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RATIONAL INHIBITOR DESIGN, SYNTHESIS AND NMR SPECTROSCOPIC STUDY BY TRANSFERRED NUCLEAR OVERHAUSER SPECTROSCOPY OF NOVEL INHIBITORS OF CINNAMYL ALCOHOL DEHYDROGENASE, A CRITICAL ENZYME IN LIGNIFICATION

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Cinnamyl alcohol dehydrogenase is one of the enzymes controlling the first two committed steps of lignification. Using a 3-dimensional similarity model of this enzyme, a series of novel phosphonates (**1**–**5**) was designed as potential inhibitors. Phosphonates **1**–**5** were synthesized in good yield by reaction of the corresponding cinnamaldehydes with tetraethylmethylene diphosphonate. Monophosphonic acids **6** and **7** were obtained by basic hydrolysis of the corresponding phosphonates while phosphonamidate **8** was synthesized by reacting benzylamine with the iminium salt intermediate of the monophosphonic acid. Using recombinant cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) the inhibitory activity of these compounds was evaluated and compared with that of the carbonyl analogues. Inhibition kinetic studies showed compounds **2** and **3** to be mixed type linear inhibitors while compound **4** was uncompetitive. ^{1}H NMR studies of inhibitor **2**, for which K_i and K_i' were 20 and 86 μM, respectively, in the presence of CAD based on selective line-broadening showed an increased interaction of the 3-OMe

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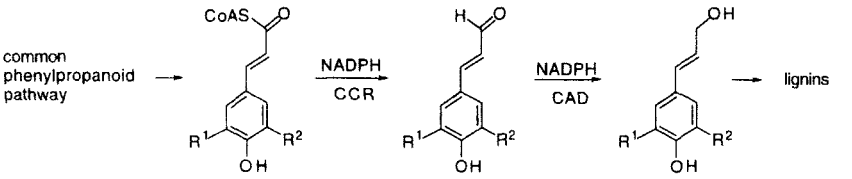
group of the aromatic ring of the inhibitor with the active site of the CAD. A transferred nuclear overhauser effect spectroscopy (TRNOESY) experiment for inhibitor **2** with CAD was used to determine the conformation of this compound bound to CAD. These results were found to be consistent with the 3-dimensional structural model of the enzyme.

Keywords: Alcohol dehydrogenase; Lignification; Phosphonates; Enzyme inhibition; TRNOESY; Inhibitor conformation

Abbreviations: CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamoyl CoA reductase; DIEA, N,N-diisopropylethylamine; DPPA, diphenylphosphorylazide; DTT, dithiothreitol; LDA, lithium diisopropylamide; THF, tetrahydrofuran; TRNOESY, transferred nuclear Overhauser effect spectroscopy

INTRODUCTION

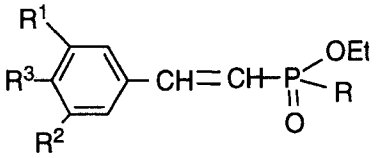
Cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) is one of the enzymes controlling the first committed steps of lignification[1] (Equation 1).



Coniferyl alcohol; $R^1 = H$, $R^2 = OCH_3$
Sinapyl alcohol; $R^1 = R^2 = OCH_3$
p-Coumaryl alcohol; $R^1 = R^2 = H$ (1)

With the availability of the sequence[2] of the gene for CAD from *Eucalyptus*, it became possible to predict the structure of this enzyme by homology modelling[3] using the coordinates of horse liver alcohol dehydrogenase[4] supported by structural data for several other dehydrogenases. From this model the active-site was predicted to contain a hydrophobically lined slot adjacent to the catalytic zinc atom. This hydrophobic slot, formed by Ile-229, Trp-119 and Phe-298, was proposed to provide the specificity for CAD by binding the *p*-hydroxycinnamyl portion of the substrate. A well-formed molecular sandwich results when a cinnamyl alcohol is docked into this region with the planar cinnamyl group face-to-face on one side with the aromatic ring of Phe-298 and on the other with that of Trp-119.[3] In the course of modelling this enzyme several readily available ligands, including some flavones, were predicted as novel inhibitors of CAD, and were found to inhibit with I_{50} values in the region of 50–700 mM. However, there are still

rather few structural classes of inhibitor described for CAD. Further analysis of the molecular model has led us to predict that appropriate phosphonate analogues of cinnamyl alcohol might provide leads to new more potent, specific inhibitors of CAD, which may be useful to manipulate lignification in experimental systems. We now present the synthesis and enzyme kinetic study of a range of phosphonates and derivatives (**1**–**8**) as potential inhibitors of CAD. We have also been able to determine by NMR spectroscopy the enzyme-bound conformation of one of these (**2**) by making use of the fast exchange of relatively weakly bound ligands and the TRNOESY.



	R^1	R^2	R^3	R
1	H	H	OH	OEt
2	H	OMe	OH	OEt
3	OMe	OMe	OH	OEt
4	H	OMe	OMe	OEt
5	H	H	OMe	OEt
6	H	OMe	OH	OH
7	H	OMe	OMe	OH
8	H	OMe	OMe	$NHCH_2Ph$

MATERIALS AND METHODS

Purification of CAD

The cDNA for CAD from *Eucalyptus* has been cloned and over-expressed in *Escherichia coli*.[2] Recombinant CAD produced as described previously[5] was purified as follows. The clear bacterial cell lysate obtained after centrifugation was applied to a column of Q-Sepharose Fast-Flow (10 × 2.6 cm) pre-equilibrated with buffer A (pH 7.5, 20 mM Tris/HCl, 5 mM DTT, 2% ethylene glycol) using a flow rate of 10 ml/min. After applying the sample, the column was washed with ten column volumes of buffer A containing 100 mM NaCl. CAD was eluted with 200 mM NaCl in buffer A. Fractions containing CAD activity were pooled and the protein precipitated with 80%

ammonium sulfate. The precipitate was centrifuged and resuspended in pH 7.5, 100 mM Tris/HCl containing 5 mM DTT and 5% ethylene glycol. After desalting using a PD-10 column (Pharmacia) the sample was applied to a column of 2′,5′-ADP-Sepharose (5 × 1.5 cm) equilibrated with the same buffer at a flow-rate of 15 ml/h. After washing the column with five column volumes of the same buffer, CAD was eluted with a gradient of 0–4 mM $NADP^+$ (total volume 40 ml). CAD activity during the course of purification was measured spectrophotometrically at 30°C by means of the increase in absorbance at 400 nm due to the formation of coniferaldehyde. The assay incubation mixture contained 2 mM coniferyl alcohol, 0.2 mM $NADP^+$ and an aliquot of protein extract in pH 8.80, 100 mM Tris/HCl.[6] CAD purity was controlled by SDS gel electrophoresis (Figure 1). The specific activity of recombinant CAD was also measured and found equal to 80 nkat/mg compared to 92 nkat/mg for the native enzyme.[5]

Inhibition Studies

Inhibition studies were carried out under the following conditions. Stock solutions of substrate (1 and 2 mM) in Tris/HCl buffer, pH 8.80, and

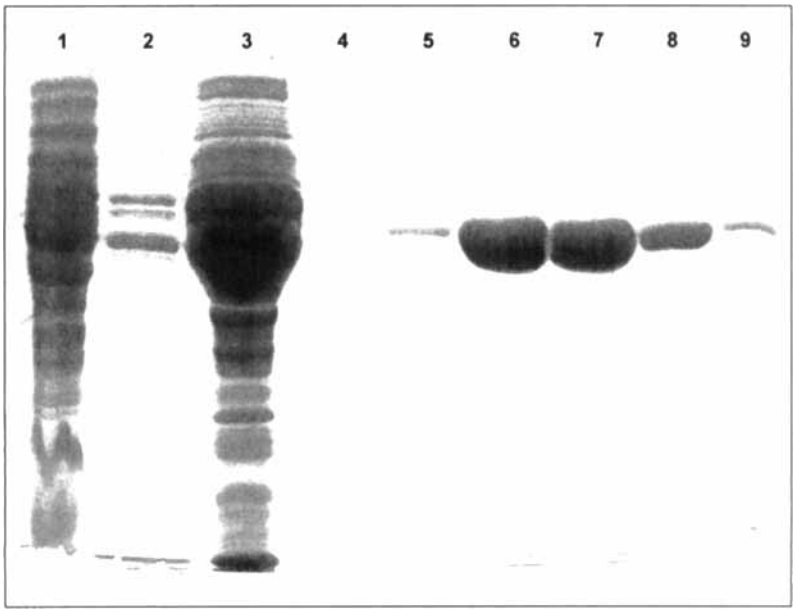


FIGURE 1 SDS-gel electrophoresis for recombinant CAD: cell lysate (lane 1); after ion-exchange on Q-sepharose (lane 2); some extract after precipitation and desalting (lane 3); final purified fraction from 2′,5′-ADP-Sepharose column (lanes 4–9). See Color Plate IV.

$NADP^+$ (1 mM) in distilled water were prepared before use. Stocks solutions of inhibitors were prepared in anhydrous CH_3CN. Under the conditions of kinetic assays the level of CH_3CN did not exceed 5% v/v and did not affect the enzyme activity. UV kinetic studies were performed using a Perkin Elmer Lambda 7 spectrophotometer thermostated to $30 \pm 0.1°C$. The initial rates of enzymic oxidation at substrate concentrations in the range 2.5–50 μM were thus obtained and fitted to a hyperbolic curve by using the Enzfitter programme by R.J. Leatherbarrow, distributed by Elsevier Biosoft. The values of k_{cat}, K_m and K_{mapp} were thus calculated. All velocity equations fit the usual Henri–Michaelis–Menten equation. K_{mapp} is the constant of this equation obtained in presence of different concentrations of inhibitor and is generally equal to the K_m term multiplied by a factor which is a function of the inhibitor concentration and the inhibition constant. At least three determinations were made for each assay, and mean values were used for subsequent calculations. Checks of the enzyme activity were performed regularly over the course of each inhibition study. The percentage of inhibition values were determined in order to provide a basis for detailed K_i determination for the better inhibitors in the series. The type of inhibition was determined by a diagnostic set of plots[7] viz., $1/v_0$ versus [I] at various values of [S], $1/v_0$ versus 1/[S] at various [I] and $[S]/v_0$ versus [I] at various [S]. According to the type of inhibition detected by these plot patterns, appropriate plots and/or replots were used to determine K_i values.

Synthesis of Phosphonate and Derivatives 1–8

IR spectra were recorded on a Perkin Elmer 883 spectrometer. ^1H, ^{13}C and ^{31}P NMR spectra were recorded with a Bruker AC-200 or AC-250 (200 or 250 MHz for ^1H, 50.3 or 62.9 MHz for ^{13}C and 81 MHz (AC-200) for ^{31}P, respectively). Chemical shifts were referenced to tetramethylsilane as internal reference for ^1H NMR and to tetramethyl silane and phosphoric acid as external references for ^{13}C and ^{31}P NMR spectra respectively. Products were purified by medium pressure liquid chromatography on a Jobin–Yvon Moduloprep apparatus by using Amicon 6–35 μm or Merck 15 μm silica. Melting points were determined with a capillary Electrothermal 9200 apparatus. Mass spectra were recorded on a Nermag R10-10 apparatus. All solvents were distilled and dried before use.

General Procedure for Synthesis of Phosphonates 1–5

A 250 ml round-bottomed, flask containing a magnetic stirring bar was charged under N_2 and at −50°C with 41.6 mmol (26 ml) of n-butyllithium

(1.6 M) and 41.6 mmol (4.21 g) of N,N-diisopropylamine in 63 ml of freshly distilled THF. Tetraethylmethylene diphosphonate (41.6 mmol, 12 g) in 63 ml of THF was then added and the mixture stirred for 1 h before adding a solution of 3,4-dimethoxybenzaldehyde, 4-methoxybenzaldehyde (41.6 mmol, 6.9 g and 5.65 g, respectively) or 4-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde, 3,5-dimethoxy-4-hydroxybenzaldehyde (20.8 mmol, 2.54 g, 3.16 g and 3.78 g, respectively) in 40 ml of THF. The mixture was stirred for 45 min at −50°C, 15 min at room temperature and heated at reflux for 2 h. Reaction was quenched with saturated aqueous NH_4Cl (100 ml), the product extracted with ether (3 × 50 ml) and the combined organic phase dried over $MgSO_4$ and concentrated *in vacuo*. Crude product was purified on silica gel (eluant:ethyl acetate/petroleum ether/EtOH; 80/15/5 for compounds **1**–**3**, or ethyl acetate/petroleum ether for compounds **4** and **5**).

Diethyl-2-(4-hydroxyphenyl)ethene phosphonate, **1** 4.25 g (80% yield) of purified **1** were obtained. m.p. 97°C. IR ($CHCl_3$) ν cm^{-1}: 3160, 1605, 1213, 1056, 1029. ^1H NMR (250 MHz, $CDCl_3$) δ (ppm): 8.85 (s, 1H), 7.41 (dd, 1H, $^3J_{H-H} = 17.5$ Hz, $^3J_{H-P} = 22.5$ Hz, C*H*CH-*P*), 7.32, 6.91 (2 × d, AA′BB′, 2 × 2H, $J = 7.5$ Hz, 4H, Ar), 6.00 (dd, 1H, $^3J_{H-H} = {}^2J_{H-P} =$ 17.5 Hz, CHP), 4.10 (m, 4H, OCH_2), 1.34 (t, 6H, $J = 7.5$ Hz, CH_2CH_3). ^{13}C NMR (62.9 MHz, $CDCl_3$) δ (ppm): 159.52 (s), 149.50 (d $^2J_{C-P} = 7.0$ Hz), 129.61 (d), 126.45 (s $^3J_{C-P} = 22.5$ Hz), 116.05 (d), 108.80 (d $^1J_{C-P} =$ 187.0 Hz), 62.06 (t $^2J_{C-P} = 6.0$ Hz), 16.35 (q $^3J_{C-P} = 5$ Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm): 21.3. MS (EI, m/e): 257 ($M^+ + 1$; 28%), 256 (M^+; 45%), 228 (5%), 200 (6%), 147 (100%), 120 (83%). $C_{12}H_{17}O_4P$ Calc. (found): C 56.3 (56.0); H 6.7 (6.7)%.

Diethyl-2-(4-hydroxy-3-methoxyphenyl)ethene phosphonate, **2** 4.45 g (75% yield) of purified **2** was obtained. m.p. 95.5°C. ^1H NMR (200 MHz, $CDCl_3$) δ (ppm): 7.39 (dd, 1H, $J_{H-H} = 17.5$ Hz, $^3J_{H-P} = 22.5$ Hz; C*H*CH-*P*), 7.00 (m, 3H, Ar), 6.03 (t, 1H, $^3J_{H-H} = {}^2J_{H-P} = 17.54$ Hz, CHP), 4.11 (m, 4H, OCH_2); 3.69 (s, 3H, OCH_3), 1.34 (t, 6H, $J_{H-H} = 7.0$ Hz, CH_2CH_3). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm): 148.99 (d $^2J_{C-P} = 7.0$ Hz), 148.02 (s), 147.04 (s), 127.33 (s $^3J_{C-P} = 23.7$ Hz), 122.47 (d), 114.82 (d), 109.43 (d), 110.23 (d $^1J_{C-P} = 188.7$ Hz), 61.85 (t $^2J_{C-P} = 6.0$ Hz), 55.97 (q), 16.41 (q, $^3J_{C-P} = 5$ Hz). ^{31}P NMR (80 MHz, $CDCl_3$) δ (ppm): 20.67 (s, 1P). $C_{13}H_{19}O_5P$ Calc. (found): C 54.5 (54.0); H 6.6 (6.7)%.

Diethyl-2-(3,5-dimethoxy-4-hydroxyphenyl)ethene phosphonate, **3** 4.9 g (75% yield) of purified **3** were obtained. m.p. 156°C. IR (CCl_4) ν cm^{-1}: 3550, 1601, 1220, 1058, 1031. ^1H NMR (250 MHz, $CDCl_3$) δ (ppm): 7.38 (dd, 1H, $^3J_{H-H} = 17.5$ Hz, $^3J_{H-P} = 22.5$ Hz), 6.71 (s, 2H), 6.05 (dd, 1H,

$^3J_{H-H} = {}^2J_{H-P} = 17.5$ Hz), 5.98 (s, 1H), 4.10 (m, 4H), 3.68 (s, 6H), 1.32 (t, 6H, $J = 7.5$ Hz). ^{13}C NMR (62.9 MHz, $CDCl_3$) δ (ppm): 148.95 (d $^2J_{C-P} =$ 7 Hz), 147.25 (s), 137.13 (s), 126.32 (s $^3J_{C-P} = 23.7$ Hz), 111.12 (d $^1J_{C-P} =$ 188.7 Hz), 104.72 (d), 61.81 (t $^2J_{C-P} = 6$ Hz), 56.3 (q), 16.43 (q $^3J_{C-P} = 5$ Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm): 20.27. MS (EI, m/e): 316 (M^+, 8%), 286 (3%), 270 (31%), 256 (74%), 147 (53%), 91 (100%). $C_{14}H_{21}O_6P$ Calc. (found): C 53.2 (53.25); H 6.7 (6.7)%.

Diethyl-2-(3,4-dimethoxyphenyl)ethene phosphonate, **4** 9.6 g (77% yield) of purified **4** were obtained. m.p. 68.9°C. ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 7.39 (dd, 1H, $^3J_{H-H} = 17.5$ Hz, $^3J_{H-P} = 22.5$ Hz, C*H*CH-*P*), 6.90 (m, 3H, Ar), 6.04 (t, 1H, $J_{H-H} = {}^2J_{H-P} = 17.5$ Hz), 4.08 (m, 4H, OCH_2), 3.86 (s, 6H, OCH_3), 1.31 (t, 6H, $J_{H-H} = 7.0$ Hz, CH_2CH_3). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm): 151.05 (s), 149.23 (s), 148.60 (d $^2J_{C-P} = 7$ Hz), 127.93 (s $^3J_{C-P} = 23.7$ Hz), 122.11 (d), 111.74 (d), 111.25 (d $^1J_{C-P} = 188.7$ Hz), 108.75 (d), 61.73 (t $^2J_{C-P} = 6$ Hz), 55.97 (q), 55.69 (q), 16.41 (q $^3J_{C-P} =$ 5 Hz). ^{31}P NMR (80 MHz, $CDCl_3$) δ (ppm): 20.40 (s, 1P). $C_{14}H_{21}O_5P$ Calc. (found): C 56.0 (55.8), H 7.05 (7.0), O 26.6 (26.7)%.

Diethyl-2-(4-methoxyphenyl)ethene phosphonate, **5** 9.09 g (80% yield) of purified **5** were obtained. IR ($CHCl_3$) ν cm^{-1}: 1607, 1172, 1057, 1032. ^{1}H NMR (250 MHz, $CDCl_3$) δ (ppm): 7.43 (dd, 1H, $^3J_{H-H} = 17.5$ Hz, $^3J_{H-P} = 22.5$ Hz), 7.42, 6.88 (2 × d, 2 × 2H, AA′BB′, $J = 9.5$ Hz), 6.06 (t, 1H, $^3J_{H-H} = {}^2J_{H-P} = 17.5$ Hz), 4.10 (m, 4H), 3.81 (s, 3H), 1.33 (t, 6H, $J = 7.5$ Hz). ^{13}C NMR (62.9 MHz, $CDCl_3$) δ (ppm): 161.34 (s), 148.49 (d $^2J_{C-P} = 7$ Hz), 129.3 (d), 127.64 (s $^3J_{C-P} = 23.7$ Hz), 114.25 (d), 110.85 (d $^1J_{C-P} = 188.7$ Hz), 61.79 (t $^2J_{C-P} = 6$ Hz), 55.42 (q), 16.42 (q $^3J_{C-P} = 5$ Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm): 20.54. MS (EI m/e): 271 ($M^+ + 1$; 12%), 270 (M; 67%), 242 (6%), 161 (97%), 134 (100%). $C_{13}H_{19}O_4P$ Calc. (found): C 57.8 (57.5); H 7.1 (7.0)%.

Synthesis of Ethyl-2-(4-hydroxy-3-methoxyphenyl)ethene hydrogen phosphonate, **6** A solution of diethyl 2-(4-hydroxy-3-methoxyphenyl)ethene phosphonate **2** (13.9 mmol, 4 g) in EtOH (15 ml) and NaOH (22 ml, 2M) was refluxed for 20 h, the mixture neutralized carefully (pH 6.7) and extracted with $CHCl_3$ (3 × 20 ml). The aqueous phase was acidified (pH 2) and re-extracted with $CHCl_3$ (3 × 20 ml) and the combined organic phase dried ($MgSO_4$) and evaporated to leave 2.5 g (9.7 mmol) of pure monoacid **6** (yield 70%). ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 9.21 (s, 2H, O*H*), 7.31 (dd, 1H, $J_{H-H} = 16.2$ Hz, $^3J_{H-P} = 24$ Hz, C*H*CH-*P*), 6.69 (m, 3H, Ar), 6.10 (t, 1H, $J_{H-H} = {}^2J_{H-P} = 16.2$ Hz), 4.11 (m, 4H, OCH_2), 3.84 (s, 3H, OCH_3), 1.33 (t, 3H, $J_{H-H} = 8$ Hz, CH_2CH_3). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm): 147.90 (d $^2J_{C-P} = 7$ Hz), 147.89 (s), 146.84 (s), 127.49 (s $^3J_{C-P} = 23.7$ Hz),

122.49 (d), 117.44 (d), 111.17 (d $^1J_{C-P} = 188.7$ Hz), 106.35 (d), 61.78 (t $^2J_{C-P} = 6$ Hz), 55.91 (q), 16.35 (q $^3J_{C-P} = 5$ Hz). ^{31}P NMR (80 MHz, $CDCl_3$) δ (ppm): 22.17 (s, 1P). MS (EI, m/e): 259 ($M^+ + 1$, 26%), 258 (M, 86%), 212 (18%), 150 (100%), 135 (71%). $C_{11}H_{15}O_5P$ Calc. (found): C 51.2 (51.3); H 5.9 (5.75)%.

Ethyl-2-(3,4-dimethoxyphenyl)ethene hydrogenophosphonate, **7** A solution of diethyl 2-(3,4-dimethoxyphenyl)ethene phosphonate **4** (7 mmol, 2.1 g), NaOH (10 ml, 2 M) and EtOH (10 ml) was refluxed for 20 h, the mixture diluted with water (10 ml) and extracted with $CHCl_3$ (2 × 20 ml). The aqueous phase was acidified (pH 2), extracted with $CHCl_3$ (3 × 20 ml) and the combined organic phase dried ($MgSO_4$) and evaporated to leave 1.5 g (5.6 mmol) of pure monoacid **7** (yield 80%). ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 9.6 (s, 1H, O*H*), 7.37 (dd, 1H, $J_{H-H} = 15.4$ Hz, $^3J_{H-P} = 21$ Hz, C*H*CH-*P*), 6.88 (m, 3H, Ar), 6.15 (t, 1H, $J_{H-H} = {}^2J_{H-P} = 15.4$ Hz), 4.10 (m, 4H, OCH_2), 3.87 (s, 6H, OCH_3), 1.32 (t, 3H, $J_{H-H} = 8.0$ Hz, CH_2CH_3). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm): 151.17 (s), 149.44 (s), 147.84 (d $^2J_{C-P} =$ 7 Hz), 128.23 (s $^3J_{C-P} = 23.7$ Hz), 122.31 (d), 112.12 (d $^1J_{C-P} = 188.7$ Hz), 111.95 (d), 108.93 (d), 61.95 (t $^2J_{C-P} = 6$ Hz), 55.22 (q), 55.14 (q), 16.61 (q $^3J_{C-P} = 5$ Hz). ^{31}P NMR (80 MHz, $CDCl_3$) δ (ppm): 21.69 (s, 1P). MS (EI, m/e): 273 ($M^+ + 1$; 15.5%), 272 (M; 100%), 162 (40%), 119 (27%). $C_{12}H_{17}O_5P$ Calc. (found): C 52.9 (53.05); H 6.3 (6.3)%.

Synthesis of Ethyl-2-N-benzyl-2-(3,4-dimethoxyphenyl)ethene phosphonamidate, **8** A solution of n-butyllithium (1.1 ml, 1.8 mmol) was added at 0°C with stirring to a solution of monoacid **7** (1.8 mmol, 0.5 g) in benzene (2 ml) and ether (10 ml). After 10 min, solvent was evaporated and the resulting white solid suspended in anhydrous benzene (10 ml). To this stirred solution was added, under argon, 4 mmol dimethylformamide (311 ml) and oxalyl chloride 3.7 mmol (322 ml) resulting in extensive bubbling and a white precipitate. The reaction mixture was stirred for 20 min then frozen and lyophilized. The resultant solid was dissolved in anhydrous CH_3CN (20 ml) and cooled to −40°C under argon. To this was added a solution of benzylamine (9.2 mmol, 0.99 g) and N,N-diisopropyl ethylamine (9.2 mmol, 1.2 g). The reaction mixture was stirred at −20°C for 30 min then warmed to room temperature and stirred for an additional 10 h. The reaction mixture was washed with saturated NH_4Cl (40 ml) and extracted with ethyl acetate. The organic phase was dried ($MgSO_4$) and concentrated to leave 0.51 mg (1.4 mmol) of pure racemate **8** (yield 78%). m.p. 95.2°C. ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 7.27 (m, 6H, Ar, C*H-P*), 6.84 (m, 3H, Ar), 6.07 (t, 1H, $J_{H-H} = {}^2J_{H-P} = 17.5$ Hz), 4.08 (m, 4H, OCH_2, CH_2-Ar), 3.82 (d, 6H, OCH_3), 3.45 (m, 1H, N*H*), 1.26 (t, 3H, $J_{H-H} = 7.0$ Hz, CH_2CH_3). ^{13}C NMR

(50 MHz, $CDCl_3$) δ (ppm): 150.68 (s), 149.12 (s), 146.40 (d $^2J_{C-P} = 7$ Hz), 140.26 (s $^3J_{C-P} = 23.7$ Hz), 128.55 (d), 128.09 (s), 127.45 (d), 127.22 (d), 121.82 (d), 114.35 (d $^1J_{C-P} = 177.6$ Hz), 110.96 (d), 109.29 (d), 60.26 (t $^2J_{C-P} = 6$ Hz), 55.94 (q), 55.86 (q), 44.80 (t $^2J_{C-P} = 5.5$ Hz), 16.48 (q $^3J_{C-P} = 5$ Hz). ^{31}P NMR (80 MHz, $CDCl_3$) δ (ppm): 23.39 (s, 1P). $C_{19}H_{24}O_4NP$ Calc. (found) C 63.2 (63.0), H 6.7 (6.6), N 3.9 (3.9)%.

^{1}H NMR Spectroscopy

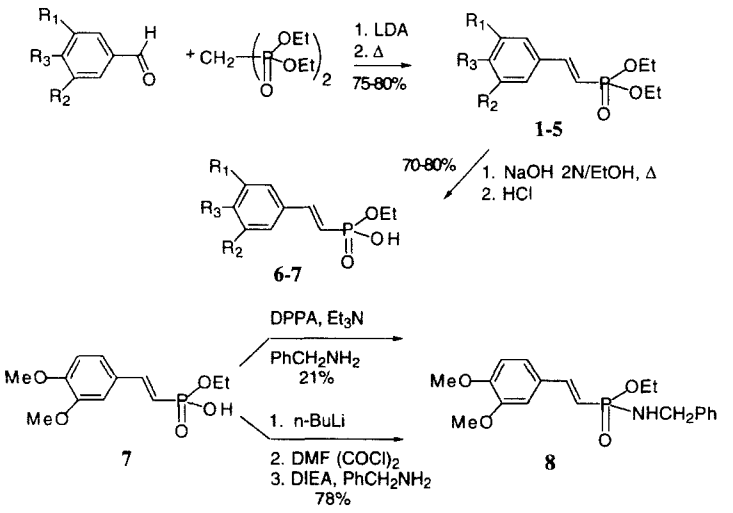
The ^{1}H NMR studies of the interaction of phosphonate **2** with recombinant CAD were carried out on a Varian Unity 500 spectrometer operating at 500 MHz and at ambient temperature. The carrier frequency was set at the frequency of the HDO solvent resonance which was suppressed using low-power, selective irradiation during the pulse delay of 1.5 s. The 1D ^{1}H spectrum of phosphonate **2** (0.5 mM) in NMR buffer (50 mM phosphate buffered D_2O at pH 7.5 containing 0.5 mM DTT) was recorded using a 90° pulse with 32 K data points. Data were processed without using any window functions and the line-width at half-peak height was measured for each resolved resonance. To this solution, recombinant CAD was added in a small amount of the same buffer to give a final protein : ligand ratio of 1 : 100. The 1D ^{1}H spectrum was recorded and processed as described above.

Pure absorption phase-sensitive NOESY spectra were acquired using the hypercomplex method of data collection.[8,9] A spectral width of 5999.7 Hz was used for NOESY spectra being acquired as 1024 points in t_2 for $2 \times 128 t_1$ increments and zero-filled to 1024 points in t_1 prior to Fourier transformation. NOESY data sets collected using 75 and 150 ms mixing times were apodized with mild Gaussian window functions in t_1 and t_2. An 80 ms NOESY experiment was also recorded for the pure phosphonate **2** under the same experimental conditions except that $2 \times 256 t_1$ increments were collected.

RESULTS AND DISCUSSION

Synthesis of Vinylphosphonates

Phosphonates and phophonamidates are known as efficient inhibitors of proteases and other enzymes.[10–16] Hydroxyphosphinyl inhibitors of HMG-CoA Reductase have been prepared based on fungal metabolites. The original inhibitors bind very tightly and served as a platform to develop mechanism-based phosphinyl inhibitors.[17,18] The design of enzyme inhibitors has been usually founded on the mechanism of the reaction and the phosphorus



SCHEME 1

moiety was postulated to provide a transition state analog in the enzymatic reaction.

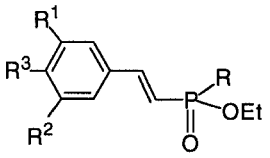
Among the methods available to obtain phosphonates[19–22] the synthesis of trans ethylenic phosphonates **1–8** (Scheme 1) was performed according to a method developed by Kiddle and Mikolajckyk.[21,22]

Tetraethylmethylene diphosphonate anion formed in the presence of LDA in THF was reacted with the corresponding aldehyde. Thermal elimination of the phosphoric diester gave exclusively trans vinylic phosphonates in good yields. The trans configuration was determined by NMR ^{1}H spectroscopy from the coupling constants of the ethylenic protons ($^3J_{H-H} = 17.5$ Hz and $^3J_{H-P} = 22.5$ Hz). Monophosphonic acids were obtained in 70–80% yield by treatment of the diesters in basic media (NaOH/EtOH) under reflux conditions. Finally phosphonamidate **8** was obtained according to a procedure developed by Duran[23] for synthesis of cinnamides (carbonyl equivalents) and by Bartlett for other phosphonamidate compounds.[11] It consisted of activating the phosphonic acid by means of an iminium salt and then treating with the corresponding amine. Compound **8** was thus obtained in 78% yield.

Enzyme Inhibition

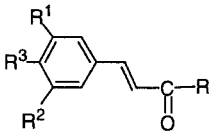
Derivatives **1–8** were found to inhibit recombinant CAD to varying extents (Table 1). A relatively good inhibitory activity against CAD was observed with diethyl phosphonates **2** and **3** in the coniferyl and sinapyl series and

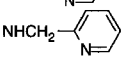
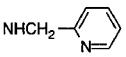
TABLE I Inhibition by phosphonates and derivatives (**1–8**) of recombinant CAD in tris buffer (100 mM, pH 8.8) at 30°C with coniferyl alcohol (0.2 mM) as substrate and $NADP^+$ (0.2 mM) as cofactor



Compound	R^1	R^2	R^3	*R*	[*I*] mM	% *Inhibition*
1	H	H	OH	OEt	2	17
2	H	OMe	OH	OEt	1.6	35
3	OMe	OMe	OH	OEt	1.8	25
4	H	OMe	OMe	OEt	2	40
5	H	H	OMe	OEt	1	30
6	H	OMe	OH	OH	2	10
7	H	OMe	OMe	OH	2	9
8	H	OMe	OMe	$NHCH_2Ph$	1.4	50

TABLE II Inhibitory potency of cinnamic esters and amides against CAD extracted from xylem poplar stems in tris (100 mM, pH 8.8) at 30°C with coniferyl alcohol (0.2 mM) as substrate and $NADP^+$ (0.2 mM) as cofactor[23]



Entry	R^1	R^2	R^3	*R*	*I* (mM)	% *Inhibition*
1	H	OMe	OH	O^tBu	2	50
3	H	H	H		1	0
4	H	H	H		2	0
5	H	OMe	OH		0.5	56
6	H	H	OMe		2	10

with the benzyl phosphonamidate analog **8**. An equally potent inhibition was found with the diethyl phosphonates **4** and **5** while hydrogen phosphates **6** and **7** do not appear to inhibit CAD. The inhibitory activity of the phosphonate derivatives can be compared with their carbonyl analogues.[24,25] Table II shows the results obtained with purified CAD from xylem poplar stems. The main trends can be summarized as follows: a tert-butyl

carboxyester presents approximately the same inhibitory activity as the diethyl phosphonate analogue. In the case of the coniferyl series there is good inhibitory activity in both cases. The absence of 4-OH, 3-OMe groups on the aromatic ring or chain, shortening of the amido group causes a dramatic decrease in activity in the amido series.

Detailed enzyme kinetic studies of inhibitors **2**–**4** and **8** were undertaken. While the results for compound **8** are extremely difficult to analyze, probably due to the racemic form of the compound, the classes of inhibition detected for compounds **2**–**4** (Figures 2–4) indicate that exclusive binding to the active site does not occur and that in some cases inhibitor and substrate can be bound simultaneously to this dimeric enzyme. Compounds **2** and **3** showed a mixed linear type inhibition[7] following Equation (2) (Table III, Figures 2 and 3). Values of K_i, K'_i and affinity constants α were determined (Table III). The high α values of this system that might be considered as a mixture of partial competitive inhibition and pure non-competitive inhibition indicate that Compounds **2** and **3** have a high affinity for the active site of the enzyme, especially Compound **3** even if it is a weaker inhibitor ($\alpha \sim 18$). Compound **2** presents the best K_i value (20 μM) being about three-fold the K_m ($K_m = 6.3\,\mu M$) value (although K_m need not reflect substrate binding). Compound **4** was found to be an uncompetitive inhibitor following Equation (3) (Table III, Figure 4) and does not act at the active site of the enzyme. Our findings are consistent with the 3-dimensional structural model of CAD. From this model the active site of CAD was predicted to be a hydrophobic cavity. Consequently charged compounds would not be expected to efficiently inhibit the enzyme. This is in accordance with the fact that hydrogen phosphates, which under the optimum pH conditions for CAD activity are charged, have no effect on the enzyme.

The region near the catalytic zinc atom is also sufficiently large to accept bulky groups. Diethoxy functions (case of phosphonate inhibitors) or tert-butoxy ones (Table II, entry 1) can fit in the substrate-binding site of CAD.

Finally, one of the most important factors is the presence of the 4-OH or better 4-OH, 3-OMe substituents on the aromatic ring of the substrate. Model building and docking of the substrate (Figure 5) indicated that the 4-hydroxy group of the substrate (or the inhibitor) is correctly placed to allow hydrogen bonding either with Tyr-113 or the backbone carbonyl group of Trp-119. The aromatic ring that is slotted in between the rings of Trp-119 and Phe-298 as a molecular sandwich can be further stabilized by another H-bond interaction between its 3-methoxy group and the NH group of Trp-119. Its presence can thus increase the specificity and the affinity of the inhibitor for binding in the active site of the CAD.

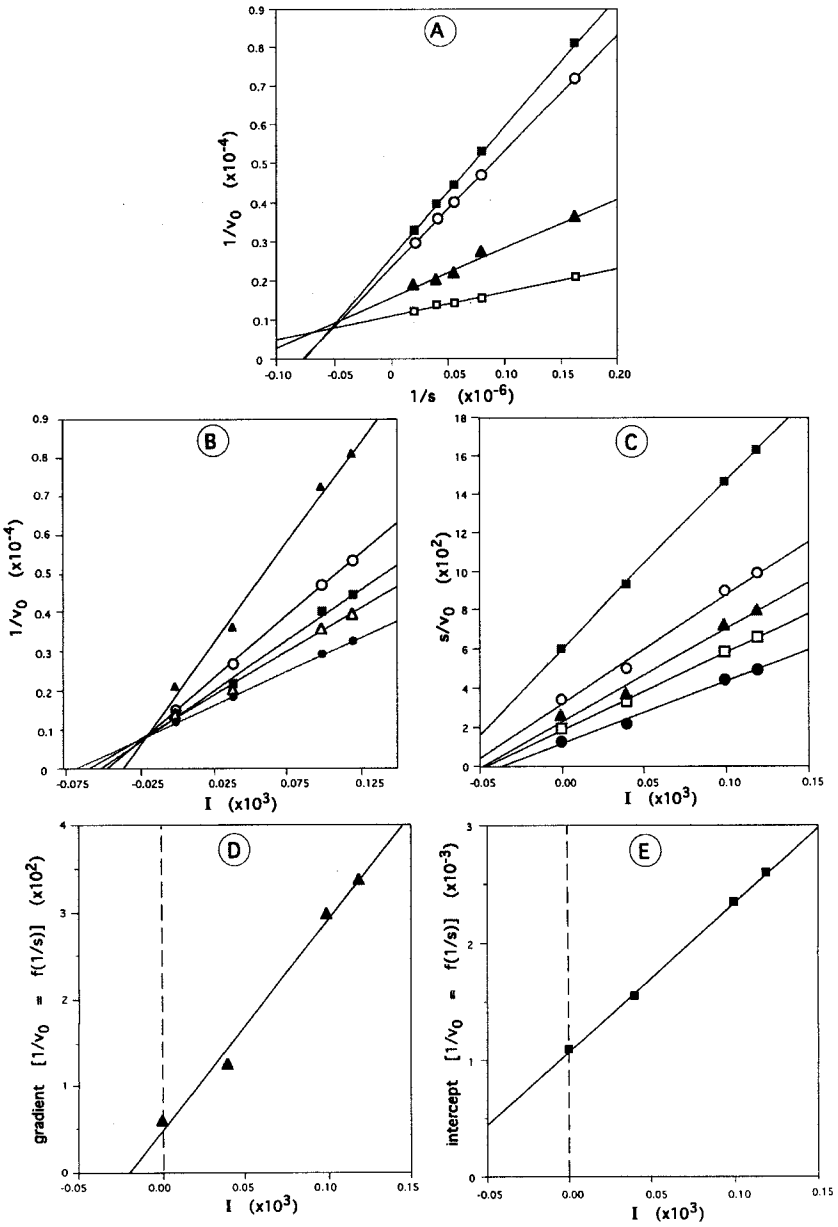


FIGURE 2 Diagnostic plots for CAD inhibition by phosphonate **2**. **A** is the double reciprocal plot of 1/initial velocity versus 1/[substrate] at fixed inhibitor concentration. **B** is the reciprocal plot of 1/initial velocity versus [inhibitor] at fixed substrate (coniferyl alcohol) concentration. **C** is the plot of [substrate]/initial velocity versus [inhibitor] at fixed substrate concentration. **D** is the replot of gradients of the curves $1/v_0 = f(1/[S]$ versus [inhibitor]. **E** is the replot of the 1/v axis intercept of reciprocal plot data ($1/v_0 = f(1/[S])$) versus [inhibitor]. For **A**, inhibitor concentrations are indicated as follows: (□) 0 mM; (▲) 0.04 mM; (○) 0.1 mM; (■) 0.12 mM. For **B** the substrate concentrations are indicated as follows: (•) 50 μM; (△) 25 μM; (■) 18 μM; (○) 12.5 μM; (▲) 6.12 μM. For **C** the substrate concentrations are indicated as follows: (•) 50 μM; (□) 25 μM; (▲) 18 μM; (○) 12.5 μM; (■) 6.12 μM.

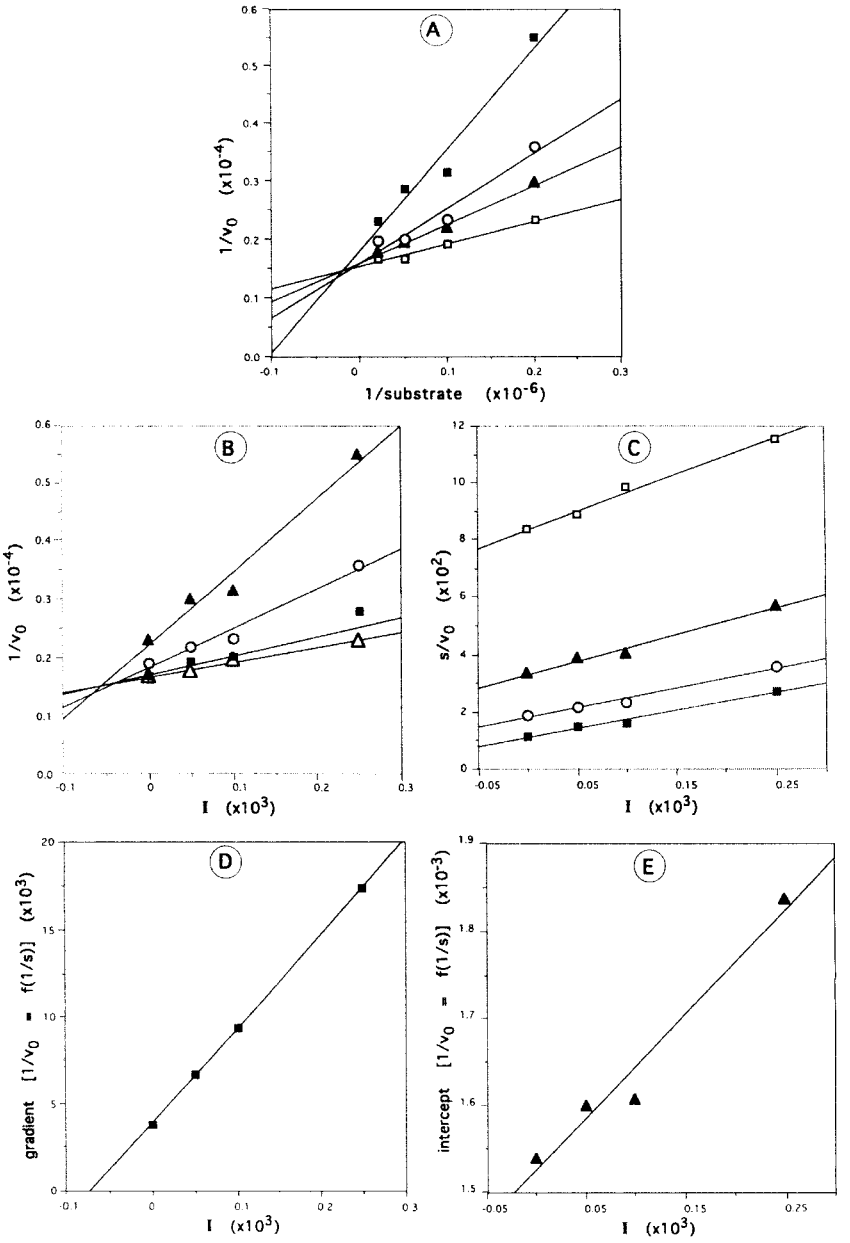


FIGURE 3 Diagnostic plots for CAD inhibition by phosphonate **3**. **A** is the double reciprocal plot of 1/initial velocity versus 1/[substrate] at fixed inhibitor concentration. **B** is the reciprocal plot of 1/initial velocity versus [inhibitor] at fixed substrate (coniferyl alcohol) concentration. **C** is the plot of [substrate]/initial velocity versus [inhibitor] at fixed substrate concentration. **D** is the replot of gradients of the curves $1/v_0 = f(1/[S])$ versus [inhibitor]. **E** is the replot of 1/v axis intercept of the reciprocal plot data ($1/v_0 = f(1/[S])$) versus [inhibitor]. For **A**, inhibitor concentrations are indicated as follows: (□) 0 mM; (▲) 0.05 mM; (○) 0.1 mM; (■) 0.25 mM. For **B** the substrate concentrations are indicated as follows: (△) 50 μM; (■) 20 μM; (○) 10 μM; (▲) 5 μM. For **C** the substrate concentrations are indicated as follows: (■) 50 μM; (○) 20 μM; (▲) 10 μM; (□) 5 μM.

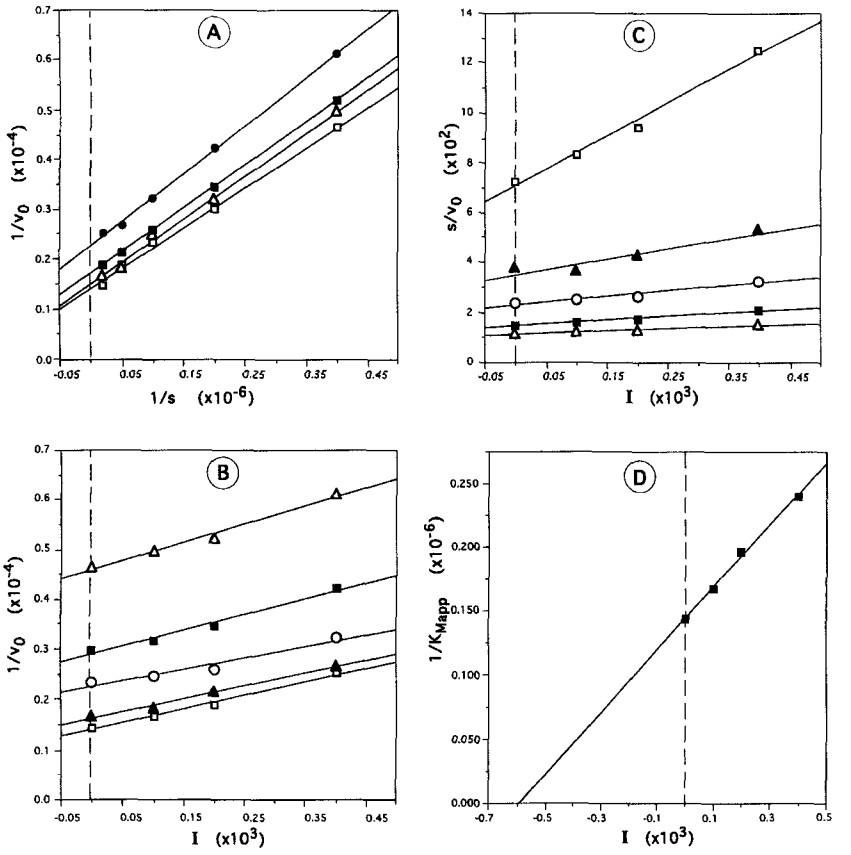


FIGURE 4 Diagnostic plots for CAD inhibition by phosphonate **4**. **A** is the double reciprocal plot of 1/initial velocity versus 1/[substrate] at fixed inhibitor concentration. **B** is the reciprocal plot of 1/initial velocity versus [inhibitor] at fixed substrate (coniferyl alcohol) concentration. **C** is the plot of [substrate]/initial velocity versus [inhibitor] at fixed substrate concentration. **D** is the plot of $1/K_{mapp}$ (apparent Michaelis constant) versus [inhibitor]. For **A**, inhibitor concentrations are indicated as follows: (□) 0 mM; (△) 0.01 mM; (■) 0.2 mM; (•) 0.4 mM. For **B** the substrate concentrations are indicated are as follows: (□) 50 μM; (▲) 20 μM; (○) 10 μM; (■) 5 μM; (△) 2.5 μM. For **C** the substrate concentrations are as follows: (△) 50 μM; (■) 20 μM; (○) 10 μM; (▲) 5 μM; (□) 2.5 μM.

The difference in the type of inhibition between Compounds **2**, **3** versus **4** indicates the importance of the 4-hydroxy group for inhibitor specificity. In fact, according to our model of the active site of the CAD,[3] and the inhibitory results obtained, it seems likely that a preferable H-bond interaction is established between the oxygen atom of the carbonyl group of Trp-119 with the hydrogen atom of the 4-OH group of the aromatic ring of the substrate (or inhibitors **2** and **3**). The lack of this interaction for Compound **4** might render it an uncompetitive inhibitor.

TABLE III Type of inhibition and inhibition constants for Compounds **2–4** and related Equations (2) and (3) for the determination of the different constants

Inhibitor	K_i (± S.D.) (μM)	K_i' (± S.D.) (μM)	α (± S.D.)	*Type of inhibition*
2	20 ± 2.0	86 ± 8	4.3 ± 0.4	Mixed linear
3	72 ± 7	1290 ± 100	18 ± 2	Mixed linear
4	630 ± 60	—	—	Uncompetitive

For Compounds **2** and **3** the reciprocal form of the velocity equation is

$$\frac{1}{v} = \frac{K_m}{v_{max}}\left(1 + \frac{I}{K_i}\right)\frac{1}{[S]} + \frac{1}{v_{max}}\left(1 + \frac{[I]}{aK_i}\right) \quad K_i' = aK_i \tag{2}$$

while for Compound **4** it is,

$$\frac{1}{v} = \frac{K_m}{v_{max}}\frac{1}{[S]} + \frac{1}{v_{max}}\left(1 + \frac{[I]}{K_i}\right). \tag{3}$$

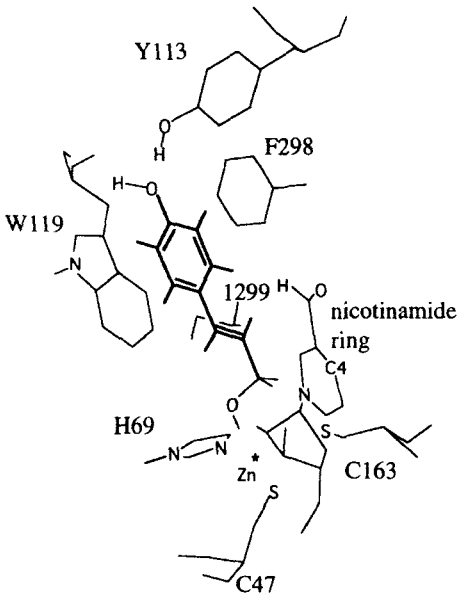


FIGURE 5 View of the substrate binding region of CAD from *Eucalyptus*. The structure shown was modelled as described previously.[3]

Conformation of Bound Phosphonate 2

The ^{1}H NMR spectrum of phosphonate **2** is shown in Figure 6(a). The addition of CAD to give a molar ratio of inhibitor : protein of 100 : 1, caused broadening of resonances throughout the spectrum (Figure 6(b), Table IV).

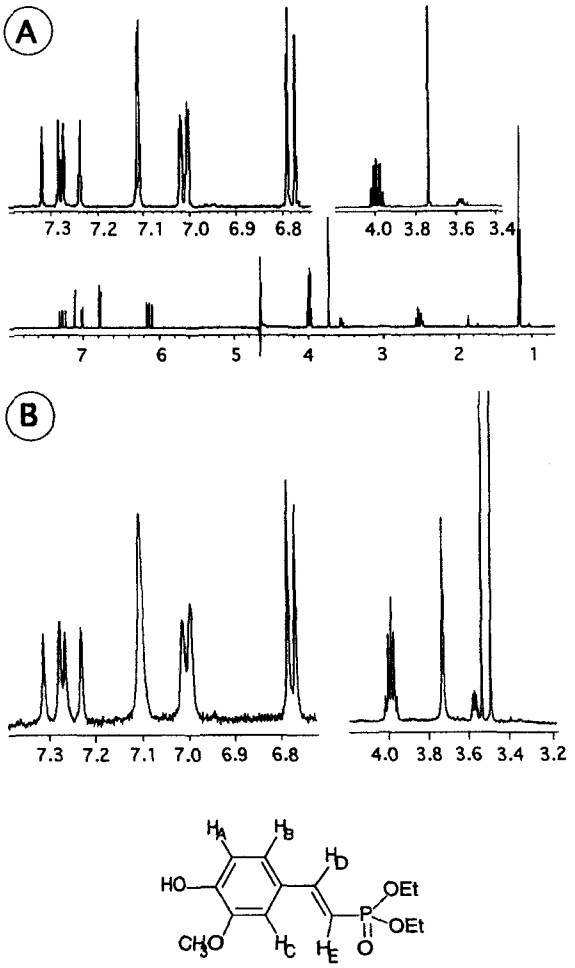


FIGURE 6 Line-broadening effects on inhibitor **2** resonances induced by binding CAD. (a) ^{1}H NMR spectra of free inhibitor **2** (0.5 mM), pH = 7.5/D_2O containing 0.5 mM DTT. 90° pulse, 32 K data points. (b) ^{1}H NMR spectra of free inhibitor **2** in presence of CAD ([**2**] : [CAD] ratio of 100 : 1).

Resolvable resonances were broadened on average by a factor of 2.07 (s.d. = 0.098) at half-height, except for the methoxy resonance, which was broadened by a factor of 3.6. The line-broadening of ligand resonances due to the addition of protein indicates a binding interaction. If non-specific binding is occurring uniform line-broadening of all resonances is expected.[26] The present case, where one resonance (the methoxy group) is selectively broadened relative to the other resonances, indicates that specific binding is

TABLE IV Line-broadening of resonance peaks of inhibitor **2** in presence of CAD

Proton	δ (ppm)	*Alone* (±0.03)	*With CAD* (±0.03)
H_A	6.78 d	1.08, 1.09	2.21, 2.17
H_B	7.02 dd	3.57, 3.49, 3.51, 3.60	n.r.
H_C	7.12 d	3.62, 3.53	n.r.
H_D	7.28 dd	1.50, 1.55, 1.46, 1.46	3.05, 3.09, 3.20, 2.98
H_E	6.13 dd	1.02, 1.08, 1.10, 1.05	n.r.
OCH_3	3.74 s	1.11	3.99
OCH_2	4.0 q	1.58, 1.55	3.21, 3.13
OCH_2CH_3	1.18 t		2.72

[**2**] = 0.5 mM, pH 7.5/D_2O containing 0.5 mM DTT with [**2**]:[CAD] ratio of 100:1. s = singlet; d = doublet; dd = double doublet; t = triplet; q = quartet; n.r. = not resolved.

occurring and that the proton(s) involved in the interaction are either undergoing different molecular motion and/or a change in chemical shift. Thus, the 1D ^1H NMR spectra indicate that the methoxy group is intimately involved in the binding process.

The weak binding of phosphonate **2** (giving fast exchange on the NMR time-scale) allows us to make use of TRNOESY[27,28] to study the CAD-bound conformation of the ligand. In conditions of fast exchange between bound and free ligand, the NOESY cross-peaks observed (using TRNOESY) between the resonances of the free ligand (which is in a great excess over the bound ligand) can be due to NOESY transferred from the bound state by chemical exchange. This is the case here as a control NOESY experiment of the phosphonate **2** alone recorded under the same conditions showed no NOE cross-peaks. Moreover, the sign of the NOE of free and bound ligands are opposite because of the slow tumbling rate of the protein : ligand complex confirming that it is the bound structure of the inhibitor that the detected NOE intensities refer to. The ligand : protein ratio used in this case (100 : 1) is relatively high for a TRNOESY experiment which commonly uses ratios of 5-30 : 1. However, the literature contains examples where ratios of 50 : 1[29] and 111 : 1[30] have been used. The TRNOESY spectrum (150 ms mixing time) of phosphonate **2** in the presence of CAD (molar ratio 100 : 1) shows strong NOE cross-peaks between H_A and H_B, and between H_C and H_E resonances (Figure 7). NOEs were also observed between H_B and H_D and between H_C and H_{Me} resonances. Integration of these cross-peak volumes and use of the isolated spin-pair approach allowed the distances between these protons to be calculated (Table V). This approach suffers from the inability to take into account the possibility of spin diffusion occurring and does not allow for multiple correlation times. In this study spin diffusion was minimised by the use of short mixing times (75 and 150 ms), the intensities of the NOEs observed at 75 ms being

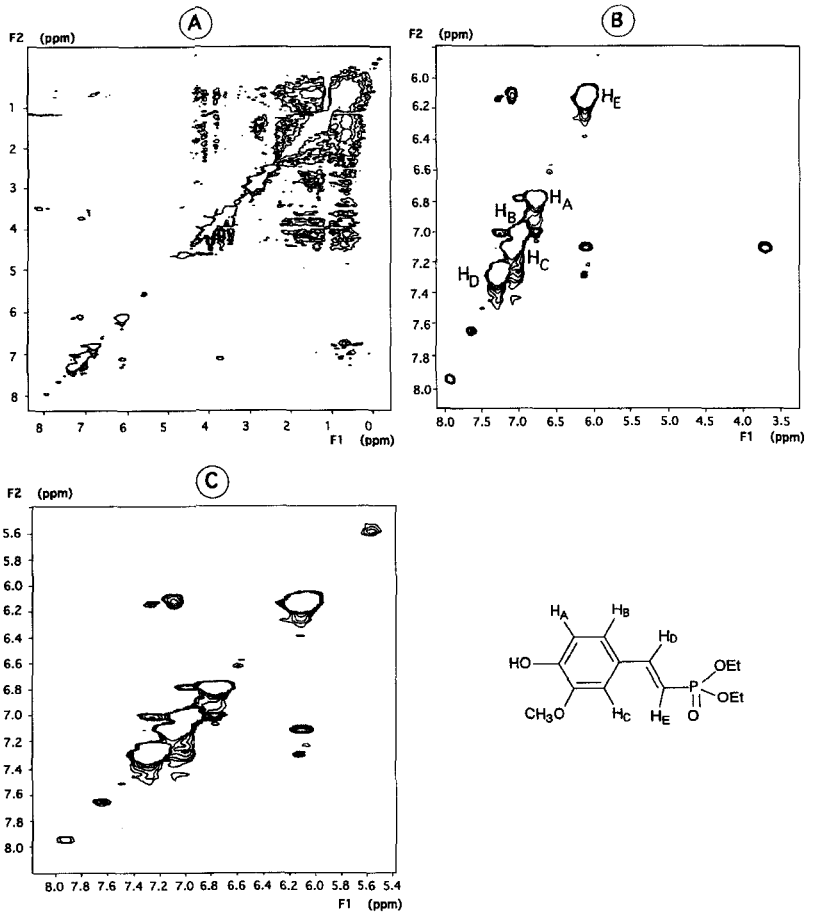
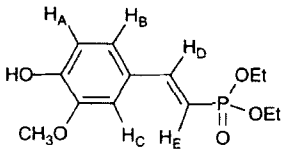


FIGURE 7 TRNOESY experiment involving inhibitor **2** in the presence of CAD. The conditions used were: [**2**] = 1 mM, [**2**]:[CAD] = 100:1, pH = 7.5/D_2O containing 0.5 mM DTT and mixing time: 150 ms. (a) Complete spectrum, (b) and (c) expansions of parts of the spectrum.

consistent with those observed at 150 ms. The reasonable assumption was made that the molecular motion experienced by the phenyl and alkene regions of the inhibitor molecule are similar as they are rigidly linked together. The NOEs between H_B and H_D, and between H_C and H_E, and the absence of H_B/H_E and H_C/H_D NOEs clearly defines the torsion angle τ_1 in the orientation $-90° > \tau_1 > 90°$ ($\tau_1 = C_{H_C}-C_{Ar}-C-H_D$). In order to refine the range of τ_i a grid-search was carried out using CHARMM molecular modelling software where the complete structure was minimised using molecular mechanics as the τ_1 was rotated in 10° increments from 0 to 350°.

TABLE V Inter-proton distances calculated for the TRNOESY experiment of phosphonate **2** bound to CAD



Involved protons	*TRNOESY distance*[a] (Å, ±0.05)
H_B–H_D	2.86
H_C–H_E	2.59
H_C–H_{CH_3}	2.91

[a] $r_{ij} = r_{kl}(R_{kl}/R_{ij})^{1/6}$; r is distance between protons; R is intensity of NOE experiment; k and l are reference protons; i and j are protons whose separation is unknown. A reference distance of 2.475 Å for H_A–H_B was used.

From analysis of H_B–H_D and H_C–H_E distance constraints the value of τ_1 was defined to be ±130°, defining in turn the preferential conformation of phosphonate **2** when bound to CAD.

To this point NOES between the ligand and the enzyme have not been detected and the binding site cannot be defined. However, the results of inhibition tests and the observed interaction between the 3-OMe group of compound **2** with CAD are in accordance with the 3-dimensional model proposed for the substrate.[3] The preferential conformation that has been determined for compound **2** bound to CAD, obtained through TRNOESY experiment, provides a pointer for the design of better inhibitors by modifying the functional groups on the phosphorus atom. Our results show that suitably chosen phosphonates can lead to inhibitors of CAD.

Acknowledgements

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