

# The Central Cleavage of $\beta,\beta$ -Carotene – A Supramolecular Mimic of Enzymatic Catalysis

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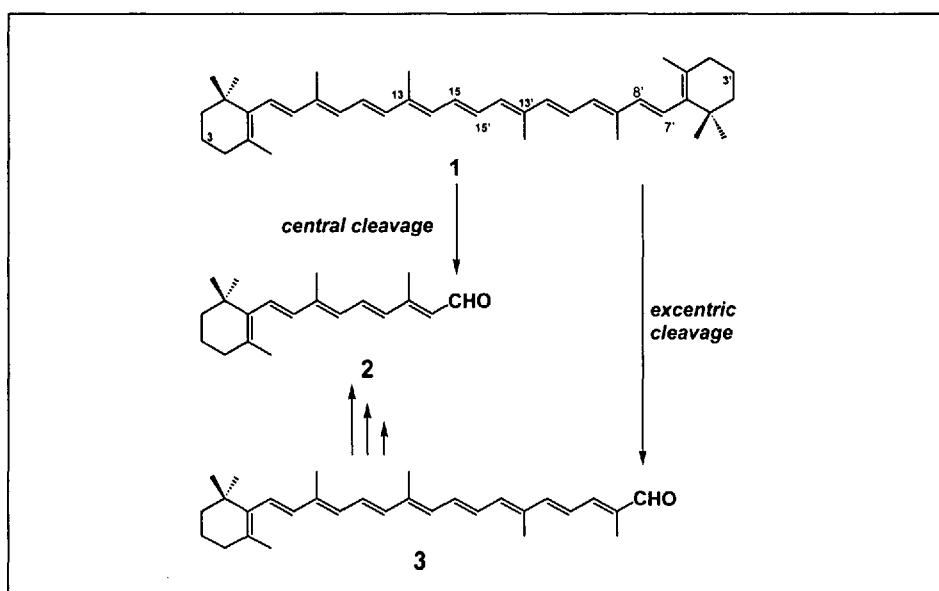
**Abstract:** The enzyme  $\beta,\beta$ -carotene 15,15'-dioxygenase which cleaves  $\beta,\beta$ -carotene to retinal (provitamin A) has been identified for the first time in chicken intestinal mucosa and subsequently sequenced and expressed in two different cell lines. To mimic this unusual metabolism a supramolecular receptor has been synthesized which binds  $\beta,\beta$ -carotene with  $K_a = 8.3 \times 10^6 \text{ M}^{-1}$  and it was shown that the corresponding oxo-ruthenium complex catalyzes the selective cleavage of carotenoids.

**Keywords:** Carotenoids · Catalysis · Cyclodextrins · Enzymes · Ru-porphyrins · Supramolecular chemistry

## Introduction

To catalyze enzymatic reactions nature employs to a great extent the features of supramolecular chemistry. Substrate binding and transition state stabilization are accomplished through hydrophobic interactions, H-bonding and Coulomb forces; the formation of covalent intermediates is the exception rather than the rule. The present account reports on the design and synthesis of a supramolecular system that catalyzes a particularly challenging reaction: the regiospecific cleavage of  $\beta,\beta$ -carotene.

$\beta,\beta$ -Carotene (**1**) is the parent structure and most important compound of the 'orange pigments of life' comprising >650 carotenoids which are abundant in the plant and animal kingdom [1][2]. Both plants and bacteria can biosynthesize carotenoids [3] but mammals rely on extraction from their diet. The function of



Scheme 1. Metabolism of  $\beta,\beta$ -carotene (**1**)

carotenoids is associated with i) color, in animals and plants; ii) photosynthesis of respective organisms; and iii) antioxidant reactivity, such as quenching free radicals and  $^1\text{O}_2$  [4]. The significance of  $\beta,\beta$ -carotene (**1**) to humans concerns its antioxidant activity and its enzymatic conversion to retinal (**2**) (provitamin A). To date, two modes of cleavage of **1** have been proposed: the *central cleavage* of **1** providing two moles of **2**, and the more recently discovered *excentric cleavage*

which yields first apocarotenals, such as **3**, which may be degraded to **2** by  $\beta$ -oxidation (Scheme 1) [5][6].

*Central cleavage* of **1** seems to be the most important metabolic pathway and enzymatic activity has been detected in various tissues since its discovery in the mid 50s. Since then many attempts failed to purify and characterize this enzyme. Nevertheless and despite the lack of solid information regarding the enzymatic reaction mechanism as well as the nature of

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the co-factor, the enzyme was termed  $\beta,\beta$ -carotene 15,15'-dioxygenase (EC 1.13.11.21).

Only very recently we have been able to establish a purification protocol of the enzyme isolated from chicken's intestinal mucosa which led to the identification of the catalytically active protein [7]. The enzyme was enriched 226-fold to a specific activity of  $2500 \text{ pmol h}^{-1}(\text{mg protein})^{-1}$ , and the gel band of the final gel filtration that best correlated with  $\beta,\beta$ -carotene 15,15'-dioxygenase activity (Fig.1) was sequenced and degenerated oligonucleotides were designed. With RT-PCR a cDNA fragment was obtained, labeled, and subsequently used to screen a chicken duodenal expression library. The isolated full-length cDNA of  $\beta,\beta$ -carotene 15,15'-dioxygenase contains a coding sequence of 1578 bp leading to a protein of 526 amino acids.

Expression of the hexa-His tailed protein in *E. coli* and BHK (baby hamster kidney) cells gave, after affinity chromatography, a catalytically active, cytosolic enzyme (60.3 kDa) which cleaves  $\beta,\beta$ -carotene (1) to retinal (2) as the only reaction product [7]. At about the same time another research group published on the expression of the  $\beta,\beta$ -carotene 15,15'-dioxygenase derived from *Drosophila melanogaster* [8].

Parallel to our efforts to purify the native protein [7][9] we investigated the substrate specificity of  $\beta,\beta$ -carotene 15,15'-dioxygenase from chicken intestinal mucosa using enriched enzyme fractions [10]. It turned out that any deviation from the rod-like  $\beta,\beta$ -carotene structure is not tolerated by the enzyme such that carotenoids containing a (Z)-configured double bond, a triple bond or a single bond at C(15)–C(15') were neither substrates nor inhibitors; the same is true for carotenoids HO-substituted in both end groups. In contrast cryptoxanthin (4) is cleaved to 2 and 3-hydroxy retinal (5) (Scheme 2); this information is important, because it is possible to investigate the enzymatic reaction mechanism for the first time [9].

The surprisingly regioselective cleavage of  $\beta,\beta$ -carotene was often attributed to the fact that the central double bond of 1 is the sterically least hindered compared to all other double bonds. To investigate this point we incubated nor-carotenoids lacking one methyl group (at C(13)), see 6, and two methyl groups (at C(13) and C(13')). Whereas the former offers three disubstituted double bonds to be cleaved, the latter even contains five identical double bonds. Interestingly

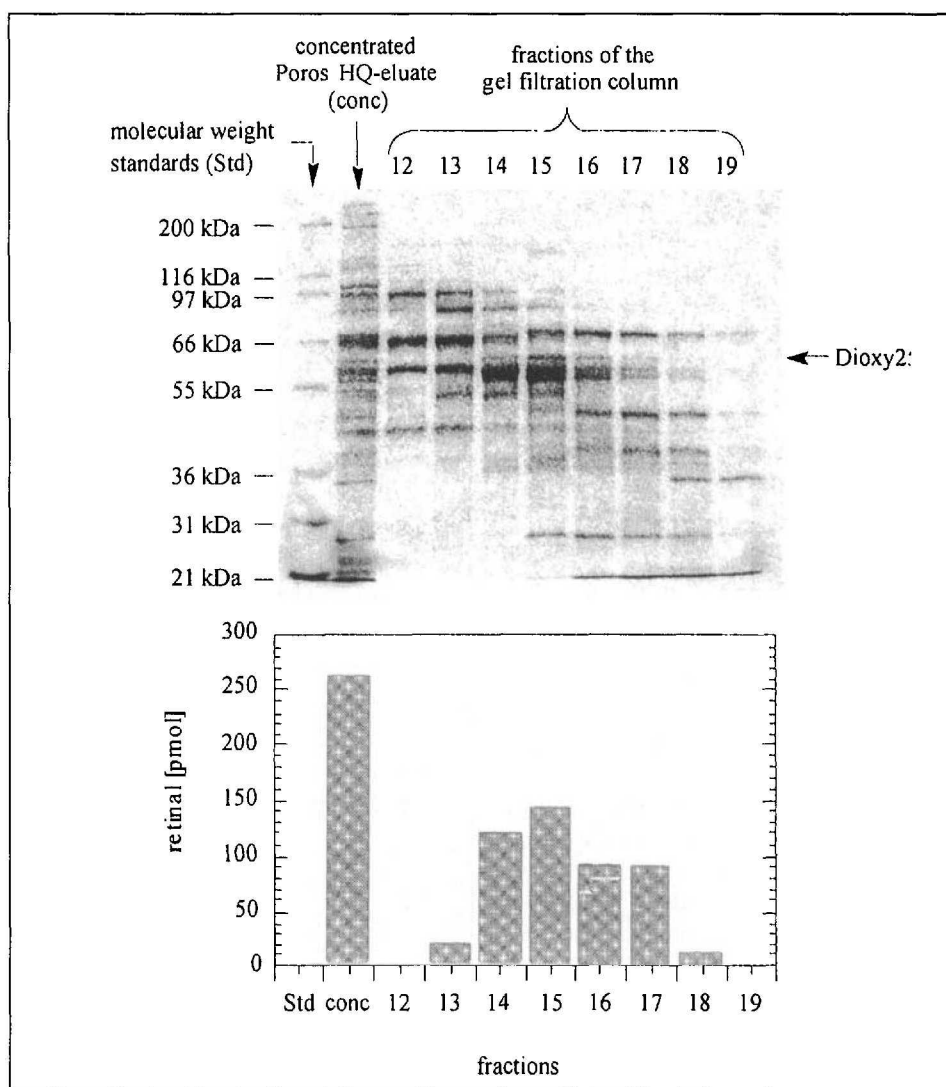
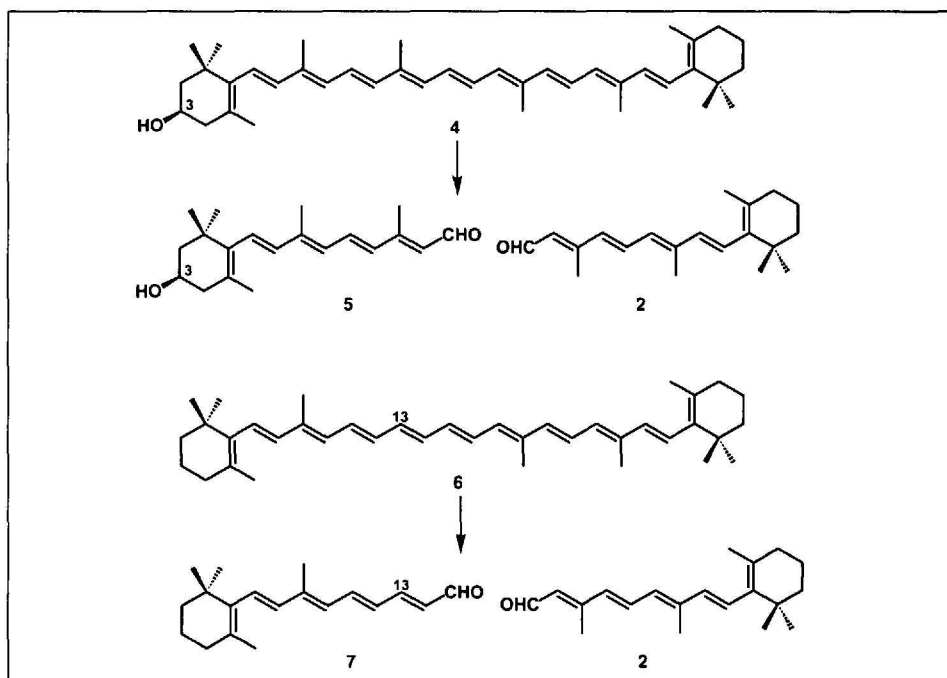


Fig. 1. Identification of the enzyme  $\beta,\beta$ -carotene 15,15'-dioxygenase isolated from chicken intestinal mucosa; the SDS gel band, assigned as Dioxy 25, is visible in GFC fractions 13–18 and correlates with the enzyme activity (below) of the same samples. This band was sequenced and the corresponding DNA fragment served as a template for the synthesis of a homologous primer.



Scheme 2. Substrate specificity of  $\beta,\beta$ -carotene.

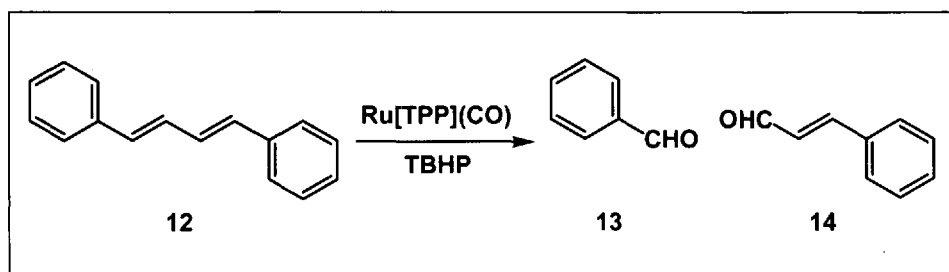
only **6** is readily cleaved to retinal (**2**) and nor-retinal **7**, and no other cleavage products are detected.

These results suggest that the enzyme has a very rigid substrate-binding pocket, *i.e.* a tube made of hydrophobic amino acids of which probably certain valines attach to the methyl groups of  $\beta,\beta$ -carotene to direct the substrate into position such that only the central double bond can be attacked by oxygen activated by the metal cofactor. Accordingly the selectivity of enzymatic double bond cleavage (one out of six of **1**) is derived purely from geometrical constraints. And since substrate binding is definitively of hydrophobic nature the system presents a formidable challenge for synthetic supramolecular chemistry.

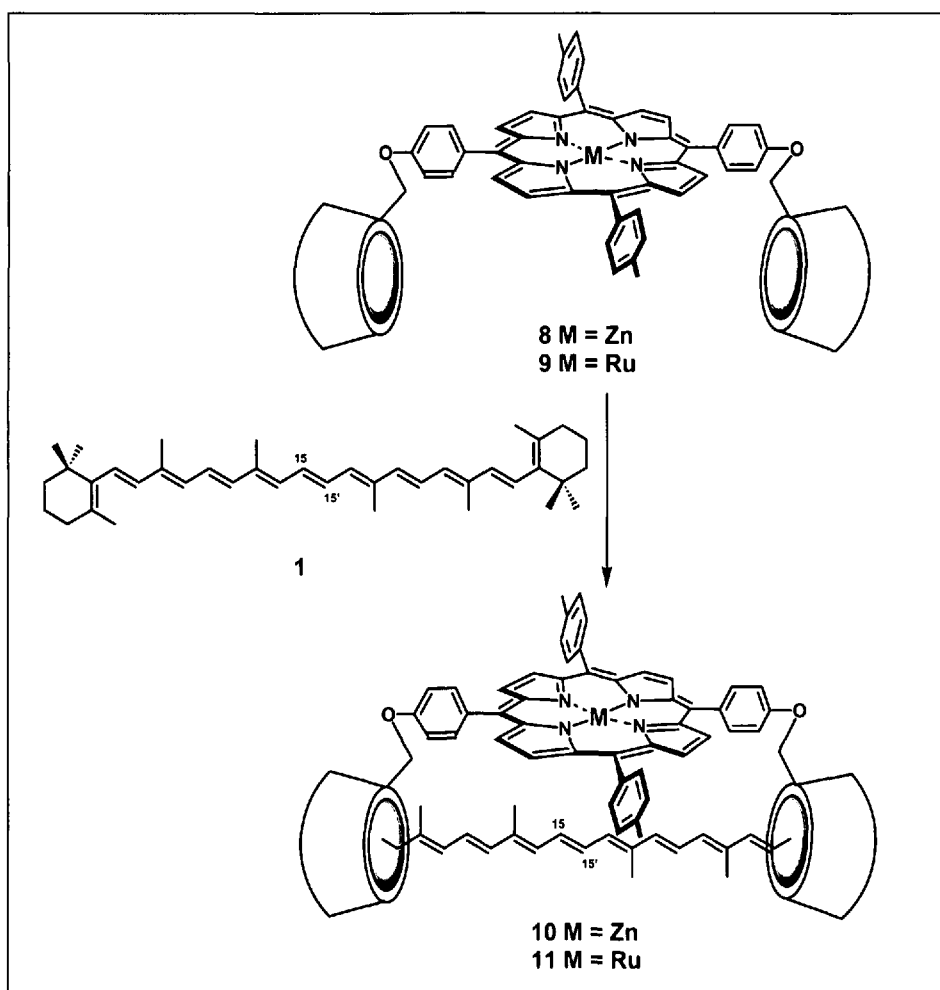
## Results and Discussion

To accomplish a supramolecular catalyst which mimics the enzymatic cleavage of carotenoids the following strategy was employed: i) the synthesis of a receptor for **1** in which the binding constant,  $K_a$  for **1** is orders of magnitude greater than that for retinal (**2**) in order to prevent product inhibition; ii) the introduction of a reactive metal center which is capable of cleaving (*E*)-configured, conjugated double bonds to aldehydes; iii) the choice of a co-oxidant which is inert towards **1** in the absence of a catalytic metal center; v) selection of optimal experimental conditions for catalytic cleavage.

The design of the supramolecular construct **8** (Scheme 3) was found, using the MOLOC program [11], to be an ideal candidate for the binding of **1**. Each of the cyclodextrins was shown to be capable of binding one of the cyclohexenoid endgroups of  $\beta,\beta$ -carotene, leaving the porphyrin to span the polyene chain, see **10**. Optimization of the complex showed that approximately half of **1** would be included in the cyclodextrin cavities and that the C(15)–C(15') double bond would be perfectly in place under a corresponding oxo metal complex.



Scheme 4. Catalytic oxidation of (*E,E*)-1,4-diphenyl-1,3-butadiene.



Scheme 3. Formation of the 1:1 inclusion complexes **10/11** from  $\beta,\beta$ -carotene **1** and the bis- $\beta$ -cyclodextrin metalloporphyrins **8/9**.

In the absence of **1** several *unproductive* conformations of **8/9** are possible due to rotation about the ether linkages; in the presence of **1**, however, an *induced fit* should be observed yielding the inclusion complexes **10/11**. As well as having the role of a spacer and potential metal ligand, the porphyrin was also chosen for its physico-chemical properties. Metalloporphyrins display a characteristic fluorescence at around 650nm 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.

The bis- $\beta$ -cyclodextrin-Zn porphyrin **8** was prepared [12][13] and the binding constant  $K_a$  for **1** was determined using fluorescence spectroscopy revealing a figure of  $K_a = 8.3 \times 10^6 \text{ M}^{-1}$ . This satisfied the first of our strategic criteria for mimicking the biological system as the binding constant for retinal **2** to  $\beta$ -cyclodextrin has been reported to be  $3.6 \times 10^3 \text{ M}^{-1}$  [14].

With regard to the choice of a metalloporphyrin capable of cleaving double bonds there was only one precedent in the literature: reaction of  $\alpha$ -methyl styrene to give acetophenone in the presence of *tert*-butyl hydroperoxide (TBHP) [15]. We have systematically studied the reactivity of *open face* as well as *face protected* ruthenium porphyrins towards substrates containing conjugated (*E*)-configured double bonds in the presence of TBHP.

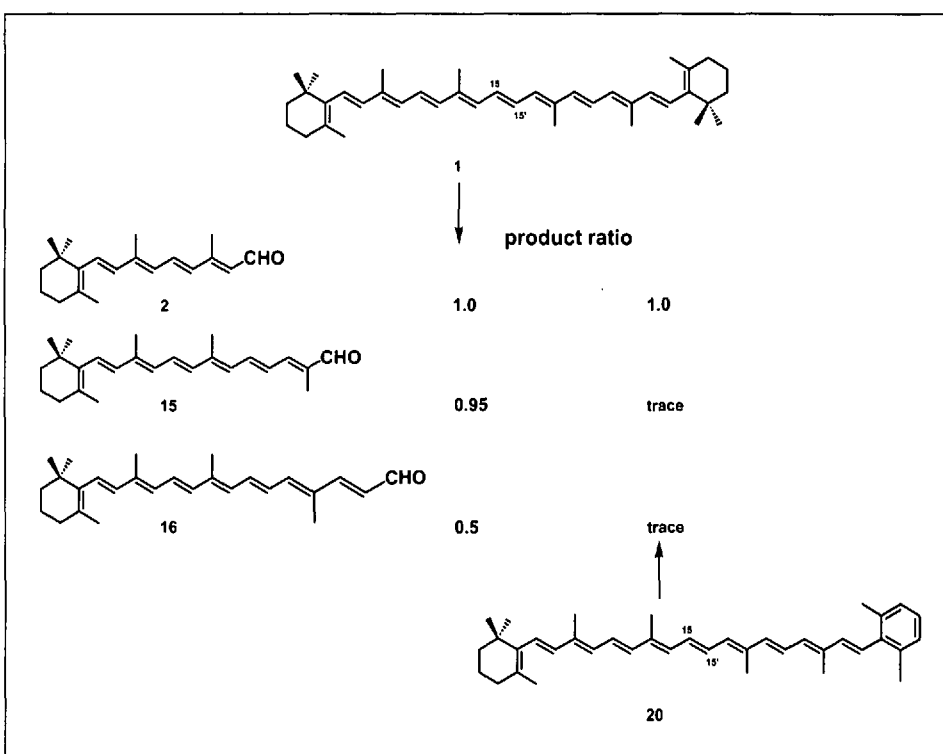
A representative example is the cleavage of (*E,E*)-1,4-diphenyl-1,3-butadiene (**12**) which is first epoxidized and subsequently gives benzaldehyde (**13**) and cinnamaldehyde (**14**) almost quantitative-

ly (Scheme 4) [16]. We have also tested the stability of **1** towards TBHP, which showed no degradation after 24 h in the absence of catalyst.

With the above prerequisites satisfied, the stage was set to employ the supramolecular system **9** (Scheme 3) to investigate the catalytic cleavage of  $\beta,\beta$ -carotene **1**. The ruthenium porphyrin **9** was obtained by the reaction of commercially available 6<sup>A</sup>-O-(*p*-tosyl)- $\beta$ -cyclodextrin with the corresponding metal porphyrin bis-phenol in DMF for three days in the presence of cesium carbonate. The reaction was followed by analytical HPLC and the desired product purified by preparative HPLC. For the catalytic cleavage of  $\beta,\beta$ -carotene **1**, a biphasic system was established in which **1** is extracted from a 9:1 mixture of hexane and chloroform into a water phase containing **9** (10%) and TBHP. The reaction products, released from the receptor, are then extracted into the organic phase, aliquots of which were subjected to HPLC conditions developed for the analysis of carotene dioxygenase enzyme studies [10]. The reaction products were identified by retention time (co-injection with authentic samples) and by their UV spectra. Quantification was by means of external calibration curves. The ratio of reaction products is given in Scheme 5. It is evident that **1** is not only cleaved at the central double bond but also at C(12')=C(11') to give 12'-apocarotenal (**15**) and at C(10')=C(9') to give 10'-apocarotenal (**16**). The combined yield of aldehydes **2**, **15** and **16** was 30%, which compares well with the efficiency of  $\beta,\beta$ -carotene 15,15'-dioxygenase which gives retinal (**2**) in 20–25% [10]. Most interestingly, the double bond in closest proximity to the central one, C(14')=C(13'), remains untouched.

At this stage we considered two possible setups leading to the cleavage of double bonds other than C(15)-C(15'): i) binding of  $\beta,\beta$ -carotene in an 'unproductive' fashion, see **17**, or ii) lateral movement of the substrate within the two cyclodextrins, see **18** (Fig. 2). To investigate the first possibility we prepared the mono-bridged Ru porphyrin **19** which prevents binding and oxidation of the substrate from the 'wrong' site by protection of one porphyrin face [17]. Using the same conditions, reaction of **19** with **1** gave the same product composition as for **9** thus excluding substrate approach as shown in **17**.

Regarding the alternative lateral movement we reasoned that the selectivity of double bond oxidation displayed by



Scheme 5. Product ratios for oxidations of  $\beta,\beta$ -carotene (**1**) and Phe- $\beta$ -carotene (**20**) with the catalyst **9**/TBHP.

the catalyst **9** should change if at least one of the endgroups of the substrate **1** is exchanged for an equally hydrophobic substituent displaying different contacts with the interior of the  $\beta$ -cyclodextrin cavity.

For this purpose we choose Phe- $\beta$ -carotene (**20**) which is also an excellent substrate of the enzyme  $\beta,\beta$ -carotene 15,15'-dioxygenase [18]. Catalytic oxidation of **20** with **9**/TBHP was indeed very regiospecific since only retinal (**2**) and the corresponding Phe analogue were detected (see Scheme 5) [13]. This suggests that stronger hydrophobic interactions between the aromatic endgroup of **20** and the  $\beta$ -cyclodextrin cavity are responsible for stabilizing the 1:1 inclusion complex with the central double bond under the reactive ruthenium center. In contrast **1** slides within the inclusion complex exposing three double bonds to the reactive ruthenium center.

The supramolecular system presented here is one of the few examples which mimics the reactivity and selectivity of an enzymatic reaction using unmodified, original substrates of an enzyme. This construct is distinct from other elegant approaches [19] which require substrates with substituents that support binding to the cyclodextrins and inevitably display product inhibition.

Furthermore the problem of epoxidizing/cleaving (*E*)-configured, conjugated double bonds has been successfully solved, and it can be envisaged that this

oxo-ruthenium porphyrin catalyzed double bond cleavage will be applicable in preparative chemistry [16].

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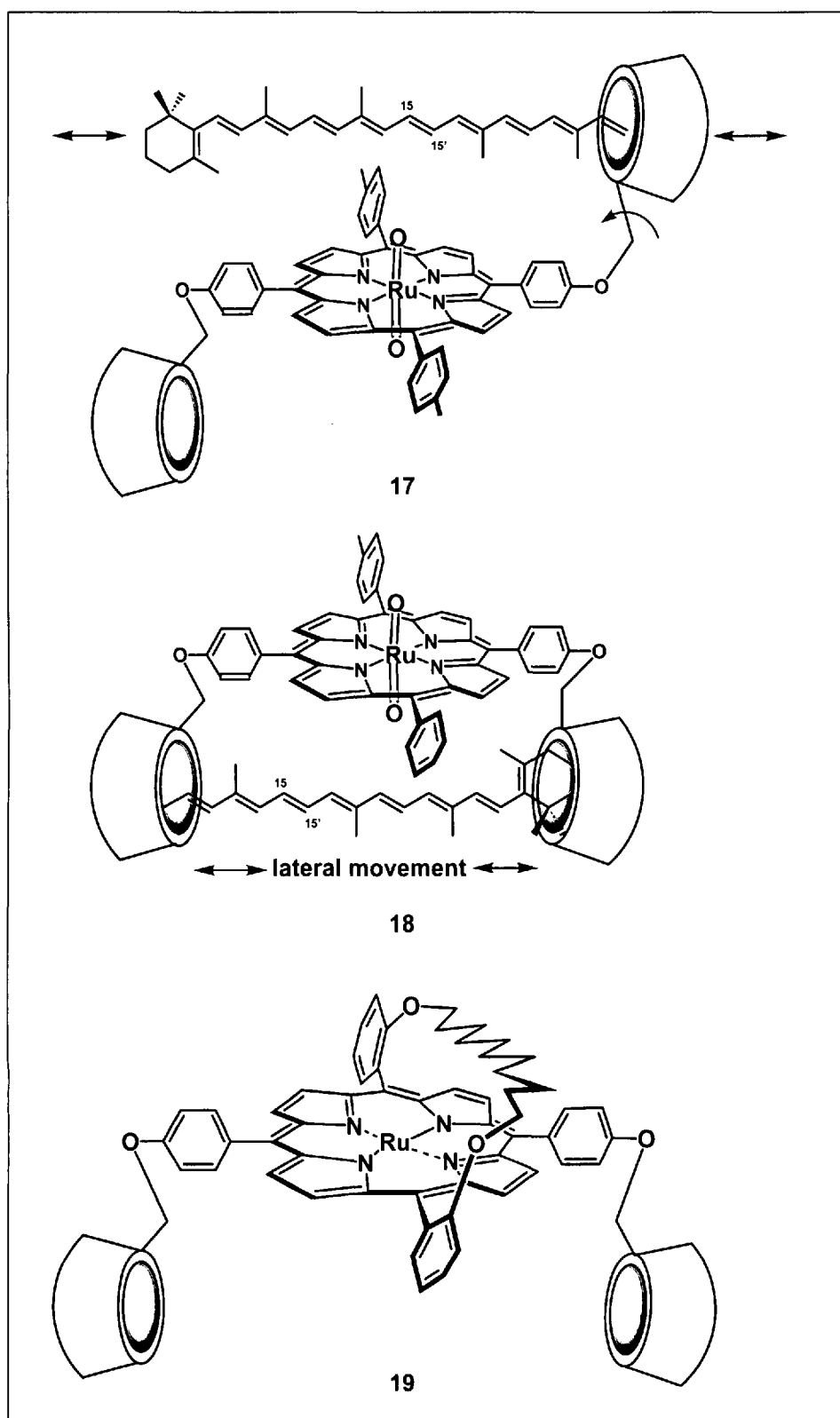


Fig. 2. Schematic representation of the conformeric inclusion complexes (17 and 18) between 1 and the dioxo ruthenium complexes derived from 9 *in situ*. Below the face-protected receptor 19.

Brugger, M. Wyss, A. Friedlein, H. Bachmann, W. Hunziker, *Biochem. Biophys. Res. Commun.* **2000**, *271*, 334–336.

[8] J. von Lintig, K. Vogt, *J. Biol. Chem.* **2000**, *275*, 11915–11920.

[9] C. Jarret, M. Leuenberger, W.-D. Woggon, in preparation.

[10] G. Wirtz, PhD Thesis, University of Basel, **1998**.

[11] P.R. Gerber, K. Müller, *J. Comput. Aided Mol. Design* **1995**, *9*, 251–257.

[12] R.R. French, J. Wirz, W.-D. Woggon, *Helv. Chim. Acta* **1998**, *81*, 1521–1527.

[13] R.R. French, P. Holzer, M.G. Leuenberger, W.-D. Woggon, *Angew. Chem. Int. Ed.* **2000**, *39*, 1267–1269.

[14] Q.-X. Guo, T. Ren, Y.-P. Fang, Y.-C. Liu, *J. Incl. Phenom. Mol. Recogn. Chem.* **1995**, *22*, 251–256.

[15] S. Takagi, T.K. Miyamoto, *Inorg. Chim. Acta* **1990**, *173*, 215–221.

[16] P. Holzer, R.R. French, W.-D. Woggon, in preparation.

[17] M. Nold, Diploma Thesis, University of Basel, **1999**.

[18] M.G. Leuenberger, PhD Thesis, University of Basel, in preparation.

[19] J. Yang, R. Breslow, *Angew. Chem.* **2000**, *112*, 2804–2806.

[1] O. Straub, 'Key to Carotenoids', Ed. H. Pfander, Birkhäuser Verlag, Basel & Boston, **1987**.

[2] 'Carotenoids', vol. 1a: Isolation and Analysis, Eds. G. Britton, S. Liaaen-Jensen, H. Pfander, Birkhäuser Verlag, Basel, Boston, Berlin **1995**.

[3] 'Carotenoids', vol. 3: Biosynthesis and Metabolism, Eds. G. Britton, S. Liaaen-

Jensen, H. Pfander, Birkhäuser Verlag, Basel, Boston, Berlin **1998**.

[4] R. Edge, T.G. Truscott, *The Spectrum* **2000**, 12–20.

[5] J.A. Olson, *J. Nutr.* **1989**, *119*, 105–108.

[6] X.-D. Wang, G.-W. Tang, J.G. Fox, N.I. Krinsky, R.M. Russel, *Arch. Biochem. Biophys.* **1991**, *285*, 8–16.

[7] A. Wyss, G. Wirtz, W.-D. Woggon, R.