JOC Articles

Assessing the Substrate Selectivities and Enantioselectivities of Eight Novel Baeyer–Villiger Monooxygenases toward Alkyl-Substituted Cyclohexanones

Brian G. Kyte,[†] Pierre Rouvière,[‡] Qiong Cheng,[‡] and Jon D. Stewart*,[†]

127 Chemistry Research Building, University of Florida, Gainesville, Florida 32611, and DuPont, Inc., Experimental Station, E328, Wilmington, Delaware 19880-0328

jds2@chem.ufl.edu

Received August 7, 2003

Genes encoding eight Baeyer–Villiger monooxygenases have recently been cloned from bacteria inhabiting a wastewater treatment plant. We have carried out a systematic investigation in which each newly cloned enzyme, as well as the cyclohexanone monooxygenase from *Acinetobacter* sp. NCIB 9871, was used to oxidize 15 different alkyl-substituted cyclohexanones. The panel of substrates included equal numbers of 2-, 3-, and 4-alkyl-substituted compounds to probe each enzyme's stereoselectivity toward a homologous series of synthetically important compounds. For all 4-alkyl-substituted cyclohexanones tested, enzymes were discovered that afforded each of the corresponding (*S*)-lactones in ≥98% ee. This was also true for the 2-alkyl-substituted cyclohexanones examined. The situation was more complex for 3-akyl-substituted cyclohexanones. In a few cases, single Baeyer–Villiger monooxygenases possessed both high regio- and enantioselectivities toward these compounds. More commonly, however, they showed only one type of selectivity. Nonetheless, enzymes with such properties might be useful as parts of a two-step bioprocess where an initial kinetic resolution is followed by a regioselective oxidation on the isolated, optically pure ketone.

Introduction

Homochiral ϵ-caprolactones are widely used building blocks in organic and polymer synthesis. Among the variety of possible routes that have been explored,[1–5] enantioselective Baeyer–Villiger oxidations of appropriately substituted cyclohexanones constitute a particularly convenient pathway to these compounds since the ketone precursors can be prepared by well-established methods. A number of metal-based Baeyer–Villiger catalysts have been reported, and some of these show impressive stereoselectivities, albeit on a limited range of substrates.[6–11] It should be noted, however, that such "chemical" Baeyer–Villiger oxidants offer a clear strategy for improving substrate- and stereoselectivities by altering ligand structures and reaction conditions and, in principle, provide access to the antipodal product if the metal ligand(s) can be obtained in mirror image form.

The stereoselectivities of enzymatic Baeyer–Villiger oxidations are almost uniformly very high for those ketones accepted as substrates. Flavoprotein monooxygenases utilize atmospheric O_2 as the oxidant and yield water as the waste product, which minimizes the environmental burden of these reactions.[12–15] Biocatalytic strategies, however, can also be problematic when it is necessary to obtain both product enantiomers. This means that a different enzyme with complementary stereoselectivity (rather than the enantiomer of an existing catalyst) must be identified. Consequently, the practical impact of enzymatic Baeyer–Villiger oxidations in preparative organic chemistry will be increased signifi-

* To whom correspondence should be addressed: Phone: (352) 846-0743. Fax: (352) 846-2095.
[†] University of Florida.
[‡] DuPont, Inc.

(1) Pirkle, W. H.; Adams, P. E. *J. Org. Chem.* **1979**, *44*, 2169–2175.
(2) Blanco, L.; Guibé-Jampel, E.; Rousseau, G. *Tetrahedron Lett.* **1988**, 1915–1918.
(3) Fouque, E.; Rousseau, G. *Synthesis* **1989**, 661–666.
(4) Fellous, R.; Lizzani-Cuvelier, L.; Loiseau, M. A.; Sassy, E. *Tetrahedron: Asymmetry* **1994**, *5*, 343–346.
(5) Shioji, K.; Matsuo, A.; Okuma, K.; Nakamura, K.; Ohno, A. *Tetrahedron Lett.* **2000**, 8799–8802.
(6) Gusso, A.; Baccin, C.; Pinna, F.; Strukul, G. *Organometallics* **1994**, *13*, 3442–3451.
(7) Bolm, C.; Schlingloff, G.; Weickhardt, K. *Ang. Chem., Int. Ed. Engl.* **1994**, *33*, 1848–1849.
(8) Bolm, C.; Schlingloff, G.; Bienewald, F. *J. Mol. Catal. A: Chem.* **1997**, *117*, 347–350.
(9) Bolm, C.; Luong, T. K. K.; Schlingloff, G. *Synlett* **1997**, 1151.
(10) Strukul, G.; Varagnolo, A.; Pinna, F. *J. Mol. Catal. A: Chem.* **1997**, *117*, 413–423.
(11) Bolm, C.; Beckmann, O.; Cosp, A.; Palazzi, C. *Synlett* **2001**, 1461–1463.
(12) Willetts, A. *Trends Biotechnol.* **1997**, *15*, 55–62.
(13) Mihovilovic, M. D.; Müller, B.; Stanetty, P. *Eur. J. Org. Chem.* **2002**, 3711–3730.
(14) Alphand, V.; Carrea, G.; Wohlgemuth, R.; Furstoss, R.; Woodley, J. M. *Trends Biotechnol.* **2003**, *21*, 318–323.
(15) Kamerbeek, N. M.; Janssen, D. B.; van Berkel, W. J. H.; Fraaije, M. W. *Adv. Synth. Catal.* **2003**, *345*, 667–678.

10.1021/jo030253l CCC: $27.50 © 2004 American Chemical Society
Published on Web 12/12/2003

cantly by expanding the existing repertoire of known monooxygenases.

Cyclohexanone monooxygenase (E.C. 1.14.13.22) from *Acinetobacter* sp. NCIB 9871 has been the most extensively studied Baeyer–Villiger monooxygenase since its initial isolation and characterization by Trudgill and coworkers in 1976.[16] This enzyme has been shown to carry out asymmetric Baeyer–Villiger oxidations on a variety of cyclic ketones with high chemo-, regio-, and enantioselectivities (for recent reviews, see refs 13 and 17). The enzyme-catalyzed pathway is analogous to the stereoelectronically governed Criegee mechanism followed in peracid-mediated Baeyer–Villiger oxidations.[18–21] This makes regioselectivity predictable by familiar rules. Preparative reactions involving *Acinetobacter* sp. NCIB 9871 cyclohexanone monooxygenase have been carried out with the purified or semipurified enzyme (with provision for regeneration of the essential NADPH cofactor), by whole cells of wild-type or mutant *Acinetobacter* strains, or with whole cells of recombinant baker's yeast or *Escherichia coli* cells (reviewed in refs 13 and 17). Genetically engineered strains are particularly convenient since they minimize the possibility of competing side-reactions and provide high enzyme levels that maximize reaction rates. With recent advances in process development that have significantly increased the volumetric productivities of these whole-cell-mediated bioconversions,[22,23] the major issue in enzymatic Baeyer–Villiger oxidations is the limited array of products that can be provided solely by the *Acinetobacter* sp. NCIB 9871 enzyme.

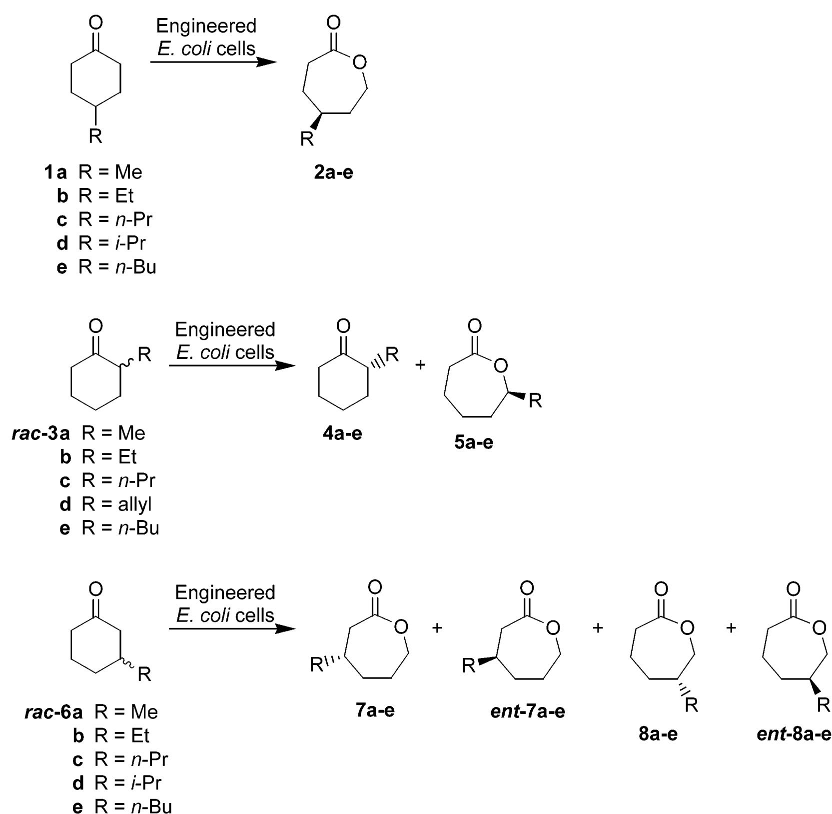
Several groups have recently addressed the lack of diversity in Baeyer–Villiger monooxygenases. Over the past 30 years, several enzymes have been purified from bacterial species;[24–26] unfortunately, the corresponding genes were not cloned, with one exception.[27] The lack of a cloned gene makes it impossible to use genetically engineered strains to overproduce the corresponding protein. This problem has been addressed by Witholt,[28] Lau,[29] Rouvière,[30–32] and Cheng,[33] who have utilized molecular biology strategies to clone a variety of genes encoding novel Baeyer–Villiger monooxygenases. Their efforts have dramatically increased the available enzyme diversity. Unfortunately, with a few exceptions,[13,29,34] this growth in monooxygenase availability has not been matched by a corresponding increase in knowledge of their substrate selectivities and enantioselectivities. Here, we report the systematic characterization of eight novel Baeyer–Villiger monooxygenases cloned by DuPont workers from organisms found in a wastewater treatment plant. While preliminary characterization experiments had verified that each was a bona fide Baeyer–Villiger monooxygenase, neither their substrate selectivities nor enantioselectivities had been established prior to these studies. We therefore used whole cells of engineered *E. coli* strains expressing each of the new enzymes to determine the outcomes of a series of reactions involving a homologous series of 2-, 3-, and 4-alkyl-substituted cyclohexanones. We have previously used this same series of ketones to profile the properties of the *Acinetobacter* sp. NCIB 9871 cyclohexanone monooxygenase, which provides a basis for comparison with the new data.[35]

Results and Discussion

Most of the substituted cyclohexanones required for this study were available commercially (Scheme 1). The remaining compounds were synthesized by standard methods.[36–38] Baeyer–Villiger oxidations of all cyclohexanones were carried out on 1 mmol scales with growing cells of *E. coli* strains that expressed a single heterologous monooxygenase enzyme. The appropriate genes were inserted into standard expression plasmids.[30–33] Monooxygenase production was induced by adding isopropylthio-β-D-galactoside (IPTG) when the cultures reached the late logarithmic phase of growth. The appropriate cyclohexanone and a stoichiometric quantity of cyclodextrin (if necessary) were added to a final concentration of 10 mM 30 min thereafter. The reactions were allowed to proceed at room temperature until complete.[39] For conversions involving kinetic resolutions, 50% completion was reached within 2–6 h. Oxidations of prochiral cyclohexanones were completed within 12 h. Traces of indole were the only byproduct detected in the reaction mixtures, and chemical yields were uniformly high (according to GC analyses that included an internal standard). Both the extent of conversion and the enantiomeric

(16) Donoghue, N. A.; Norris, D. B.; Trudgill, P. W. *Eur. J. Biochem.* **1976**, *63*, 175–192.
(17) Stewart, J. D. *Curr. Org. Chem.* **1998**, *2*, 211–232.
(18) Schwab, J. M. *J. Am. Chem. Soc.* **1981**, *103*, 1876–1878.
(19) Schwab, J. M.; Li, W.-b.; Thomas, L. P. *J. Am. Chem. Soc.* **1983**, *105*, 4800–4808.
(20) Ryerson, C. C.; Ballou, D. P.; Walsh, C. T. *Biochemistry* **1982**, *21*, 2644–2655.
(21) Sheng, D.; Ballou, D. P.; Massey, V. *Biochemistry* **2001**, *40*, 11156–11167.
(22) Walton, A. Z.; Stewart, J. D. *Biotechnol. Prog.* **2002**, *18*, 262–268.
(23) Doig, S. D.; Avenell, P. J.; Bird, P. A.; Gallati, P.; Lander, K. S.; Lye, G. J.; Wohlgemuth, R.; Woodley, J. M. *Biotechnol. Prog.* **2002**, *18*, 1039–1046.
(24) Griffin, M.; Trudgill, P. W. *Biochem. Soc. Trans.* **1973**, *1*, 1255–1258.
(25) Britton, L. N.; Brand, J. M.; Markovetz, A. J. *Biochim. Biophys. Acta* **1974**, *369*, 45–49.
(26) van der Werf, M. J. *Biochem. J.* **2000**, *347*, 693–701.
(27) Kamerbeek, N. M.; Moonen, M. J. H.; van der Ven, J. G. M.; van Berkel, W. J. H.; Fraaije, M. W.; Janssen, D. B. *Eur. J. Biochem.* **2001**, *268*, 2547–2557.
(28) van Beilen, J. B.; Mouriane, F.; Seeger, M. A.; Kovac, J.; Li, Z.; Smits, T. H. M.; Fritsche, U.; Witholt, B. *Environ. Microbiol.* **2003**, *5*, 174–182.
(29) Iwaki, H.; Hasegawa, Y.; Wang, S.; Kayser, M. M.; Lau, P. K. C. *Appl. Environ. Microbiol.* **2002**, *68*, 5671–5684.
(30) Brzostowicz, P. C.; Gibson, K. L.; Thomas, S. M.; Blasko, M. S.; Rouviere, P. E. *J. Bacteriol.* **2000**, *182*, 4241–4248.
(31) Brzostowicz, P. C.; Blasko, M. S.; Rouvière, P. E. *Appl. Microbiol. Biotechnol.* **2002**, *58*, 781–789.
(32) Brzostowicz, P. C.; Walters, D. M.; Thomas, S. M.; Nagarajan, V.; Rouvière, P. E. *Appl. Environ. Microbiol.* **2003**, *69*, 334–342.
(33) Kostichka, K.; Thomas, S. M.; Gibson, K. J.; Nagarajan, V.; Cheng, Q. *J. Bacteriol.* **2001**, *183*, 6478–6486.
(34) Mihovilovic, M. D.; Rudroff, F.; Müller, B.; Stanetty, P. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1479–1482.
(35) Stewart, J. D.; Reed, K. W.; Martinez, C. A.; Zhu, J.; Chen, G.; Kayser, M. M. *J. Am. Chem. Soc.* **1998**, *120*, 3541–3548.
(36) Stork, G.; Brizzolara, A.; Landesman, H.; Szmuszkovicz, J.; Terrell, R. *J. Am. Chem. Soc.* **1963**, *85*, 207–222.
(37) Eschenmoser, A.; Schreiber, J.; Julia, S. A. *Helv. Chim. Acta* **1953**, *36*, 482–488.
(38) Stork, G.; Danheiser, R. L. *J. Org. Chem.* **1973**, *38*, 1775–1776.
(39) A control reaction in which cyclohexanone and isopropyl-thio-β-D-galactoside were added to a culture of *E. coli* TOP10 cells lacking a plasmid showed no ϵ-caprolactone formation, demonstrating that the cloned monooxygenases were responsible for all of the Baeyer–Villiger oxidations observed.

SCHEME 1



purity of the residual starting material and the lactone product(s) were assessed by GC under conditions that allowed baseline resolution of the racemic materials. Parallel GC analysis of authentic standards revealed the absolute configurations of most species and their order of elution from a chiral-phase GC column. For reactions involving kinetic resolutions, at least five substrate enantiomeric excess values were determined during the first 50% of the reaction; then, these data were analyzed by computer fitting to an equation that allowed calculation of the enantioselectivity value (E).[40,41]

4-Alkyl-Substituted Cyclohexanones. Depending on the substitution pattern, Baeyer–Villiger oxidations of cyclohexanones can either involve desymmetrization (in the case of mesomeric cyclohexanones **1a**–**e**) or kinetic resolutions (**3a**–**e** and **6a**–**e**) (Scheme 1). The former case is relatively straightforward since only a single lactone regioisomer is possible. *Acinetobacter* sp. NCIB 9871 cyclohexanone monooxygenase generally shows good (*S*)-selectivity in conversions of 4-alkyl-substituted cyclohexanones; however, two cases are problematic: (*S*)-**2c** is available in only 92% ee, and *n*-butyl-substituted cyclohexanone **1e** is accepted as a substrate only at very high enzyme concentrations and with low enantioselectivity.[35,42–44] We hoped that one or more of the new Baeyer–Villiger monooxygenases would provide solutions to both of these problems.

The results of enzymatic Baeyer–Villiger oxidations of **1a**–**e** are shown in Figure 1. Nearly all of the enzyme–substrate pairs exhibited predominantly (*S*)-selectivity. In the few cases where (*R*)-lactones were formed preferentially, the enantiomeric purities were not synthetically useful (<60%). On the other hand, the expanded collection of enzymes did provide the two (*S*)-ϵ-caprolactone building blocks that were not produced effectively by the original *Acinetobacter* enzyme (**2c** and **2e**). Cyclohexanone monooxygenases from *Brevibacterium* sp. (ChnB1), *Acidovorax* CHX, and *Rhodococcus* SC1 all oxidized **1c** with very high (*S*)-enantioselectivity, and the *Brevibacterium* sp. ChnB1 and *Rhodococcus* SC1 enzymes also showed complete stereoselectivity in the oxidation of 4-*n*-butylcyclohexanone **1e**.

2-Akyl-Substituted Cyclohexanones. As expected, only the "normal" lactone regioisomer was formed in all biological oxidations of cyclohexanones **3a**–**e**. *Acinetobacter* sp. NCIB 9871 cyclohexanone monooxygenase exhibits very high E values favoring the (*S*)-ketones in conversions of racemic **3b**–**e** where substituents are larger than a methyl group.[41] It shows only limited enantioselectivity toward **3a**, however ($E = 10$). Finding a solution to this problematic reaction was therefore a key goal.

We were gratified to discover that the cyclododecanone monooxygenase from *Rhodococcus* SC1 oxidized **3a** with almost complete (*S*)-selectivity ($E \geq 200$). This is an unusual trait since all of the other enzymes showed poor

(40) Chen, C.-S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294–7299.

(41) Stewart, J. D.; Reed, K. W.; Zhu, J.; Chen, G.; Kayser, M. M. *J. Org. Chem.* **1996**, *61*, 7652–7653.

(42) Taschner, M. J.; Black, D. J. *J. Am. Chem. Soc.* **1988**, *110*, 6892–6893.

(43) Taschner, M. J.; Black, D. J.; Chen, Q.-Z. *Tetrahedron: Asymmetry* **1993**, *4*, 1387–1390.

(44) The presence of an isopropyl substituent in **1d** inverts the (*R*)-/(*S*)- designation because of a change in priority assignments. The absolute sense of stereoselectivity is the same as in the majority of cases. This is also true for **3d** and **6d**. Data for these substrates are marked by an asterix to highlight this phenomenon.

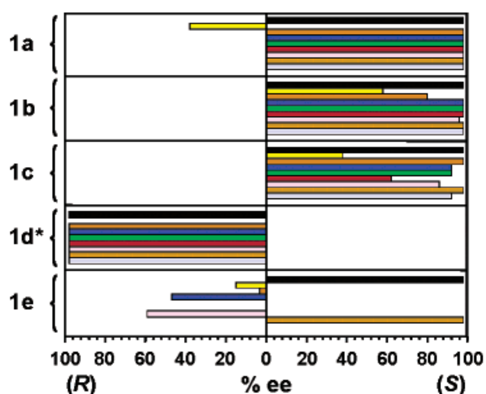


FIGURE 1. Baeyer–Villiger oxidations of 4-alkyl-substituted cyclohexanones **1a**–**e**. Enantiomeric excess values for reactions allowed to proceed to completion are indicated. For each substrate, the bar colors indicate the identities of the organisms from which the Baeyer–Villiger monooxygenase was originally cloned: *Brevibacterium* sp. ChnB1, black; *Brevibacterium* sp. ChnB2, yellow; *Acidovorax* CHX, orange; *Acinetobacter* SE19, dark blue; *Arthrobacter* BP2, green; *Rhodococcus* phi1, red; *Rhodococcus* phi2, pink; *Rhodococcus* SC1, brown; *Acinetobacter* sp. NCIB 9871, light blue. Where a bar is absent, no reaction occurred and only starting material was observed by GC. The asterix next to **1d** indicates that the apparent selectivity change is due to a change in substituent priority numbers.

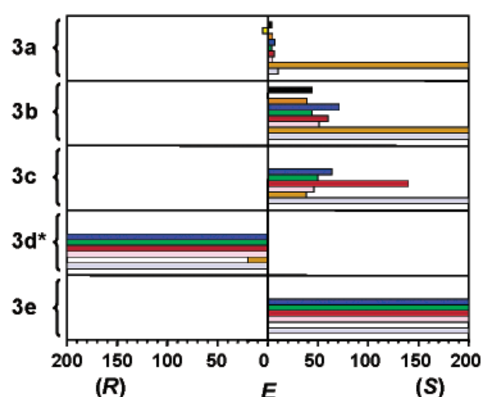


FIGURE 2. Baeyer–Villiger oxidations of 2-alkyl-substituted cyclohexanones **3a**–**e**. Enantioselectivity values (E) were determined by computer fitting of GC data obtained from the complete time course of each reaction to an equation derived from the theoretical expression relating the enantiomeric excess of residual substrate (ee_S) with the fractional conversion (c): $c = ([\{1 + ee_S\}^E]/[1 - ee_S])^{1/(1-E)}$. The bar colors indicate the identities of the organisms from which the Baeyer–Villiger monooxygenases were cloned (same as in Figure 1). Where a bar is absent, no reaction occurred and only starting material was observed by GC. The asterix next to **3d** indicates that the apparent selectivity change is due to a change in substituent priority numbers.

enantiomeric discrimination toward this substrate (Figure 2). In fact, most of the newly cloned Baeyer–Villiger monooxygenases oxidized 2-alkyl-substituted cyclohexanones with lower stereoselectivities than the *Acinetobacter* sp. NCIB 9871 enzyme. This monooxygenase and that from *Rhodococcus* SC1 are the most useful for synthetic purposes since together they allow both the (*R*)-ketones and (*S*)-lactones to be prepared in very high enantiomeric purities from all of the 2-substituted cyclohexanones examined here.

3-Aklyl-Substituted Cyclohexanones. Baeyer–Villiger oxidations of cyclohexanones **6a**–**e** are more complicated than the examples discussed above since both bonds flanking the carbonyl have nearly equal electron density (Scheme 1). In the absence of enzymatic guidance, oxidations of such ketones afford almost equal amounts of both regioisomers; when carried out on racemic ketones, four different lactone products result. *Acinetobacter* sp. NCIB 9871 cyclohexanone monooxygenase shows very interesting behavior toward this group of substrates. In the case of methyl- and ethyl-substituted **6a** and **6b**, respectively, the two ketone enantiomers are oxidized cleanly to divergent lactone regioisomers, although the process occurs with E values only slightly favoring the (*R*)-ketones (2 and 18, respectively).[35] By contrast, as the steric size of the substituent is increased (**6c**–**e**), high regioselectivity is observed (favoring **8** and *ent*-**8**), although E values are modest.[35] For this series of substrates, it is thus important to specify not only the E value but also the regioselectivity of the oxidations. The latter property is reported as percent regioisomeric excess (% re), which was calculated after all of the ketone had been consumed. The definition of this quantity is analogous to those for enantiomeric and diastereomeric excess values.[45]

There are four possible outcomes for enzymatic Baeyer–Villiger oxidations of 3-alkyl-substituted cyclohexanones: (1) high enantio- and regioselectivity ($E \geq 60$, % re ≥ 98), (2) high enantioselectivity but poor regioselectivity ($E \geq 60$, % re < 98), (3) poor enantioselectivity with high regioselectivity ($E < 60$, % re ≥ 98), and (4) low enantio- and regioselectivity ($E < 60$, % re < 98). While case one is clearly the most desirable, cases two and three also have synthetic value. Highly enantioselective oxidations provide access to optically pure ketones by removal of the undesired antipode (case two). After separation from the unwanted lactone(s), the remaining ketone can be converted to a homochiral lactone by subsequent oxidation using an enzyme with poor enantioselectivity but high regioselectivity (case three). In our study of newly cloned Baeyer–Villiger monooxygenases (Figure 3), we found examples of all four classes of behavior, with type three being most common (15 occurrences). Interestingly, none of the enzymes studied here favored formation of regioisomer **7**. Lactones with the general structure of **7** were either produced in a 1:1 ratio with **8** or they were the minor component in the final product mixture.

Having access to pairs of enzymes that selectively deliver either product enantiomer is a key goal in biocatalysis. The oxidation of **6e** by the ChnB1 and ChnB2 enzymes from *Brevibacterium* sp. provides an excellent example of this phenomenon. Both conversions are highly enantio- and regioselective, affording (*R*)- or (*S*)-**8e**, respectively. Unfortunately, this behavior was not general and these two enzymes showed relatively poor enantioselectivity toward the other 3-substituted cyclohexanones with the exception of the ChnB1-mediated oxidation of **6a**, which favored the (*S*)-ketone with an E value of 60. The only other oxidation that combined high regio- and enantioselectivity involved *n*-propyl-substi-

(45) Percent regioisomeric excess (% re) is defined as the percentage of the major regioisomer (both enantiomers) minus the percentage of the minor regioisomer (both enantiomers).

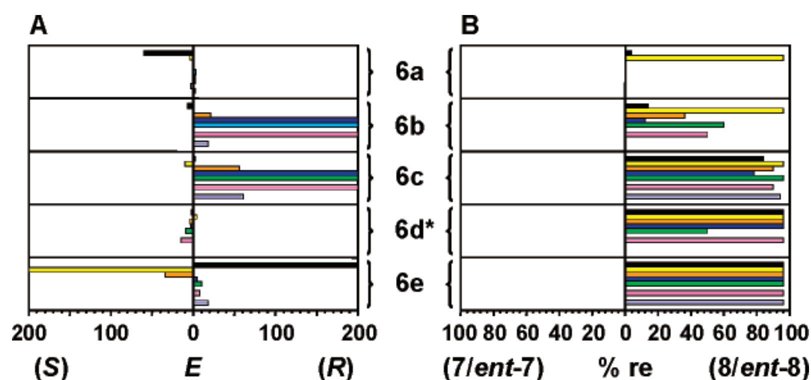


FIGURE 3. Baeyer–Villiger oxidations of 3-alkyl-substituted cyclohexanones **6a**–**e**. The bar colors indicate the identities of the organisms from which the monooxygenases were cloned (same as in Figure 1). Where a bar is absent, no reaction occurred and only starting material was observed by GC. (A) Enantioselectivity values for reactions of **6a** were determined as described previously (see legend to Figure 2). For other cases, the same equation was applied to data obtained from a single time point during the reaction time course after derivatization of the residual ketone. (B) The regioselectivities of Baeyer–Villiger oxidations of **6a**–**e** were determined by allowing reactions to proceed to completion and then subtracting the percentage of the minor regioisomer (**7** + *ent*-**7**) from the percentage of the major regioisomer (**8** + *ent*-**8**). This value, defined as the regioisomeric excess (% re), is depicted for each enzyme–substrate pair. The asterix next to **6d** indicates that the apparent selectivity change is due to a change in substituent priority numbers.

		R	R	R
Me	(R)		+	+
	(S)			+
Et	(R)			
	(S)		+	
n-Pr	(R)		+	
	(S)	+	+	
i-Pr / allyl*	(S)			
	(R)			
n-Bu	(R)		+	
	(S)	+	+	

FIGURE 4. Summary of substituted ϵ-caprolactones available in homochiral form by enzymatic Baeyer–Villiger oxidations. Compounds depicted with a solid green box can be produced in ≥98% ee directly by Baeyer–Villiger oxidations using at least one of the monooxygenases investigated here. Those shown with a white/green striped pattern are available by an initial kinetic resolution with one monooxygenase followed by a peracid-mediated Baeyer–Villiger oxidation or a second enzymatic oxidation. Plus signs denote ϵ-caprolactones newly available in homochiral form using one of the eight novel monooxygenases explored here. Compounds depicted with red boxes are not yet available in homochiral form using Baeyer–Villiger monooxygenases. The asterix next to the entries for R = *i*-Pr/allyl indicates that the apparent selectivity change is due to a change in substituent priority numbers.

tuted **6c**, which was converted cleanly by the *Arthrobacter* BP2 monooxygenase to (*R*)-**8c**.

Conclusion

Our long-term goal is to identify one or more Baeyer–Villiger monooxygenases capable of stereo- and regioselectively oxidizing all of the substituted cyclohexanones studied here to afford all possible alkyl-substituted ϵ-caprolactones in homochiral form. Our results are summarized in Figure 4, which indicates the spectrum of substituted ϵ-caprolactones that can be produced in ≥98% ee using either a single enzymatic oxidation or a chemoenzymatic or two-enzyme combination. Of the 21 available lactones in this series, just under half are novel building blocks made available by one or more of the newly cloned Baeyer–Villiger monooxygenases.

Baeyer–Villiger oxidations of 4-alkyl-substituted cyclohexanones are straightforward reactions that can only afford single lactone products. We were unable to identify monooxygenases that provided (*R*)-lactones from ketones **1a**–**e** with acceptable optical purities, although we were able to produce (*S*)-**2a**–**e** with uniformly high % ee values using biocatalysts identified in this study. Discovering (*R*)-selective Baeyer–Villiger monooxygenases remains an important goal that will require a further expanded set of enzymes.

The stereoselective oxidation of 2-alkyl-substituted cyclohexanones is now an essentially solved problem for the substrates investigated here. The intrinsic bias toward migration of the more-substituted carbon ensures high regioselectivity and the use of either the *Rhodococcus* SC1 (for **3a**) or *Acinetobacter* sp. NCIB 9871 monooxygenases (for **3b**–**e**) ensures efficient kinetic resolutions that yield homochiral lactones. The antipodes can be prepared by subsequent chemical oxidation of the recovered optically pure ketone, which occurs with retention of stereochemistry.

The newly cloned Baeyer–Villiger monooxygenases contribute most significantly to stereoselective oxidations of 3-alkyl-substituted cyclohexanones. These are also the most challenging class of substrates for stereoselective Baeyer–Villiger reactions. In some cases, e.g., **6c** and **6e**, single Baeyer–Villiger monooxygenases display both high enantio- and regioselectivities, thereby directly providing homochiral lactones from the corresponding racemic ketones. In other cases, judicious combinations of enzymes with differing selectivities could be employed sequentially to yield homochiral products. For example, (*S*)-**8b** could be prepared by oxidizing racemic **6b** with the *Acinetobacter* SE19 enzyme until 50% completion (to remove the undesired antipode), and then subjecting the isolated (*S*)-**6b** to a regioselective Baeyer–Villiger reaction catalyzed by the *Brevibacterium* sp. ChnB2 enzyme.

Sequences leading to (*R*)-**8a** and (*S*)-**8c** could be devised along similar lines.

Our focus in this study was to characterize the substrate selectivities and enantioselectivities of newly discovered enzymes, rather than on isolating each product. Previous efforts using either growing or nongrowing cells of an *E. coli* strain overexpressing *Acinetobacter* sp. NCIB 9871 cyclohexanone monooxygenase yielded product titers of 1–2[46] and 11 g/L,[22,47] respectively. Analogous applications of the strains investigated here are likely to be equally straightforward. The use of these novel Baeyer–Villiger monooxygenases in asymmetric synthesis will be reported in due course.

Experimental Section

Standards of racemic lactones were prepared by oxidation of ketones with *m*-CPBA as reported previously.[35] Biotransformations were monitored by GC using DB-17 and Chirasil-Dex CB columns for nonchiral and chiral separations, respectively. The absolute configurations of products and the order of elution during chiral-phase GC analysis were determined by comparisons with authentic standards.[35] All of the strains used in these studies can be obtained by contacting Drs. Pierre Rouvière and Qiong Cheng at DuPont, Inc.

General Procedure for Preparing Ketones 1e, 3b, and 3e. Pyridinium chlorochromate (67 mmol, 14.4 g) and methylene chloride (100 mL) were added to a 300 mL round-bottom flask. Neat alcohol (45 mmol) was added dropwise at room temperature. After TLC analysis (4:1 hexanes/ethyl ether) indicated that the reactions were complete (ca. 3 h), the mixtures were filtered through Celite 545 and washed with saturated $CuSO_4$ (4 × 100 mL). After drying with Na_2SO_4, the solvent was removed by rotary evaporation. No further purification was required.

General Procedure for Preparation of 3-Alkyl-Substituted Cyclohexanones 6b–e. Enone intermediates were prepared from 1,3-cyclohexanedione according to literature procedures.[37,38] The appropriate Grignard reagent (45 mmol) was added to a solution of 3-isobutoxy-2-cyclohexen-1-one (30 mmol, 5.0 g) in 50 mL of anhydrous diethyl ether at room temperature over 30 min. After GC analysis indicated that the reaction had reached completion, the reaction was quenched by slowly adding 100 mL of 10% H_2SO_4. The aqueous layer was extracted with ethyl acetate (3 × 100 mL), and then the combined organic layers were washed with brine (200 mL), dried with anhydrous Na_2SO_4, and concentrated by rotary evaporation. The enones were purified by vacuum distillation. Saturated ketones were prepared by catalytic hydrogenation over palladium on carbon as described previously.[47] No further purification was required.

General Procedure for Baeyer–Villiger Oxidations Using Whole Cells of Engineered *E. coli* Strains. Overnight cultures were grown by adding a single colony of the appropriate strain to 10 mL of LB medium supplemented with 100 μg/mL ampicillin. After the cultures had shaken overnight at 37 °C, a 1 mL aliquot was added to a 100 mL portion of LB medium supplemented with 200 μg/mL of ampicillin (except for the strain expressing the *Rhodococcus* SC1 enzyme, which required 100 μg/mL ampicillin) in a 250 mL Erlenmeyer flask. The cultures were shaken at 150–200 rpm at 37 °C until they reached an OD_{600} between 1.8 and 2.0; then, isopropyl-β-D-galactoside (IPTG) was added to a final concentration of 0.10 mM. The cultures were shaken at 150 rpm for an additional 30 min at room temperature. If cyclodextrins were required (to solubilize the ketone), they were added to a concentration of 10 mM at this time. The appropriate ketone substrate was also added to a final concentration of 10 mM. Baeyer–Villiger oxidations were carried out by shaking the cultures at 150 rpm at room temperature. Samples for GC analysis were prepared by taking 100 μL aliquots of the reaction mixture and extracting with 900 μL of EtOAc that also contained 0.01% methyl benzoate as an internal standard. A 1.0 μL portion of the organic phase was used for GC analysis.

In reactions of 4-alkyl-substituted cyclohexanones **1a–e**, conversions were allowed to proceed to completion (as determined by normal-phase GC analysis); then, the optical purities of the lactones were determined by chiral-phase GC measurements.

Baeyer–Villiger oxidations of 2-alkyl-substituted cyclohexanones **3a–e** were initially carried out using normal-phase GC monitoring to determine whether a given enzyme showed activity toward that substrate and to establish an approximate time course for the conversion. The bioconversions were then repeated in order to determine *E* values accurately. At least five samples were taken between 0 and 50% fractional conversion (as determined by normal-phase GC analysis), and the enantiomeric purities of both the remaining ketone and the lactone product were determined by chiral-phase GC analyses. These data were fit directly to an analytical expression derived from Sih's theoretical analysis of kinetic resolutions.[40,41] The fits were inspected visually to ensure that no systematic deviations occurred (which would indicate that secondary processes such as enantioselective degradation had taken place during the bioconversion). No deviations were observed.

Baeyer–Villiger oxidations of 3-alkyl-substituted cyclohexanones **6a–e** were first monitored by normal-phase GC to determine whether a substrate was accepted by a given monooxygenase. These bioconversions were continued until all of the ketone substrate had been consumed to provide data for calculating the regioisomeric excess values.[45] All successful reactions were then repeated to determine the corresponding *E* values. The enantiomers of ketone **6a** could be separated cleanly by chiral-phase GC, and *E* values for this substrate were determined as described in the previous paragraph. The enantiomers of **6b–e** could not be separated under our conditions for chiral-phase GC analysis. These reactions were therefore run to 35–50% completion (as determined by normal-phase GC analysis), and then remaining substrate and lactone product(s) were removed by extraction with ethyl acetate (3 × 100 mL). After concentration by rotary evaporation, the residue was mixed with 1.5 equiv (relative to starting ketone concentration) of (2*R*,3*R*)-butanediol (1.5 mmol, 140 mg), a catalytic amount of *p*-toluenesulfonic acid, and 25 mL of benzene. The reaction mixture was held at reflux for 2 h using a Dean–Stark trap; then, it was cooled to room temperature, and an aliquot was analyzed by chiral-phase GC. The enantiomeric excess value determined for the residual ketone substrate, along with the fractional conversion value (assessed as described above by normal-phase GC), was used to calculate the *E* value.[40]

Acknowledgment. We thank Cerestar, Inc., for their generous donation of cyclodextrins. Financial support by the National Science Foundation (CHE-0130315) is also gratefully acknowledged.

Supporting Information Available: Detailed experimental procedures for all compounds synthesized in this study, GC analysis conditions and chromatograms for all racemic ketones and lactones with elution patterns indicated, procedures for strain storage, and a description of the methods used to construct an overexpression plasmid for the *Rhodococcus* SC1 cyclododecanone monooxygenase. This material is available free of charge via the Internet at http://pubs.acs.org.

JO030253L

(46) Mihovilovic, M. D.; Chen, G.; Wang, S.; Kyte, B.; Rochon, F.; Kayser, M. D.; Stewart, J. D. *J. Org. Chem.* **2001**, *66*, 733–738.
(47) Walton, A. Z.; Stewart, J. D. *Biotechnol. Prog.* **2003**, in press.