Kinetic deuterium isotope effect in the oxidation of veratryl alcohol promoted by lignin peroxidase and chemical oxidants

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The intramolecular kinetic deuterium isotope effects (k_H/k_D = 4.6–4.9) determined in the H_2O_2-induced oxidation of the racemic and enantiomeric forms of α-monodeuterated veratryl alcohol catalysed by lignin peroxidase (LiP) are very similar to those determined in the oxidation of racemic α-monodeuterated veratryl alcohol promoted either by a LiP model compound (a water soluble iron porphyrin using *m*-chloroperbenzoic acid as the oxidant) (k_H/k_D = 4.2) or by potassium 12-tungstocobalt(III)ate, a genuine one-electron oxidant (k_H/k_D = 4.5). These results indicate that very likely veratryl alcohol radical cation, once generated by the LiP–H_2O_2 system, is released from the enzyme and is deprotonated by the medium.

Introduction

Lignin peroxidase (LiP, EC 1.11.1.7) is an extracellular heme enzyme isolated from ligninolytic cultures of the white-rot basidiomycetous fungus *Phanerochaete chrysosporium*.[1,2] The importance of this enzyme is related to lignin degradation which is a fundamental process in the pulp and paper industry. Accordingly LiP catalyses the oxidative depolymerization of dilute solutions of polymeric lignin *in vitro*.[3] LiP is also able to promote the H_2O_2-dependant oxidation of several classes of compounds characterized by relatively high redox potential (< 1.4 V).[4–15] The catalytic cycle of LiP involves the initial reaction of the ferric heme in the native state with H_2O_2 to yield compound I, the active enzymatic species, an oxy-ferryl heme porphyrin π-cation radical ($P^{+\bullet}Fe(IV)=O$) (eqn. 1), then the

$$\underset{\text{LiP}}{\text{P-Fe(III)}} + H_2O_2 \rightarrow \underset{\text{LiP Compound I}}{\overset{+\bullet}{\text{P}}\text{-Fe(IV)=O}} + H_2O \quad (1)$$

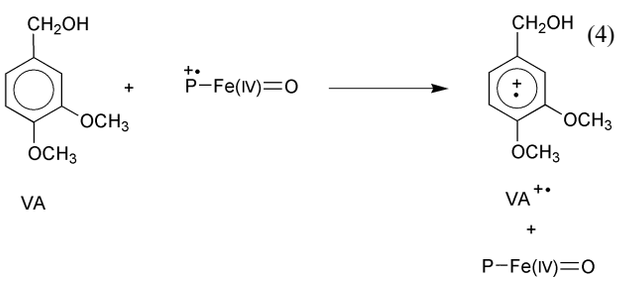
resting state of the enzyme is regenerated after two single electron oxidations of substrates with formation of the intermediate compound II, which retains the oxy-ferryl heme ($PFe(IV)=O$) (eqn. 2–3).

$$\overset{+\bullet}{\text{P}}\text{-Fe(IV)=O} + S \rightarrow \underset{\text{LiP-Compound II}}{\text{P-Fe(IV)=O}} + S^{+\bullet} \quad (2)$$

$$\text{P-Fe(IV)=O} + S + 2\,H^+ \longrightarrow \text{P-Fe(III)} + S^{+\bullet} + H_2O \quad (3)$$

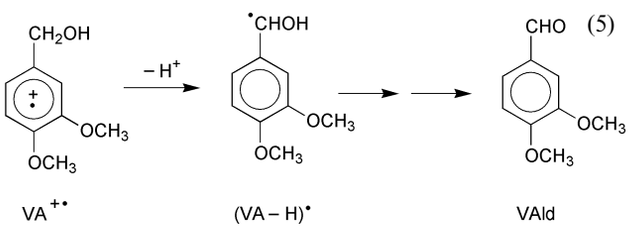
The LiP-catalysed degradation of lignin appears to require the presence of veratryl alcohol, 3,4-dimethoxybenzyl alcohol (VA), a secondary metabolite of the fungus *Phanerochaete chrysosporium*, as a cofactor.[16–18] This observation has led to a lively discussion about the actual role played by VA in this respect and several hypotheses (redox mediation, protection of the enzyme from irreversible inactivation by H_2O_2, completion of the catalytic cycle) have been presented.[17,19,20] The knowledge of the detailed mechanism of the oxidation of VA catalysed by LiP is certainly essential to this discussion.

There is wide consensus that the oxidation of VA involves the transfer of one electron to LiP I leading to the formation of $VA^{+\bullet}$ and the reduced form of the oxo complex, compound II (eqn. 4).[21,22] Subsequently $VA^{+\bullet}$ undergoes C_α-H deproton-



(4)

ation,[11] a typical reaction of alkylaromatic radical cations,[23,24] to form a carbon centred radical which is eventually converted to veratryl aldehyde, VAld, (eqn. 5).[25]



(5)

However, it has not yet been clearly established whether such deprotonation is an enzymatic process or if it occurs in the medium after $VA^{+\bullet}$ has been released from the enzyme. The latter possibility was considered the most likely being consistent with the proposal that VA may act as a redox mediator in LiP-catalysed oxidations.[17,27,10] However, the finding that in the LiP-catalysed oxidation of VA the formation of VAld is not

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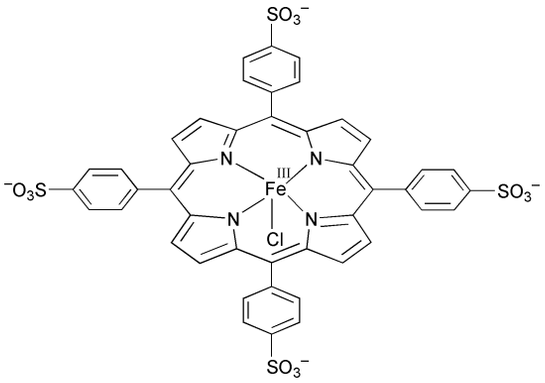

Fig. 1 Metalloporphyrin 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine-*p*,*p*′,*p*″,*p*‴-tetrasulfonic acid iron(III) chloride.

accompanied by that of dimeric products[28] has suggested that there should be no significant diffusion of $VA^{+\bullet}$ into the reaction medium and that therefore its deprotonation probably occurs while the radical cation is still bound to the enzyme. Moreover, it has recently been proposed that once $VA^{+\bullet}$ is formed by LiP compound I, a strong $VA^{+\bullet}$–LiP compound II complex is created which should be the species playing the key role in the redox mediation process.[29] Return of this complex to the native state may release $VA^{+\bullet}$ to the medium, however, the possibility that deprotonation takes place in the $VA^{+\bullet}$–LiP II complex must be considered.[29–31] On the other hand, very little is known about the VA binding site which might be close to the heme-edge[32–34] or also, as recently proposed, on the LiP surface in proximity of a tryptophan residue (Trp 171).[35,36]

In this context, we have felt that a better insight in this mechanistic problem might be obtained by specifically investigating the deprotonation step, and namely one of its most important properties, the kinetic deuterium isotope effect (KDIE). Thus, we have determined the intramolecular KDIE of the LiP-catalysed oxidation of both (*S*) and (*R*) forms of the α-monodeuterated veratryl alcohol [(*S*)-VA-*d* and (*R*)-VA-*d*] promoted by H_2O_2. The results of the LiP-catalysed reactions have been compared with those obtained in the oxidation of racemic α-monodeuterated veratryl alcohol [(±)-VA-*d*] promoted by potassium dodecatungstocobalt(III)ate, $K_5Co(III)W_{12}O_{40}$, [Co(III)W] a genuine outer-sphere one-electron chemical oxidant[37,38] and by *m*-chloroperbenzoic acid (MCPBA) in the presence of the water soluble metalloporphyrin 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine-*p*,*p*′,*p*″,*p*‴-tetrasulfonic acid iron(III) chloride (FeTPPSCl) (Fig. 1). The latter system is expected to mimic the LiP reactivity,[39] since the active species, formed by reaction of FeTPPSCl with MCPBA can be described as an iron(IV)-oxo porphyrin radical cation and closely resembles LiP I, the active species of LiP.

The KDIE value is expected to be significantly influenced by the nature of the proton abstracting base. Thus, if the deprotonation of $VA^{+\bullet}$ in the LiP-catalysed oxidation is promoted by the enzyme, it is very likely that the KDIE value is different from those determined in the chemical ([FeTPPSCl–MCPBA] or [Co(III)W]) reactions. Moreover, different properties of the two enantiomeric alcohols with respect to the KDIE value can also be expected, due to the enzyme chirality.[40,41] Conversely, if $VA^{+\bullet}$ is deprotonated as a free radical cation in the medium no significant differences in the KDIE values between chemical and enzymatic reactions, as well as between the (*R*) and (*S*) enantiomers in the enzymatic reaction, are predicted.

Results and discussion

All the oxidations were carried out at pH 4 (50 mM sodium tartrate buffer), at 25 °C, using equimolar amounts of the oxidant and the substrate, under an argon atmosphere. As expected, veratryl aldehyde and α-deuterated veratryl aldehyde were the only reaction products with the enzymatic[25] and the chemical oxidizing systems so indicating that deprotonation is the only observed route for $VA^{+\bullet}$. No products were formed when the oxidation was carried out in the absence of the oxidant [H_2O_2 or MCPBA or Co(III)W] or in the absence of LiP. The intramolecular KDIE (k_H/k_D) values were calculated *via* GC-MS analysis by the ratios of the intensities of their molecular ion peaks (166 and 167, respectively) corrected for the ^{13}C contribution. In order to exclude any equilibration process for $VA^{+\bullet}$, which might affect the interpretation of our results it was verified that in the enzymatic oxidation of racemic VA-*d* the unreacted substrate had the same deuterium content of the starting material and that there was no enantiomeric enrichment. The intramolecular KDIE values obtained in the enzymatic and chemical reactions are reported in Table 1.

Table 1 Intramolecular deuterium kinetic isotope effect in the oxidation of [α-$^2H_1$]veratryl alcohols catalysed by LiP, FeTPPSCl or Co(III)W[a]

Substrate	Oxidizing system	k_H/k_D[b]
(*R*)-VA-*d*	LiP–H_2O_2	4.6
(*S*)-VA-*d*	LiP–H_2O_2	4.9
(±)-VA-*d*	LiP–H_2O_2	4.7
(±)-VA-*d*	FeTPPSCl–MCPBA	4.2
(±)-VA-*d*	Co(III)W	4.5

[a] For the reaction conditions see the Experimental section. [b] Determined *via* GC-MS by the ratio between the corrected signal intensities of the two molecular ions at *m*/*z* 167 and 166. Average of at least three determinations, the error is in all cases *ca.* ±0.2.

Inspection of the data in Table 1 immediately reveals that the values of KDIE for the enzymatic and chemical oxidations are very close to one another, the differences being practically within the experimental error. This observation immediately suggests that $VA^{+\bullet}$ deprotonation is very likely promoted by the same basic species in the oxidation catalysed by LiP and in those promoted by FeTPPSCl and Co(III)W. The most reasonable hypothesis is that this base is in all cases the reaction medium and that therefore deprotonation of $VA^{+\bullet}$ generated by LiP is not an enzymatic reaction. In line with this conclusion is also the observation that almost identical k_H/k_D are found in the LiP-induced oxidations of (*R*)-VA-*d* and (*S*)-VA-*d*. Clearly, the relative reactivity of the C_α-H and C_α-D bonds is the same either when we compare the pro-*S* hydrogen with the pro-*R* deuterium or *vice versa*, indicating that in the $VA^{+\bullet}$ deprotonation process there is no differentiation between the two enantiotopic hydrogens of the CH_2OH group. This finding is also consistent with a deprotonation reaction which does not involve a veratryl alcohol radical cation still bound to the enzyme. In this case, the two enantiomeric radical cations should form two diastereomeric complexes and diastereomeric transition states are expected for the cleavage reactions of the prochiral hydrogens. This should lead to somewhat different values of k_H/k_D for the (*R*) and (*S*) forms of VA-*d*, which is not observed.

Summing up, the present results allow us to conclude that a classical enzymatic pathway for the deprotonation of veratryl alcohol radical cation in the LiP promoted oxidation of VA is very unlikely; even though the formation of a $VA^{+\bullet}$–LiP II complex takes place, as suggested,[29] such a complex should not play any significant role in the $VA^{+\bullet}$ deprotonation process. This conclusion may also lend support to the suggestion that VA binds to Trp 171 on the enzyme surface rather than to the heme-edge.[35,36] Accordingly, in the former case a fast release from the enzyme to the medium of $VA^{+\bullet}$, once it is formed by a long range electron transfer, is a very reasonable possibility. It should finally be noted that the similar k_H/k_D values for the LiP and FeTPPSCl catalysed oxidation of VA confirm the capacity of the iron porphyrin to efficiently mimic the enzymatic reaction.

Experimental

Methods

^{1}H-NMR spectra were recorded on a Bruker AC300P spectrometer in $CDCl_3$. GC-MS analyses were performed on an HP5890 GC (OV1 capillary column, 12 m × 0.2 mm) coupled with an HP5970 MSD. GC analyses were performed on a Varian 3400 GC (OV1 capillary column, 25 m × 0.2 mm).

Substrates and reagents

All the reagents and solvents were of the highest purity available and used without further purification (unless otherwise specified). The concentration of H_2O_2 (Carlo Erba Reagents) was determined by titration with permanganate.[42] MCPBA was purified by washing it with phosphate buffer and assayed by iodometric titration.[43] 5,10,15,20-Tetraphenyl-21*H*,23*H*-porphine-*p*,*p*′,*p*″,*p*‴-tetrasulfonic acid tetrasodium salt dodecahydrate (Aldrich) was metallated according to the literature procedure.[44] Co(III)W was prepared using the literature procedure[37] with some modifications.[38] LiP was prepared and purified as described in the literature.[45] The concentration of the enzyme solution was determined spectrophotometrically (ε_{409nm} = 169 mM^{-1} cm^{-1}).[46]

α-Deuterated veratryl aldehyde was prepared by oxidation of [α-$^2H_2$]veratryl alcohol (from 3,4-dimethoxybenzoic acid and $LiAlD_4$[47]), with pyridinium chlorochromate (PCC) in anhydrous CH_2Cl_2.[48]

(*R*)-[α-$^2H_1$]Veratryl alcohol [(*R*)-VA-*d*] and (*S*)-[α-$^2H_1$]-veratryl alcohol [(*S*)-VA-*d*] were synthesized by the asymmetric reduction of α-deuterated veratryl aldehyde with *B*-(3α-pinanyl)-9-borabicyclo[3.3.1]nonanes [obtained by hydroboration of enantiomerically pure (*S*)-α-pinene and (*R*)-α-pinene, respectively, with 9-borabicyclo[3.3.1]nonane (9-BBN)] according to a literature procedure.[49,50]

The products were purified by silica-gel chromatography (petroleum ether 30–50–ethyl acetate in gradient of elution) and identified by GC-MS and ^{1}H-NMR analysis. The products were obtained with isotopic purity >99%, as determined by GC-MS. The enantiomeric excess of the two alcohols was determined by ^{1}H-NMR analysis of their esters with (*S*)-2-acetoxy-2-phenylethanoic acid:[50,51] [(*R*)-[α-$^2H_1$]veratryl alcohol] (*S*)-2-acetoxy-2-phenylethanoate: δ 6.7–8.2 (m, 8H, Ar-H), 5.95 (s, 1H, CH), 5.11 (t, 1H, CH_SD_R), 3.86 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3), 2.19 (s, 3H, CH_3).

[(*S*)-[α-$^2H_1$]veratryl alcohol] (*S*)-2-acetoxy-2-phenylethanoate: δ 6.7–8.2 (m, 8H, Ar-H), 5.95 (s, 1H, CH), 5.01 (t, 1H, CH_RD_S), 3.86 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3), 2.19 (s, 3H, CH_3).

The enantiomeric excess was 96% for (*R*)-VA-*d* and 94% for (*S*)-VA-*d*.

The racemic [α-$^2H_1$]veratryl alcohol [(±)-VA-*d*] was prepared by reduction of veratryl aldehyde with $NaBD_4$.[47]

Product analysis

Reaction products were analysed by GC, GC-MS and ^{1}H-NMR. Yields were determined by GC (using 4-methoxyacetophenone as the internal standard) and referred to the starting material. A good recovery of materials (>95%) was observed in all the experiments. The intramolecular KDIE values were determined by GC-MS analysis by the ratio between the signal intensities of the two molecular ions at *m*/*z* 167 and 166 corrected for the ^{13}C contribution.

Oxidations

Enzymatic oxidation. The alcohol (18 μmol) and LiP (0.38 units, 0.64 nmol) were magnetically stirred in 5 mL of argon-degassed 50 mM sodium tartrate buffer solution, pH = 4.0 at 25 °C. H_2O_2 (18 μmol) in 0.25 mL of buffer solution was gradually added in 1 h using a syringe pump. The products of the reaction were extracted with CH_2Cl_2 and dried over anhydrous Na_2SO_4. The yields in VAld, referred to the starting material, which is equimolecular with the oxidant, were as follows: (*R*)-VA-*d* (13%), (*S*)-VA-*d* (16%), (±)-VA-*d* (16%).[52]

In the oxidation of (±)-VA-*d* the deuterium content of the unreacted substrate was the same (>99%) as that of the starting material with no enantiomeric enrichment.

Biomimetic oxidation. The oxidant MCPBA (18 μmol) was added to a magnetically stirred argon-degassed solution of the substrate (18 μmol) and FeTPPSCl (0.54 μmol) in 5 mL of 50 mM sodium tartrate buffer solution, pH = 4.0, at 25 °C. After 1 h, a saturated solution of $NaHCO_3$ was added to the reaction mixture which was extracted with CH_2Cl_2 and dried over anhydrous Na_2SO_4. The yield in VAld, referred to the starting material, which is equimolecular with the oxidant was 12%.

Chemical oxidation. The alcohol (18 μmol) and the oxidant (18 μmol) were magnetically stirred for 4 h, at 25 °C in 5 mL of argon-degassed 50 mM sodium tartrate buffer solution, pH = 4.0. The products of the reaction were extracted with CH_2Cl_2 and dried over anhydrous Na_2SO_4. The yield in VAld, referred to the starting material, which is equimolecular with the oxidant was 37%.

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References

1 M. Tien and T. K. Kirk, *Science*, 1983, **221**, 661.
2 J. K. Glenn, M. A. Morgan, M. B. Mayfield, M. Kuwahara and M. H. Gold, *Biochem. Biophys. Res. Commun.*, 1983, **114**, 1077.
3 K. E. Hammel and M. A. Moen, *Enzyme Microb. Technol.*, 1991, **13**, 15.
4 K. E. Hammel, M. Tien, B. Kalyanaraman and T. K. Kirk, *J. Biol. Chem.*, 1985, **260**, 8348.
5 H. E. Schoemaker, P. J. Harvey, R. M. Bowen and J. M. Palmer, *FEBS Lett.*, 1985, **183**, 7.
6 P. J. Kersten, M. Tien, B. Kalyanaraman and T. K. Kirk, *J. Biol. Chem.*, 1985, **260**, 2609.
7 K. E. Hammel, B. Kalyanaraman and T. K. Kirk, *Proc. Natl. Acad. Sci. USA*, 1986, **83**, 3708.
8 K. Miki, V. Renganathan and M. H. Gold, *Biochemistry*, 1986, **25**, 4790.
9 V. Renganathan, K. Miki and M. H. Gold, *Arch. Biochem. Biophys.*, 1986, **246**, 155.
10 P. J. Harvey, R. Floris, T. Lundell, J. Palmer, H. E. Schoemaker and R. Wever, *Biochem. Soc. Trans.*, 1992, 345.
11 M. Bietti, E. Baciocchi and S. Steenken, *J. Phys. Chem. A*, 1998, **102**, 7337.
12 S. D. Haemmerli, M. S. A. Leisola, D. Sanglard and A. Fiechter, *J. Biol. Chem.*, 1986, **261**, 6900.
13 D. K. Joshi and M. H. Gold, *Biochemistry*, 1994, **33**, 10969.
14 B. Kalyanaraman, *Xenobiotica*, 1995, **25**, 667.
15 G. V. B. Reddy, M. D. S. Gelpke and M. H. Gold, *J. Bacteriol.*, 1998, **180**, 5159.
16 K. Lundquist and T. K. Kirk, *Phytochem.*, 1978, **17**, 1676.
17 P. J. Harvey, H. E. Schoemaker and J. M. Palmer, *FEBS Lett.*, 1986, **195**, 242.
18 D. C. Goodwin, S. D. Aust and T. A. Grover, *Biochemistry*, 1995, **34**, 5060.
19 K. Valli, H. Wariishi and M. H. Gold, *Biochemistry*, 1990, **29**, 8535.
20 R. S. Koduri and M. Tien, *Biochemistry*, 1994, **33**, 4225.
21 G. Labat and B. Meunier, *Bull. Soc. Chim. Fr.*, 1990, **127**, 553.
22 H. B. Dunford, *Heme Peroxidases*, John Wiley & Sons, New York, 1999, Ch. 12.
23 M. S. Schmittel and A. Burghart, *Angew. Chem., Int. Ed. Engl.*, 1997, **36**, 2550.
24 E. Baciocchi, M. Bietti and O. Lanzalunga, *Acc. Chem. Res.*, 2000, **33**, 243.

25 VAld is the only reaction product when operating under an inert atmosphere. In the presence of O_2 other products are formed.[26]
26 R. Have and M. C. R. Franssen, *FEBS Lett.*, 2001, **487**, 313.
27 G. Gilardi, P. J. Harvey, A. E. G. Cass and J. M. Palmer, *Biochim. Biophys. Acta*, 1990, **1041**, 129.
28 D. K. Joshi and M. H. Gold, *Eur. J. Biochem.*, 1996, **237**, 45.
29 P. J. Harvey, J. M. Palmer, H. E. Schoemaker, H. L. Dekker and R. Wever, *Biochem. Biophys. Acta*, 1988, **994**, 59.
30 A. Khindaria, I. Yamazaki and S. D. Aust, *Biochemistry*, 1995, **34**, 16860.
31 A. Khindaria, I. Yamazaki and S. D. Aust, *Biochemistry*, 1996, **35**, 6418.
32 S. L. Edwards, R. Raag, H. Wariishi, M. H. Gold and T. L. Poulos, *Proc. Natl. Acad. Sci. USA*, 1993, **90**, 750.
33 K. Piontek, T. Glumoff and K. Winterhalter, *FEBS Lett.*, 1993, **315**, 119.
34 T. Choinowski, W. Blodig and K. H. Winterhalter, *J. Mol. Biol.*, 1999, **286**, 809.
35 W. A. Doyle, W. Blodig, N. C. Veicht, K. Piontek and A. T. Smith, *Biochemistry*, 1998, **37**, 15097.
36 K. Ambert-Balay, S. Fuchs and M. Tien, *Biochem. Biophys. Res. Commun.*, 1998, **251**, 283.
37 L. Eberson, *J. Am. Chem. Soc.*, 1983, **105**, 3192.
38 E. Baciocchi, M. Crescenzi, E. Fasella and M. Mattioli, *J. Org. Chem.*, 1992, **57**, 4684.
39 B. Meunier, *Metalloporphyrins in Catalytic Oxidations*, ed. R. A. Sheldon, Marcel Dekker, Inc, New York, 1994, Ch. 5.
40 D. B. Northrop, *Isotope Effects on Enzyme-Catalysed Reactions*, eds. W. W. Cleland, M. H. O'Leary and D. B. Northrop, University Park Press, Baltimora, MD, 1977, pp. 122–152.
41 L. Melander and W. H. Saunders, *Reaction Rates of Isotopic Molecules*, John Wiley, New York, 1980, Ch. 4, 9.
42 H. A. Flascka, A. J. Jr. Barnard and P. E. Stwrock, *Quantitative Analytical Chemistry*, Harper & Row, New York, 1969, vol. 2, pp. 149–167.
43 N. N. Schwartz and J. H. Blumbergs, *J. Org. Chem.*, 1964, **29**, 1976.
44 E. B. Fleisher, J. M. Palmer, T. S. Srivastava and A. Chatterjee, *J. Am. Chem. Soc.*, 1971, **93**, 3162.
45 M. Tien and T. K. Kirk, *Methods Enzymol.*, 1988, **161**, 238.
46 M. Tien, T. K. Kirk, C. Bull and J. A. Fee, *J. Biol. Chem.*, 1986, **261**, 1687.
47 M. J. Coon and A. D. N. Vaz, *Biochemistry*, 1994, **33**, 6442.
48 E. J. Corey and J. W. Suggs, *Tetrahedron Lett.*, 1975, 2647.
49 M. M. Midland, A. Tramontano and S. A. Zderic, *J. Am. Chem. Soc.*, 1977, **109**, 5211.
50 K. Ramalingam, P. Nanjappan, D. M. Kalvin and R. W. Woodward, *Tetrahedron*, 1988, **44**, 5597.
51 D. Parker, *J. Chem. Soc., Perkin Trans. 2*, 1983, 83.
52 Substantially higher yields in VAld have been reported by D. K. Joshi and M. H. Gold[28] but their conditions were significantly different from ours.