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Application of newly available bio-reducing agents to the synthesis of chiral hydroxy-β-lactams: model for aldose reductase selectivity

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Abstract—Homochiral 3-hydroxy-4-substituted β-lactams serve as precursors to the corresponding α-hydroxy-β-amino acids—key components of many biologically and therapeutically important compounds. We have developed a short synthetic sequence for these targets using a biocatalytic reduction to install the desired chirality. By employing 3-oxo-4-phenyl-β-lactam as a probe, 19 individual yeast reductases were screened for their efficiency and enantioselectivity with respect to this substrate. Four highly selective candidates all belonging to the aldose reductase (AKR) superfamily were identified. Two are *Re*-face selective and two are *Si*-face selective. The modelled three-dimensional structures of these enzymes provide a rationale for this behaviour and suggest strategies for their further improvement.

1. Introduction

Homochiral 3-hydroxy-4-substituted β-lactams are convenient precursors for β-amino-α-hydroxy acids, which are key building blocks for pharmaceutically important compounds such as phenylisoserine analogues used in the synthesis of new taxane anticancer drugs[1,2] and inhibitors of proteases such as renin[3] and HIV-1 protease.[4] Consequently, development of practical, stereoselective methods for their preparation continues to be of general interest.

Our approach to these building blocks has utilized bioreductions of α-keto-β-lactams in order to obtain the desired chirality by simple and inexpensive means.[5–8] The general strategy is illustrated in Scheme 1. Clearly, the stereoselectivity of the reduction step has the greatest impact on the synthetic utility of the overall route. We originally employed commercial bakers' yeast cells as well as engineered *Saccharomyces cerevisiae* strains with altered levels of specific intracellular reductases to reduce several α-keto-β-lactams substituted with aryl and alkyl groups in the 4-position. This approach was quite successful in preparing the enantiopure *cis*-(3*R*,4*S*) and *trans*-(3*R*,4*R*) diastereomers of 4-*tert*-butyl-3-hydroxy β-lactam; however, reductions of other substituted β-lactams were not adequately selective.[7]

Our examination of *S. cerevisiae* genome revealed that a large number of genes (⩾49) appeared to encode reductases.[9] If multiple yeast reductases accept a single substrate, but operate with divergent stereoselectivities, the result will be a mixture of products. The solution is to examine each enzyme separately. Previously, we had overexpressed four yeast reductases in *Escherichia coli* and used whole cells of these strains to reduce a series of 3-oxo-4-substituted β-lactams. One enzyme—Ara1p—was diastereoselective vis à vis aryl substituted substrates and allowed a dynamic kinetic resolution that yielded highly enantioenriched alcohol products.[8,10] Since whole cell screening can be complicated by toxicity and membrane permeability, we have created a collection of 19 yeast reductases, expressed in *E. coli* as glutathione (S)-transferase (GST) fusion proteins. All of these can be purified by a common affinity matrix, which allows the properties of each catalyst to be defined in the absence of competitors.[11–14] Herein, we report the application of this library to reduce α-keto-β-lactam **1**, which can be used as a precursor to the Taxol® side chain.[1] Our previous attempts to use whole bakers' yeast

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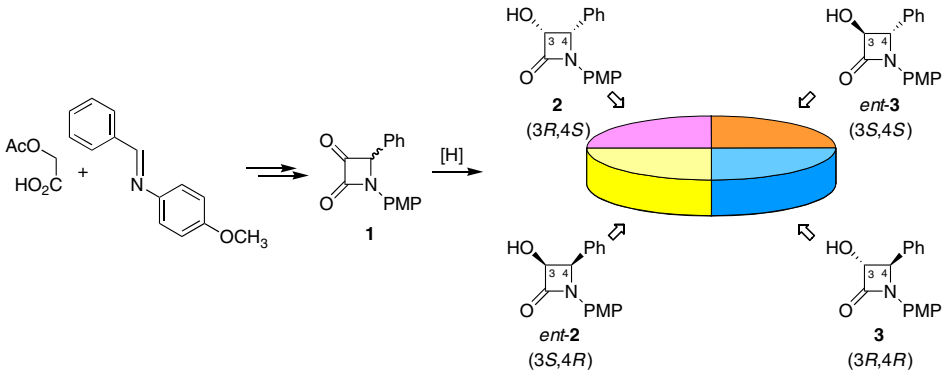

Scheme 1.

cells to convert **1** to **2** met with only limited success,[5,6] and we hoped that individual yeast reductases might prove more synthetically useful. In addition, the data accumulated herein have allowed us to begin understanding the structure–function relationships of some of the reductases examined.

2. Results and discussion

α-Keto-β-lactam **1** was prepared as described previously.[15] Homochiral samples of alcohols **2**, *ent*-**2** were obtained via lipase-mediated resolution of the corresponding racemic *cis*-acetate (3*R*,4*S*/3*S*,4*R*),[16] while yeast-mediated reduction of **1** provided a chromatographically separable, single *trans*-(3*R*,4*R*)-isomer **3**.[17] All four diastereomeric alcohols derived from **1** were cleanly separated by chiral-phase HPLC and the peaks assigned by co-injection with the homochiral standards. Small-scale reductions were carried out with each of the 19 GST-fusion proteins in the presence of NADPH. The cofactor was regenerated by a glucose-6-phosphate/glucose-6-phosphate dehydrogenase couple. Reactions were carried out at both pH 7 and pH 8 and the results are summarized in Table 1.

All the yeast enzymes examined readily accepted **1** as a substrate, in contrast to previous studies where ketones were typically only reduced by a subset of the library.[12,14] On the other hand, the stereoselectivities elicited by **1** were generally only modest. This behaviour is also relatively atypical. Only four reductases showed noteworthy stereoselectivities toward **1** (Yjr096w, Ydl124w, Ybr149w, and Ycr107w). In all of these cases, better selectivity was obtained at pH 7. At a higher pH (8), the rate of conversion increased but the selectivity dropped. The product distribution of the four-stereoselective reactions requires that dynamic kinetic resolution occurs during the bioconversion.[10]Apparently, the increase in the rate of enzymatic reduction is much higher than the increase in the rate of epimerization at pH 8.

2.1. Analysis of enzyme stereoselectivity

Interestingly, the four most selective enzymes are all members of the aldose reductase (AKR) superfamily.[18] Even within this family of sequence-related proteins, however, three different classes of behaviour are evident. Both the Ybr149w and Ycr107w proteins gave primarily *cis*-alcohol *ent*-**2** with 90% and 83% ee, respectively. On the other hand, the Yjr096w and Ydl124w reductases predominantly produced **2**. The remaining aldose reductases (Ynl331c, Yor120w, Yhr104w, and Ydr369w) were relatively non-selective. This information provided a unique opportunity for unravelling structure–function relationships that governs the interactions of aldose reductases with non-natural substrates.

Aldose reductases are monomeric proteins of approximately 325 residues found throughout all three kingdoms that bind NADPH without a Rossmann-fold motif.[19] Human aldose reductase has been particularly well studied in connection with its potential role in diabetes- and galactosemia-related blindness.[20] Aldose reductases follow an ordered mechanism in which NADPH binds first and departs last. The *pro*-*R* H of NADPH is transferred to the substrate carbonyl with concomitant protonation of the oxygen by an enzyme residue acting as a general acid.[18] Four conserved residues (Asp, Tyr, Lys, and His) have been shown to be critical in the function of human aldose reductase,[21,22] as well as in aldehyde reductase[23] and 3-α-hydroxysteroid dehydrogenase.[24] X-ray crystallography has demonstrated that the spatial arrangement of these four residues, as well as the overall β-barrel structure of aldose reductase family members, is highly conserved, strongly implying a common catalytic mechanism.

The four possible alcohol stereoisomers derived from **1** require a hydride transfer from either the *Re* or *Si* faces (Fig. 1). There is also the possibility of diastereoselectivity if an enzyme prefers the pre-existing (4*R*)- or (4*S*)-configuration. This allows us to characterize the Ybr149w and Ycr107w enzymes as catalyzing *Re* face hydride addition with a preference for (4*R*)-**1** to give mainly *ent*-**2**. By contrast, both Yjr096w and Ydl124w facilitate hydride transfer to the *Si* face of (4*S*)-**1**, yielding **2** as the major product. Other members of the aldose reductase superfamily such as Ynl331c, Yor120w, Yhr104w, and Ydr368w allow **1** to bind in multiple orientations that permit hydride transfer to either the *Re* or *Si* faces with similar probabilities.

Table 1. Reduction of **1** by *S. cerevisiae* GST-fusion proteins

Yeast gene	pH 7			pH 8		
	Conversion (%)		Product composition[a]	Conversion (%)		Product composition[a]
	GC	HPLC		GC	HPLC	
Yjr096w	63	83	8*SS*, 15*SR*, 77*RS*	89	100	15*SS*, 18*SR*, 67*RS*
Ydl124w	68	90	10*SS*, 7*SR*, 83*RS*	93	99	32*SS*, 9*SR*, 59*RS*
Ybr149w	73	92	4*SS*, 93*SR*, 3*RS*	91	100	4*SS*, 88*SR*, 8*RS*
Ycr107w	67	79	5*SS*, 89*SR*, 6*RS*	93	99	10*SS*, 78*SR*, 11*RS*
Ynl331c	63	39	24*SS*, 50*SR*, 26*RS*	94	100	24*SS*, 47*SR*, 9*RS*
Yor120w	62	39	17*SS*, 53*SR*, 30*RS*	30	100	19*SS*, 54*SR*, 27*RS*
Yhr104w	67	30	24*SS*, 50*SR*, 26*RS*	91	100	25*SS*, 47*SR*, 28*RS*
Ydr368w	52	68	9*SS*, 43*SR*, 47*RS*	88	100	16*SS*, 45*SR*, 39*RS*
Ygl185c	63	51	13*SS*, 22*SR*, 65*RS*	93	100	14*SS*, 21*SR*, 65*RS*
Ynl247c	66	71	8*SS*, 25*SR*, 66*RS*	82	100	15*SS*, 23*SR*, 62*RS*
Ypl275w	66	36	21*SS*, 42*SR*, 37*RS*	88	100	23*SS*, 44*SR*, 34*RS*
Ypl113c	65	30	24*SS*, 49*SR*, 27*RS*	93	100	24*SS*, 47*SR*, 29*RS*
Ylr070c	67	47	15*SS*, 42*SR*, 43*RS*	94	99	17*SS*, 35*SR*, 47*RS*
Yal060w	74	29	24*SS*, 49*SR*, 27*RS*	94	100	24*SS*, 47*SR*, 29*RS*
Yal061w	69	37	20*SS*, 39*SR*, 41*RS*	93	98	17*SS*, 29*SR*, 54*RS*
Ygl157w	66	37	25*SS*, 43*SR*, 32*RS*	96	100	24*SS*, 47*SR*, 29*RS*
Ydr541c	60	36	24*SS*, 47*SR*, 28*RS*	97	98	24*SS*, 47*SR*, 29*RS*
Ygl039w	60	53	14*SS*, 42*SR*, 44*RS*	93	100	20*SS*, 46*SR*, 34*RS*
Yol151w	70	32	32*SS*, 55*SR*, 13*RS*	92	100	24*SS*, 47*SR*, 29*RS*

Enzymes are grouped by superfamily (in order, aldose reductases, D-hydroxyacid dehydrogenases, medium-chain dehydrogenases, and short-chain dehydrogenases) with boundaries indicated by hair lines.

[a] Product compositions were determined by chiral-phase HPLC and depicted according to the colours shown in Scheme 1.

We attempted to uncover the structural feature(s) that correlated with the observed stereoselectivities of the eight aldose reductases examined here. Both we[9] and others[25] have previously carried out primary sequence alignments of all eight of the yeast aldose reductases and shown that the catalytic tetrad is conserved in each. Moreover, the locations of gaps and insertions between the yeast protein sequences and family members with known three-dimensional structures are consistent with the notion that all possess the same overall fold with differences confined largely to the length and conformations of surface loops. Therefore, we focussed our attention on these portions of the proteins; particularly loop A (residues 113–135, human aldose reductase numbering), located near the active site and possibly involved in determining substrate specificity.[18] A multiple sequence alignment of the eight yeast aldose reductases with that of human aldose reductase revealed that the loop A sequences could be divided into two categories on the basis of length (Fig. 2). The Yjr096w

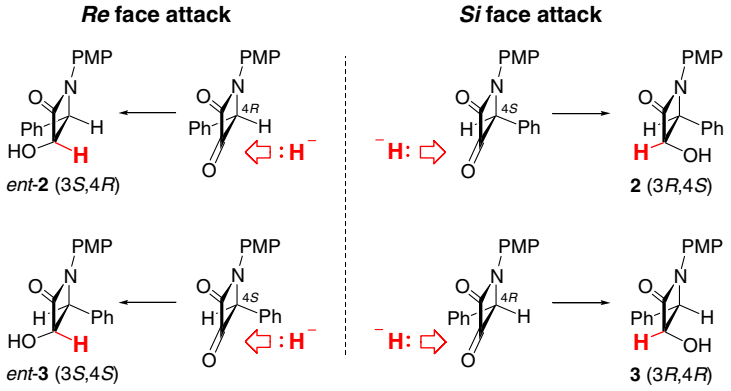

Figure 1. Relationship between the stereochemistry of hydride addition and the configurations of alcohol products. The hydride donated by NADPH is depicted in red.

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                        |<---------- Loop A ----------->|

1ADS        DYLDLYLIHWPTGFK--PGKEFFP--------LDESGNVVPSDTNIL

Yjr096W     QYIDLLLIHSP----------------------------LEGSKLRL
Ydl124W     DYVDLYLLHSPFVSK------------------------EVNGLSLE

Ybr149W     EYVDLLLQHWPLCFEKIKDPKGISGLVKTPV-DDSGKTMYAADGDYL
Ycr107W     DWIDILYVHWWDYMS---SIEEFMDSLHILVQQGKVLYLGVSDTPAW
Ynl331C     DWIDILYIHWWDYMS---SIEEVMDSLHILVQQGKVLYLGVSDTPAW
Yor120W     DYVDLYLMHWPARLDPAYIKNEDILSVPTKK-DGS-RAVDITNWNFI
Yhr104W     DYLDLYYIHFPIAFKYVPFEEKYPPGFYTGADDEKKGHITEAHVPII
Ydr368W     DYVDLYLMHWPVPLKTDRVTDGNVLCIPTLE-DGT-VDIDTKEWNFI
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Figure 2. CLUSTAL W multiple sequence alignment[26] of yeast aldose reductase loop A sequences along with human aldose reductase (1ADS). All sequences were aligned globally and dashes indicate gaps introduced into the sequences to maximize overlap. The catalytically essential histidine (His 110, human aldose reductase numbering) is depicted in blue.

and Ydl124w sequences have the shortest loop A portions (5 and 9 amino acids, respectively) while the remaining yeast reductases have much longer loop A regions.

To examine these sequence differences more carefully, structural models were constructed, using CPHmodel 2.0,[27] for the four representative yeast aldose reductases: Yjr096w, Ydl124w, Ybr149w, and Ydr368w. These proteins were chosen since the first two catalyze hydride transfer to the *Si* face of **1**, the third to the *Re* face of **1**, and the last allows **1** to bind in two different orientations that afford both (*R*)- and (*S*)-alcohol products with similar facility. Models were constructed using the human aldose reductase structure (1ADS) that also includes bound NADPH.[19]

As anticipated, the (α_8/β_8) cores of all four modelled yeast proteins were essentially identical to that of the human aldose reductase and the positions of all four catalytically essential residues were superimposable.[28] On the other hand, the shapes of the loop A regions displayed significant differences (Fig. 3). For example, models of Ydl124w and Ybr149w, two enzymes with antipodal substrate specificities, show not only the expected difference in loop A sizes, but also that the loops occupy mirror image positions with respect to the bound NADPH and the key catalytic residues. The high degree of overall sequence similarity makes it highly unlikely that these two enzymes would bind the NADPH cofac-

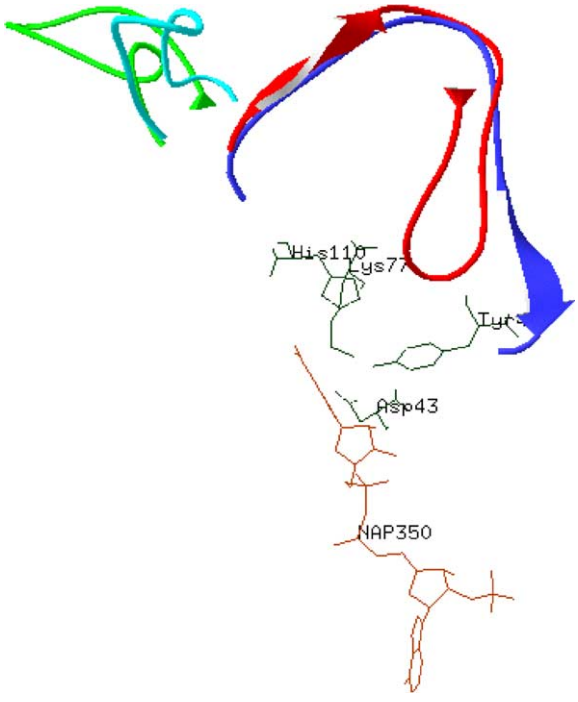

Figure 3. Loop A regions of modelled yeast reductases. The location of the bound NADPH (shown in orange) and the four catalytically essential residues (yellow) are derived from the X-ray crystal structure of human aldose reductase (1ADS). The modelled loop A conformations of yeast aldose reductases Yjr096w (turquoise), Ydl124w (green), Ybr149w (blue), and Ydr368w (red) are shown in ribbon form.

tor differently; instead, the substrate likely occupies mirror image orientations. As noted above (Fig. 1), this would lead to antipodal products, consistent with the experimental observations. The loop A sizes and conformations in models of Yjr096w and Ydl124w are similar, consistent with both mediating *Si*-face hydride transfer to **1**. It is also interesting to compare the loop A portions of the stereoselective reductases with that of a non-selective reductase such as Ydr368w. In this case, the conformation of the loop A region of Ydr368w resembles that of Ybr149w; however, its slightly shorter length makes it tempting to speculate the fact that it is insufficient to enforce a single binding mode for **1**.

Although the length and spatial position of A loops appear to play an important role in the selectivity of aldose reductases, their amino acid composition is clearly also a factor. For example, A loops of Ycr107W and Ynl331C are identical except for a single amino acid change: phenylalanine in the selective Ycr107W to valine in the only marginally selective Ynl331C.

3. Conclusion

This study examined the behaviour of a series of yeast reductases toward a probe α-keto-β-lactam **1**. The results differed from those observed in our previous studies of this library in several respects.[11–14] First, all of the reductases examined accepted **1**. This was unusual since even for small substrates, it was generally found that a given substrate would be reduced by only a subset of the library. The prior exception we had observed was ethyl 4-chloroacetoacetate, whose carbonyl was activated by inductive effects of the neighboring chlorine atom.[12] Likewise, the ketone carbonyl of **1** is also highly activated both by geometric strain and Coulombic repulsion, and it is likely that these effects enhance its ability to act as a substrate for a broad array of enzymes.

The relative lack of stereoselectivity in enzymatic reductions of **1** was also somewhat surprising. Initial experiments on the prochiral α- and β-ketoesters suggested that when a substrate is accepted by a reductase, the reaction tends to proceed with a high stereoselectivity.[11,12] Clearly, the reductions of **1** did not follow this trend. Instead, even the most highly selective reactions afforded alcohols with ca. 90% ee. It is important, however, to note the similarity between reductions of **1** and those of α-ketoesters investigated earlier.[12] Three α-ketoesters, ethyl 2-oxobutyrate, ethyl 2-oxopentanoate and ethyl 2-oxo-3-phenylbutyrate, accepted by seven (out of eight) aldose reductases listed in Table 1, were reduced with lower than anticipated enantiomeric excess. Furthermore, a clear change in selectivity was parallel to that observed for α-keto-β-lactam **1**; Yjr096w and Ydl124w catalyzed *Si*-face and Ybr149w and Ycr107w *Re*-face hydride transfer.[12]

Differences in loop A conformations provide one possible reason for the divergent stereoselectivities observed, at least within the family of aldose reductases. The enticing possibility that more stereoselective reductions might be obtainable by further alterations in this region remains to be tested experimentally.

4. Experimental

4.1. General

α-Keto-β-lactam **1** was prepared as described previously.[15] Authentic samples of **2**, *ent*-**2**, and **3** were prepared by literature methods.[15] Yeast GST-fusion proteins were isolated as described previously[13] and stored at −20 °C in buffer containing 50% glycerol. Screening reactions were sampled by mixing 100 μL of the reaction with an equal quantity of EtOAc containing methyl benzoate (internal standard), followed by GC analysis with a DB-1301 column (15 m × 0.53 mm). The enantiomeric compositions of reduction reactions were determined by chiral-phase HPLC on a Chiracel OD-H column (4.6 × 250 mm) operated isocratically (90:10 hexanes/*i*-PrOH) at a flow rate of 1.0 mL/min. UV detection (254 nm) was used and peaks were identified by co-injection with authentic standards of **2**, *ent*-**2**, and **3**.

4.2. Small-scale reductions with bakers' yeast reductases

Screening was carried out on 0.5 mL scales at 30 °C in 100 mM KP_i (pH 7.0 or 8.0). Reaction mixtures contained 5 mM **1** (25 μL of a 100 mM stock in EtOH), 5 mM hydroxypropyl-β-cyclodextrin (125 μL of a 20 mM stock in KP_i buffer), 0.10 mM $NADP^+$ (1.5 μL of a 34 mM stock in KP_i buffer), 7.5 mM glucose-6-phosphate (2.6 μL of a 1.5 M solution in KP_i buffer), 0.5 U of glucose-6-phosphate dehydrogenase (2.0 μL of a 0.25 U/μL stock in KP_i buffer) plus 344 μL of KP_i buffer. After vortex mixing, 5 μg of the appropriate yeast GST-fusion protein was added. The reactions were then rotated gently and sampled periodically for GC and HPLC analysis.

Acknowledgments

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