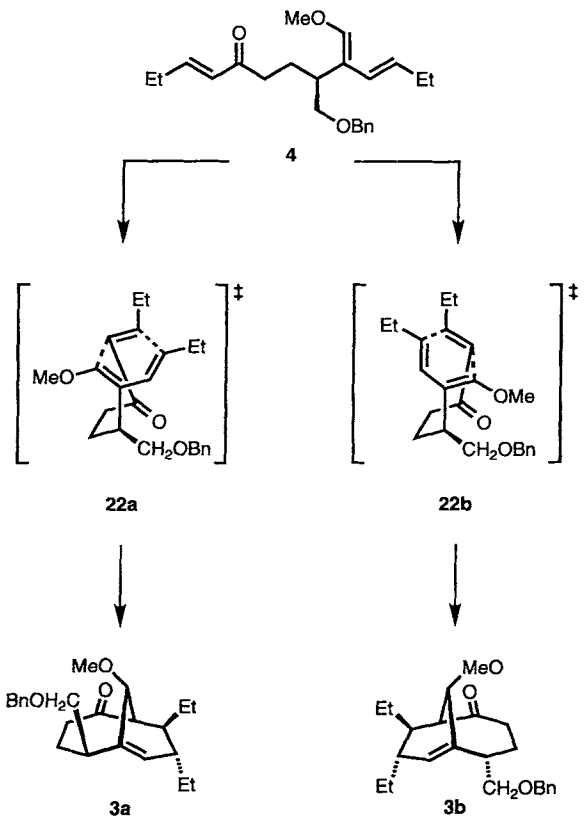
Although **4** was rather stable under thermal conditions (toluene, 110 °C, several hours), it smoothly underwent intramolecular Diels–Alder reaction in the presence of Me_2AlCl in CH_2Cl_2 at −10 °C. The resulting mixture of cycloadducts **3a, b** was chromatographically separated (silica gel, 6% acetone in hexane). The components **3a** and **3b** (R_f 0.25, 66% yield and R_f 0.28, 20% yield, stereochemistry unassigned) are presumably formed via the favored transition states **22a** and **22b**, respectively (Scheme 3). Variation of the substituents on the backbone of **4** and of the reaction conditions and catalyst is expected to improve the stereochemical outcome of the cycloaddition reaction, as desired for a total synthesis of the target molecules.



Scheme 3. Favored Diels–Alder transition states **22a** and **22b** leading to **3a** and **3b**, respectively (a racemic mixture was used; only one enantiomer is shown).

The chemistry described defines a possible strategy for the total synthesis of **1** and **2**, and opens the way for construction of simpler biological mimics of this class of compound for biological investigations.

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[10] All new compounds exhibited satisfactory spectral and/or exact mass data. Yields refer to chromatographically and spectroscopically homogeneous materials.

Metal-Free Bacterial Haloperoxidases as Unusual Hydrolases: Activation of H_2O_2 by the Formation of Peracetic Acid**

Martin Picard, Jürgen Gross, Ellen Lübbert, Sabine Tölzer, Susanne Krauss, Karl-Heinz van Pée, and Albrecht Berkessel*

Dedicated to Professor Waldemar Adam on the occasion of his 60th birthday

Haloperoxidases catalyze the formation of hypohalites from hydrogen peroxide and chloride, bromide, or iodide [Eq. (1)].

$$H_2O_2 + Hal^- \xrightleftharpoons[\text{Hal: I, Br, Cl}]{\text{haloperoxidase}} OHal^- + H_2O \qquad (1)$$

The electrophiles thus formed are able to halogenate suitable organic substrates[1–3] and can thus play an important role in the biosynthesis of halogenated natural products. Haloperoxidases can also catalyze the transfer of oxygen from hydrogen peroxide to organic substrates such as olefins or thioethers.[4] Therefore, this class of enzymes has been intensively studied with respect to preparative transformations, for example, the asymmetric epoxidation of olefins and sulfoxidation of thioethers.[4] Furthermore, hydrogen peroxide is a readily available, mild, and environmentally friendly terminal oxidant.

Most haloperoxidases require a cofactor to catalyze the redox reaction shown in Equation 1. The type of cofactor is used to classify these enzymes into heme-containing, vanadium-containing, and metal-free haloperoxidases. So far only the heme-containing haloperoxidases have proven suitable for preparative applications, for example the chloroperoxidase from the fungus *Caldariomyces fumago*.[4] Although good yields and enantioselectivities could be achieved with some substrates, for a wider application the limited stability of this enzyme (temperature, cosolvents, pH, H_2O_2) is a serious drawback.

[*] Prof. Dr. A. Berkessel,[+] Dipl.-Chem. M. Picard, Dr. J. Gross
Organisch-chemisches Institut der Universität
Im Neuenheimer Feld 270, D-69120 Heidelberg (Germany)
Dipl.-Chem. E. Lübbert, Dipl.-Lebensmittelchem. S. Tölzer,
Prof. Dr. K.-H. van Pée
Institut für Biochemie der Technischen Universität Dresden (Germany)
S. Krauss
Institut für Mikrobiologie der Universität Stuttgart (Germany)
[+] New address:
Institut für Organische Chemie der Universität
Greinstrasse 4, D-50939 Köln (Germany)
Fax: Int. code +(221) 470-5102
e-mail: berkessel@uni-koeln.de

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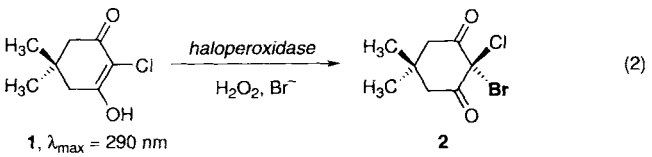
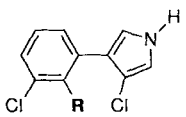
About ten years ago, van Pée et al. described the first metal-free haloperoxidases from bacteria: the chloroperoxidases from *Pseudomonas pyrrocinia* (CPO-P)[5] and from *Streptomyces aureofaciens* Tü24 (CPO-T).[6] The monochlorodimedone assay was used to trace the haloperoxidase activity [Eq. (2)]. In this



1, λ_{max} = 290 nm — **2** (2)

assay, the loss of extinction of the enolone band of the substrate 2-chlorodimedone (**1**) is followed spectrophotometrically at $\lambda = 290$ nm.[7]

In further publications, the halogenating activity of these enzymes was also shown for natural substrates, for example, in the course of the biosynthesis of the antimycotic pyrrolnitrin **4**.[5, 8] In the presence of hydrogen peroxide, CPO-P/CPO-T do not only catalyze the introduction of the two chlorine atoms, but also effect the oxidation of the amino group of the precursor **3** to the nitro group in pyrrolnitrin **4**.[9]

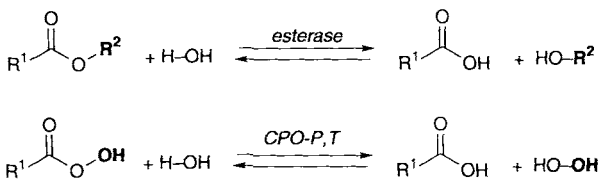


3: R = NH_2
4: R = NO_2

Since CPO-P und CPO-T were characterized as very stable enzymes, they appeared to be interesting potential catalysts for preparative applications.[5, 6] At the beginning of our investigations, the mechanisms of catalysis of heme- and vanadium-containing enzymes were relatively clear.[1-3] In contrast, the mode of action of the metal-free enzymes was absolutely unknown. One hypothesis involved the intermediary formation of a methionine sulfoxide in the enzyme's active site.[10] However, a recent X-ray structural analysis[11] on the bromoperoxidase A2 from *Streptomyces aureofaciens* (ATCC 10762) rendered this proposal unlikely. Instead, the X-ray crystal structure revealed the presence of the "catalytic triad" Ser-His-Asp in the enzyme's active site. The question arose how this catalytic triad, which normally catalyzes the hydrolysis of ester and amide bonds,[12] can activate hydrogen peroxide! Herein we describe first our attempts to use the chloroperoxidases from *Pseudomonas pyrrocinia* and *Streptomyces aureofaciens* (CPO-P, CPO-T) as catalysts for oxidation reactions and second our experiments aimed at the clarification of the mechanism of catalysis of the above enzymes. We present a mechanistic proposal that is able to explain all experimental data available so far.

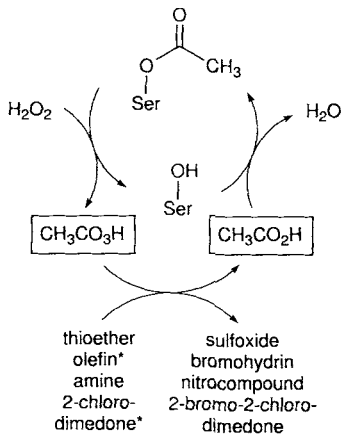
The following was observed when CPO-P und CPO-T were used as oxidation catalysts: a) Formation of halohydrins: In the presence of hydrogen peroxide and bromide, styrene, (*E*)-β-methylstyrene and 1,2-dihydronaphthalene were converted rapidly and quantitatively. By comparison with authentic samples, all reaction products were identified as the expected bromohydrins, for example 2-bromo-1-phenylethanol in the case of styrene. Quite remarkably, all bromohydrins were formed as racemic mixtures. When bromide was exchanged for chloride, chlorohydrins did not form analogously. b) Epoxidation of olefins: We found that neither CPO-P nor CPO-T catalyzed the epoxidation of olefins with H_2O_2. No reaction occurred with either electron-rich (styrene, 1,2-dihydronaphthalene) or electron-deficient olefins (*E*-crotonic or *E*-cinnamic acid, 2-cyclohexenone, 2-isopropylidenecyclopentanone). (c) Oxidation of thioethers: CPO-P und CPO-T catalyzed the quantitative conversion of thioethers such as methyl, ethyl, and octyl phenyl sulfide, to afford the corresponding sulfoxides. The sulfoxidations, too, afforded totally racemic products. d) Oxidation of aniline derivates: In the presence of hydrogen peroxide and CPO-P or CPO-T, 2-, 3- and 4-chloroaniline were smoothly converted into the corresponding nitrobenzenes.

The formation of racemic products does not suggest an oxidation within the enzyme's active site. On the contrary, these results point to a diffusible oxidizing entity. CPO-P and CPO-T are active only in acetate or propionate buffer, and not, for example, in phosphate buffer. The enzymatic activity is a function of the buffer concentration and shows saturation characteristics (not depicted). Thus, it seemed that the oxidation of halide does not take place in the active sites of CPO-P and CPO-T. Instead, an enzyme-catalyzed equilibration of hydrogen peroxide with acetate (propionate) affording peracetate (perpropionate) appeared much more likely. As mentioned above, the active site of the enzyme contains the catalytic triad of serine esterases. As shown in Scheme 1, the hydrolysis of an ester (top equation from left to right) closely resembles the reaction of a carboxylic acid with H_2O_2 (bottom reaction from right to left).



Scheme 1. Comparison of ester hydrolysis with the reaction of a carboxylic acid with H_2O_2.

According to this hypothesis, the peracid formed in very low stationary-state concentration oxidizes thioethers to sulfoxides and bromide to bromine, which accounts for the formation of halohydrins from olefins. Analogous to the mechanism of serine esterase catalyzed ester hydrolyses, the hydroxyl group of the "catalytic" serine would be acetylated during turnover (Scheme 2).[12] Indeed, the X-ray crystal structure of the haloperoxidase from *Streptomyces aureofaciens* (crystallized from acetate buffer) showed additional electron density in the vicinity of this hydroxyl group which was interpreted as an acetate residue.[11]



Scheme 2. Postulated mechanism of the catalysis by metal-free haloperoxidases. The conversion of substances indicated by an asterisk requires the presence of bromide.

The assumption of an enzyme-catalyzed formation of peracetic acid as the diffusible oxidizing entity is further supported by the following observations: a) Incubation of CPO-T with H_2O_2 in acetate buffer and subsequent ultrafiltration yielded a protein-free filtrate that gave a positive monochlorodimedone assay (Figure 1). b) All the oxidations (formation of halohydrins, sulfoxidation) that can be performed with H_2O_2 and CPO-P/CPO-T took place in just the same way when dilute peracetic acid was used. This is true for the peroxidase assay shown in Equation 2, too. It is well-known that peracids oxidize bromide to bromine,[13] and that the Br_2 transforms **1** into **2**

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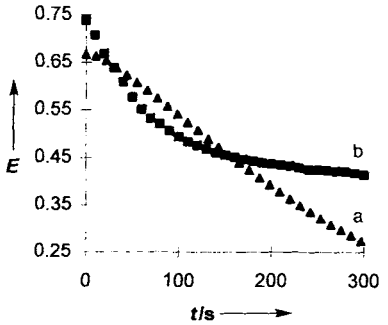

Figure 1. Monochlorodimedone assay in acetate buffer in the presence of NaBr with a) CPO-T and H_2O_2 and b) the protein-free ultrafiltrate obtained from the incubation of CPO-T with H_2O_2 in acetate buffer. E = extinction.

[Eq. (2)] and effects the formation of halohydrins from olefins in aqueous medium. c) Under the reacton conditions employed, dilute peracetic acid did not effect the epoxidation of olefins (see above). d) In the presence of H_2O_2/chloride, the enzymes CPO-P/CPO-T did not give a positive monochlorodimedone assay.[5,6] In the presence of peracetic acid and chloride, no positive assay was observed either. e) Phenylmethanesulfonyl fluoride (PMSF) is a well-known inhibitor for serine-proteases und -esterases. Its inhibitory activity results from the sulfonation of the serine-hydroxyl group in the active site.[14] PMSF also inhibits oxidation reactions catalyzed by CPO-P and CPO-T. As an example, the inhibition of CPO-T (monochlorodimedone assay, Eq. 2) as a function of time and acetate concentration is shown in Figure 2.

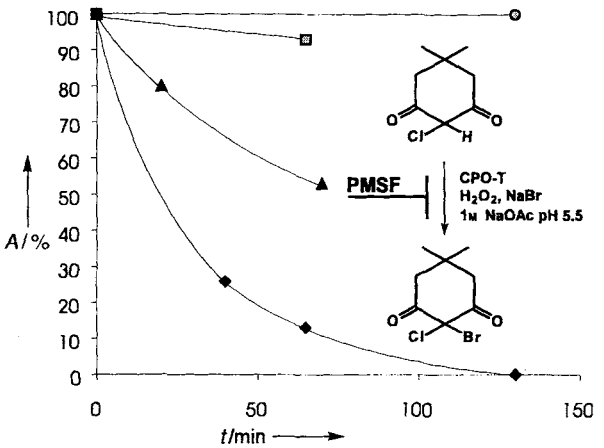

Figure 2. Time course of the inhibition of the oxidizing activity of CPO-T by PMSF in water and in sodium acetate buffer. o Control CPO-T/water, □ control CPO-T/buffer, • inhibition CPO-T/water, ▲ inhibition CPO-T/buffer; t = incubation time, A = relative activity.

Obviously, the irreversible inhibition by PMSF proceeded much faster in water than in acetate buffer.[15] This effect can easily be explained by the competition of acetate with PMSF in the reaction with the serine hydroxyl group in the enzyme's active site.

Until now, all attempts to prove the presence of peracetic acid in the equilibrium mixture by spectroscopic techniques were frustrated by its low equilibrium concentration relative to the much higher contents of water and acetate. With this in mind, we decided to prove the enzyme-catalyzed equilibration *starting from peracetic acid* (second Equation in Scheme 1, from left to right). Indeed, this approach proved successful: When an approximately 30 mM solution of peracetic acid in 1 M acetate buffer (pH 5.5) was acidified to pH = 1 and extracted with ether, the presence of peracetic acid in the extract could unambiguously be proven by high-resolution CI-MS. When CPO-T was added to the same solution of peracetic acid in acetate buffer, and the same extraction procedure was carried out, the HR-CI-MS of the extract did not show the presence of peracetic acid! As a control experiment, the equivalent amount of trypsin was added instead of CPO-T: No measurable decrease of the peracetic acid concentration took place within hours.

Our mechanistic hypothesis implies that the haloperoxidases CPO-P/CPO-T should have esterase activity as well. We confirmed this assumption with 4-nitrophenyl acetate as substrate: at 20 °C and pH 5.5, the ester was hydrolyzed rapidly, whereas no reaction occurred in the absence of the enzymes. The observed esterase activity is in agreement with the categorization of the (structurally characterized) bromoperoxidase from *Streptomyces* as an α/β-hydrolase.[11] Similar to the irreversible inhibition of the enzymatic activity by PMSF (see above), the hydrolysis of 4-nitrophenyl acetate in acetate buffers of higher concentration proceeds more slowly than at lower acetate concentrations: For example, changing from 0.05 M to 0.5 M decreased the rate of reaction to about one quarter. Clearly, the hydrolytic activity of CPO-P/CPO-T was also inhibited by PMSF.

If the catalytic triad of CPO-P and CPO-T catalyzes the equilibration between acetate/H_2O_2 and peracetic acid, other serine-esterases should be able to do the same.[16] We checked this assumption on chymotrypsin, trypsin, elastase, alkaline protease from *Streptomyces griseus* and acetylcholine esterase from *Torpedo californica*. Whereas the first four enzymes proved inactive in the monochlorodimedone assay [Eq. (2) and MS-experiment, see above], the acetylcholine esterase showed a catalytic activity comparable to that of CPO-T. However, the former enzyme was rapidly and irreversibly deactivated by H_2O_2.

Our investigations have shown that the oxidizing activity of the metal-free bacterial haloperoxidases CPO-P and CPO-T is in fact due to an unusual hydrolase activity, namely, the formation of peracetic acid from acetate and hydrogen peroxide (Schemes 1 and 2). Thus, we have introduced a novel enzymatic system (besides lipases[17]) for the in situ activation of hydrogen peroxide by peracid formation.

Experimental Section

General: Chloroperoxidase from *Streptomyces aureofaciens* Tü24 (CPO-T) was isolated from pHM621-containing *S. lividans*. CPO-P from *Pseudomonas pyrrocinia* was isolated from pHW321-containing *E. coli* [18,19]. Chymotrypsin, trypsin, elastase, and acetylcholine esterase from *Torpedo californica* were purchased from Sigma, and protease alkaline from ICN. *n*-Octyl phenyl sulfide and all sulfoxides were prepared according to literature procedures [20,21]. The identification of the reaction products was done by comparison with authentic samples.

Enzyme-catalyzed oxidations (typical experiments): Standard assay for haloperoxidase activity according to Hewson and Hager [7] with monochlorodimedone (44 μM), H_2O_2 (7.2 mM), NaBr (82 mM) in 1 M acetate buffer, pH 5.5, 25 °C (this reaction was also used for the serine-hydrolases stated in the text): The reaction was started by the addition of the enzyme and followed spectrophotometrically at λ = 290 nm. The analysis of the solution for low-molecular weight oxidant was done as follows: CPO-T and H_2O_2 were incubated in acetate buffer for 30 min, then the enzyme was removed by ultrafiltration (Centricon-10, *Amicon*). The filtrate (500 μL) was added to a solution (500 μL) of monochlorodimedone (88 μM) and NaBr (164 mM) in 1.0 M acetate buffer (pH 5.5). The reaction was followed spectrophotometrically. Formation of bromohydrins: 1 M sodium acetate buffer (3.0 mL, pH 4.0), 1,4-dioxane (0.5 mL), *tert*-butyl alcohol (0.5 mL), 1 M NaBr (1.0 mL), H_2O_2 (30%, 150 μL), and the olefin (100 μmol) were homogenized by sonication (10 s). The reaction was started by the addition of 4 u (determined by the monochlorodimedone assay) of CPO-P or CPO-T and performed at 50 °C (3 h). The reaction mixture was extracted with ether (5 mL, 60 s sonication), and the ether phase was analyzed by GC and GC/MS. Control experiments were carried out in the same way, but without the addition of enzyme. Epoxidation of olefins: As described under bromohydrin formation, but without addition of NaBr. Under these conditions, no transformation of the olefins was observed. *Oxidation of thioethers*: 1 M sodium acetate buffer (4.0 mL, pH 5.5), 1,4-dioxane (1.0 mL), H_2O_2 (150 μL, 30%) and the thioether (100 μmol) were homogenized by sonication (10 s). The reaction was started by the addition of 8 u (determined by the monochlorodimedone assay) CPO-P or CPO-T and carried out at 22 °C (2 h). The reaction mixture was extracted with ether (5 mL, 60 s ultrasound treatment), and the ether phase was analyzed by GC, GC/MS, or HPLC (*n*-octyl phenyl sulfide). Control experiments were carried

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out in the same way, but without the addition of enzyme. Oxidation of amines: 2-, 3-, or 4-chloroaniline (78 μM), H_2O_2 (44.4 μM), and 1 M sodium acetate buffer (pH 4.5). The reaction was started by the addition of 0.5 u (determined by the monochlorodimedone assay) CPO-P or CPO-T to 1 mL of the mixture. The reaction was carried out at 30 °C (3-chloroaniline: 30 min; 2-, 4-chloroaniline: 50 min). The products were identified by HPLC-coinjection.

Oxidations with peracetic acid: Under identical experimental conditions, peracetic acid was used instead of H_2O_2/enzyme, (200 μmol CH_3CO_3H instead of 150 μmol H_2O_2). In the monochlorodimedone assay, peracetic acid was used in a concentration of 72 μM (instead of 7200 μM H_2O_2).

Proof of the enzymatic transformation of peracetic acid by mass spectrometry: sector-field mass spectrometer Jeol JMS-700 MS, high-resolution CI-MS with isobutane as reactant gas, positive ion mode, resolution $R = 8000$, accumulation of 5–10 scans of the accelerating voltage (m/z 56–90, 8 s per cycle), quasi-molecular ion $[M+H]^+$ (calcd. for $C_2H_5O_3$: 77.0239, found: 77.0261), internal mass calibration with ions derived from isobutane and the solvent. Aliquots (2.5 mL) of a 37 mM solution of peracetic acid in 1 M sodium acetate buffer (pH 5.5) were a) incubated with 5.5 mg (ca. 50 u) CPO-T for 10 min at room temperature (RT), b) incubated with 5.5 mg trypsin for 1 h at RT, or c) kept at RT without the addition of enzyme. The solutions were then acidified with H_2SO_4 to pH 1 and extracted with ether (1 mL). The extracts were concentrated to approximately 50 μL in a stream of N_2 and introduced into the mass spectrometer through the reference inlet. Whereas peracetic acid was clearly present in approximately the same concentration in experiments (b) and (c), no peracetic acid could be detected by MS in the extract from (a). Furthermore, whereas the pungent smell of peracetic acid persisted in the experiments (b) and (c), this typical odor vanished immediately after the addition of enzyme in experiment (a).

Hydrolase activity (typical experiments): The enzymes were added to a solution of 0.1 μmol *p*-nitrophenylacetate in 2.0 mL 1 M sodium acetate buffer (pH 5.5) and 10 μL *tert*-butyl alcohol. The reaction was followed by the decrease of extinction at $\lambda = 317$ nm.

Inhibition experiments: In a typical experiment, 0.04 u CPO-T (determined by the monochlorodimedone assay) in 500 μL of water or sodium acetate buffer (pH 5.5, various concentrations) were incubated with a solution of PMSF (4 μmol) in *tert*-butyl alcohol (20 μL) at 50 °C. The remaining enzymatic activity was determined at various incubation times as described above.

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Synthesis of Rotaxanes by Brief Melting of Wheel and Axle Components

Mirko Händel, Marcus Plevoets, Sven Gestermann, and Fritz Vögtle*

Dedicated to Professor Sigrid D. Peyerimhoff on the occasion of her 60th birthday

In 1995 we synthesized the first amide-type rotaxanes by forming the amide bond of the axle in the presence of the wheel.[1] The triphenylmethyl stoppers used were sterically demanding enough to prevent wheels of type **1**[2] from slipping off the axle. We have been searching for a way to thread such a macrocycle onto a preassembled axle by using a "slipping approach" analogous to that described previously for other types of rotaxanes.[3] In the course of this research we have synthesized amide-type rotaxanes **2** by simply melting the preassembled wheel and axle components for about one minute.

In order to synthesize amide rotaxanes in this way (Scheme 1), it is necessary to tune the size complementarity of stoppers and wheels. At high temperatures the wheel must be able to slip over the bulky stoppers onto the axle, whereas at low temperatures the barrier should be high enough to prevent the reverse process, thus resulting in stable mechanical bonding between wheel and axle. A synthesis in which wheels were slipped over triphenylmethyl stoppers did not appear promising to us, since even when such rotaxanes are heated to 100 °C in tetrachloroethane no disassembly into a wheel and axle is observed.[4] Hence in order to investigate a "slipping-on" process we synthesized axles **3** with smaller di-*tert*-butylphenyl stoppers, which according to space-filling models should pass through the cavities of wheels like **1** more readily.[5] Attempts to slip tetralactams **1** onto the *meta*-phenylene axle **3α** in high boiling solvents like tetrachloroethane were not successful: We recovered most of the starting materials **1a** and **3α**, but did not detect the rotaxane **2aα**.

Thus, it appeared reasonable not to work under solvent conditions any longer but to turn to experiments in the melt in order to increase the concentration and to achieve higher temperatures. Furthermore, instead of **3α** we employed the slightly longer axle **3β** in order to reduce the steric hindrance between the wheel and axle.[3b] The pulverized components **1a** and **3β** were melted down in a tube heated to 350 °C with a hot air

[*] Prof. Dr. F. Vögtle, Dr. M. Händel, Dipl.-Chem. M. Plevoets, Dipl.-Chem. S. Gestermann
Institut für Organische Chemie und Biochemie der Universität
Gerhard-Domagk-Strasse 1, D-53121 Bonn (Germany)
Fax: Int. code +(228)73-5662
e-mail: voegtle@snchemie1.chemie.uni-bonn.de