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Pichia stipitis OYE 2.6 variants with improved catalytic efficiencies from site-saturation mutagenesis libraries

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ABSTRACT

An earlier directed evolution project using alkene reductase OYE 2.6 from *Pichia stipitis* yielded 13 active site variants with improved properties toward three homologous Baylis–Hillman adducts. Here, we probed the generality of these improvements by testing the wild-type and all 13 variants against a panel of 16 structurally-diverse electron-deficient alkenes. Several substrates were sterically demanding, and as hoped, creating additional active site volume yielded better conversions for these alkenes. The most impressive improvement was found for 2-butylidenecyclohexanone. The wild-type provided less than 20% conversion after 24 h; a triple mutant afforded more than 60% conversion in the same time period. Moreover, even wild-type OYE 2.6 can reduce cyclohexenones with very bulky 4-substituents efficiently.

1. Introduction

In 1932, Warburg and Christian isolated a protein from brewers' bottom yeast with a tightly associated yellow pigment (Farbstoffkomponente[1]).[2,3] The yellow substance, later identified as flavin mononucleotide (FMN), was the first example of a catalytically essential enzyme component that was not part of the polypeptide chain itself. The enzyme later became known as old yellow enzyme (OYE) and it has served as the prototype for this class of flavoproteins as well as an important model system for understanding protein—flavin interactions and flavoproteins in general. In particular, the Massey group carried out extensive biophysical studies of OYE over the years[4] and the enzyme's crystal structure was solved by Fox and Karplus in 1994.[5] The FMN cofactor of OYE can be efficiently reduced by NADPH and subsequently reoxidized by electron transfer to O_2 as well as a variety of non-physiological partners such as ferricyanide. Because O_2 reduction was relatively slow, however, this reaction was judged unlikely to be physiologically relevant. Despite intensive investigations, the biological role of OYE in yeast is still unknown, as is the identity of its 'normal' electron acceptor(s).

In 1995, Massey reported that the reduced FMN of OYE could transfer electrons to the alkene moieties of conjugated aldehydes and ketones and the stereochemical course was shown to involve net *trans*-addition of H_2.[6] Stereoselective alkene reductions are valuable transformations in asymmetric synthesis; moreover, because typical organometallic hydrogenation catalysts generally perform poorly with enones and enals, interest in these enzymatic alternatives has grown rapidly (recently reviewed in[7–9]). Homologs of the original brewers' bottom yeast (*Saccharomyces pastorianus*) OYE have been found in nearly all bacteria, fungi and many plants. Several have been cloned, overexpressed and evaluated for synthetic potential (for recent examples, see[10–12] and references therein). Finding alkene reductases with both broad substrate acceptance and good catalytic rates, particularly for larger alkenes, has been the goal of many of these studies. Several groups have improved alkene reductases using protein engineering (for recent examples, see[13] and references therein.

OYE 2.6 from *Pichia stipitis* is an OYE homolog that is more stable than *S. pastorianus* OYE under process conditions, which increases its utility in asymmetric synthesis. The enzyme also shows very high stereoselectivities toward a series of Baylis–Hillman adducts whose reductions yield useful synthetic building blocks.[14] We have solved the X-ray crystal structure of OYE 2.6, in free form and complexed with nicotinamide (an NADPH analog) and *p*-chlorophenol (a substrate analog).[15] These studies provided important insights into substrate discrimination and the stereochemical courses of alkene reductions. In addition, they also suggested fruitful targets for mutagenesis in order to improve the catalytic performance of OYE 2.6 for synthetic applications. We recently completed an extensive directed evolution study of OYE 2.6 that focused on altering its catalytic efficiencies for three representative Baylis–Hillman adducts **1**–**3** (Fig. 1).[16] Three successive rounds of active site-targeted mutagenesis were carried out, and a number of second- and third-generation library members

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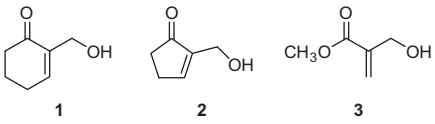

Figure 1. Structures of Baylis–Hillman substrates employed in the earlier directed evolution study. A total of 13 OYE 2.6 active site residues were examined by site-saturation mutagenesis and variants with inverted stereoselectivity for alkenes **2** and **3** were identified after two and three successive rounds, respectively.[16]

showed interesting changes in catalytic properties for these alkenes. Here, we ask the question, were these improved properties limited only to the three substrates actually used to screen the libraries, or can these enzyme variants also reduce other, structurally unrelated alkenes with greater efficiencies? To answer this, we assembled a panel of representative alkenes (Fig. 2) and tested each as a substrate for the wild-type and 13 key OYE 2.6 variants. Our goal was to probe changes in accessible active site volume by systematic variations in substrate structure. Because the conformational preferences of 2-cyclohexenones are easily predictable, we focused particular attention on this subclass.

Alterations at three active site residues had the greatest impact on OYE 2.6 catalytic properties toward alkenes **1**–**3**. When viewed from above (the direction from which substrates enter the active site), the side-chains of Tyr 78 and Ile 113 form one side of the substrate binding pocket and that of Phe 247 interacts with the other (Fig. 3). To form a catalytically productive enzyme–substrate complex, two atoms must occupy specific positions: the carbonyl oxygen forms hydrogen bonds with the side-chains of His 188 and His 191 that polarize the conjugated alkene to accept a hydride from N_5 of reduced FMN, whose distance and angle with respect to the substrate β-carbon are tightly constrained.[17]

Replacing Tyr 78 with Trp dramatically impacted substrate binding interactions[16], so this mutant was assessed singly and the change was retained in all subsequent second- and third-generation variants. In a Tyr 78 Trp background, replacing Ile 113 with Cys or with hydrophobic residues (Phe, Leu, Met or Val) significantly impacted reductions of alkenes **1**–**3**. Likewise, substituting Ala, His or Trp for Phe 247 in a Tyr 78 Trp background improved the catalytic properties of OYE 2.6 when assessed against alkenes **1**–**3**. Finally, we also examined third-generation variants derived from site-saturation mutagenesis libraries at position 113 in proteins already containing Tyr 78 Trp/Phe 247 Ala or Tyr 78 Trp/Phe 247 His mutations. That both mutagenesis pathways converged to the same pair of amino acids at position 113 (Cys or Val), substitutions previously found to be beneficial in a second-generation library suggests that we have adequately covered the available sequence space within the active site of OYE 2.6.

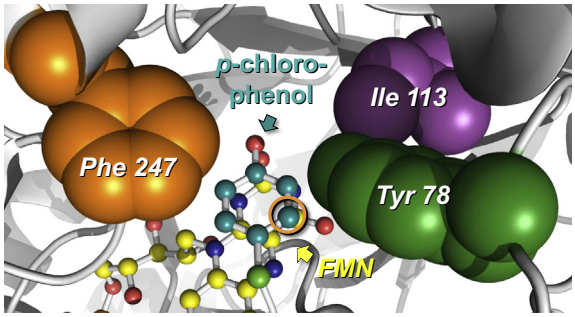

Figure 3. X-ray crystal structure *P. stipitis* OYE 2.6 with bound *p*-chlorophenol (PDB code 4DF2).[15] *p*-Chlorophenol binds between the side-chains of Tyr 78 (green), Ile 113 (purple) and Phe 247 (orange), shown in space-filling representations. Alkene substrates place their carbonyl oxygen at the location of the phenolic hydroxyl moiety and their electron-deficient β-carbon at the position indicated by the orange circle in order to achieve the proper geometry for efficient hydride transfer from N_5 of the reduced FMN (carbons shown in yellow).

2. Results and discussion

2.1. Protein isolation and catalytic assays

During the earlier directed evolution campaign, alkene reductions were carried out by mixing intact cells of the appropriate *Escherichia coli* overexpression strains with the substrate. Whole cell-mediated reactions are experimentally simple and lend themselves to medium-throughput screening methods. One drawback is that the observed conversion efficiencies depend on both the enzyme's intrinsic catalytic ability and its overexpression level. We eliminated the latter as a potential influence by overproducing wild-type OYE 2.6 and all 13 variants in *E. coli* as fusion proteins with glutathione *S*-transferase and purifying them individually by one-step affinity chromatography on glutathione–agarose.[14] SDS–PAGE analysis confirmed that all proteins were at least 90% pure. All reactions in this study used a consistent protein quantity (ca. 100 μg/reaction), so the observed fractional conversions roughly reflect relative reaction rates.

Most of the alkene substrates were commercially available; those that were not (**4**–**7** and **12**–**15**) were prepared by literature methods.[18] Authentic standards of racemic and/or diastereomeric mixtures of reduction products were prepared by individually reducing all substrates with H_2 in the presence of Pd/C.

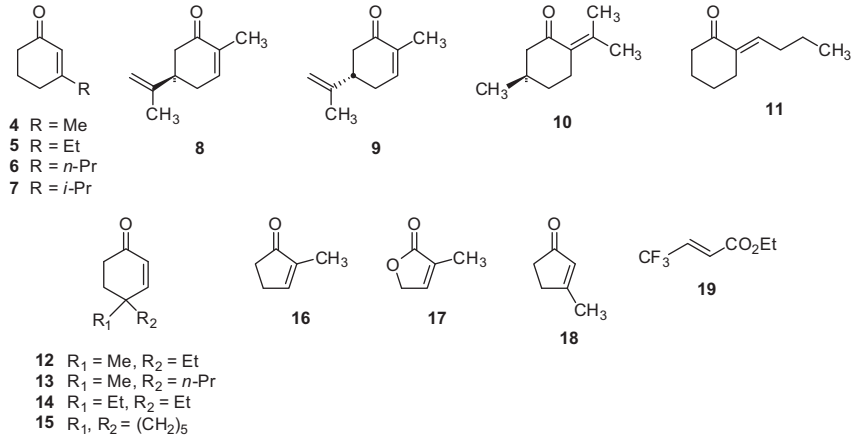

Figure 2. Structures of alkene substrates examined in this study.

Enzyme-catalyzed alkene reductions were carried out at room temp for 24 h at pH 7.0 in total volumes of 0.30 mL with 10 mM substrate and 4.6 μM fusion protein. Conversions and stereoselectivities (when appropriate) were assessed by GC after extracting with EtOAc.

2.2. Cyclohexyl substrates

In common with all conjugate additions, enzyme-catalyzed alkene reductions are acutely sensitive to steric hindrance at the β-carbon. This is one factor that makes alkenes **4**–**7** challenging substrates. Beyond their intrinsically disfavored β,β-disubstitution patterns, reduction rates can be further depressed by steric clash between the exocyclic β-substituent and active site protein moieties. Indeed, an earlier study using *S. pastorianus* OYE to reduce 2-cyclohexenones **4**–**7** found a very significant negative correlation between β-substituent size and reduction rates and usable conversions were only obtained for **4** and **5.**[18a] A similar trend was observed for wild-type *P. stipitis* OYE 2.6, where even the best substrate (**4**) gave only 55% conversion after 24 h (Fig. 4). Replacing Tyr 78 with Trp, either singly or with concomitant changes at position 113 and/or 247 gave >98% conversion of **4** within the same time period. This is a significant improvement. Similar trends were observed for **5** and **6**, although the optimal replacements for Ile 113 differed (Met and Leu, respectively). The Tyr 78 Trp/Ile 113 Met double mutant reduced **7** to the greatest extent; however, the low conversion for even this 'best' mutant lessens the practical utility. In each case, better rates were obtained from enzymes with smaller side-chains at position 113, consistent with a steric explanation of the results. It might be possible to obtain even better conversions by exploring additional, smaller Ile 113 replacements. It is important to note that the mutations did not erode stereoselectivity, and only the (*S*)-products were detectable by GC in all cases.

In contrast to β-substituted 2-cyclohexenones, α-substitution does not significantly impair reduction rates by alkene reductases. This was readily apparent in conversions of (*R*)- and (*S*)-carvone (**8** and **9**, respectively), which were reduced almost quantitatively by all proteins examined (Fig. 5). In addition, no change in stereochemical preference was observed for reductions of **8** and **9** when Ile 113 was substituted by other amino acids. This contrasts sharply with the behavior of analogous mutants of *S. pastorianus* OYE.[19]

We tested two exocyclic enones, (*R*)-pulegone **10** and *n*-butyl-substituted **11** since these have proven to be challenging substrates for alkene reductases. The presence of a tetrasubstituted alkene in **10** makes this enone especially resistant to enzyme-catalyzed reductions. As noted above, efficient reduction requires that the ketone oxygen and alkene β-carbon occupy specific locations and while it is not possible to place **10** and **11** exactly congruently with the most preferred positions, the arrangements shown in Figure 6 are reasonable approximations. The wild-type and most mutants reduced **10** with good efficiencies and with consistent stereoselectivities that could be explained by the binding orientation shown in Figure 6. When compared with the parent 2-cyclohexenone, most of the added steric bulk of (*R*)-pulegone **10** resides on the 'western' side of the active site. Even without additional mutagenic sculpting, this region of the OYE 2.6 active site is relatively open, consistent with the observed behavior. By contrast, alkene **11** presents a more difficult challenge since the *n*-butyl chain impinges on the locations of side-chains at positions 78 and 113 and the ring requires space on the 'western' side, near the side-chain at position 247. Not unexpectedly, alkene **11** is a poor substrate for wild-type OYE 2.6 (ca. 20% conversion after 24 h); however, conversion was increased four-fold when the Tyr 78 Trp/Ile 113/Phe 247 His triple mutant was substituted. This is a very significant improvement and clearly demonstrates the value of combining mutations on both the 'eastern' and 'western' sides of the active site.

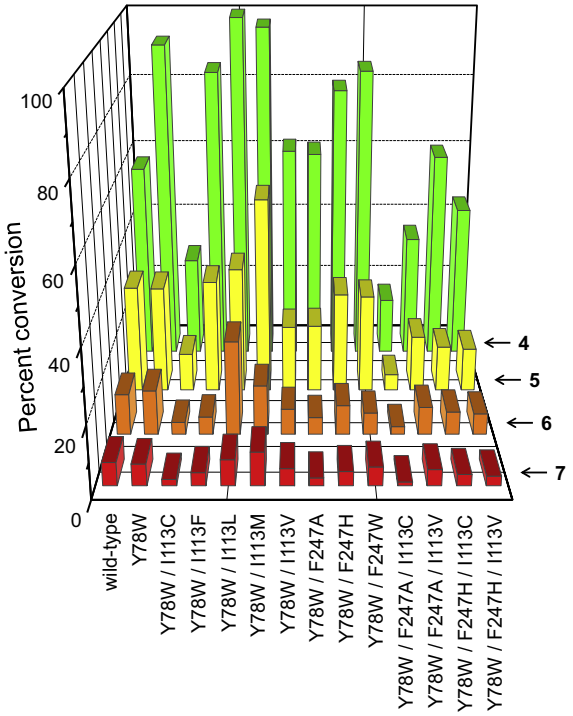

Figure 4. Catalytic activities of OYE 2.6 and mutants for alkenes **4**–**7**. Data are grouped by substrate (**4**, green; **5**, yellow; **6**, orange; **7**, red). In all cases, (*S*)-products were obtained in ⩾98% ee.

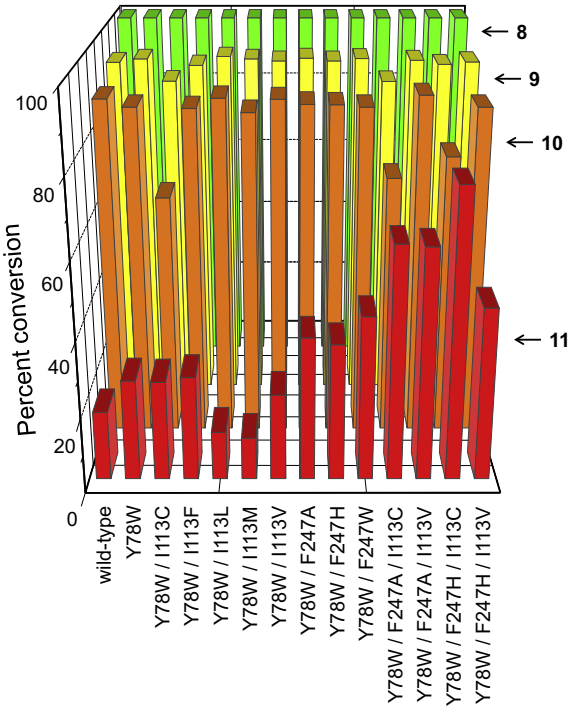

Figure 5. Catalytic activities of OYE 2.6 and mutants for alkenes **8**–**11**. Data are grouped by substrate (**8**, green; **9**, yellow; **10**, orange; **11**, red). In all cases, products were obtained in ⩾98% de (**8**–**10**) or ⩾98% ee (**11**).

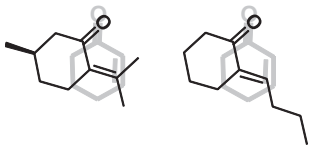

Figure 6. Approximate overlay of substrates 10 (left) and 11 (right) on the parent 2-cyclohexenone substrate (grey lines). Only the binding orientation show was considered since the alternative, flipped binding mode would be impossible to accommodate within the available active site volume.

The crystal structure of OYE 2.6 with bound *p*-chlorophenol shows significant accessible active site volume in the vicinity of the chlorine moiety (Fig. 3). Consistent with this notion, the wild-type and all 13 mutants examined reduced four representative 4,4-disubsituted 2-cyclohexenones with very bulky substituents (**12**–**15**), providing ⩾98% conversions after 24 h. Since the steric demands for these substrates are directed away from the positions of the three amino acids examined in our libraries, there was essentially no impact from changes at positions 78, 113 and/or 247. Nonetheless, these results underscore the ability of this enzyme to accommodate even relatively bulky alkenes, which is an important asset for synthetic applications.

2.3. Other substrates

α-Methyl cyclopentenone **16** was an excellent substrate for wild-type OYE 2.6 and all 13 mutants examined here, and quantitative conversions were observed in all cases (Fig. 7). While lactone analog **17** was also well-accepted by the wild-type enzyme (70% conversion after 24 h), several variants provided even higher conversions within the same time period. The acceptance of an ester such as **17** is valuable since acrylate derivatives are generally poor substrates for alkene reductases of the OYE superfamily. Consistent with the behavior encountered in 2-cyclohexenones **4**–**7**, a

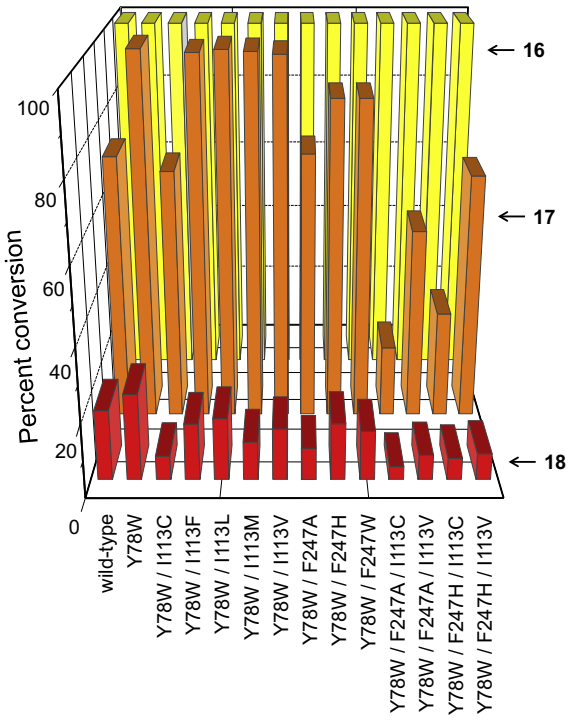

Figure 7. Catalytic activities of OYE 2.6 and mutants for alkenes **16**–**18**. Data are grouped by substrate (**16**, yellow; **17**, orange; **18**, red).

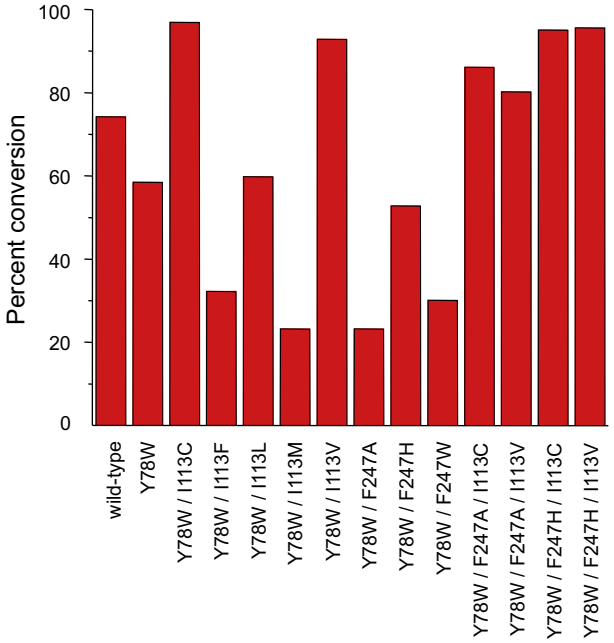

Figure 8. Catalytic activities of OYE 2.6 and mutants for alkene 19.

β-methyl group drastically reduced the conversion efficiency for **18**, and in this case, none of the mutants provide significant improvements.

Enzymes of the old yellow enzyme family generally reduce acyclic α,β-unsaturated esters with poor efficiencies unless an additional electron-withdrawing group is present (for recent examples, see[20,21] and references therein). To the best of our knowledge, a trifluoromethyl substituent had not been evaluated in this regard, and we therefore tested **19** as a representative example. The extent of reduction depended significantly on the identities of residues at position 113, and Cys and Val were particularly beneficial (Fig. 8). Wild-type OYE 2.6 gave ca. 75% conversion of 19 after 24 h. Replacing Tyr 78 with Trp lessened the conversion somewhat, but this could be reversed by compensating replacements for Ile 113. Interestingly, changes at position 247 were also deleterious in this case, but these could also be rescued by Cys or Val at position 113 (Fig. 8).

3. Conclusions

Given the time and effort required for directed evolution programs, it is important that they deliver enzyme variants with improved properties for a range of applications beyond the limited number that guided the mutagenesis. The major change by residues examined here was to increase the active site volume near the β- and β′-carbons of bound substrates. As anticipated, this increased the size of acceptable substrates for OYE 2.6. In principle, computer docking studies could be used to predict the acceptable substrate/protein pairs. On the other hand, many of the substrates examined here were conformationally mobile. Moreover, uncertainties in computing catalytically productive poses make the results of such calculations difficult to interpret. We therefore concentrated on making screening reactions as simple as possible, so that experimental answers can be obtained quickly and with minimal effort. It would be a simple matter to extend studies of the purified mutants described here to additional substrates of synthetic interest. Likewise, given the high sequence similarity between alkene reductases of the old yellow enzyme family, amino

acid variations identified in this study may well translate to other, related enzymes and may yield equally-useful mutants.

4. Experimental section

4.1. Protein overexpression and isolation

Strains overexpressing wild-type OYE 2.6 or a mutant fused to GST were grown overnight in LB medium[22] supplemented with 200 μg/mL ampicillin at 37 °C with shaking. Cultures were diluted 1: 100 into 500 mL of LB medium containing 200 μg/mL ampicillin, then shaken at 37 °C until they reached $O.D._{600} \approx 0.7$. IPTG and glucose were added to final concentrations of 0.5 mM and 0.4%, respectively, then cultures were shaken at 30 °C for an additional 3 h. Cells were collected by centrifugation and lysed using a French pressure cell. Insoluble debris was removed by centrifuging for 60 min at 30,000*g* at 4 °C. The GST-fusion proteins were purified by affinity chromatography on glutathione–agarose as described previously.[14] Following purification, proteins were concentrated by ultrafiltration, glycerol was added to a final concentration of 50% and the solutions were stored at −20 °C. Protein concentrations were estimated by Bradford assays using BSA as a standard.[23]

4.2. Substrate and product synthesis

Alkenes available commercially (**8**–**10**, **16**–**19**) were used without further purification. The remaining substrates were synthesized by literature methods. β-Substituted cyclohexenones **4**–**7** were prepared in an earlier study.[18a] Alkenes **12**–**15** were prepared by acid-catalyzed annelation of the appropriate aldehyde with methyl vinyl ketone using a literature procedure.[18b]

Authentic standards for each reduction product by stirring ethyl acetate solutions under an atmosphere of H_2 in the presence of Pd/C. Reactions were stirred at room temp until GC/MS indicated complete consumption of starting materials, the solvent was removed by rotary evaporation.

4.3. Screening reactions

Reaction mixtures (0.30 mL total volume) contained wild-type or a mutant OYE 2.6 (ca. 100 μg, 4.6 μM final concentration), glucose dehydrogenase (75 μg), $NADP^+$ (0.20 mM), glucose (220 mM) and alkene substrate (10 mM) in KP_i, pH 7.0. Reactions were incubated at room temp for 24 h, then extracted with an equal quantity of EtOAc and analyzed by GC.

Acknowledgment

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.07.001.

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