DOI: 10.1002/cbic.200700689

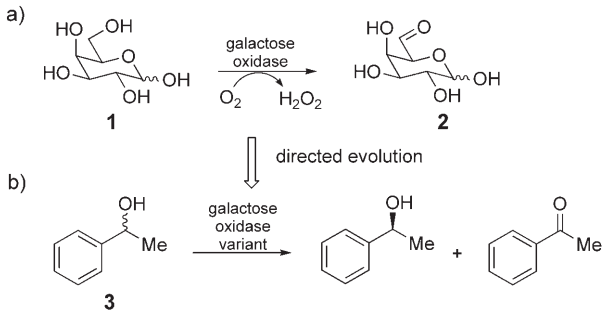
Directed Evolution of Galactose Oxidase: Generation of Enantioselective Secondary Alcohol Oxidases

Franck Escalettes and Nicholas J. Turner*[a]

Optically pure secondary alcohols are highly valuable chiral building blocks that can be prepared by a number of methods, especially asymmetric reduction of ketones or resolution based approaches. In the latter case, lipases or esterases are frequently used as catalysts for the acylation/deacylation of racemic alcohols[1] and recently methods have been developed for recycling the unreactive enantiomer leading to dynamic kinetic resolution (DKR) processes.[2] An alternative strategy to DKR which we have recently pursued is deracemisation[3] in which for our purposes an enantioselective oxidase enzyme is combined with a nonselective chemical reductant[4] to generate optically pure products in high yield (⩾50%). In order to extend this approach beyond amino acids[5] and amines[6] to encompass alcohols we required access to enantioselective alcohol oxidases with appropriate substrate specificity.

Certain alcohol oxidases (for example, from *Pichia* species) are well documented but tend to have rather restricted substrate ranges and are generally specific for the oxidation of primary alcohols to the corresponding aldehydes.[7] Whilst some secondary alcohol oxidases are known,[8] for example, pyranose oxidase and glycolate oxidase, they do not possess the broad substrate specificity required for general application. Recently, progress has been made in the development of chemocatalysts for the enantioselective oxidation of secondary alcohols.[9] Against this background we sought to develop enantioselective alcohol oxidase enzymes of broad synthetic utility using galactose oxidase (GOase) as our starting point.[10] GOase is a soluble, Cu-dependent enzyme that catalyses the O_2 dependent oxidation of the C6-OH of D-galactose **1** to the corresponding aldehyde **2** (Scheme 1 A). Wild-type GOase has a relatively narrow substrate specificity,[7,11] possessing highest activity towards specific sugars (D-galactose **1**, raffinose, guar gum) and simple primary alcohols (for example, dihydroxyacetone, benzyl alcohol). A striking example of its high specificity is given by its activity towards D-glucose which is about 10^6-fold less reactive than D-galactose. Moreover, in the context of our interest, the wild-type enzyme displays no activity towards secondary alcohols.

As a model system we sought to identify variants of GOase that could catalyse the enantioselective oxidation of 1-phenylethanol **3** (Scheme 1 B). We recognised that it would be necessary to change three key features of the enzyme to achieve this goal, namely 1) change in specificity from a 1° to a 2° alcohol oxidase, 2) capability for enantioselective oxidation, and 3) the ability to bind nonpolar rather than polar substrates.



Scheme 1. A) Reaction catalysed by wild-type galactose oxidase (GOase); B) Enantioselective oxidation of 1-phenylethanol **3** using variant GOases.

The starting points for our studies were the GOase variants M_1 and M_3 recently reported by Sun et al.[12,13] The M_1 variant contains six mutations compared to the wild-type enzyme (Ser10Pro, Met70Val, Pro136, Gly195Glu, Val494Ala, Asn535Asp). This variant has been shown to give higher expression levels of a more stable and active enzyme in *E. coli* although its substrate specificity is essentially the same as the wild type. The M_3 variant was derived from M_1 and contains a further three mutations (Trp290Phe, Arg330Lys, Gln406Thr), all located at the active site. This variant, which had been selected on the basis of its improved activity towards D-glucose,[13] was also characterised against a range of substrates and reported to have very weak activity towards racemic but-3-en-2-ol and butan-2-ol although no data on enantioselectivity was given.

The genes encoding GOase M_1 and M_3 variants, which were generated from the wild-type gene[14] by site-directed mutagenesis, were cloned into the pET16b vector followed by expression in *E. coli*. Both variants were purified by Ni-affinity chromatography and then assayed against racemic 1-phenylethanol as substrate. Although no activity was observed with the M_1 variant, we were encouraged to observe weak activity with the M_3 variant. Moreover, the M_3 variant also appeared to be enantioselective with the *R* enantiomer showing approximately 30-fold greater activity than the *S* enantiomer (data not shown). We therefore decided to subject the M_3 variant to further rounds of random mutation coupled with screening to optimise the activity toward 1-phenylethanol.

Based upon earlier reports,[6,15] we developed a colorimetric solid-phase assay (see below) which allowed us to assess the activity of individual clones when grown on agar plates. This screening method, which relies upon capture of the hydrogen peroxide biproduct produced in the oxidation reaction, is versatile, in that different substrates can easily be introduced into

[a] *Dr. F. Escalettes, Prof. N. J. Turner*
School of Chemistry, University of Manchester
Manchester Interdisciplinary Biocentre
131 Princess Street, Manchester M1 7DN (UK)
Fax: (+44) 161-275-1311
E-mail: nicholas.turner@manchester.ac.uk

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

the assay, and also reasonably high-throughput allowing up to 100 000 clones to be readily screened per round of evolution. Libraries of M_3 GOase were generated using error-prone PCR (ep-PCR) in which the average number of amino acid mutations per gene was about 3–4. These libraries were used to transform *E. coli* and the resulting colonies (approximately 80 000) screened on solid-phase against but-3-en-2-ol (substrate concentration = 200 mM) as substrate rather than 1-phenylethanol in view of the greater solubility of the former substrate. Approximately 120 colonies were picked which showed improved activity compared to the parent M_3. One of them, M_{3-5}, appeared to show good activity and high enantioselectivity when reassayed against (*R*)-1-phenylethanol using the solid-phase assay (Figure 1).

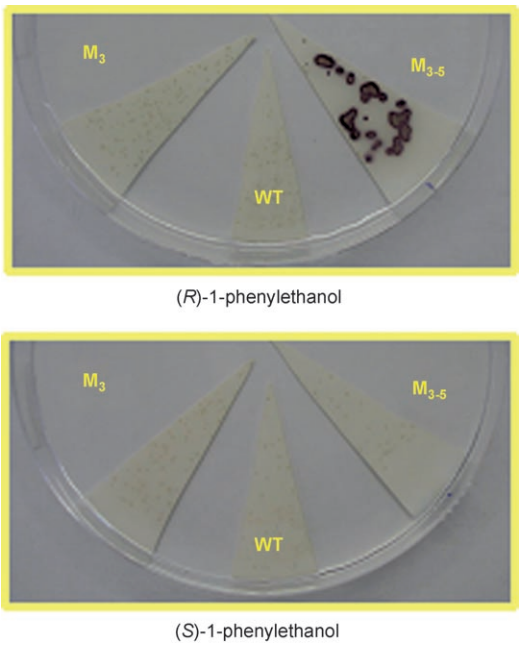


Figure 1. Solid-phase assay of wild type, M_3 and M_{3-5} GOases using 1-phenylethanol **3** as substrate A) *R* enantiomer B) *S* enantiomer.

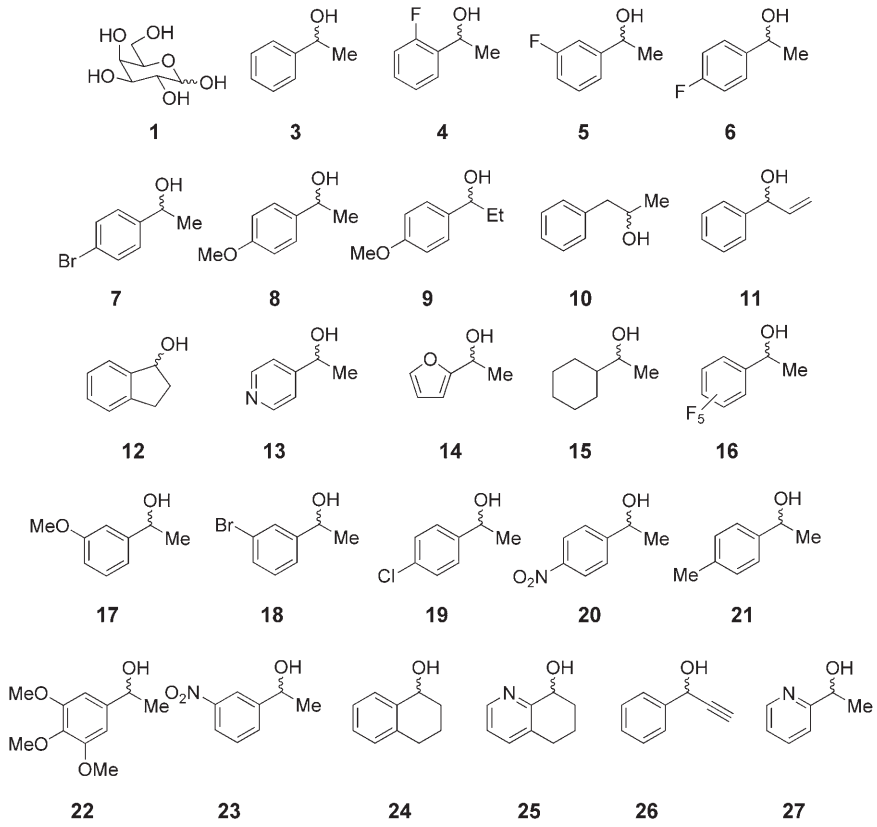
The wild-type (WT) GOase, M_3, and M_{3-5} variants were all purified by Ni-affinity chromatography and characterised initially against D-galactose **1** and 1-phenylethanol **3** as substrates (Table 1).

The M_{3-5} variant was found to possess a significantly higher k_{cat} (12-fold) and lower K_M (eightfold) value towards 1-phenylethanol **3** compared with the parent M_3 variant resulting in an increase in specificity constant (k_{cat}/K_M) of >100-fold. Correspondingly, the relative decrease in activity towards D-galactose in going from WT to M_3 to M_{3-5} was approximately 2000-fold (data not shown) implying a significant change in substrate specificity. The purified M_3 and M_{3-5} variants were then characterised against a broader panel of secondary alcohols.

Table 1. Kinetic (k_{cat} and K_M) values for GOase variants using either D-gal **1** or 3-fluoro-1-phenylethanol **5** as substrate.

GOase	Substrate	k_{cat} [s^{-1}]	K_M [mM]	k_{cat}/K_M [$M^{-1}s^{-1}$]
WT	D-gal **1**	156 ± 6	15.0 ± 2.0	10400 ± 1440
M_3	D-gal **1**	54 ± 7	1800 ± 400	31 ± 7
M_3	(±)-**3**	0.3 ± 0.06	178 ± 62	1.5 ± 0.6
M_{3-5}	(±)-**3**	3.6 ± 0.05	22.0 ± 1.0	163 ± 8
M_{3-5}	(*R*)-**3**	3.5 ± 0.04	7.4 ± 0.6	479 ± 39
M_{3-5-24}	(±)-**3**	3.6 ± 0.07	16.3 ± 1.3	220 ± 18
M_{3-5-32}	(±)-**3**	3.1 ± 0.05	13.8 ± 1.0	227 ± 16
$M_{3-5-215}$	(±)-**3**	2.9 ± 0.05	10.6 ± 0.7	270 ± 20

The M_{3-5} variant was found to exhibit good activity against a range substituted 1-phenylethanol analogues **4**–**8**, often with higher rates of oxidation (Figure 2). Other secondary alcohols containing an aryl group (1-phenylallyl alcohol **11**, indanol **12**, and 1-pyridine ethanol **13**) were also oxidised at useful rates. However, alcohols **9**, **10**, **14**, and **15** were poor substrates. It is interesting to note that a vinyl group is tolerated (**11**), but not



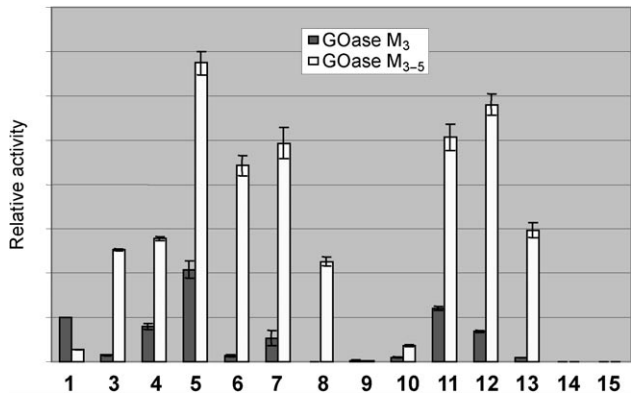


Figure 2. Substrate specificity of M_3 and M_{3-5} GOase variants towards a range of secondary alcohols.

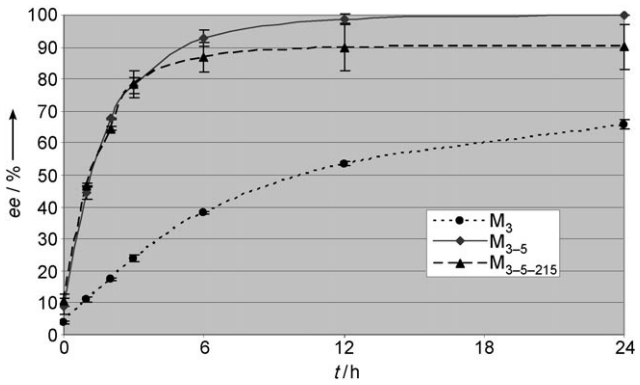
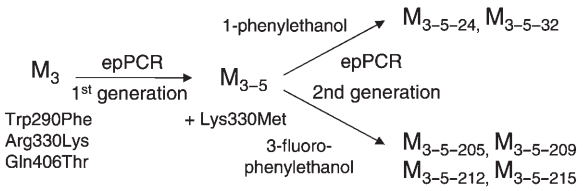


Figure 3. Enantioselective oxidation of (±)-3-fluoro-1-phenylethanol **5** using M_3, M_{3-5}, and $M_{3-5-215}$ variants.

the sterically similar ethyl group (**9**), indicating subtle differences in reactivity.

In order to obtain further improvements in activity, a second library of variants was generated, using M_{3-5} as parent, and screened separately against both 1-phenylethanol **3** ([S] = 100 mM; 240 000 clones) and 3-fluoro-1-phenylethanol **5** ([S] = 50 mM; 120 000 clones) as substrates. Two variants were isolated with improved activity towards **3** (M_{3-5-24} and M_{3-5-32}) and four with enhanced activity towards **5** ($M_{3-5-205}$, $M_{2-5-209}$, $M_{3-5-212}$, $M_{3-5-215}$), (Scheme 2). Although no significant improvements in k_{cat} were observed, three variants possessed reduced K_M values (Table 1), including the $M_{3-5-215}$ variant (K_M = 10.6 mM compared to 22.0 mM for the M_{3-5} parent), indicating the value of lowering the substrate concentration in the solid-phase assay in order to select for K_M variants.

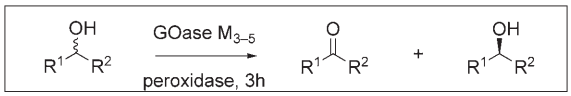


additional mutations present

M_{3-5-24}	+ Thr130Ser
M_{3-5-32}	+ Met278Thr, Asp517Val, Tyr576His
$M_{3-5-205}$	+ Thr138Ala, Ser425Pro, Ile498Val, Asp517Gly
$M_{3-5-209}$	+ Lys370Asn, Ala391Ser
$M_{3-5-212}$	+ Tyr43Phe, Ser291Cys
$M_{3-5-215}$	+ Asn413Tyr, Tyr436Phe

Scheme 2. GOase variants derived from 1 st and 2nd round random mutagenesis/screening experiments.

The M_3, M_{3-5}, and $M_{3-5-215}$ variants were then directly compared for their ability to enantioselectively oxidise racemic 3-fluoro-1-phenylethanol **5** (monitored by chiral HPLC). These reactions were carried out in the presence of O_2 and also horse radish peroxidase, both of which are known to stimulate the activity of GOase.[16] In all cases, selective oxidation of (*R*)-**5** to the corresponding ketone was observed although with different rates (Figure 3). The reaction involving the M_3 variant gave *ee* = 67% after 24 h whereas both the M_{3-5} (12 h; 99% *ee*) and $M_{3-5-215}$ (12; 90% *ee*) variants were considerably more active. Increasing the concentration of horse radish peroxidase had a dramatic effect resulting in an *ee* of 99% at 50% conversion after only 2 h under optimised conditions with the M_{3-5} variant. Finally, these conditions were then applied to the kinetic resolution of a range of secondary alcohols. After 3 h, the *ee* of the remaining alcohol was determined and in many cases found to be 99% indicating very high enantioselectivity at a conversion of approximately 50%. For some substrates (**4**, **11**, **16**, **24**, **27**) the enantiomeric excesses after 3 h were lower although longer reaction times led to higher values. However for alcohols **12** (*ee* = 61%) and **25** (*ee* = 78%) prolonged incubation with the enzyme resulted in no further increase in *ee* suggesting lower intrinsic enantioselectivity (Scheme 3).



alcohol	*ee*/%	alcohol	*ee*/%
3	99	**18**	99
4	61	**19**	99
5	99	**20**	96
6	99	**21**	99
7	99	**22**	99
8	99	**23**	99
11	45	**24**	15
12	50	**25**	45
16	65	**26**	99
17	99	**27**	67

Scheme 3. Kinetic resolution of a range of secondary alcohols using the GOase M_{3-5} variant (*ee* values determined by chiral HPLC).

The observed selectivity of these GOase variants for the *R* enantiomer of **5** is consistent with the report by Minasian et al.,[17] who demonstrated by isotopic labelling that during the oxidation of both D-galactose **1** and benzyl alcohol **16** it is the pro-*S* hydrogen atom that is selectively removed.

The M_{3-5} variant contains a single mutation at position 330 compared to M_3 (Lys→Met; Arg in WT). This active-site residue has previously been identified as important in controlling substrate recognition. Mutation from Arg (WT) to Lys improves

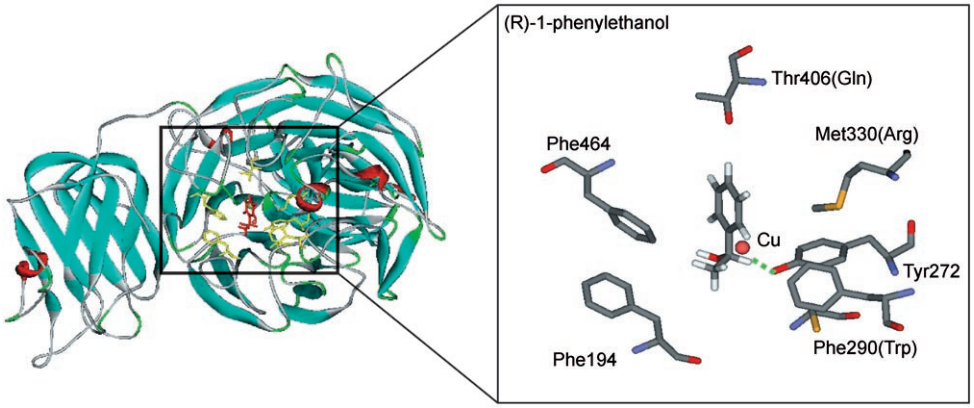


Figure 4. Model showing (*R*)-1-phenylethanol **3** docked into active site of GOase M_{3-5} variant (wild-type residues in brackets).

activity towards D-glucose[13] and D-fructose.[18] Arg330 is believed to interact directly with the substrate D-galactose by forming two hydrogen bonds to the C3- and C4-OH groups.[19] Figure 4 shows a model of the GOase M_{3-5} variant generated from the available crystal structure of the wild-type enzyme. The *R* enantiomer of 1-phenylethanol **3** appears to bind very well with the C1 methyl group occupying a small hydrophobic pocket generated by amino acid residues Phe194 and Phe464. Binding of the *S* enantiomer would require interpolation of the C1 methyl and phenyl groups which is highly unfavourable. Introduction of Met at 330 clearly results in a more hydrophobic active site that is well suited to binding 1-phenylethanol and structurally related compounds. The mutations present in the second generation variants derived from M_{3-5} are difficult at this stage to interpret in that they are varied in nature and indeed a number occur on the surface of the protein (Figure 4).

In summary we have identified variants of GOase that possess good activity ($k_{cat} > 200\ min^{-1}$) towards a range of secondary alcohols based upon the 1-phenylethanol template and high enantioselectivity (*ee* = 99%) in the kinetic resolution of (±)-3-fluoro-1-phenylethanol. We are currently examining application of these catalysts in the preparative deracemisation of racemic secondary alcohols.

Acknowledgement

We are grateful to the EPSRC for funding. We also thank the Wellcome Trust for financial support.

Keywords: alcohol oxidase · biocatalysis · directed evolution · enantioselective catalysis · screening

[1] U. T. Bornscheuer, R. J. Kazlauskas, *Hydrolases in Organic Synthesis* 2nd ed., Wiley, Weinheim, **2005**.
[2] B. Martin-Matute, M. Edin, K. Bogar, F. B. Kaynak, J.-E. Backvall, *J. Am. Chem. Soc.* **2005**, *127*, 8817; M.-J. Kim, Y. I. Chung, Y. K. Choi, H. K. Lee, D. Kim, J. Park, *J. Am. Chem. Soc.* **2003**, *125*, 11494.
[3] C. C. Gruber, I. Lavandera, K. Faber, W. Kroutil, *Adv. Synth. Catal.* **2006**, *348*, 1789.
[4] N. J. Turner, *Curr. Opin. Chem. Biol.* **2004**, *8*, 114.
[5] G. J. Roff, R. C. Lloyd, N. J. Turner, *J. Am. Chem. Soc.* **2004**, *126*, 4098; A. Enright, F.-R. Alexandre, G. J. Roff, I. G. Fotheringham, M. J. Dawson, N. J. Turner, *Chem. Commun.* **2003**, 2636.
[6] C. J. Dunsmore, R. Carr, T. Fleming, N. J. Turner, *J. Am. Chem. Soc.* **2006**, *128*, 2224; R. Carr, M. Alexeeva, A. Enright, T. S. C. Eve, M. J. Dawson, N. J. Turner, *Angew. Chem.* **2003**, *115*, 4955; *Angew. Chem. Int. Ed.* **2003**, *42*, 4807; M. Alexeeva, A. Enright, M. J. Dawson, M. Mahmoudian, N. J. Turner, *Angew. Chem.* **2002**, *114*, 3309; *Angew. Chem. Int. Ed.* **2002**, *41*, 3177.
[7] A. Siebum, A. van Wijk, R. Schoevaart, T. Kieboom, *J. Mol. Catal. B* **2006**, *41*, 141.
[8] W. Kroutil, H. Mang, K. Edegger, K. Faber, *Adv. Synth. Catal.* **2004**, *346*, 125.
[9] S. D. Rychnovsky, T. L. McLernon, H. Rajapakse, *J. Org. Chem.* **1996**, *61*, 1194; A. T. Radosevich, C. Musich, F. D. Toste, *J. Am. Chem. Soc.* **2005**, *127*, 1090; S. K. Mandal, M. S. Sigman, *J. Org. Chem.* **2003**, *68*, 7535; E. M. Ferreira, B. M. Stoltz, *J. Am. Chem. Soc.* **2001**, *123*, 7725.
[10] N. Ito, S. E. V. Phillips, C. Stevens, Z. B. Ogel, M. J. McPherson, J. N. Keen, K. D. S. Yadav, P. F. Knowles, *Nature* **1991**, *350*, 87; J. W. Whittaker, M. M. Whittaker, *J. Biol. Chem.* **1988**, *263*, 6074.
[11] M. H. Mendonça, G. T. Zancan, *Arch. Biochem. Biophys.* **1987**, *252*, 507.
[12] L. Sun, I. P. Petrounia, M. Yagasaki, G. Bandara, F. H. Arnold, *Protein Eng.* **2001**, *14*, 699.
[13] L. Sun, T. Bulter, M. Alcalde, I. P. Petrounia, F. H. Arnold, *ChemBioChem* **2002**, *3*, 781.
[14] We are grateful to Prof. H. Kiramatsu for the gift of the wild-type GOase gene. ((move to Acknowledgement?))
[15] S. Delagrave, D. J. Murphy, J. L. Rittenhouse Pruss, A. M. Maffia III, B. L. Marrs, E. J. Bylina, W. J. Coleman, C. L. Grek, M. R. Dilworth, M. M. Yang, D. C. Youvan, *Protein Eng.* **2001**, *14*, 261.
[16] P. Tressel, D. J. Kosman, *Biochem. Biophys. Res. Commun.* **1980**, *92*, 781.
[17] S. G. Minasian, M. M. Whitaker, J. W. Whitaker, *Biochemistry* **2004**, *43*, 13683.
[18] S. E. Deacon, K. Mahmoud, R. K. Spooner, S. J. Fairbank, P. F. Knowles, S. E. V. Phillips, M. J. McPherson, *ChemBioChem* **2004**, *5*, 972.
[19] R. M. Wachter, B. P. Branchaud, *J. Am. Chem. Soc.* **1996**, *118*, 2782; S. J. Firbank, M. S. Rogers, C. M. Wilmot, D. M. Dooley, M. A. Halcrow, P. F. Knowles, M. J. McPherson, S. E. Phillips, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 12932.

Received: November 13, 2007
Published online on March 10, 2008

