Margaret M. Kayser

Department of Physical Sciences, University of New Brunswick, Saint John, New Brunswick, Canada

J. Heterocyclic Chem., **36**, 1533 (1999).

Introduction.

Oxidizing "designer yeast" is a new recombinant strain of baker's yeast that can be "switched on" to perform oxidations [1] rather than the more familiar reductions [2]. The cyclohexanone monooxygenase enzyme (CHMO) from soil bacteria *Acinetobacter* sp. NCIB 9871 [3] has been shown to oxidize ketones to lactones, and sulfides to sulfoxides, often with very high enantioselectivities [4]. Unfortunately, cyclohexanone monooxygenase has a half life of only 24 hours at 4°, the yield from the parent organism is relatively low, and it requires reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor [5]. These characteristics discourage large-scale reactions and more general use in organic synthesis. To make this useful enzyme more accessible, the new yeast reagent was constructed by cloning a gene expressing cyclohexanone monooxygenase from *Acinetobacter* into *Saccharomyces cerevisiae* [6]. A growing culture of engineered yeast produces a higher quantity of the protein and provides a constant supply of the NADPH cofactor. Biotransformations can be carried out in the same way as the ordinary yeast-catalyzed reactions well familiar to organic chemists.

In earlier communications we reported "designer yeast"-catalyzed oxidations of cyclohexanones and cyclopentanones to the corresponding lactones [1,6,7]. In these reactions, prochiral 4-alkyl cyclohexanones were converted to optically pure lactones [1], while 2-substituted racemic cyclohexanones and cyclopentanones were kinetically resolved, often with enantioselectivities higher than 95% *ee* [6,7]. Isolated yields in all yeast-catalyzed oxidations were acceptably high for the substrates investigated, and the products were surprisingly pure. Thus, "oxidizing yeast" has been established as an effective, enantioselective, bioorganic equivalent of organic peracids.

More recently, we have expressed the same cyclohexanone monooxygenase in *Escherichia coli* [8]. The recombinant strain, [BL21(DE3)(pMM4)], has a strong promoter thus the enzyme is produced at about 20% total protein expression. This is considerably higher than the previous *E. coli* overexpression strain [9], or the wild type *Acinetobacter* [5]. This engineered bacteria was used alongside the recombinant yeast, as a complementary bioreagent, in testing the efficiency of oxidations of 4-substituted cyclohexanones with functionalized side chains, and the oxidations of sulfides, dithiolanes and dithianes to sulfoxides.

4-Substituted Cyclohexanones.

Optically pure ε-caprolactones are useful fragments for the construction of a variety of organic compounds. For example, enantiomerically pure 4-(*S*)-methyl-ε-caprolactone from the yeast-catalyzed oxidation of 4-methylcyclohexanone was polymerized and used in the investigation of macrostructures such as coiled coils [10].

Earlier studies indicating that a variety of 4-substituted cyclohexanones are suitable substrates for cyclohexanone monooxygenase [1,4] encourage further investigation of this class of compounds as substrates for our new bioreagents, particularly those that are substituted with functional groups or functionalized chains. The initial results nicely parallel those obtained with the isolated enzyme. For example, the transformation of 4-hydroxycyclohexanone gave the corresponding lactone but the optical purity was low and it spontaneously rearranged to the γ-lactone, which is consistent with the results reported by Taschner for the reaction with purified cyclohexanone monooxygenase [11]. 4-Methoxycyclohexanone was also oxidized to the corresponding lactone; here the enantioselectivity was higher (72% *ee*) and again almost identical to that reported for the enzyme-catalyzed reaction. Since compounds with chains longer than four carbon atoms in the 4-position are not acceptable substrates for cyclohexanone monooxygenase, it was not surprising that 4-allyloxycyclohexanone was not oxidized. After 120 hours incubation only the corresponding alcohol, the product of reduction by native yeast reductases, was recovered. The absence of any oxidation by the Baeyer-Villiger yeast of 4-ethoxycyclohexanone was more unexpected. In a parallel reaction with the engineered *E. coli*, unchanged starting material was recovered after 72 hours incubation. Since 4-ethoxycyclohexanone does not inhibit the growth of either the yeast or *E. coli* it appears that it is not an acceptable substrate for cyclohexanone monooxygenase.

Model Studies.

Cyclohexanone monooxygenase is a flavin dependent enzyme. Flavins are remarkably versatile cofactors [12]. Flavin 4a-hydroperoxide functions either as a nucleophile or as an electrophilic oxygen transfer reagent [13].

All mechanistic studies on enzymatic Baeyer-Villiger reactions support the hypothesis that chemical and enzymatic reactions are closely related and involve a two-step process: attack by peroxide to form a tetrahedral "Criegee intermediate" followed by a [1,2] sigmatropic shift [14]. Several models for the active site of cyclohexanone

monooxygenase have been proposed [15-18]. Cyclohexanone, in the model [17,18] binds in a specific chair conformation so that in a developing tetrahedral intermediate hydroperoxide is delivered equatorially and the O(H) substituent is axial. Based on the assumption that in the active site flavin occupies a well-defined position, only the specific C-C bond (antiperiplanar to the O-O peroxide bond) can migrate. In view of the above, and after the analysis of results form oxidations of numerous substrates, we have proposed that the enantioselectivity of cyclohexanone monooxygenase arises from a combination of modest intrinsic chiral discrimination coupled with the conformational behavior of bound substrates [6]. Additional insights on the nature of the active site of cyclohexanone monooxygenase can be deduced from the study of substrates with functionalized substituents.

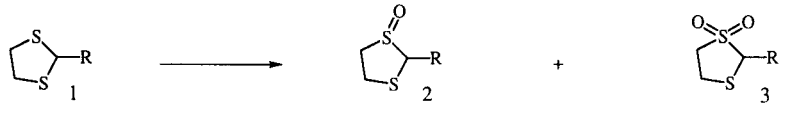
Oxidation at Sulfur.

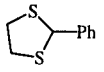
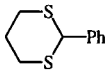
Optically pure sulfoxides are powerful stereodirecting groups, particularly important as chiral synthons for the asymmetric C-C bond formation [19-22]; numerous approaches to their preparation have been investigated in recent years [23]. The limitations encountered in chiral chemical oxidations [24-26], combined with efforts to develop ecologically more benign processes, inspired a search for bioorganic oxidation methods. A large number of microorganisms [27-30], including natural baker's yeast [31,32], catalyze oxidations at sulfur. Limited substrate acceptability and low enantioselectivity, however, are often a problem. Since cyclohexanone monooxygenase has been shown to perform highly enantioselective oxidation of several sulfides to sulfoxides [32], our engineered baker's yeast and *E. coli* appeared to be promising candidates for development as "reagents" for these reactions.

A background study of several 1,3-dithiolane and 1,3-dithianes, previously tested as substrates for isolated cyclohexanone monooxygenase and *Acinetobacter* [33,34], was conducted to compare the performance of the two recombinant organisms with that of the purified enzyme (Table 1). The analysis of the results obtained identified several problems that may occur during these transformations.

The most obvious complication is associated with overoxidation of these substrates by cyclohexanone monooxygenase. The purified enzyme oxidizes 1,3-dithiolane to the (*R*)-sulfoxide in 94% yield and >98% *ee*. In addition 6% of the sulfone is produced. The optical purity of the sulfoxide increases during the second oxidation, which favors conversion of (*S*)-sulfoxide to sulfone [34]. Reaction with *Acinetobacter* gave similar results, but the proportion of sulfone was higher and enantiomeric purity

Table 1
Biocatalytic Oxidations of Dithiolanes and Dithianes



Substrate	Biocatalyst	Ratio (GC) 1:2:3 [a]	Sulfoxide (2) *trans:cis*	Sulfoxide (2) Yield % [b]	Sulfoxide (2) *ee* %	Sulfone (3) Yield % [b]	Sulfone (3) *ee* %	References
	Isolated enzyme	0:94:6	-	94	>98 (*R*)	6	NA	34
	Acinetobacter	NR	-	71	95 (*R*)	16	-	33c
	15C (host strain)	100:0:0	-	-	-	-		
	15C (pKR001)	5:25:70	-	20	75 (*R*)	45	-	
	E. coli (host strain)	100:0:0	-	-	-	-	-	
	E. coli (pMM4)	0:15:85	-	13	86 (*R*)	47		
	15C	81:19:0	1:2	ND	70 [c]	-	-	
	15C (pKR001)	6:94:0	32:1	74	20	-		
	E. coli	100:0:0	-	-	-	-	-	
	E. coli (pMM4)	9:67:24	40:1	60	20	-	-	
	Isolated enzyme	0:81:19	-	81	>98 (*R*)	19	NA	34
	Acinetobacter	NR	-	76	>98 (*R*)	20	-	33c
	15C	82:1:17	-	ND	ND	ND		
	15C (pKR001)	1:42:57	-	18	90 (*R*)	19	-	
	E. coli	100:0:0	-	-	-	-	-	
	E. coli (pMM4)	3:91:6	-	73	84 (*R*)	ND		
	Isolated enzyme	0:100:6	50:1	NR	28 [c]	-	-	33b
	Acinetobacter	NR	-	-	-	-	-	
	15C	ND	9:1			-	-	
	15C (pKR001)	66:34:0	-	30	30	-	-	
	E. coli	100:0:0	19:1	-	-	-	-	
	E. coli (pMM4)	34:66:0		ND	12	-	-	

[a] Determined by chromatography; [b] Isolated yield after purification; [c] Optical purity of the major diastereomer. NR = not reported; ND = not determined; NA = not applicable.

of the sulfoxide slightly lower [33a]. Oxidations by engineered yeast cells and *E. coli* produced a higher proportion of sulfone and a lower optical purity of the sulfoxide. Similar results were obtained during the oxidations of 1,3-dithiane. It is important to note, however, that for 2-phenyl-1,3-dithiane, a substrate not oxidized to sulfone by cyclohexanone monooxygenase, enantiomeric purity of the sulfoxide from transformations with yeast and *E. coli* was very close to that obtained with the purified enzyme. This suggests that the discrepancy between the purified enzyme and the whole cells may be associated with transport through the membrane. If the diffusion of a highly polar sulfoxide is delayed by sluggish migration through the membrane it remains in the vicinity of the enzyme competing with sulfide for the active site.

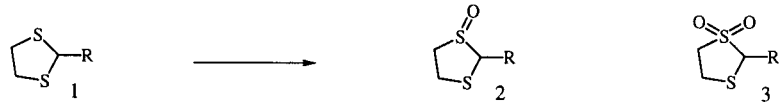
A second problem surfaced during oxidations of 1,3-dithiane, 2-phenyl-1,3-dithiane and 2-phenyl-1,3-dithiolane. Control experiments with unmodified host-yeast cells showed that they also can oxidize these compounds to sulfoxides and, in the case of dithianes, further to sulfones. None of the substrates tested (thioethers or sulfoxides), however, was oxidized by the host *E. coli* strain. These findings underscored the importance of control experiments and suggested that in some cases the engineered *E. coli* may be the bioreagent of choice.

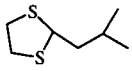
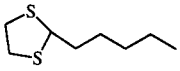
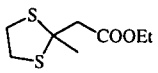
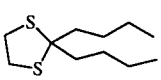
Several other difficulties associated with biooxidations of thioethers became evident during this study. Low solubility and occasional toxicity of the substrate presented problems in some transformations. These problems were generally successfully surmounted by performing the reactions in the presence of an equivalent of β- or γ-cyclodextrin. In addition, during prolonged reactions significant quantities of poorly soluble, slightly volatile starting materials were lost due to evaporation. In several cases, we observed that the loss through evaporation was as fast as, or faster than, the oxidation reaction, particularly when an insoluble substrate formed a film on the surface of the reaction mixture. Here again, the addition of an equivalent of β- or γ-cyclodextrin was usually beneficial. To minimize further losses caused by evaporation several reactions were carried out in baffled flasks covered with aluminum foil and sealed with parafilm. To insure an adequate supply of oxygen the size of the reaction flasks was doubled. Without extensive optimization, these strategies allowed the initially low isolated yields to be significantly improved.

These problems may be quite general in sulfide biotransformations; therefore, only sulfides that are relatively soluble and stable in water, and are oxidized rapidly by cyclohexanone monooxygenase to sulfoxides and slowly, if at all, to sulfones are "good" substrates for yeast-catalyzed reactions.

The following survey of several substituted dithiolanes, dithianes and thioethers (Tables 2, 3 and 4) shows that,

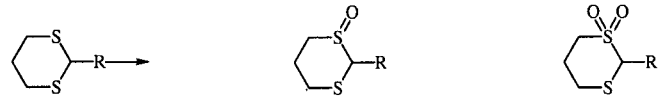
Table 2
Biooxidations of Dithiolanes

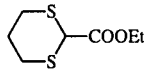
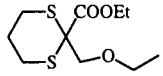
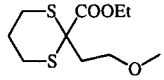
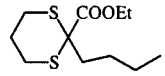


Substrate	Biocatalyst	Time (hours)	Ratio(GC) 1:2:3	Sulfoxide (2) *trans:cis*	Sulfoxide (2) Yield % [a]	Sulfoxide (2) *ee* %	Sulfone (3) Yield % [a]	Sulfone (3) *ee* %
	15C(pKR001)	28	1:22:77	10:1	16	20 [b]	15	76
	E. coli (pMM04)	20	34:31:35	5:1	35	40 [b]	16	73
	15C(pKR001)	36	0:81:19	NA	42	64	12	NA
	E. coli (pMM04)	20	9:67:24		46	69	6	NA
	15C(pKR001)	48	37:63:0	43:1	57	84	NA	NA
	E. coli (pMM04)	112	38:62:0	>50:1	22	>99	NA	NA
	15C(pKR001)	48	2:98:0	>50:1	96	36	NA	NA
	E. coli (pMM04)	23	70:30:0	>50:1	12	35	NA	NA
	15C(pKR001)	160	44:56:0	3:1	ND	>99 [b]	NA	NA
	E. coli (pMM04)	65	ND	3:1	50	>99 [b]	NA	NA
	15C(pKR001)	110	100:0:0	-	-	-	-	-
	E. coli (pMM04)	ND						

[a] Isolated yield after chromatography; [b] Optical purity of the major diastereomer. ND = not determined; NA = not applicable.

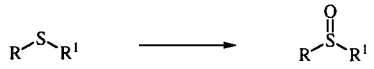
Table 3
Biooxidations of Dithianes

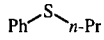


Substrate	Biocatalyst	Time (hours)	Ratio(GC) 1:2:3	*trans:cis*	Sulfoxide Yield % [a]	*ee* %	Sulfone Yield % [a]
	15C(pKR001)	90	71 [b]	-	0	-	-
	E. coli (pMM04)	15	12	2:1	37 [c]	-	10
	15C(pKR001)	130	-	19:1	64 [c]	>99	-
	E. coli (pMM04)	ND					
	15C(pKR001)	130	-	>50:1	63	>99	-
	E. coli (pMM04)	48	60	>50:1	12	>99	-
	15C(pKR001)	130	100	-	-	-	-
	E. coli (pMM04)	48	100	-	-	-	-

[a] Isolated yield after chromatography; [b] Hydrolyzed material, 17% recovery; [c] Optical purity of the major diastereomer.

Table 4
Biooxidations of Dithianes



Substrate	Biocatalyst	Time (hours)	Sulfide Recovery %	Sulfoxide Yield % [a]	Sulfoxide *ee* %
	15C(pKR001)	40	0	95	>99 (*R*)
	15C(pKR001)	120	20	10	25 (*R*)
	15C(pKR001)	40	10	57	97 (*R*)
	E. coli (pMM04)	21	0	24	79 (*R*)
	15C(pKR001)	23	0	53	74 (*R*)

[a] Isolated yield after chromatography.

despite the limitations indicated above, a variety of compounds can be oxidized by either engineered yeast or *E. coli*, or both, in good isolated yields and with high enantioselectivity and diasteroselectivity.

At this time, the results for biooxydations at sulfur are still in the purely empirical stage and we are not able to predict which compounds are going to be successful substrates.

In conclusion, the present study shows that oxidations at sulfur carried out by engineered yeast or *E. coli* can be highly enantioselective and efficient for a variety of substrates. When the results are compared with those obtained with the purified enzyme, low cost, accessibility and experimental simplicity makes them an attractive alternative to the enzyme or *Acinetobacter*-catalyzed transformations.

Acknowledgments.

This work would not be possible without the untiring and enthusiastic efforts of my collaborators: my student Gang Chen who has recently completed his Ph.D. dissertation, Dr. Marko Mihovilovic (Vienna University of Technology) and my colleague and collaborator Professor Jon Stewart (University of Florida, Gainesville) and his students Keith Reed and Carlos Martinez. Financial support by the Natural Sciences and Engineering Research Council (Canada) and the National Science Foundation (USA) is gratefully acknowledged. We also are indebted to Cerestar, Inc. for supplying the cyclodextins used in this work.

REFERENCES AND NOTES

[1a] J. D. Stewart, K. W. Reed and M. M. Kayser, *J. Chem. Soc., Perkin Trans. 1*, 755, (1996); [b] J. D. Stewart, K. W. Reed, J. Zhu, G. Chen and M. M. Kayser, *J. Org. Chem.*, **61**, 7652 (1996).

[2] S. Servi, *Synthesis*, 1, (1990); R. Csuk and B. I. Glanzer, *Chem. Rev.*, **91**, 49 (1991).

[3] N. A. Donoghue, D. B. Norris and P. W. Trudgill, *Eur. J. Biochem.*, **63**, 175 (1976).

[4] J. D. Stewart, *Curr. Org. Chem.*, **2**, 211 (1998).

[5a] O. Abril, C. C. Ryerson, C. T. Walsh and G. M. Whitesides, *Bioorg. Chem.*, **17**, 41 (1989); [b] G. Carrea, B. Redigolo, S. Riva, S. Colonna, N. Gaggero, E. Battistel and D. Bianchi, *Tetrahedron: Asymmetry*, **3**, 1063 (1992).

[6] J. D. Stewart, K. W. Reed, C. A. Martinez, J. Zhu, G. Chen, and M. M. Kayser, *J. Am. Chem. Soc.*, **120**, 3541 (1998).

[7] M. M. Kayser, G. Chen and J. D. Stewart, *J. Org. Chem.*, **63**, 7103 (1998).

[8] G. Chen, M. M. Kayser, M. Mihovilovic, M. E. Mrstik, C. A. Martinez and J. D. Stewart, *New J. Chem.*, **8**, 827 (1999).

[9] Y.-C. J. Chen, O. P. Peoples and C. T. Walsh, *J. Bacteriol.*, **170**, 781 (1988).

[10] H. M. Janssen, Ph.D. Thesis, Technische Universiteit Eindhoven, 1997.

[11] M. J. Taschner, D. J. Black and Q.-Z. Chen, *Tetrahedron: Asymmetry*, **4**, 1387 (1993).

[12] C. Walsh, *Acc. Chem. Res.*, **13**, 148 (1980).

[13] J. M. Schwab, W.-B. Li and L. P. Thomas, *J. Am. Chem. Soc.*, **105**, 4800 (1983).

[14] I. O. Sutherland, in Comprehensive Organic Chemistry, Vol **2**, D. Barton and W. D. Ollis, eds, Pergamon Press, Oxford, 1979, pp 869-956.

[15] V. Alphand and R. Furstoss, *J. Org. Chem.*, **57**, 1306 (1992).

[16] D. R. Kelly, *Tetrahedron: Asymmetry*, **7**, 1149 (1996).

[17] G. Ottolina, P. Pasta, G. Carrea, S. Colonna, S. Dallavalle and H. L. Holland, *Tetrahedron: Asymmetry*, **6**, 1375 (1995).

[18] G. Ottolina, G. Carrea, S. Colonna, S. Ruckemann, *Tetrahedron: Asymmetry*, **7**, 1123 (1996).

[19] A. J. Walker, *Tetrahedron: Asymmetry*, **3**, 961 (1992).

[20] M. Mikolajczyk and J. Drabowicz, *Top. Stereochem.*, **13**, 333 (1982); J. Drabowicz, P. Kielbasinski and M. Mikolajczyk in The Chemistry of Sulfones and Sulfoxides, S. Patai, Z. Rappoport and C. J. M. Stirling, eds, John Wiley & Sons Ltd. 1988, pp 233-278.

[21] G. Solladié, *Synthesis*, 185 (1981); G. H. Posner, *Acc. Chem.* Res., **20**, 72 (1987).

[22] H. L. Holland, *Chem. Rev.*, **88**, 473 (1988); M. R. Barbachyn and C. K. Johnson, in Asymmetric Synthesis, Vol **4**, J. D. Morrison and J. W. Scott, eds, Academic Press, Inc., New York 1984, Chapter 2.

[23] S.-H. Zhao, O. Samuel and H. B. Kagan, *Tetrahedron*, **43**, 5135 (1987); H. B. Kagan, E. Dunach, C. Nemecek, P. Pitcher, O. Samuel and S.-H. Zhao, *Pure Appl. Chem.*, **57**, 1911 (1985).

[24] R. F. Bryan, F. A. Carey, O. D. Dailey, R. J. Maher and R.W. Miller, *J. Org. Chem.*, **43**, 90 (1978).

[25] P. B. C. Page, R. D. Wilkes, E. S. Namwindwa and M. J. Witty, *Tetrahedron*, **52**, 2125 (1996) and references therein.

[26] V. K. Aggarwal, G. Evans, E. Moya and J. Dowden, *J. Org. Chem.*, **57**, 6390 (1992).

[27] B. J. Auret, D. R. Boyd, H. B. Henbest and J. Rass, *J. Chem. Soc.(C)*, 237 (1968).

[28] H. L. Holland, H. Popperl ,R. W. Ninniss and P. C. Chenchaiah, *Can. J. Chem.*, **63**, 1118 (1985).

[29] H. Ohta, Y. Okamoto and G. I. Tsuchihashi, *Agric. Biol. Chem.*, **49**, 671 (1985).

[30] S. Colonna, N. Gaggero, L. Casella, G. Carrea and P. Pasta, *Tetrahedron: Asymmetry*, **3**, 95 (1992).

[31] J. Beecher, P. Richardson, S. Roberts and A. Willetts, *Biotechnology Letters*, **17**, 1069 (1995).

[32] J. Tang, I. Brackenridge, S. M. Roberts, J. Beecher and A. Willetts, *Tetrahedron*, **51**, 13217 (1995).

[33a] V. Alphand, N. Gaggero, S. Colonna, P. Pasta and R. Furstoss, *Tetrahedron*, **53**, 9695 (1997); [b] S. Colonna, N. Gaggero, P. Pasta and G. Ottolina, *J. Chem. Soc. Chem. Commun.*, 2303 (1996); [c] V. Alphand, N. Gaggero, S. Colonna and R. Furstoss, *Tetrahedron Letters*, **37**, 6117 (1996).

[34] S. Colonna, N. Gaggero, A. Bertinotti, G. Carrea, P. Pasta and A. Bernardi, *J. Chem. Soc., Chem. Commun.*, 1123 (1995).