,4'-[10,20-Bis(4-methylphenyl)porphyrin-5,15-diyl]bisphenol (**8**). To a soln. of **13** (20 mg; 0.028 mmol, 1 equiv.) in CH_2Cl_2 (1.5 ml), EtSH 1.5 ml was added, followed by $AlCl_3$ (151 mg, 1.13 mmol, 40 equiv.). The

resulting green soln. was stirred at r.t. overnight and then quenched by the slow addition of sat. aq. $NaHCO_3$ soln. The mixture was then extracted with three portions of AcOEt and the combined org. phase dried (Na_2SO_4) and evaporated. Purification by CC (silica gel, CH_2Cl_2/MeOH 99:1) gave 13 mg (70%) of **8**. Purple solid. R_f (CH_2Cl_2/MeOH 19:1): 0.26. UV/VIS (CH_2Cl_2): 420. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.84 (*s*, H–C(2), H–C(8), H–C(12), H–C(18)); 8.82 (*s*, H–C(3), H–C(7), H–C(13), H–C(17)); 8.08 (*d*, J = 7.82, 4 H_o (Me*Ar*)); 8.01 (*d*, J = 8.73, 4 H_m (*Ar*OH)); 7.52 (*d*, J = 7.84, 4 H_m (MeAr)); 7.12 (*d*, J = 8.73, 4 H_o (*Ar*OH)); 4.9–4.1 (br. *s*, 2 H, ArO*H*); 2.69 (*s*, 6 H, *Me*Ar). MALDI-TOF-MS: 674.8 (M^+).

6^A,6'^{A}-O-{[10,20-Bis(4-methylphenyl)porphyrin-5,15-diyl]di-4,1-phenylene}bis[β-cyclodextrin] (**4**). To a soln. of **8** (5 mg, 7.4 mmol, 1 equiv.) in DMF (0.5 ml), anh. Cs_2CO_3 (24 mg, 74 mmol, 10 equiv.) was added. The mixture was stirred at r.t. for 2 h, then 6^A-deoxy-6^A-iodo-β-cyclodextrin (**6**; 46 mg, 37 mmol, 5 equiv.) added in one portion, and the mixture heated to 80° for 8 h. Another portion of **6** was then added as a soln. in DMF (1 ml) and the mixture stirred at 80° overnight. A third portion of **6** was added and stirring continued for further 24 h. The DMF was then evaporated and the crude residue purified by RP-CC (EtOH/H_2O 2:3 (→ polar by-products and residual **6**), then EtOH/H_2O 1:1): 14 mg (66%) of **4** as a purple solid. A sample (5 mg) was further purified by HPLC (40–60% MeCN/H_2O within 20 min, 1.5 ml/min): **4** of 99% purity; t_R 13.7 min. R_f 0.37 (PrOH/H_2O/AcOEt/25% aq. NH_3 soln. 5:3:3:1). UV/VIS (DMSO): 418. UV/VIS (H_2O): 420. ^{1}H-NMR (600 MHz, (D_6)DMSO): 8.88 (*s*, H–C(2), H–C(8), H–C(12), H–C(18) (porph)); 8.81 (*s*, H–C(3), H–C(7), H–C(13), H–C(17) (porph)); 8.09 (2*d*, J = 7.69, 4 H_o (Me*Ar*), 4 H_m (*Ar*O)); 7.63 (*d*, J = 7.97, 4 H_m (Me*Ar*)); 7.39 (*d*, J = 7.96, 4 H_o (O*Ar*)); 5.84 (br. *s*, 28 OH), 5.1–4.7 (*m*, 14 anom. H); 4.7–4.3 (*m*, 12 OH), 3.9–3.1 (*m*, 14 H–C(4), 14 H–C(5), 14 CH_2(6), 14 H–C(3) (β-CD)). MALDI-TOF-MS: 2909.8 (M^+). ESI-MS: 1454 (M^{2+}).

Determination of K_a. To a 3-ml aliquot of 0.25 μM cyclodextrin 'dimer' **4** in H_2O in a quartz cuvette which had been fitted with a magnetic stirring bar, THF (60 μl) was added. The soln. was equilibrated at 25° with stirring for 5 min and was then allowed to stand for further 5 min at 25°. The emission spectra (λ_{exc} 420 nm) was measured to afford a value for I_0 (λ_{em} 654 nm).

Samples containing increasing concentrations of β,β-carotene (**1**; 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2, 3, and 4 equiv.) were added as a soln. in THF (60 μl) to 3-ml aliquots of 0.25 μM **4** in H_2O. After addition, each sample was subjected to identical equilibration conditions as those used for the I_0 measurement. Emission spectra were then recorded, as above, to give values for I_f (λ_{em} 654 nm). All experiments were repeated four times. Analysis of the data as described in the text gave a value of $(2.4 \pm 0.1) \cdot 10^6\ M^{-1}$ for K_a.

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