University of Groningen

Biocatalytic Potential of the Epoxide Hydrolase from *Agrobacterium radiobacter* AD1 and a Mutant with Enhanced Enantioselectivity

Lutje Spelberg, Jeffrey H.; Rink, Rick; Archelas, Alain; Furstoss, Roland; Janssen, Dick B.

Published in:
Advanced Synthesis & Catalysis

DOI:
[10.1002/1615-4169\(200210\)344:9<980::AID-ADSC980>3.0.CO;2-A](https://doi.org/10.1002/1615-4169(200210)344:9<980::AID-ADSC980>3.0.CO;2-A)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2002

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Lutje Spelberg, J. H., Rink, R., Archelas, A., Furstoss, R., & Janssen, D. B. (2002). Biocatalytic Potential of the Epoxide Hydrolase from *Agrobacterium radiobacter* AD1 and a Mutant with Enhanced Enantioselectivity. *Advanced Synthesis & Catalysis*, 344(9), 980-985. [https://doi.org/10.1002/1615-4169\(200210\)344:9<980::AID-ADSC980>3.0.CO;2-A](https://doi.org/10.1002/1615-4169(200210)344:9<980::AID-ADSC980>3.0.CO;2-A)

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Biocatalytic Potential of the Epoxide Hydrolase from *Agrobacterium radiobacter* AD1 and a Mutant with Enhanced Enantioselectivity

Jeffrey H. Lutje Spelberg,^a Rick Rink,^a Alain Archelas,^b Roland Furstoss,^b Dick B. Janssen^{a,*}

^a Department of Biochemistry, Groningen Biomolecular Sciences & Biotechnology Institute, Nijenborgh 4, 9747 AG, Groningen, Netherlands

Fax: (+31)-50-3634165, e-mail: d.b.janssen@chem.rug.nl

^b Groupe Biocatalyse et Chimie Fine, Université de la Méditerranée, Faculté des Sciences de Luminy, Case 901, 163 Avenue de Luminy, 13288 Marseille Cedex 9, France

Fax: (+33)-491-829145, e-mail: furstoss@luminy.univ-mrs.fr

Received: May 18, 2002; Accepted: July 28, 2002

Dedicated to Prof. Roger Sheldon on the occasion of his 60th birthday.

Abstract: Optically pure epoxides are useful synthons for a variety of biologically active compounds. The epoxide hydrolase obtained from *Agrobacterium radiobacter* AD1 hydrolyses racemic aryl epoxides with moderate and aliphatic epoxides with low enantioselectivity. The three-dimensional structure of this enzyme indicates that two tyrosine residues interact with the epoxide oxygen. Mutating one of these, tyrosine 215, to a phenylalanine (Y215F)

resulted in an enzyme with increased enantioselectivity towards aryl epoxides. The relatively strong decrease in activity towards the remaining enantiomers makes this enzyme a much better biocatalyst than the wild-type enzyme for the preparation of optically pure (*S*)-styrene oxide derivatives.

Keywords: biotransformation; enzyme catalysis; epoxides; kinetic resolution; protein engineering

Introduction

Epoxide hydrolases catalyze the hydrolysis of an epoxide to yield the corresponding *vicinal* diol. In the past decade this class of enzymes has been explored for the preparation of optically active epoxides and diols.^[1,2,3] The most common application of epoxide hydrolases is the preparation of optically pure epoxides by enantioselective hydrolysis of a racemic epoxide. Recently, the usefulness of these enzymes in organic synthesis has been demonstrated by various preparative-scale conversions.^[4,5] A variety of biologically active products were synthesized by implementing such enzymatic or chemo-enzymatic reaction steps.^[2]

Previously, the epoxide hydrolase from *Agrobacterium radiobacter* AD1, an organism able to grow on epichlorohydrin, was investigated.^[6] The enzyme was purified and overexpressed in *E. coli*, which made it available in large amounts.^[7,8] X-ray crystallography and site-directed mutagenesis studies showed that the residues Asp107, His275 and Asp246 function as a catalytic triad. The two-step catalytic mechanism involved nucleophilic attack on the primary carbon of the epoxide ring by Asp107, resulting in the formation of

a covalent intermediate, and the hydrolysis of this ester by an activated water molecule, yielding the diol (Scheme 1). An initial determination of the enantioselectivity of kinetic resolutions showed that this enzyme is moderately enantioselective towards styrene oxide derivatives.^[9] *para*-Chlorostyrene oxide was hydrolyzed with the highest enantioselectivity, followed by *ortho*-chloro- and *meta*-chloro-substituted styrene oxides. With styrene oxide derivatives, the remaining epoxide was generally of (*S*)-configuration, except for α -methylstyrene oxide where, surprisingly, the result was opposite.^[10]

The X-ray structure shows that the enzyme consists of two domains, an α/β -hydrolase fold main domain and a cap domain.^[11] The active site with the catalytic triad is located in a hydrophobic cavity between the two domains. Two tyrosine residues (Tyr215 and Tyr152) are positioned in the cap domain in such a manner that their phenolic hydroxy groups point towards the expected binding position of the oxygen atom of the epoxide ring (Figure 1). These residues were proposed to stabilize the transition state towards the formation of the intermediate by protonation of the leaving oxygen group. On basis of the structure, mutants were con-

Scheme 1. Reaction mechanism of the epoxide hydrolase from *A. radiobacter* AD1. (A) alkylation half reaction and (B) hydrolysis half reaction. Which tyrosine residue donates a proton is not known.

Figure 1. Active site of the epoxide hydrolase from *A. radiobacter* AD1 as observed in the X-ray structure.

structured in which either tyrosine residue was replaced by a phenylalanine (Y215F and Y152F). The influence of these mutations on the kinetic parameters of hydrolysis

was investigated for both enantiomers of styrene oxide and *para*-nitrostyrene oxide.^[12] Another study showed that the enantioselectivity with substituted styrene oxides was higher for the Y215F mutant.^[13] These results prompted us to investigate the enantioselectivity towards a wider variety of aromatic and aliphatic substrates in order to obtain insight in the potential use the mutant enzyme for the preparation of optically pure epoxides.

Results and Discussion

Enantioselectivity of Wild-Type and Y215F Epoxide Hydrolase

The wild-type and Y215F epoxide hydrolase were brought to overexpression in *E. coli* and purified to homogeneity. To investigate the enzymatic conversions, 3 mM solutions of racemic epoxides were subjected to the wild-type and the Y215F epoxide hydrolase. The concentration of both enantiomers was followed in time by withdrawing samples from the reaction mixture and analyzing them using chiral GC and chiral HPLC. The enantioselectivity (E value) of the conversions was determined by progress curve analysis as described before.^[9]

Kinetic resolutions with the racemic epoxides, styrene oxide, phenyl glycidyl ether, epichlorohydrin and 1,2-epoxyhexane, which represent different classes of epoxides, were chosen to investigate the enantioselectivity of the hydrolysis catalyzed by the wild-type and Y215F epoxide hydrolase. The hydrolysis of the aliphatic epoxides epichlorohydrin and 1,2-epoxyhexane catalyzed by the wild-type and mutant enzyme occurred with no difference in enantioselectivity ($E < 2$). A significant increase in enantioselectivity was observed with phenyl glycidyl ether. The E value increased from 11 using the wild-type to 16 with mutant enzyme. The hydrolysis of styrene oxide showed the most significant increase in enantioselectivity, the E value increased from 16 with the wild-type to 32 with the mutant enzyme.

To further explore this class of substrates, a range of racemic styrene oxides, substituted with electron-donating and -withdrawing groups, were subjected to the wild-type and Y215F epoxide hydrolase (Table 1). The mutant enzyme showed an increased enantioselectivity towards all substituted styrene oxides. Kinetic resolutions with both enzymes resulted in optically pure epoxides with an (*S*)-configuration. The kinetic resolution of styrene oxide using wild-type enzyme showed a remarkable progress curve. After conversion of the (*R*)-enantiomer, the (*S*)-enantiomer was converted at a much higher rate (Figure 2A). A previous study of the kinetics showed that K_m and k_{cat} had an opposite effect on the enantioselectivity.^[14] The 46-fold lower K_m for

Table 1. Enantioselectivity of kinetic resolutions of *para*-substituted styrene oxides with the epoxide hydrolase from *A. radiobacter* AD1 and the Y215F mutant.

Epoxide	E value wild-type ^[a]	E value Y215F ^[a]
<i>para</i> -methylstyrene oxide	26 (<i>S</i>)	63 (<i>S</i>)
styrene oxide	16 (<i>S</i>)	32 (<i>S</i>)
<i>para</i> -chlorostyrene oxide	35 (<i>S</i>)	130 (<i>S</i>)
<i>para</i> -cyanostyrene oxide	90 (<i>S</i>)	130 (<i>S</i>)
<i>para</i> -nitrostyrene oxide	65 (<i>S</i>)	> 200 (<i>S</i>)

^[a] Between parentheses is the absolute configuration of the remaining enantiomer.

Figure 2. Kinetic resolution of styrene oxide (A) and *para*-methylstyrene oxide (B) by wild-type (●,○) and Y215F (▲,△) epoxide hydrolase. Closed symbols: (*R*)-enantiomer; open symbols (*S*)-enantiomer.

(*R*)-styrene oxide caused a preferred conversion of this enantiomer. However, an almost 3-fold higher k_{cat} for the (*S*)-enantiomer caused its higher hydrolysis rate.

Styrene oxide was hydrolyzed by mutant enzyme with a 2-fold higher enantioselectivity. The kinetic resolutions catalyzed by the Y215F mutant showed more standard progress curves than those with wild-type

enzyme. After conversion of (*R*)-styrene oxide, conversion of the (*S*)-styrene oxide also occurred, but at a lower rate (Figure 2A). The progress curve of the hydrolysis of *para*-methylstyrene oxide using wild-type epoxide hydrolase showed that both enantiomers were hydrolyzed at an almost equal rate (Figure 2B). With the mutant enzyme, the hydrolysis rate of the remaining (*S*)-enantiomer was substantially lower than that of the (*R*)-enantiomer.

Kinetics of Wild-Type and Mutant Enzyme with Substituted Styrene Oxide Enantiomers

To determine initial rates, optically pure enantiomers were subjected to wild-type and Y215F enzyme. With the wild-type enzyme, the initial rates at high substrate concentrations were equal to V_{max} . However, with the exception of styrene oxide, no saturation of the reaction velocity was observed with Y215F epoxide hydrolase. The low solubility of the epoxides limits the measurement of initial activities to concentrations lower than 2.5 mM.

Previous work elucidated the kinetic mechanism of hydrolysis of both enantiomers of styrene oxide by the wild-type enzyme using steady state and pre-steady state techniques. The first kinetic step involves an attack on the β -carbon atom of the epoxide, resulting in the covalent ester intermediate (alkylation half reaction). In the second catalytic step the covalent intermediate is hydrolyzed by a water molecule that attacks the carbonyl carbon of Asp107, yielding the diol (hydrolysis half reaction). With both enantiomers of styrene oxide the rate-limiting step is the hydrolysis of the covalent intermediate, which has a rate constant approximately equal to the overall k_{cat} . An electron-donating or -withdrawing substituent on the aromatic ring can have a large influence on the alkylation rate, but since this step is more than 100-fold faster than the rate-limiting hydrolysis step, an effect on the overall rate (k_{cat}) cannot be observed. The influence of a substituent on the aromatic ring on the rate of hydrolysis of the covalent intermediate will be less significant. Thus, the reaction rate is expected to be only slightly dependent on the nature of the substituent. This hypothesis coincides with V_{max} values obtained from the initial rate measurements using the substituted optically pure (*S*)-enantiomers (Table 2). Increasing the electron-withdrawing nature of the substituent resulted in only a minor increase in initial rate. These results are visualized in Figure 3, in which the logarithm of the relative V_{max} for each substituent ($V_{\text{max}}^{\text{X}}/V_{\text{max}}^{\text{H}}$) is plotted against the Hammett constant σ_{p} . For the hydrolysis of the (*S*)-enantiomers, the same plot showed a slightly positive slope $\rho_{V_{\text{max}}} (+0.12)$, confirming that either a nucleophilic mechanism is unlikely to occur in the rate-determining step, or that the nature of

Table 2. Activities of wild-type and Y215F epoxide hydrolase towards optically pure *para*-substituted styrene oxides at 2.5 mM substrate concentration.

Substituent	Configuration	Activity [$\mu\text{mol min}^{-1} \text{mg}^{-1}$]		% relative activity ^[b]
		wild-type ^[a]	Y215F	
methyl	<i>R</i>	9.2	2.9	32%
	<i>S</i>	5.4	0.1	2%
H	<i>R</i>	6.7	4.6 ^[c]	69%
	<i>S</i>	18.9	0.6	3%
chloro	<i>R</i>	11.9	8.3	70%
	<i>S</i>	17.4	0.1	< 1%
cyano	<i>R</i>	27.2	21.7	80%
	<i>S</i>	22.7	0.2	< 1%
nitro	<i>R</i>	21.0	14.6	70%
	<i>S</i>	21.8	0.3	1%

^[a] Activity is equal to V_{max} , with the exception of (*S*)-*para*-methyl styrene oxide.

^[b] Relative activity of Y215F enzyme compared to the wild-type enzyme (100%).

^[c] Activity is equal to V_{max} .

Figure 3. Hammett plot representing $\log(V_{\text{max}}^X/V_{\text{max}}^H)$ of the hydrolysis of (*R*)-*para*-substituted styrene oxides (○) and (*S*)-*para*-substituted styrene (△) oxides versus σ_p , catalyzed by wild-type enzyme.

the substituent does not have an effect on this step. Surprisingly, V_{max} values with the (*R*)-enantiomers were significantly influenced by the nature of the substituent. The rates increased when the ring substituent was more electron-withdrawing. The high value of $\rho_{V_{\text{max}}}$ (+0.55) suggests that a nucleophilic step is rate determining during the conversion of the (*R*)-enantiomer.

What could be the explanation for the difference between the two enantiomers? Rink et al. described the conversion of the (*S*)-styrene oxide by a three-step and the (*R*)-styrene oxide by a four-step mechanism to explain the observed transient kinetics.^[14] The extra step involved a unimolecular isomerization of the covalent enzyme-substrate complex. Three possible mechanistic steps were considered: a) a (re)protonation step of a tyrosine residue or the substrate oxygen, b) a slow

conformational change needed to set up the covalent intermediate for hydrolysis or c) an intramolecular rearrangement of the covalent intermediate due to an acyl transfer. The occurrence of such an intramolecular migration was considered previously with rat microsomal epoxide hydrolase.^[15] Besides this, acyl migrations have been reported during reactions catalyzed by lipases, baker's yeast and antibodies.^[16,17,18] In such a type of rearrangement, the covalently bound Asp107 migrates from the β -position of the substrate to the α -position. Assuming that this migration step would be rapid (thus having no effect on the overall rate of the process), the subsequent hydrolysis of this intermediate is expected to give a major and positive Hammett effect. The chemical base-catalyzed hydrolysis of *para*-substituted benzyl acetate derivatives, which can be regarded as structural analogues of the covalent intermediate, showed a ρ value of +0.743.^[19] This kind of acyl migration will not cause an inversion at the chiral center and does not influence the configuration of the diol. The products with or without the occurrence of the acyl transfer are identical, making the proof for such a mechanism difficult. Although the kinetically observed unimolecular isomerization step can also correlate with a conformational change or a protonation of a residue, a significant influence of the nature of the ring-substituents on the subsequent ester-cleavage step would then not be expected, because of the substantial distance to the aromatic group. It can also not be excluded that the larger substituent effect on the rate of conversion of (*R*)-enantiomers is caused by a different positioning of the covalent intermediates in the active site, or by a differential effect of the protonation state of the active site cavity.

In other studies, the kinetic mechanisms of epoxide hydrolases from fungi were investigated using the same range of substrates.^[20,21] With the epoxide hydrolase

from *Aspergillus niger*, the small magnitude of ρ (+0.3) suggested that the rate-limiting step was the ring opening of the epoxide. Similar experiments with a crude enzymatic extract prepared from *Syncephalastrium racemosum* suggested that the enzymatic mechanism was a concerted process implying a general acid activation of the oxirane ring, together with a nucleophilic attack.

The initial activities of the Y215F mutant towards (*R*)-styrene oxide derivatives (2.5 mM) were lower than those measured with the wild-type enzyme. With the exception of *para*-methylstyrene oxide, the remaining activity was around 70 to 80% of that of wild-type activity. The mutant enzyme showed a drastic decrease in activity towards the (*S*)-enantiomers. The effect was most pronounced with styrene oxides bearing an electron-withdrawing group, resulting in a residual activity of less than 1%. The low initial rates towards the remaining (*S*)-enantiomer improve the applicability of this enzyme for kinetic resolutions.

Conclusion

The wild-type epoxide hydrolase from *A. radiobacter* AD1 is a moderately enantioselective biocatalyst for the preparation of optically pure epoxides. The enantioselectivity towards aliphatic epoxides is low. Aromatic epoxides were hydrolyzed with moderate enantioselectivity. Mutation of tyrosine 215 to a phenylalanine resulted in an increased enantioselectivity and in this study we showed that this improved performance holds for a range of substituted styrene oxides. The increased enantioselectivity and strong decrease in activity towards the remaining (*S*)-enantiomer make the Y215F enzyme very useful for biocatalytic applications. Indeed, it allows circumventing the non-classical two phase kinetic behavior of the wild-type enzyme that seriously hampers its applicability. With the Y215F enzyme this problem no longer exists, which in combination with the increased enantioselectivity, makes the mutant a much better biocatalyst for the preparation of optically pure styrene oxide derivatives.

Experimental Section

General

Purified wild-type and the Y215F mutant enzyme were prepared as described before.^[9] The enantiomeric excess (ee) and the yields of the epoxides were determined using a gas chromatograph with an FID-detector or an HPLC both equipped with chiral columns. NMR spectra were recorded in CDCl₃. The racemic epoxides styrene oxide, phenyl glycidyl ether, epichlorohydrin and 1,2-epoxyhexane are commercially available (Aldrich). Racemic *para*-methylstyrene oxide, *para*-

chlorostyrene oxide, *para*-cyanostyrene oxide and *para*-nitrostyrene oxide were prepared as described previously.^[9,22] Optically pure enantiomers (ee > 98%) of *para*-methylstyrene oxide, *para*-chlorostyrene oxide, and *para*-nitrostyrene oxide and *para*-cyanostyrene oxide were prepared as described before.^[21]

Kinetic Resolution Experiments

A closed reaction vessel containing 20 mL Tris buffer (50 mM, pH 9.0) was incubated in a water bath at 30 °C. The epoxide was added to a final concentration of 3 mM. *para*-Substituted styrene oxides were added from stock solution in DMSO to a final concentration of 0.5% DMSO. The enzyme was added to such a concentration that allowed the first reacting enantiomer to be converted within 50 min. The reaction was monitored by periodically taking samples from the reaction mixture. The samples were extracted with diethyl ether containing mesitylene as an internal standard and analyzed by chiral GC or chiral HPLC.

Initial Rate Experiments

A closed reaction vessel containing 20 mL Tris buffer (50 mM, pH 9.0) was incubated in a water bath at 30 °C. The epoxide was added from a stock solution in DMSO to a final concentration of 1.5 mM to 2.5 mM and the enzyme was added immediately afterwards. The reaction was monitored by periodically taking samples from the reaction mixture, followed by GC or HPLC analysis.

Chiral Analysis of Epoxides

The enantiomeric excess of the epoxides was determined using the following columns: Chiraldex G-TA (Astec): styrene oxide, epichlorohydrin and 1,2-epoxyhexane; Chirasil Dex CB (Chrompack): *para*-cyanostyrene oxide, *para*-chlorostyrene oxide and *para*-nitrostyrene oxide; Chiralcel OD (Daicel): phenyl glycidyl ether.

Calculation of E Values

The E values of the kinetic resolutions were calculated from the Michaelis–Menten parameters for both enantiomers.^[9] To estimate the kinetic parameters, the equations describing competitive Michaelis–Menten kinetics were fitted by numerical integration to the data obtained from the kinetic resolution experiments. With the epoxides styrene oxide, *para*-methylstyrene oxide and *para*-chlorostyrene oxide the first order chemical hydrolysis rate constants of respectively $8.6 \times 10^{-6} \text{ s}^{-1}$, $1.2 \times 10^{-5} \text{ s}^{-1}$ and $6.2 \times 10^{-6} \text{ s}^{-1}$ were taken into account.

Acknowledgements

This research was financially supported by Innovation Oriented Research Program (IOP) on Catalysis (no. 94007a) of the Dutch

Ministry of Economic Affairs. Part of this work has been achieved in the context of the EC BIOC4-CT95-0005 and QLK-CT-2000-00426 contracts.

References

- [1] I. V. J. Archer, *Tetrahedron* **1997**, 53, 15617.
- [2] A. Archelas, R. Furstoss, *Curr. Opin. Chem. Biol.* **2001**, 5, 112.
- [3] A. Archelas, R. Furstoss, in *Biocatalysis. From discovery to application*, (Ed.: W.-D. Fessner), *Topics in Current Chemistry*, 200, Springer, Berlin, **1998**, p. 159.
- [4] K. M. Manoj, A. Archelas, J. Baratti, R. Furstoss, *Tetrahedron* **2001**, 57, 695.
- [5] R. V. A. Orru, I. Osprian, W. Kroutil, K. Faber, *Synthesis* **1998**, 1259.
- [6] A. J. van den Wijngaard, D. B. Janssen, B. Witholt, *J. Gen. Microbiol.* **1989**, 135, 2199.
- [7] M. H. J. Jacobs, A. J. van den Wijngaard, M. Pentenga, D. B. Janssen, *Eur. J. Biochem.* **1991**, 202, 1217.
- [8] R. Rink, M. Fennema, M. Smids, U. Dehmelt, D. B. Janssen, *J. Biol. Chem.* **1997**, 272, 14650.
- [9] J. H. Lutje Spelberg, R. Rink, R. M. Kellogg, D. B. Janssen, *Tetrahedron: Asymmetry* **1998**, 9, 459.
- [10] A. Archelas, R. Furstoss, *J. Org. Chem.* **1999**, 64, 6112.
- [11] M. Nardini, I. S. Ridder, H. J. Rozeboom, K. H. Kalk, R. Rink, D. B. Janssen, B. W. Dijkstra, *J. Biol. Chem.* **1999**, 274, 14579.
- [12] R. Rink, J. Kingma, J. H. Lutje Spelberg, D. B. Janssen, *Biochemistry* **2000**, 39, 5600.
- [13] R. Rink, J. H. Lutje Spelberg, R. J. Pieters, J. Kingma, M. Nardini, R. M. Kellogg, B. W. Dijkstra, D. B. Janssen, *J. Am. Chem. Soc.* **1999**, 121, 7417.
- [14] R. Rink, D. B. Janssen, *Biochemistry* **1998**, 37, 18119.
- [15] H. -F. Tzeng, T. Laughlin, R. N. Armstrong, *Biochemistry* **1998**, 37, 2905.
- [16] N. Murakami, T. Morimoto, H. Imamura, A. Nagatsu, J. Sakakibara *Tetrahedron* **1994**, 50, 1993.
- [17] R. Hayakawa, M. Shimizu, T. Fujisawa, *Tetrahedron Asymmetry* **1997**, 8, 3201.
- [18] E. Fernholz, D. Schloeder, K. K. C. Liu, C. W. Bradshaw, H. Huang, K. Janda, R. A. Lerner, C. H. Wong, *J. Org. Chem.* **1992**, 57, 4757.
- [19] H. H. Jaffe, *Chem. Rev.* **1953**, 53, 191.
- [20] A. Pedragosa-Moreau, C. Morisseau, J. Zylber, A. Archelas, J. Baratti, R. Furstoss, *J. Org. Chem.* **1996**, 61, 7402.
- [21] P. Moussou, A. Archelas, J. Baratti, R. Furstoss, *J. Org. Chem.* **1998**, 63, 3532.
- [22] R. B. Westkaemper, R. P. Hanzlik, *Arch. Biochem. Biophys.* **1981**, 208, 195.