*Pure Appl. Chem.*, Vol. 74, No. 8, pp. 1397–1408, 2002.


# Oxidative cleavage of carotenoids catalyzed by enzyme models and beta-carotene 15,15´-monooxygenase*

Wolf-D. Woggon

*Institute of Organic Chemistry, University of Basel, St. Johanns-Ring 19, CH-4056 Basel, Switzerland*

*Abstract*: The enzyme that catalyzes the central cleavage of β-carotene is an iron monooxygenase. The protein was isolated from chicken intestinal mucosa and overexpressed in two different cell lines. Inductively coupled plasma (ICP) emission analysis revealed that the hydrophobic 60.3-kDa enzyme contains one iron/mole protein. The substrate specificity was investigated, and the reaction mechanism elucidated incubating α-carotene in the presence of highly enriched $^{17}O_2$ and $H_2{}^{18}O$. A supramolecular enzyme model was synthesized, binding carotenoids $K_a > 10^6\ mol^{-1}$, which mimics the regiospecific enzymatic cleavage of carotenoids.

## INTRODUCTION

β-Carotene **1** is the parent structure and most important compound of the "orange pigments of life" comprising >650 carotenoids, which are abundant in photosynthetic bacteria and 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 significance of β-carotene **1** to humans concerns its antioxidant activity [4], and its enzymatic conversion to retinal **2** (pro-vitamin A). To date, two modes of cleavage of **1** have been proposed: the *central cleavage* of **1** providing two moles of **2** [5], and the more recently discovered *excentric cleavage,* which yields first apocarotenals, such as 8-apocarotenal **3**, which may be degraded to **2** by β-oxidation (Fig. 1) [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-1950s. Since then, many attempts failed to purify and charcterize this enzyme. Nevertheless, and despite the lack of solid information regarding the enzymatic reaction mechanism, as well as the nature of the cofactor, the enzyme was termed β-carotene 15,15′-dioxygenase (EC 1.13.11.21).

Only very recently, have we been able to establish a purification protocol of the enzyme isolated from chicken 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 $pmol/h^{-1}/(mg\ protein)^{-1}$. The gel band of the final gel filtration that best correlated with enzyme activity (Fig. 2) was sequenced, and degenerate oligonucleotides were designed. With reverse transcriptase-polymerase chain reaction (RT-PCR), a cDNA fragment was obtained, labeled, and subsequently used to screen a chicken duodenal expression library. The isolated full-length cDNA of the β-carotene cleaving enzyme contains a coding sequence of 1578 bp leading to a protein of 526 amino acids. Expression of the hexa-histidine tagged

*Lecture presented at the 13th International Symposium on Carotenoids, Honolulu, Hawaii, USA, 6–11 January 2002. Other presentations are presented in this issue, pp. 1369–1477.

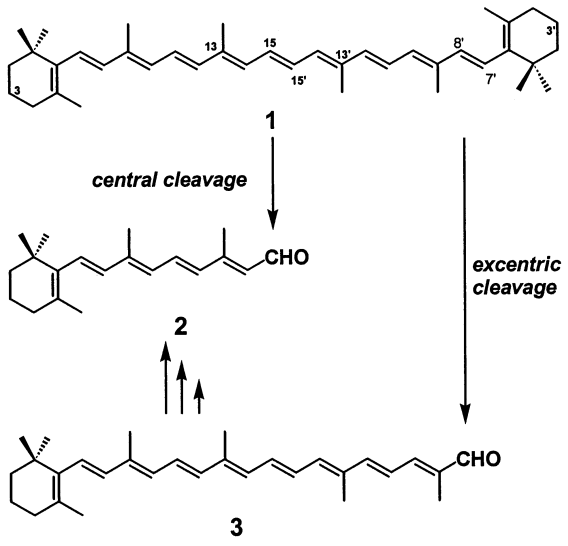


**Fig. 1** Central and excentric cleavage of β-carotene.

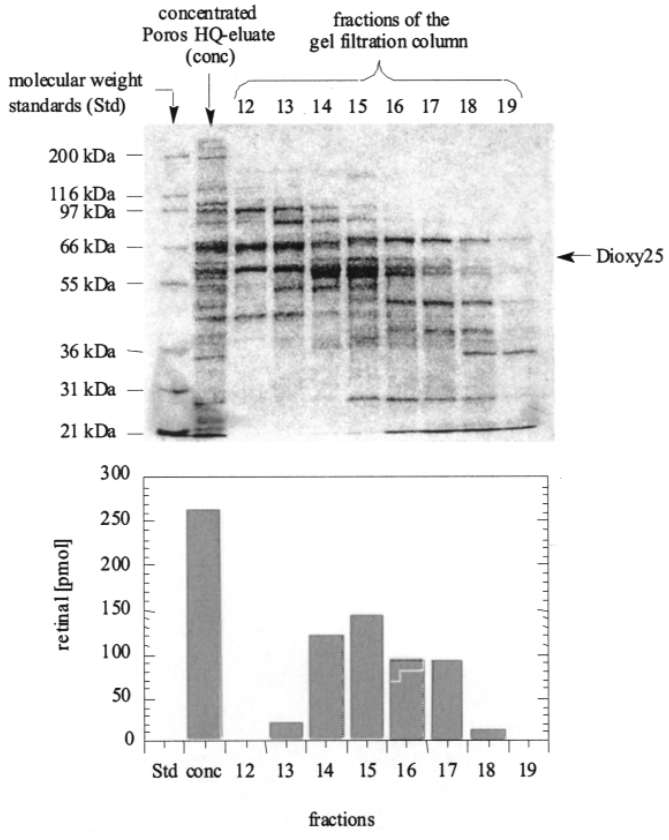


**Fig. 2** Identification of the enzyme (see Dioxy25) that catalyzes the central cleavage of **1**. Upper panel: sodium dodecyl sulfate (SDS) gels of fractions of gel filtration chromatography. Lower panel: activity profile of the corresponding fractions.

protein in *E. coli* and baby hamster kidney (BHK) cells gave, after affinity chromatography, a catalytically active, cytosolic enzyme (60.3 kDa), which cleaves β-carotene **1** to retinal **2** as the only reaction product [8]. At about the same time, another research group published on the expression of the enzyme from *Drosophila melanogaster* [9], and later a mouse kidney cDNA was identified encoding for a 64-kDa protein that displayed enzymatic activity [10].

In the present account, we wish to report on the reaction mechanism of the enzyme catalyzing the central cleavage, its substrate specificity, and the design, synthesis, and application of a supramolecular enzyme model.

## SUBSTRATE SPECIFICITY OF THE ENZYME CATALYZING THE CENTRAL CLEAVAGE OF β-CAROTENE

The suprisingly regioselective cleavage of β-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 synthetic nor-carotenoids lacking one methyl group [at C(13)], see **4**, and two methyl groups [at C(13) and C(13′)], see **5** (Fig. 3). Whereas the former offers three disubstituted double bonds to be cleaved, the latter contains five identical double bonds. Interestingly, only **1** and **4** are readily cleaved to retinal **2** and nor-retinal **6**, respectively, and no other cleavage products are detected [11].

Further investigation of the substrate specificity of the enzyme revealed [11] that any deviation from the rod-like structure of β-carotene is not tolerated by the enzyme such that carotenoids containing a (*Z*)-configured double bond, **7**, a triple bond, **8**, or a single bond at C(15)-C(15′), **9**, were neither substrates nor inhibitors (Fig. 4). The same is true for carotenoids HO-substituted at both endgroups, see **10** (Fig. 5). In contrast, cryptoxanthin **11** is cleaved to **2** and 3-hydroxy retinal **12**. Interestingly, α-carotene **13** is also accepted and cleaved in good yield to **2** and α-retinal **14**.

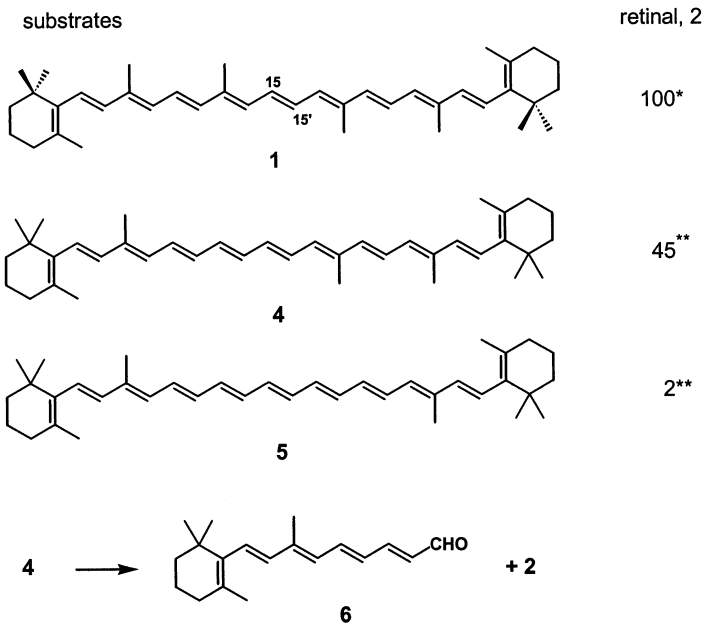


**Fig. 3** Enzymatic formation of aldehydes from **1** and substrate analogs **4** and **5**; * absolute yield 30 %; ** relative to yield from **1**, set at 100 % for comparison.

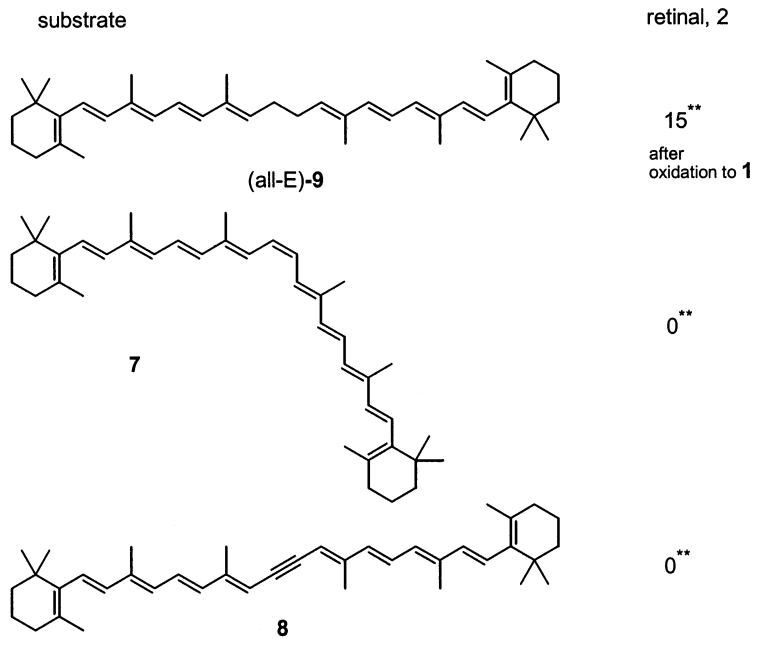


**Fig. 4** Enzymatic formation of retinal **2** from carotenoids (all-*E*)-**9**, **7**, and **8**.

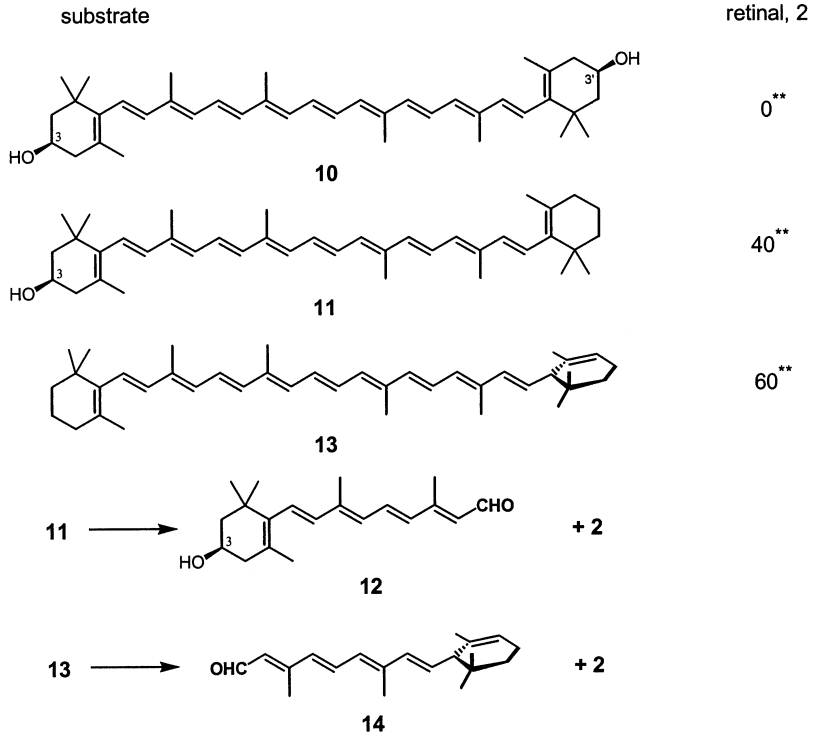


**Fig. 5** Enzymatic formation of aldehydes from natural carotenoids **10–13** with one or both end-groups modified.

To further investigate the specificity of the enzyme toward central cleavage, we synthesized the non-natural carotenoids Ph-β-carotene **15**, Ph-*retro*-carotene **16**, and Py-*retro*-carotene **17** (Fig. 6). **15** was believed to be tolerated by the enzyme since only one end-group of **1** is replaced by an equally hydrophobic subunit with methyl groups placed at positions corresponding to those of the cyclohexenoid moiety of **1**. In contrast, **16** and **17** were prepared to explore the acceptance of the enzyme of structural changes in the vicinity of the central double bond of substrates. In fact, we hoped **16** and **17** would be inhibitors, in particular that **17** could bind to the metal cofactor of the active site replacing one of the coordinating aminoacids. **15** was indeed readily cleaved by the enzyme to give Ph retinal **18** and **2** with yields comparable to α-carotene **13**. Surprisingly, both **16** and **17** were found to be substrates yielding the aldehydes **19** and **20**, and **21** and **20**, respectively. Obviously, in both cases, the double bond conjugated to the aromatic ring is attacked. This site of oxidation corresponds to the C(14′)-C(13′) double bond of **1**. In view of the observed regiospecificity of cleavage of the *nor*-and *bis-nor* carotenoids **4** and **5**, this result was quite unexpected. The presence of aromatic rings in **16** and **17** may lead to a π–π

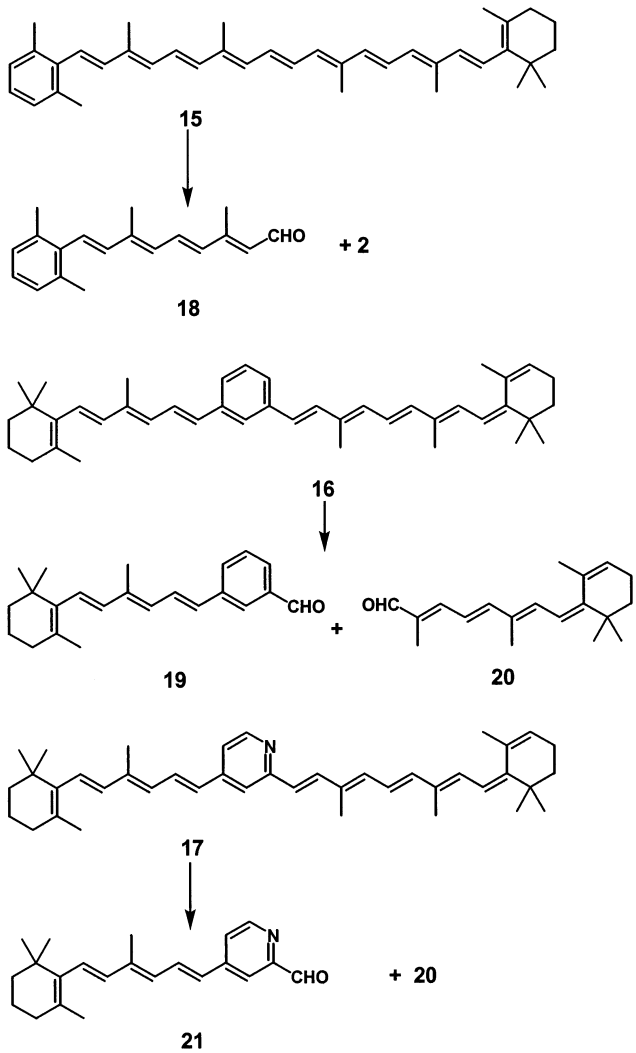


**Fig. 6** Enzymatic cleavage of non-natural carotenoids **15–17**.

interaction with aromatic aminoacids coordinating to the metal center, which forces the metal oxo intermediate to shift toward attacking the adjacent double bond.

These results suggest that the enzyme has a very rigid substrate binding pocket, e.g., a tube made of hydrophobic amino acids which certain valines attach to the methyl groups of β-carotene to direct the substrate into position, such that only the central double bond can be attacked, except for those substrates, like **16** and **17**, carrying aromatic rings built in the central part of the substrate and thus preventing the oxidation of C(15)-C(15′) double bond. Accordingly, the selectivity of enzymatic double bond cleavage of **1**, and most of substrate analogs, is derived purely from geometrical constraints [12].

## REACTION MECHANISM OF THE ENZYME CATALYZING THE CENTRAL CLEAVAGE OF CAROTENOIDS

Investigation of the substrate specificity led to the identification of three nonsymmetric carotenoids, cryptoxanthin **11**, α-carotene **13**, and Ph-β-carotene **15**, suitable for determining the reaction mechanism of the enzyme-catalyzed central cleavage. The significance of non-symmetrical substrates yielding different aldehydes that can provide exact information of the incorporation of oxygen from water and/or air into cleavage products was mainly overlooked in the past. The general consensus, that the enzyme is a dioxygenase, was based on experiments with β-carotene **1** [13], which are unsuitable to distinguish a monooxygenase- from a dioxygenase-mechanism. α-Carotene **13** was chosen as the best candidate because it was available isomerically pure, and it was expected that the aldehydes **2** and **14** would behave similar in subsequent reactions required for mass spectrometry (MS) analysis of the distribution of the labeled oxygen in both cleavage products.

In this context it is important to note that aldehydes **2** and **14** are not suitable for isotopic analysis of an oxygen label in the carbonyl group [14], because this label easily exchanges with the medium at pH of incubation (pH 7.8). Thus, we decided on a combined enzyme assay by addition of horse liver alcohol dehydrogenase (HLADH) in order to reduce **2** and **14** in situ to the corresponding alcohols, retinol **22** and α-retinol **23**.

Control experiments revealed that the rate of reduction of **2** and **14** are the same and oxygen exchange of **2** is about 5 % under these conditions. **22** and **23** are also unsuitable for Gas chromatography-mass spectrometry (GC-MS) analysis because both eliminate water. Accordingly, after quenching of the incubation and high-performance liquid chromatography (HPLC) purification of the alcohols **22** and **23**, derivatization to the silyl ethers **24** and **25** was required (Fig. 7). For the decisive incubation experiment of **13** with the enzyme, highly enriched oxygen sources were used, such as 85 % $^{17}O_2$ and 95 % $H_2{}^{18}O$ [15]. GC-MS analysis of the silylethers **24** and **25**, focusing on the molecular ion area revealed, within experimental error, equal enrichment of the $^{17}O$- and $^{18}O$-label in both derivatives of metabolites **2** and **14** (Fig. 8). This result proves the incorporation of one $^{17}O$ atom of molecular oxygen and the concomitant incorporation of $^{18}O$ from labeled water. Accordingly, and in contrast to earlier belief, the reaction mechanism of enzymatic, central β-carotene cleavage is not in agreement with a dioxygenase-catalyzed procedure.

Note that a dioxygenase mechanism would require the [2 + 2] addition of $^{17}O_2$ to the central double bond of **13**, followed by fragmentation of the intermediate dioxetane-yielding aldehydes labeled only with $^{17}O$ to the same extent; the incorporation of $^{18}O$ from water is not expected.

Experimental evidence provided here accounts for a monooxygenase-type mechanism (Fig. 9), in which the first step is an epoxidation of the central double bond of **13** followed by unselective ring opening by water and final diol cleavage to yield the aldehydes **2** and **14**.

The nature of the metal complex, involved in $O_2$ cleavage, epoxidation, epoxide hydrolysis, and oxidative C–C bond cleavage, has to be elucidated. At present, it is certain that this carotene metabolism is not a P450-catalyzed reaction because the respective heme-thiolate chromophore is absent in the purified protein as well as in the overexpressed enzyme. The availability of mg quantities of pure overexpressed protein, however, made it possible to determine for the first time the metal involved in catal-

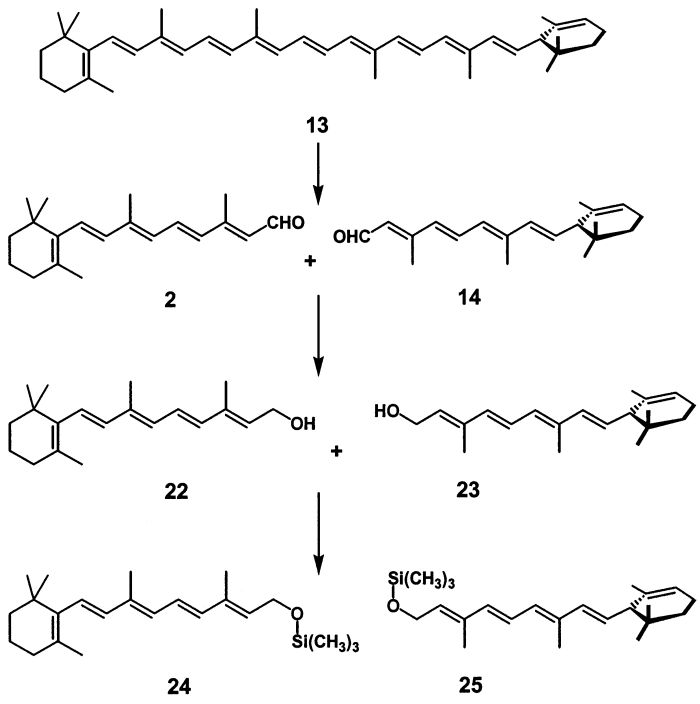


**Fig. 7** Incubation of α-carotene **13** followed by derivatization of cleavage products **2** and **14**.

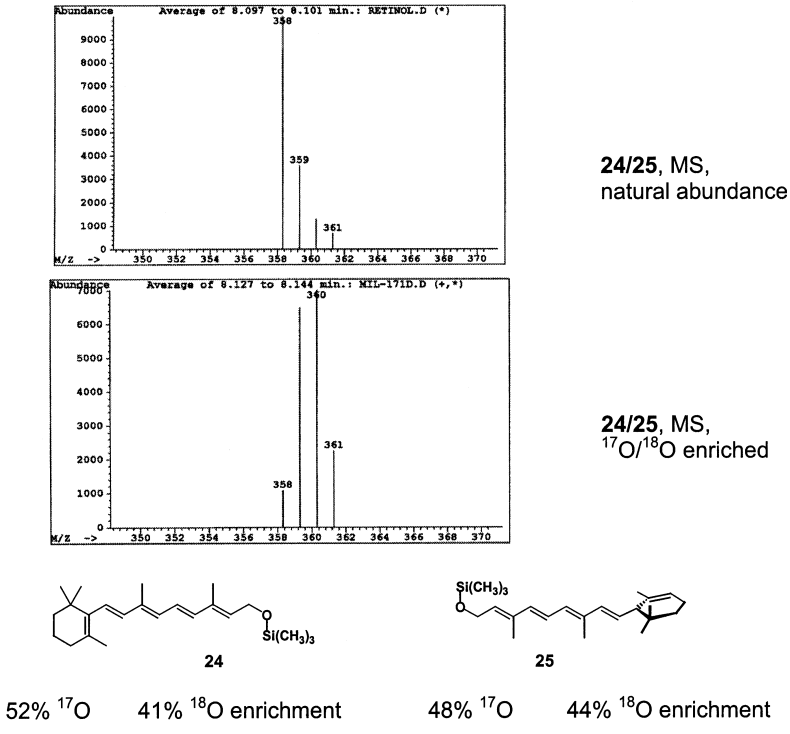


**Fig. 8** MS-spectra of derivatives of cleavage products **2** and **14** displaying $^{17}O$ and $^{18}O$ incorporation to the same extent.

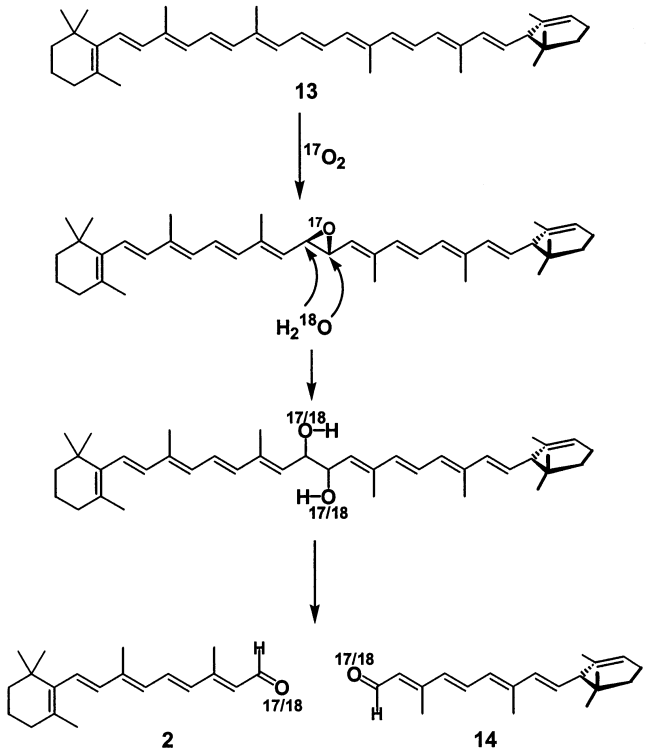


**Fig. 9** Enzymatic cleavage of α-carotene **13** in the presence of $^{17}O_2$ and $H_2{}^{18}O$.

ysis. Inductively coupled plasma (ICP) emission analysis revealed one iron per mol of protein [16]. Accordingly, the enzyme that catalyzes the central cleavage of carotenoids is an iron monooxygenase, and the absence of any chromophore suggests a coordination of iron with amino acids such as histidine, aspartate, and several water molecules. It is important to note that earlier conclusions [10] regarding the participation of iron in catalysis ("iron dioxygenase") are not convincing since they are based on incubations in the presence of chelators, such as *ortho* phenantrolin, which rendered the enzyme inactive but do not prove the presence of iron.

## DESIGN, SYNTHESIS, AND APPLICATION OF ENZYME MODELS

The fact that β-carotene 15,15′-monooxygenase controls the regiospecific cleavage of one C=C bond out of a possible six within the substrate is an intriguing and challenging one. In order to mimic such a regioselective system, the following strategy was employed: (i) 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) introduction of a reactive metal complex which is capable of cleaving *(E)*-configured, conjugated double bonds to aldehydes; (iii) use of a co-oxidant which is inert toward **1** in the absence of the metal complex.

The supramolecular construct **26** (Fig. 10) consisting of two β-cyclodextrin moieties linked by a porphyrin spacer, was designed by means of the MOLOC program 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 β,β-carotene, leaving the porphyrin to span the polyene chain. In this complex, approximately half of **1** would be included in the cyclodextrin cavities and the C(15)-C(15′) double bond would be directly under any metal that could be subsequently inserted into the porphyrin. In the absence of **1**, sev-

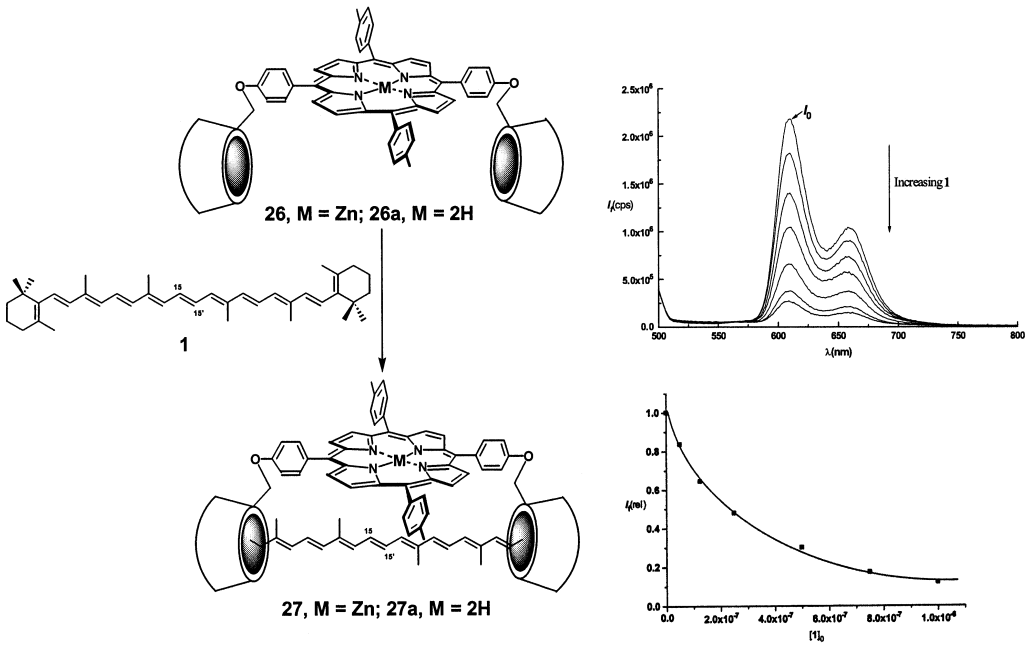


**Fig. 10** Quenching of the fluorescence of the porphyrin receptor **26** on binding the substrate **1**.

eral unproductive conformations of **26** 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 **27**. As well as having the role of a spacer and potential metal ligand, the porphyrin in **27** is also useful for the determination of the binding constant $K_a$ of **1** to **26/26a**. Porphyrins display a characteristic fluorescence at around 600–650 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. It could be reasonably postulated that a cyclodextrin dimer such as **26** should be capable of providing a $K_a$ for **1** in the region of $10^5$–$10^7$ $M^{-1}$.

The synthesis of **26** was pursued following established procedures in our laboratory [17]. After purification of the final product **26** by reverse-phase HPLC, the $K_a$ for **1** was determined by fluorescence quenching (Fig. 10).

The graphs in Fig. 10 revealed a binding constant $K_a$(**1** ◊ **26**) = $8.3 \times 10^6$ $M^{-1}$ [17]. Due to its saddle-shaped conformation, the metal-free porphyrin **26a** displays a smaller binding constant $K_a$(**1** ◊ **26a**) = $2.4 \times 10^6$ $M^{-1}$. This satisfied the first of our strategic criteria for mimicking the biological system, as the binding constant for retinal **2** to β-cyclodextrin is smaller by three orders of magnitude. Accordingly, product inhibition should not be observed if cleavage of the central double bond of **1** could be accomplished.

With regard to the choice of a metalloporphyrin capable of cleaving double bonds, we chose a ruthenium porphyrin because preliminary experiments with (*E*,*E*)-1,4-diphenyl-1,3-butadiene looked promising [18]. We have also tested the stability of **1** toward various cooxidants, finally *tert*-butyl hydroperoxide (TBHP) was chosen, which showed no degradation of **1** within 24 h in the absence of catalyst. With the above prerequisites satisfied, the stage was set to employ the supramolecular system **28** to investigate the catalytic cleavage of β-carotene **1** (Fig. 11). A biphasic system was established in which **1** is extracted from a 9:1 mixture of hexane and chloroform into a water phase containing **28** (10 mol %) and TBHP. The reaction products, released from the receptor, are then extracted into the

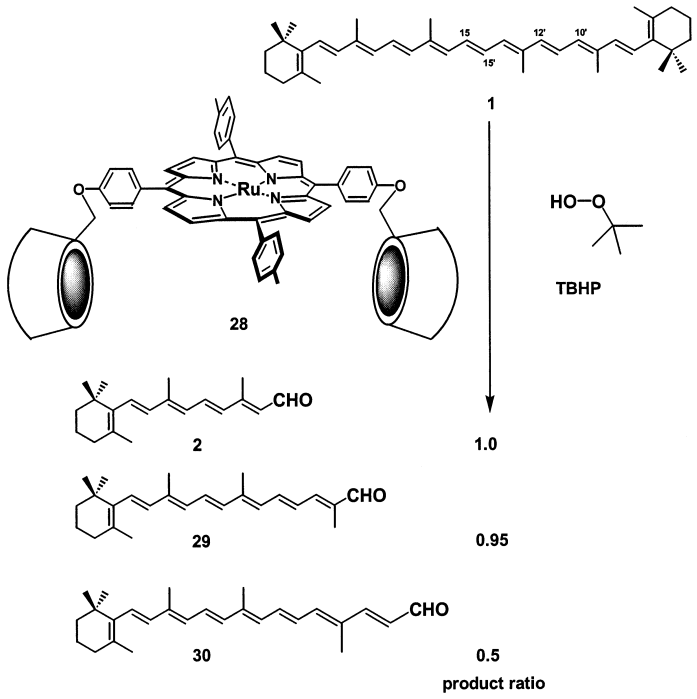


**Fig. 11** Cleavage of β-carotene **1** by **28**/TBHP.

organic phase, aliquots of which were subjected to HPLC conditions developed for the analysis of enzymatic reactions [7,8].

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 Fig. 11. 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 **29** and at C(10′) = C(9′) to give 10′-apocarotenal **30** [18]. The combined yield of aldehydes **2**, **29**, and **30** was 30 %, which compares well with the efficiency of β-carotene 15,15′-monooxygenase which gives retinal **2** in 20–25 % yield [7].

At this stage, we considered two possible setups leading to the cleavage of double bonds other than C(15)-C(15′): (i) binding of β,β-carotene in an "unproductive" fashion, see **31**, or (ii) lateral movement of the substrate within the two cyclodextrins, see **32** (Fig. 12).

The first possibility was investigated with the synthetic mono-bridged Ru porphyrin **33** (Fig. 12), for which the approach of **1** is impossible at the porphyrin face that is protected by the alkane strap [19]. Reaction of β-carotene **1** with **33**/TBHP under the same conditions as described for **28** gave the same ratio of aldehydes **2**, **29**, and **30** as shown in Fig. 11. Thus, it was concluded that the production of **29** and **30** is not due to substrate binding as shown in **31**, but rather due to lateral movement of **1** within the cavities of the cyclodextrins, see **32**. Regarding the latter aspect, we reasoned that the selectivity of double bond oxidation displayed by catalyst **28** should change if at least one of the end-groups of the substrate **1** is exchanged for an equally hydrophobic substituent displaying different contacts with the interior of the β-cylodextrin cavity. For this purpose, we chose cryptoxanthin derivatives **34** and **35**. Both compounds were oxidized with the same regioselectivity in favor of retinal formation (Fig. 13) and are more selectively cleaved than β-carotene **1** [20]. The best substrate, however, was Ph-β-carotene **15**, a substrate analog of β-carotene 15,15′-monooxygenase (see Fig. 6). Catalytic oxidation of **15** with the enzyme model **28**/TBHP was indeed very regiospecific since only retinal **2** and the corresponding

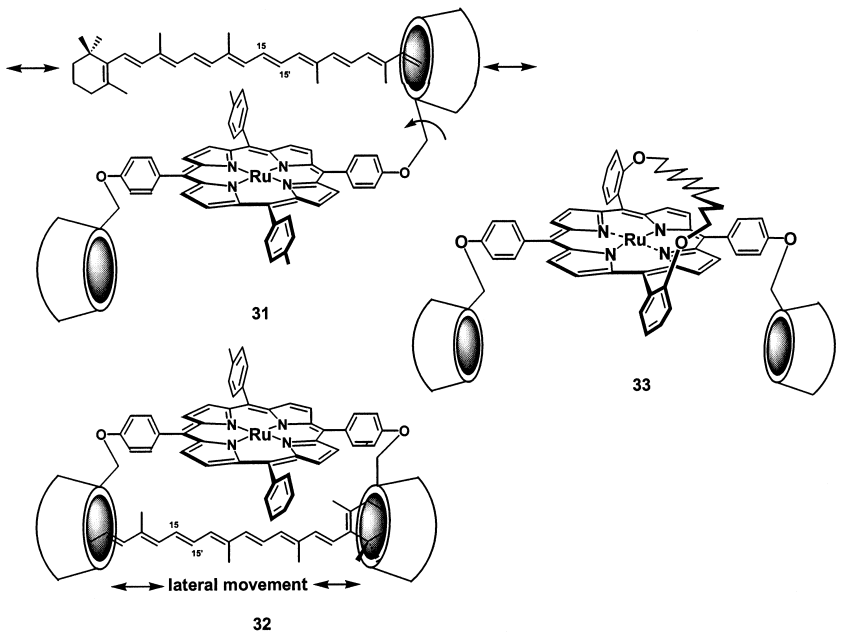


**Fig. 12** Binding modes of β,β-carotene **1** to the receptor **28**.

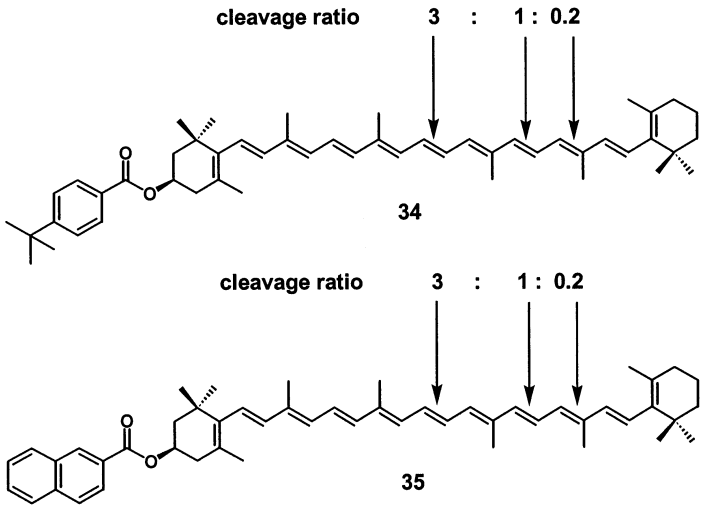


**Fig. 13** Oxidative cleavage of cryptoxanthin derivatives **34** and **35** using the catalytic system **28**/TBHP.

Phe analog **18** were detected. This suggests that stronger hydrophobic interactions between the aromatic end-group of **15** and the β-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 rather than one to the reactive Ru = O. Determination of the binding constant of **15** to the receptor **26a** supports this interpretation, i.e., $K_a$(**15** ◊ **26a**) = $5.0 \times 10^6$ $M^{-1}$ is about two times larger than $K_a$(**1** ◊ **26a**) = $2.4 \times 10^6$ $M^{-1}$.

The supramolecular system, presented here, is one of the few examples that mimic the reactivity and selectivity of an enzymatic reaction using unmodified, original substrates of an enzyme. Furthermore, the problem to epoxidize/cleave (*E*)-configured, conjugated double bonds has been successfully solved, and it can be envisaged that this oxo ruthenium porphyrin catalyzed double bond clevage will be applicable in preparative chemistry [20].

## ACKNOWLEDGMENTS

This work was generously supported by F. Hoffmann–La Roche Ltd. (Vitamins & Fine Chemicals Division). I am indebted to members of the group of Drs. W. Hunziker and R. Goralczyk for many discussions and Drs. R. K. Müller and F. Bräm for support at the outset of this project. I am also very grateful to my coworkers, mentioned in the references, in particular to Gabi Wirtz, who started this adventure during her thesis.

## REFERENCES

1. O. Straub. In *Key to Carotenoids*, H. Pfander (Ed.), Birkhäuser, Basel (1987).
2. G. Britton, S. Liaaen-Jensen, H. Pfander (Eds.). *Carotenoids*, *Vol. 1a: Isolation and Analysis*, Birkhäuser, Basel (1995).
3. G. Britton, S. Liaaen-Jensen, H. Pfander (Eds.). *Carotenoids*, *Vol. 3: Biosynthesis and Metabolism*, Birkhäuser, Basel (1998).
4. R. Edge, D. J. McGarvey, T. G. Truscott. *J. Photochem. Photobiol., B* **41**, 189–200 (1997).
5. J. A. Olson and O. Hayaishi. *Proc. Natl. Acad. Sci. USA* **54**, 1365–1370 (1965).
6. X.-D. Wang, G.-W. Tang, J. G. Fox, N. I. Krinsky, R. M. Russell. *Arch. Biochem. Biophys.* **285**, 8–16 (1991).
7. G. Wirtz. Ph.D. thesis, University of Basel, 1998.
8. A. Wyss, G. Wirtz, W.-D. Woggon, R. Brugger, M. Wyss, A. Friedlein, H. Bachmann, W. Hunziker. *Biochem. Biophys. Res. Commun.* **271**, 334–336 (2000); A. Wyss, G. Wirtz, W.-D. Woggon, R. Brugger, M. Wyss, A. Friedlein, H. Bachmann, W. Hunziker. *Biochem. J.* **354**, 521–529 (2001).
9. J. von Lintig and K. Vogt. *J. Biol. Chem.* **275**, 11915–11920 (2000).
10. J. Paik, A. During, E. H. Harrison, C. L. Mendelsohn, K. Lai, W. S. Blaner. *J. Biol. Chem.* **276**, 32160–32168 (2001).
11. G. M. Wirtz, C. Bornemann, A. Giger, R. K. Müller, H. Schneider, G. Schlotterbeck, G. Schiefer, W.-D. Woggon. *Helv. Chim. Acta* **84**, 2301–2315 (2001).
12. M. Leuenberger. Ph.D. thesis, University of Basel, 2002.
13. B. B. Vartapetyan, A. A. Dmitrovskii, D. G. Alkhazov, I. Kh. Lemberg, A. B. Girshin, G. M. Gusinskii, N. A. Starikova, N. N. Erofeeva, I. P. Bogdanova. *Biokhimiya (USSR) Engl. Transl.* **31**, 759–763 (1966).
14. J. Retéy, A. Umani-Ronchi, J. Seibel, D. Arigini. *Experientia* **22**, 502–503 (1966).
15. M. Leuenberger, C. Engeloch-Jarret, W.-D. Woggon. *Angew. Chem., Int. Ed.* **40**, 2614–2617 (2001).
16. W.-D. Woggon. In preparation.
17. R. R. French, J. Wirz, W.-D. Woggon. *Helv. Chim. Acta* **81**, 1521–1527 (1998).
18. R. R. French, P. Holzer, M. G. Leuenberger, W.-D. Woggon. *Angew. Chem., Int. Ed.* **39**, 1267–1269 (2000).
19. R. R. French, P. Holzer, M. Leuenberger, M. C. Nold, W.-D. Woggon. *J. Inorg. Biochem.* **88**, 295–304 (2002).
20. P. Holzer. Ph.D. thesis, University of Basel, 2002.