Advanced Synthesis & Catalysis

DOI: 10.1002/adsc.201500818

Ene Reductase Enzymes for the Aromatisation of Tetralones and Cyclohexenones to Naphthols and Phenols

Paul P. Kelly,[a,*] David Lipscomb,[a] Derek J. Quinn,[a] Ken Lemon,[a] Jill Caswell,[a] Jenny Spratt,[a] Birgit Kosjek,[b] Matthew Truppo,[b] and Thomas S. Moody[a]

[a] Almac, Department of Biocatalysis Isotope Chemistry, 20 Seagoe Industrial Estate, Craigavon, BT63 5QD, Northern Ireland, U.K.
Fax: (+44)-(0)28-3833-2299, phone: (+44)-(0)28-3833-2200; e-mail: paul.kelly@almacgroup.com
[b] Merck & Co., Inc., Department of Process Research, Merck Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065, USA

Received: September 2, 2015; Revised: December 17, 2015; Published online: February 17, 2016

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201500818.

Abstract: Ene reductases (EREDs) have great potential as oxidation biocatalysts, as demonstrated by their efficient conversion of a number of tetralones to the corresponding naphthols. Of 96 enzymes tested, 57 were able to produce 2-naphthol in this way. Further tests with substituted tetralones revealed typically high conversions up to >99%. The reactions were performed under mild conditions in aqueous buffer with only co-solvent, biocatalyst and oxidation substrate required for conversion. Production of a methoxy-substituted naphthol was also successfully performed on a gram scale, with 91% yield. This methodology provides a new avenue to produce substituted naphthols as valuable building blocks, with the possibility to extend the approach to the production of phenols also being demonstrated.

Keywords: aromatisation; biocatalysis; ene reductases; enzyme catalysis; naphthols; oxidation; phenols

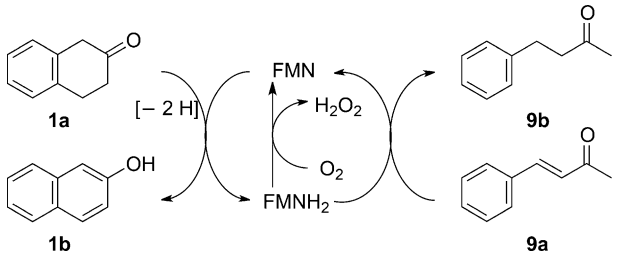
The application of biocatalysis in organic synthesis continues to grow year on year for a number of reasons including the high chemoselectivity, regioselectivity and enantioselectivity that enzyme catalysis offers to the organic chemist.[1] In addition, the application of enzymatic processes translates into hard cost savings for the fine chemical and pharmaceutical industry, by increasing net yield and purity and reducing waste for enantiopure products.[2]

The synthetic potential of flavin-dependent ene reductases (EREDs) in the asymmetric bioreduction of activated olefins has been well studied to date. They accept a wide range of activated alkene substrates including α,β-unsaturated ketones, aldehydes and nitriles, as well as carboxylic acids and their esters and nitroalkenes, as discussed in detail by several reviewers.[3] Examples of synthetically relevant chiral ERED products include (*R*)-levodione,[4] (*S*)-citronellal,[5] Roche ester derivatives[6] and γ-butyrolactones (when coupled with alcohol dehydrogenase).[7] ERED enzymes are also highly amenable to scale-up as demonstrated by the one-pot, three-enzyme system used to produce a chiral intermediate on a 70-g scale within our laboratories.[8]

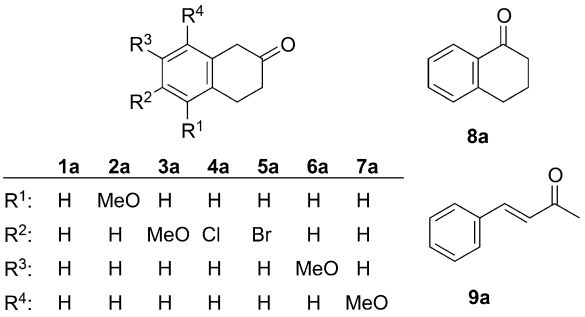
Besides alkene reduction, EREDs also exhibit a flavin-catalysed, disproportionation activity. During this reaction the flavin mononucleotide (FMN) prosthetic group is reduced by a suitable substrate (typically a conjugated enone), rather than NAD(P)H. Subsequent reduction of an activated alkene or of molecular oxygen (generating H_2O_2) by the FMN group completes the reaction cycle.[9]

Recently, Faber and colleagues[9a] demonstrated how a range of cyclohexenones and cyclic ketones could be used, as an alternative to NAD(P)H, to drive the asymmetric reduction of a number of industrially relevant, activated alkenes. This approach resulted in two products: the reduction product from the main target substrate and the oxidation product from the sacrificial co-substrate. Removal of [2H] from the co-substrate was typically followed by tautomerisation to give the aromatic (phenolic) compound, as illustrated in Scheme 1.

In the present work, the potential of EREDs as 'aromatases' in their own right is investigated, focusing on the production of naphthols. Naphthols are valuable building blocks for a wide variety of synthetic drugs and natural products. Several well-known pharmaceuticals including Propranolol, Naproxen and



Scheme 1. Disproportionation of an example substrate (2-tetralone), as catalysed by flavin-dependent EREDs. The reduced flavin can be re-oxidised by molecular oxygen to give peroxide, or *via* typical ERED reduction of an activated alkene.



	1a	2a	3a	4a	5a	6a	7a
R^1:	H	MeO	H	H	H	H	H
R^2:	H	H	MeO	Cl	Br	H	H
R^3:	H	H	H	H	H	MeO	H
R^4:	H	H	H	H	H	H	MeO

Scheme 2. Range of tetralones tested for the production of naphthols using EREDs.

Agomelatine are naphthol derived, highlighting the importance of this moiety in commercial drugs (Figure 1).[10] A proposed route to the anti-Ebola compound BCX4430 uses (*S*)-1-(α-aminobenzyl)-2-naphthol as a key chiral auxiliary in the synthesis.[11]

Substituted naphthols provide a route to biologically active naphthopyrans, similar to the natural product mollugin.[12] The methoxylated naphthyl compound guieranone A (isolated from the leaves of *Guiera senegalensis*), has been shown to have potent antifungal activity.[13] Furthermore, synthetic approaches to oxidative coupling of naphthols have attempted to mimic the natural biosynthesis of compounds such as nigerone, hypocrellin, calphostin D, phleichrome, and cercosporin.[14]

Towards the ERED-catalysed production of naphthols, a screen of 96 enzymes from Almac's selectA-Zyme collection was initially undertaken with 2-tetralone **1a** and 4-phenyl-3-buten-2-one **9a** as model substrates (Scheme 2). Compound **9a** was an inexpensive, readily available starting material and found to be well accepted by the ERED panel in previous work (unpublished data). Of the enzymes tested here, 57 produced detectable levels of 2-naphthol **1b**, and 3 of these hits were taken forward in further tests. EREDs 61, 69 and 82 all gave >67% of the naphthol and varied in their levels of conversion of substrate **9a**, giving 19%, 48% and 70%, respectively (Table 1).

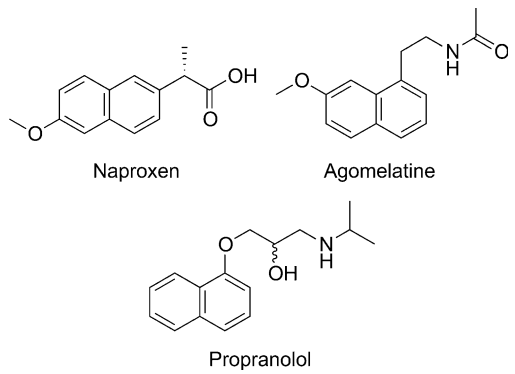


Figure 1. Pharmaceuticals derived from naphthols.

As the reactions were conducted in air there was no expectation that the oxidation and reduction product conversions would match, and indeed this was the case. Atmospheric oxygen provides an alternative route for FMN oxidation as shown in Scheme 1, and can therefore uncouple naphthol production from reduction of **9a**. As the primary aim was to promote naphthol production, the fate of the compound **9a** was of less concern in any case.

In subsequent experiments a range of substituted tetralones was used (Scheme 2). Small-scale reactions were carried out with a number of 2-tetralones **1a**–**7a** and with 1-tetralone **8a**, either with or without **9a** as a target substrate for reduction. The results, summarised in Table 1, showed that tetralones were very well accepted in general, with moderate to excellent conversions (up to >99%).

For the 2-tetralone series, the position of the aromatic substituent did appear to influence the tetralone acceptance. Specifically, position 6 was sensitive to substitutions, with drops in conversions most noticeable for the 6-methoxy compound **3a**, although the 6-chloro (**4a**) and 6-bromo (**5a**) compounds were more readily accommodated, with up to 91% and >99% conversions respectively (ERED-69 – see Table 1). Methoxy derivatives **2a**, **6a** and **7a** performed similarly to the unsubstituted compound 2-tetralone **1a**. The 7-methoxy compound **6a** in particular gave the best conversions overall, with consistently over 90% and up to >99% of the corresponding naphthol produced.

The lowest conversions were evident with 1-tetralone **8a** (although 44% conversion was still achieved), indicating that the position of the ketone moiety is important. This is consistent with the fact that substrate binding and orientation in EREDs is promoted by hydrogen bonding of the activating group with conserved histidine/histidine or histidine/asparagine residue pairs.[3d]

In light of the above results it would be interesting to test EREDs 61, 69 and 82 with more highly substi-

Table 1. Naphthol production from various tetralones using selected EREDs as oxidation catalysts.

Oxidation Substrate	Enzyme	With Reduction Substrate (**9a**) Conv. [%] (**9b**)[a]	With Reduction Substrate (**9a**) Conv. [%] (**1b–8b**)[a]	Without Reduction Substrate (**9a**) Conv. [%] (**1b–8b**)[a]
1a[b]	ERED-61	19	67	84
	ERED-69	48	67	>99
	ERED-82	70	88	>99
2a[c]	ERED-61	16	72	70
	ERED-69	35	95	94
	ERED-82	48	97	96
3a[b]	ERED-61	1	23	16
	ERED-69	14	41	53
	ERED-82	7	39	23
4a[c]	ERED-61	15	47	53
	ERED-69	45	60	91
	ERED-82	48	69	67
5a[c]	ERED-61	14	36	43
	ERED-69	39	78	>99
	ERED-82	46	50	71
6a[b]	ERED-61	31	70	93
	ERED-69	44	>99	>99
	ERED-82	73	>99	95
7a[c]	ERED-61	21	81	71
	ERED-69	32	84	>99
	ERED-82	68	91	96
8a[b]	ERED-61	25	43	44
	ERED-69	13	19	10
	ERED-82	16	19	2

[a] Conversions determined by GC-FID peak area.
[b] Corresponding naphthol products determined by comparison to reference standards on GC-FID.
[c] Corresponding naphthol products determined by GC-MS to confirm loss of [2H] in the molecular ion.

tuted tetralones, perhaps affording access to natural products such as guieranone A. Screening of the additional 54 EREDs shown to produce 2-naphthol, may also reveal even better activities with novel tetralone substrates.

For a given ERED, the ratio of the conversion of the reduction target **9a** to that of the tetralone varied depending on the tetralone substrate used. This ratio should be constant if it is assumed that, following flavin reduction by the tetralone, the likelihood of **9a** being reduced by the flavin is always the same. As the ratio is not consistent, this may indicate that different tetralones vary in their ability to promote $FMNH_2$ oxidation by O_2, or in their propensity to occupy the enzyme active site to the exclusion of **9a**.

The requirement for the reduction substrate **9a** was also of interest. Alongside the reactions that included **9a**, reactions were also run with the tetralone as the sole substrate in each case. The results (also given in Table 1) show that the absence of **9a** generally does not affect the ability of the enzyme to oxidise the tetralones. Indeed, conversions were often improved in many cases. In a separate experiment, ERED-69 was incubated in sealed vials under nitrogen, with substrate **6a**, in the presence and absence of reduction substrate **9a** (see the Supporting Information, S4). Under these conditions <1% conversion to the naphthol was seen without the reduction target whereas, in the presence of **9a**, the conversion was 92%, in line with 94% conversion to the saturated ketone **9b** (see Scheme 1). This result supports the idea that, in air, the dominant pathway for re-oxidation of the flavin, following reduction by the tetralone, is by oxygen (giving H_2O_2). In the absence of oxygen, **9a** serves as an efficient reduction target to drive the oxidation of the tetralone.

In choosing a reaction scheme to move forward, it seemed preferable to omit the additional substrate **9a**,

thus avoiding the need for sealed reactions under inert atmosphere and simplifying subsequent attempts at scale-up and product isolation.

With this in mind, the best performing substrate (7-methoxy compound **6a**) and the best performing enzyme (ERED-69) were selected for preparation of the corresponding naphthol on a gram scale. In order to increase the substrate loading, a number of co-solvents were screened at the 500-μL scale. In initial reactions, up to 17% (v/v) DMSO, *t*BME, toluene or THF was tested, with up to 42 mM (for DMSO) or 500 mM substrate (Table 2, entries 1–13). Toluene and THF were the least suitable solvents, giving at best 47% and 3% conversion, respectively, at 31 mM substrate (Table 2, entries 12 and 13).

Further tests focused on the better co-solvents, *t*BME and DMSO, at 60 mM substrate concentration. The reaction buffer was adjusted to pH 7.5 (as normal) or pH 9.0. Improved conversions were observed at pH 9.0, with values of 60% for 20% (v/v) DMSO co-solvent, 70% for 30% (v/v) DMSO and 83% with 17% (v/v) *t*BME (Table 2, entries 15, 17 and 19, respectively).

The effect of adding catalase was also tested at this stage, based on the assertion that any H_2O_2 produced might be removed and thus help to drive catalysis and protect against oxidative damage. In practice the addition of catalase did not improve conversions under the conditions tested (see the Supporting Information, S5). Finally, at 60 mM substrate the effects of reaction duration and temperature were examined, although no substantial difference in conversions was seen beyond 16 h reaction time or by varying the temperature to 25 °C or 37 °C from the original 30 °C (see the Supporting Information, S5).

With the optimised conditions in hand, the reaction was scaled to 190 mL (158 mL buffer, 32 mL *t*BME) with 2 g of substrate (60 mM or 10.6 g L^{-1}) and 3.8 g of freeze-dried ERED-69 enzyme powder. Following extraction, 1.82 g of product was isolated, giving 91% yield, with product confirmed by ^{1}H NMR (Supporting Information, S7).

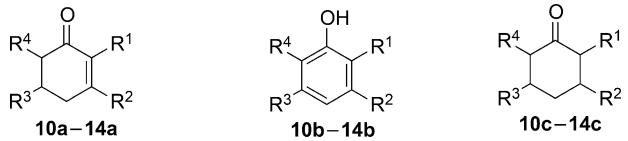
Extending the above approach, the results in Table 3 show how EREDs from the selectAZyme panel can also convert substituted cyclohexenones to the corresponding phenols (note: as with the tetralones, omission of **9a** did not seem to have a detrimental effect on conversion – see the Supporting Information, S6). Starting with 3-methyl-2-cyclohexenone **10a**, up to 96% conversion to *meta*-cresol **10b** was observed with selected EREDs. Compound **10a** can also serve as a target for reduction and the saturated product **10c** was observed at low levels (typically $<1\%$). Cyclohexenones with alternative substitution patterns were less efficient for phenol production. A methyl group at the α-carbon greatly reduced the conversions of **11a** and **13a**, with reduction to the saturated ketone favoured. A comparison of **11a** with **13a** and

Table 2. Optimisation conditions towards the scale-up of 7-methoxy-2-naphthol **6b**.

Entry	**6a** Conc. [mM]	Co-solvent (% v/v)	pH	Conv. [%][a]
1	24	DMSO (5)	7.5	79
2	48	DMSO (5)	7.5	52
3	23	DMSO (9)	7.5	75
4	46	DMSO (9)	7.5	60
5	21	DMSO (17)	7.5	77
6	42	DMSO (17)	7.5	53
7	31	*t*BME (17)	7.5	>99
8	63	*t*BME (17)	7.5	71
9	125	*t*BME (17)	7.5	32
10	250	*t*BME (17)	7.5	15
11	500	*t*BME (17)	7.5	5
12	31	toluene (17)	7.5	47
13	31	THF (17)	7.5	3
14	60	DMSO (20)	7.5	54
15	60	DMSO (20)	9.0	60
16	60	DMSO (30)	7.5	39
17	60	DMSO (30)	9.0	70
18	60	*t*BME (17)	7.5	62
19	60	*t*BME (17)	9.0	83

[a] Conversions determined by GC-FID peak area.

Table 3. Phenol production from enones **10a–14a** using selected EREDs.



	10a	11a	12a	13a	14a
R^1:	H	Me	H	Me	H
R^2:	Me	H	Me	H	Me
R^3:	H	H	Me	CH-CH_2	H
R^4:	H	H	H	H	*i*-Pr

Substrate	Enzyme	Conv. [%] **10b–14b**[a]	Conv. [%] **10c–14c**[a]
10a	ERED-66	96	<1
11a	ERED-61	13	18
12a	ERED-61	18	n.d.
13a	ERED-71	n.d.	10
14a	ERED-71	<1	n.d.

[a] Conversions determined by GC-FID peak area; n.d. = not detected.

10a with **14a** shows that additional ring substituents curtail the aromatisation reaction. Proposed explanations for this include altered (unproductive) substrate binding modes and fewer accessible hydrogens. That being the case, molecular modelling studies and targeted mutagenesis should afford access to productive binding modes that allow the efficient aromatisation of these and other cyclohexenones, while mitigating the competing reductive reaction.

Methylphenols find applications in the synthesis of drugs such as gemfibrozil[15] and α-tocopherol (vitamin E),[16] and also in the production of plastics and resins.[17] Other alkylphenols such as thymol, carvacrol and eugenol are important flavour and fragrance compounds, besides having anti-microbial properties.[18] Substituted phenols are therefore of great synthetic value and improving this new ERED-based route *via* enzyme engineering is an ongoing area of research in our laboratories.

In summary, an efficient enzymatic method for the production of substituted naphthols from the corresponding tetralones was presented. This approach takes advantage of the natural ability of ene reductases of the Old Yellow Enzyme family to work 'in reverse'. This ability was shown to be widespread among the selectAZyme panel of EREDs, with 60% of the panel giving 2-naphthol when presented with 2-tetralone as a substrate. Selected EREDs from the panel proved highly effective in the production of a set of substituted naphthols, with moderate to excellent conversions of up to >99%. A representative reaction was performed on a 2-g scale with 91% isolated yield, demonstrating the robustness of these enzyme catalysts towards solvents and elevated substrate loadings. The selectAZyme EREDs are therefore an invaluable tool for synthetic organic chemists seeking clean, safe and efficient ways to make substituted naphthols, for incorporation into new and existing synthetic APIs and natural products. In addition, the methodology can also easily be applied to the production of phenols, an area of ongoing active research within Almac.

Experimental Section

General experimental approaches are described below. For more specific details see the Supporting Information, including section S1.

Chemicals and Enzymes

Chemicals were purchased from Sigma Aldrich UK. All enzymes were obtained from Almac as freeze dried cell free extracts of *Escherichia coli*.

Analytical Methods

For quantitative conversion calculations GC-FID analysis was performed using either a Perkin–Elmer AUTOSYSTEM XL Gas Chromatograph fitted with a Zebron ZB-5 column (30 m, 0.25 mm I.D., 0.25 μm film thickness) or a Finnigan Trace GC fitted with an Agilent HP5-MS column (30 m, 0.25 mm I.D., 0.25 μm film thickness).

For tetralone substrates with no commercially available naphthol product standard (5-methoxy-2-naphthol, 6-chloro-2-naphthol, 6-bromo-2-naphthol and 8-methoxy-2-naphthol), GC-MS analysis was carried out to allow assignment of product peaks. For this purpose, a Thermo Finnigan Trace GC with PolarisQ mass spectrometer was used, and fitted with a Zebron ZB-5 column (30 m, 0.25 mm I.D., 0.25 μm film thickness).

Following product isolation from the scaled up 7-methoxy-2-naphthol reaction, the naphthol product was dissolved in $CDCl_3$ and analysed by ^1H NMR using a Bruker 500 MHz Ultrashield system.

Conditions for the Enzymatic Oxidation/Reduction of Tetralones and Enones

For small-scale screening, enzymatic reactions were carried out in 2-mL 96-well deep-well plates containing 10 mg per well of lyophilised cell free extract for each ERED in the selectAZyme panel. To each well, 500 μL of 100 mM Tris buffer, pH 7.5, were added. Substrates were dissolved in DMSO to 80 mM and 12.5 μL added to the reaction mix. Where the reduction substrate **9a** was included, 12.5 μL of an 80 mM DMSO stock were also added or alternatively 12.5 μL of DMSO when **9a** was omitted. Plates were covered with a SealPlate film (Sigma Aldrich) and left to shake overnight (16–18 h) at 30 °C and 1400 rpm in an Aosheng MB 100–4 A Thermo Shaker.

During optimisation of the ERED-69 reaction with 7-methoxy-2-tetralone, test reactions were carried out as above, except that 2 mL microfuge tubes were used (laid horizontally during shaking), and reaction parameters varied as described (see the Supporting Information, S5).

For GC analysis, an equal volume of ethyl acetate was added to each reaction and samples moved into 2 mL microfuge tubes. Tubes were shaken vigorously by hand for 1 min, vortexed briefly and then centrifuged at 16,000 × *g* for 5 min. The organic layer was pipetted off to fresh tubes and dried over $MgSO_4$.

Acknowledgements

Almac wish to thank Invest NI for research and development funding that contributed to this body of work. Invest NI's Grant for Research and Development programme is part financed by the European Regional Development Fund under the Investment for Growth and Jobs Programme 2014–2020.

References

[1] W.-D. Fessner, N. J. Turner, M. X. Wang, *Adv. Synth. Catal.* **2011**, *353*, 2189–2190.
[2] J. M. Woodley, *Trends Biotechnol.* **2008**, *26*, 321–327.
[3] a) K. Faber, M. Hall, *Biocatalysis in Organic Synthesis 2*, in: *Science of Synthesis*, (Eds.: K. Faber, W.-D. Fessner, N. J. Turner), Georg Thieme Verlag, Stuttgart, New York, **2015**, pp 213–260; b) H. S. Toogood, N. S. Scrutton, *Curr. Opin. Chem. Biol.* **2014**, *19*, 107–115; c) C. K. Winkler, G. Tasnádi, D. Clay, M. Hall, K. Faber, *J. Biotechnol.* **2012**, *162*, 381–389; d) H. S. Toogood, J. M. Gardiner, N. S. Scrutton, *ChemCatChem* **2010**, *2*, 892–914.
[4] S. Raimondi, D. Romano, A. Amaretti, F. Molinari, M. Rossi, *J. Biotechnol.* **2011**, *156*, 279–285.
[5] D. J. Bougioukou, A. Z. Walton, J. D. Stewart, *Chem. Commun.* **2010**, *46*, 8558–8560.
[6] C. Stueckler, C. K. Winkler, M. Bonnekessel, K. Faber, *Adv. Synth. Catal.* **2010**, *352*, 2663–2666.
[7] M. Korpak, J. Pietruszka, *Adv. Synth. Catal.* **2011**, *353*, 1420–1424.
[8] D. Mangan, I. Miskelly, T. S. Moody, *Adv. Synth. Catal.* **2012**, *354*, 2185–2190.
[9] a) C. K. Winkler, D. Clay, M. Entner, M. Plank, K. Faber, *Chem. Eur. J.* **2014**, *20*, 1403–1409; b) C. K. Winkler, D. Clay, E. van Heerden, K. Faber, *Biotechnol. Bioeng.* **2013**, *110*, 3085–3092; c) C. Stueckler, T. C. Reiter, N. Baudendistel, K. Faber, *Tetrahedron* **2010**, *66*, 663–667.
[10] a) H. S. Bevinakatti, A. A. Banerji, *J. Org. Chem.* **1991**, *56*, 5372–5375; b) K. Arai, Y. Chara, T. Iizumi, Y. Takakuwa, *Tetrahedron Lett.* **1983**, *24*, 1531–1534; c) S. K. Vujjini, V. R. K. R. Datla, K. R. Badarla, V. N. K. V. P. R. Vetukuri, R. Bandichhor, M. Kagga, P. Cherukupally, *Tetrahedron Lett.* **2014**, *55*, 3885–3887.
[11] X. Xu, *Chinese Patent Publication* CN104513249 A, **2015**.
[12] A. H. F. A. El-Wahab, H. M. Mohamed, A. M. El-Agrody, A. H. Bedair, *Eur. J. Chem.* **2013**, *4*, 467–483.
[13] O. Silva, E. T. Gomes, *J. Nat. Prod.* **2003**, *66*, 447–449.
[14] M. C. Kozlowski, B. J. Morgan, E. C. Linton, *Chem. Soc. Rev.* **2009**, *38*, 3193–3207.
[15] R. G. Glushkov, A. I. L′vov, O. S. Sizova, N. K. Davydova, G. M. Sanzharova, M. Ya. Polyakova, I. A. Balashova, *Pharm. Chem. J.* **1995**, *29*, 290–293.
[16] F. Schager, W. Bonrath, *J. Catal.* **1999**, *182*, 282–284.
[17] A. Gardziella, L. A. Pilato, A. Knop, *Phenolic Resins: Chemistry, Applications, Standardization, Safety and Ecology*, Springer-Verlag, Berlin, Heidelberg, **2000**.
[18] W. T. Langeveld, E. J. A. Veldhuizen, S. A. Burt, *Crit. Rev. Microbiol.* **2014**, *40*, 76–94.

