ARTICLE

OBC
www.rsc.org/obc

Inhibition of thiamin diphosphate dependent enzymes by 3-deazathiamin diphosphate

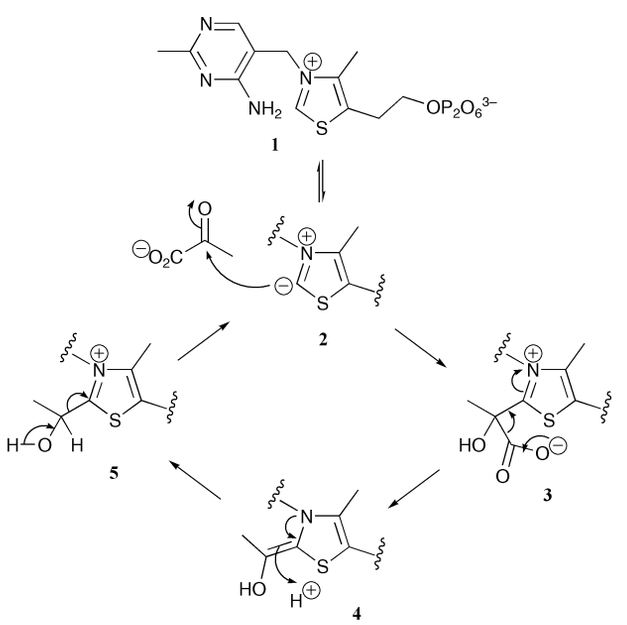
Stéphane Mann, Concepcion Perez Melero, Dan Hawksley and Finian J. Leeper *
University Chemical Laboratory, Lensfield Road, Cambridge, UK CB2 1EW

Received 17th March 2004, Accepted 22nd April 2004
First published as an Advance Article on the web 25th May 2004

3-Deazathiamin diphosphate (deazaTPP) and a second thiamin diphosphate (TPP) analogue having a benzene ring in place of the thiazolium ring have been synthesised. These compounds are both extremely potent inhibitors of pyruvate decarboxylase from *Zymomonas mobilis*; binding is competitive with TPP and is essentially irreversible even though no covalent linkage is formed. DeazaTPP binds approximately seven-fold faster than TPP and at least 25,000-fold more tightly (K_i less than 14 pM). DeazaTPP is also a potent inhibitor of the E1 subunit of α-ketoglutarate dehydrogenase from *E. coli* and binds more than 70-fold faster than TPP. In this case slow reversal of the inhibition could be observed and a K_i value of about 5 nM was calculated (*ca.* 500-fold tighter binding than TPP).

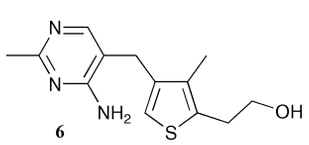
Introduction

Thiamin diphosphate (TPP) is a coenzyme used by a range of different enzymes which have one thing in common: they make and break bonds to the carbon atom of a carbonyl group. Examples include α-keto acid decarboxylases, dehydrogenases and oxidases, transketolase, deoxyxylulose synthase, benzaldehyde lyase and acetohydroxy acid synthase.[1] The mechanisms for all TPP-dependent enzymic reactions are similar and are illustrated in Scheme 1 by the mechanism for pyruvate decarboxylase (PDC). In all cases the reaction is initiated by deprotonation of the TPP **1** to give the ylid **2**. This then nucleophilically attacks the carbonyl group of the substrate, giving in the case of PDC lactyl-TPP **3**. Bond cleavage can then occur with the thiazolium ring of the TPP moiety acting as an electron sink; in enzymes like PDC which operate on α-ketoacids it is CO_2 that is lost but in some enzymes (*e.g.* transketolase, benzaldehyde lyase) an aldehyde molecule can be lost instead. The product of the bond cleavage is an enamine **4**, often called the "active aldehyde" intermediate. This then reacts with an electrophile, which in the case of PDC is simply a proton giving hydroxyethyl-TPP **5**. In other examples a ketone or aldehyde group of another substrate molecule is the electrophile, while in oxidases and dehydrogenases oxidation of the enamine can occur. Finally release of the product, acetaldehyde in this case, occurs with regeneration of the ylid **2**.



Scheme 1 Mechanism of pyruvate decarboxylase.

Although the mechanisms of TPP-dependent reactions are well known, the details of how the enzymes catalyse these mechanisms are not really understood. Many crystal structures of such enzymes have been obtained with TPP bound but in general crystal structures that show the substrate bound or intermediates in the reaction are lacking. Exceptions are structures of pyruvate bound to pyruvate:ferredoxin oxidoreductase[2] (although it may be in a non-productive complex) and separate structures of erythrose 4-phosphate[3] and the active aldehyde intermediate[4] bound to transketolase. In addition structures have been solved of a radical species related to 2-acetylTPP bound to pyruvate:ferredoxin oxidoreductase[5] and of a cyclised form of 2-hydroxyethylTPP **5** bound to acetolactate synthase[6] but in neither case is it clear that it is a true reaction intermediate that is present. The reason why it is difficult to get such crystal structures is that the substrate and intermediates normally undergo reactions too fast to be detected by crystallography. One solution to this problem would be to use an inactive analogue of the coenzyme. With this in mind, we recently reported the synthesis of 3-deazathiamin **6**, which is isoelectronic with thiamin and therefore has the same size and shape but differs only in its charge. Here we report the conversion of 3-deazathiamin into its diphosphate (deazaTPP) **15** and studies of the interaction of this compound with two TPP-dependent enzymes, pyruvate decarboxylase from *Zymomonas mobilis* and the E1 subunit of α-ketoglutarate dehydrogenase from *E. coli*. We also report the synthesis and studies of a second inactive analogue of TPP which has a benzene ring in place of the thiazolium ring.



Results

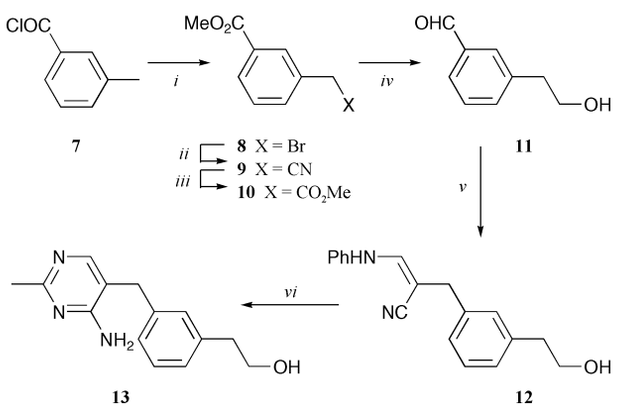
Synthetic studies

Although our previously reported synthesis of deazathiamin[7] was relatively efficient, the synthesis of the thiophene ring inevitably involved a number of steps. We thought it would be interesting therefore to compare the inhibitory properties of deazathiamin pyrophosphate (deazaTPP) with an analogue of

DOI: 10.1039/b403619k

TPP which was simpler to make. Accordingly, we synthesised **13**, an analogue of thiamin having a benzene ring in place of the thiazolium ring.

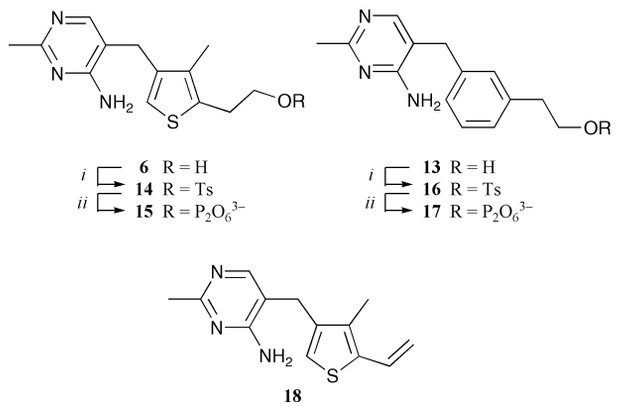
The synthesis of **13** started with *m*-toluoyl chloride **7** (Scheme 2). Bromination of the methyl group[8] followed by quenching the reaction mixture with methanol gave the bromo ester **8**. Nucleophilic substitution of the bromide by cyanide followed by methanolysis of the nitrile **9** then gave the diester **10**. Reduction of both esters with $LiAlH_4$ followed by reoxidation of the benzylic alcohol with MnO_2 gave the aldehyde **11**. Formation of the pyrimidine ring then followed the method we reported earlier:[7] base-catalysed condensation of **11** with 3-anilinopropionitrile gave the acrylonitrile **12** as a mixture of *cis* and *trans* isomers. Finally reaction with acetamidine and base gave the pyrimidine **13**.



Scheme 2 *Reagents*: *i*, Br_2 then MeOH, 65%; *ii*, KCN, 18-crown-6, CH_3CN, 91%; *iii*, MeOH, HCl, 87%; *iv*, $LiAlH_4$, Et_2O, 60% then MnO_2, $CHCl_3$, 91%; *v*, $PhNHCH_2CH_2CN$, NaOMe, DMSO–MeOH, 48%; *vi*, $CH_3C(=NH)NH_2$, NaOMe, EtOH, 81%.

With deazathiamin **6** and the benzene analogue **13** in hand, we now turned our attention to formation of the diphosphates. Initially we tried the method that has been used for the pyrophosphorylation of thiamin and related compounds such as thiamin thiazolone.[9,10] This method is to heat concentrated phosphoric acid to drive off some water and then add thiamin (or an analogue) and continue the heating. The resulting mixture contains mono-, di- and tri-phosphates of the thiamin analogue which need to be separated from each other and the vast excess of inorganic phosphates by anion exchange chromatography. With the benzene analogue this procedure did give the desired diphosphate, albeit in a low 19% isolated yield. However, with deazathiamin decomposition apparently occurred under these extremely acidic conditions and none of the desired diphosphate could be isolated. A different approach was clearly needed.

The alternative method that was now tried employed S_N2 displacement of a good leaving group by a pyrophosphate ion. This method is well known for allylic diphosphates but can also be extended to other compounds, such as nucleoside diphosphates.[11] Accordingly the alcohol groups of deazathiamin **6** and the benzene analogue **13** were tosylated and the tosylates **14** and **16** (Scheme 3) were treated with tris(tetra-*n*-butylammonium) hydrogen pyrophosphate in acetonitrile at room temperature. Purification of the diphosphates by anion exchange chromatography was much easier in this case and both deazaTPP **15** and its benzene analogue **17** could be isolated in pure form, although still in rather low yield (33% and 24% respectively). The major by-product in the formation of deazaTPP was found to be the elimination product **18** (up to 70%). On varying the temperature of the reaction it was found that the proportion of elimination product increased with increasing temperature whereas the proportion of the desired substitution product increased on lowering the temperature and also on raising the concentration of the pyrophosphate salt. The optimum



Scheme 3 *Reagents*: *i*, TsCl, pyr, 80–86%; *ii*, $(Bu_4N)_3HP_2O_7$, CH_3CN, 72% (for **15**).

conditions gave a 72% yield of deazaTPP **15** after anion exchange chromatography, along with the elimination product **18** in 12% yield.

Studies with pyruvate decarboxylase

Inactivation of holoenzyme. Our first set of enzymic studies employed pyruvate decarboxylase (PDC) from *Zymomonas mobilis*, which had been overexpressed in *E. coli* and purified to homogeneity as described previously.[12] The holoenzyme in a TPP-containing buffer was incubated with various concentrations of TPP analogue (**15** or **17**) and samples were taken at intervals and assayed for residual activity. The inactivation of the enzyme was slow – typically it was followed for 5 to 7 hours and the activity was continuing to decrease at the end of this time (Fig. 1). The enzyme activity decayed exponentially in these experiments and apparent first order rate constants (k_{app}) were obtained by fitting exponential curves to the data. It was found that at relatively low concentrations of TPP (*e.g.* 1.38 μM) the rate of inhibition was essentially the same for all concentrations of deazaTPP (1 to 15 μM) (not shown), whereas with higher concentrations of TPP (*e.g.* 15 μM) the rate of

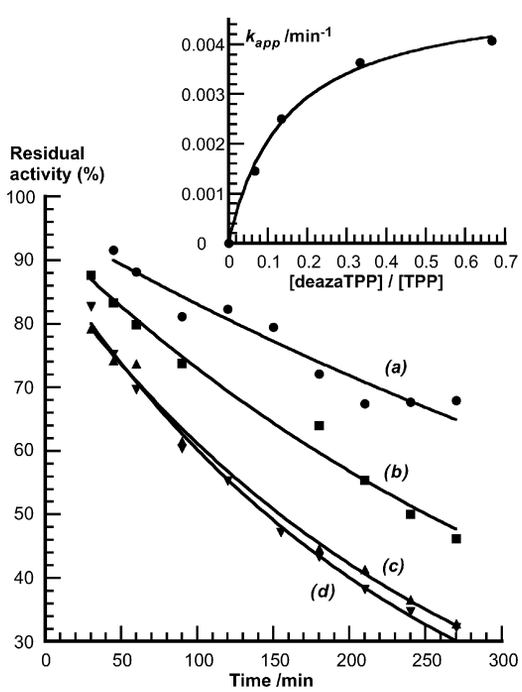


Fig. 1 Time-dependent inactivation of holo-PDC by deazaTPP (*a*) 1 μM, (*b*) 2 μM, (*c*) 5 μM, (*d*) 10 μM; in each case TPP (15 μM) was present. Inset: plot of k_{app} for the inactivation *vs.* [deazaTPP]/[TPP]; the expected saturation kinetics are seen (eqn. 2), giving k_{off} for TPP = 5.1×10^{-3} min^{-1} and the ratio of k_{on} for deazaTPP (k_i) to k_{on} for TPP to be 6.9.

inhibition by the lower concentrations of deazaTPP was significantly slower (Fig. 1). This is as expected if the binding of deazaTPP is competitive with that of TPP, as shown in Scheme 4. Starting with PDC with TPP bound, TPP has to dissociate first before deazaTPP can bind and inhibit the enzyme (hence the very slow inactivation). Once the TPP has dissociated then there is competition between deazaTPP and TPP for rebinding to the apoenzyme. At the lowest concentration of TPP, deazaTPP binds faster than TPP at all concentrations of deazaTPP tested; therefore the rate of inactivation is approximately equal to the rate of dissociation of TPP and independent of [deazaTPP]. However if the [TPP] is raised then it can compete effectively with the lower concentrations of deazaTPP for rebinding to the apoenzyme and thus the rate of inactivation is slower.

$$\text{E.TPP} \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} \text{E} \xrightarrow{k_i} \text{E.deazaTPP}$$

Scheme 4 Kinetic scheme for the inactivation of the holo-PDC by deazaTPP.

The competition between deazaTPP and TPP was further demonstrated by adding additional TPP, up to a concentration of 50 or 100 μM, half way through one of these time-course experiments. The result was that inactivation of the enzyme was virtually halted but no activity that had already been lost was recovered, suggesting that binding of deazaTPP is essentially irreversible.

The kinetic scheme of Scheme 4 would give an apparent first order rate constant for inactivation that is given by equation 1:

$$k_{app} = k_{off} \frac{k_i[\text{deazaTPP}]}{k_i[\text{deazaTPP}] + k_{on}[\text{TPP}]} \quad (1)$$

Rearranging equation 1, one gets equation 2:

$$k_{app} = \frac{k_{off}[\text{deazaTPP}]/[\text{TPP}]}{[\text{deazaTPP}]/[\text{TPP}] + k_{on}/k_i} \quad (2)$$

Hence a graph of k_{app} *vs.* [deazaTPP]/[TPP] should show Michaelis–Menten-like saturation kinetics and fitting the appropriate curve to the experimental data will give values for k_{off} and k_i/k_{on}. This graph is shown in the inset to Fig. 1. From the curve-fitting a value for k_{off} of 5.1×10^{-3} min^{-1} and for k_i/k_{on} of 6.9 can be obtained. This means that deazaTPP binds nearly seven times faster than TPP does at the same concentration. The rate constant for binding of TPP has previously been reported [13] to be 0.035 μM^{-1} min^{-1}, which gives a value for k_i of 0.24 μM^{-1} min^{-1}.

Binding to apoenzyme. In view of the fact that binding of the deazaTPP to holoPDC is so slow, because of the slow dissociation of the already bound TPP, we decided to study the binding of the analogues to apoPDC. The apoenzyme can be prepared by adjusting the pH to 8.2 and then separating the cofactor from the protein by either dialysis [13] or gel filtration [12] or, in this case, by repeated ultrafiltration. Two methods were then used to follow binding of the TPP analogues, fluorescence quenching and enzyme activity.

It has previously been shown that binding of TPP to apoPDC from *Z. mobilis* results in a decrease in the fluorescence due to the tryptophan residues in the protein.[13] The fluorescence decreases over time with an apparent first-order rate constant given by $k_{app} = k_{on}[L] + k_{off}$, where L is the ligand that is binding to the protein. We found that binding of the TPP analogues, **15** and **17**, to apoPDC also causes a decrease in fluorescence. The change in fluorescence was followed over time at 30 °C for a range of different concentrations of deazaTPP **15**,

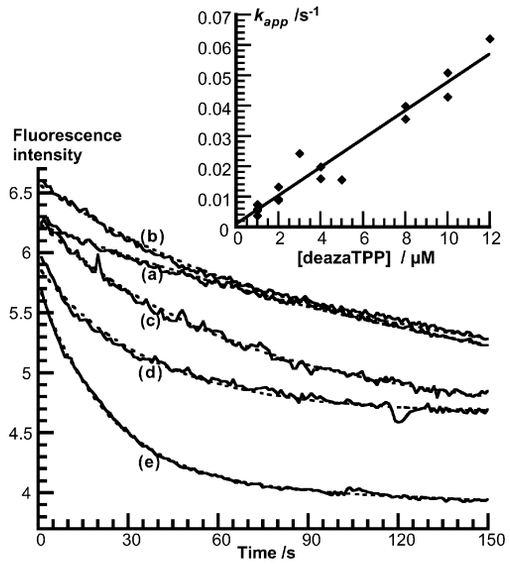


Fig. 2 Change in fluorescence when deazaTPP binds to apo-PDC; the solid lines are the experimental data and the dotted lines are the best-fit exponential decay curves; deazaTPP concentrations: (a) 1 μM, (b) 2 μM, (c) 4 μM, (d) 8 μM, (e) 10 μM. Inset: plot of k_{app} for the fluorescence change *vs.* [deazaTPP]; the slope of the graph gives k_{on} for deazaTPP (k_i) and the intercept on the *y*-axis gives k_{off} for deazaTPP.

as shown in Fig. 2. The graph of k_{app} *vs.* [deazaTPP] is given in the inset to Fig. 2. The slope gives a value of k_i for deazaTPP of 0.28 μM^{-1} min^{-1}, similar to the value found by the competitive inhibition experiments. The k_{off} value for dissociation of deazaTPP is given by the intercept on the *y*-axis. It can be seen that this intercept is within experimental error of zero, again indicating that the binding of deazaTPP is more or less irreversible.

The total decrease of fluorescence associated with binding of deazaTPP was roughly constant whenever the molar ratio of deazaTPP to active sites (assuming four active sites per tetramer) was 1 : 1 or greater and addition of TPP at the end of the incubation caused no further decrease in fluorescence. If the molar ratio of deazaTPP to active sites was less than 1 : 1, however, then a smaller decrease of fluorescence was observed and addition of TPP at the end of the incubation did cause a further decrease in fluorescence. This shows that the stoichiometry of bound deazaTPP to active sites is 1 : 1.

In order to find out whether the binding shows any reversibility at all, apoPDC was incubated with deazaTPP until there was no measurable activity (after addition of TPP to reconstitute holoenzyme from any remaining apoenzyme). Excess deazaTPP was removed by ultrafiltration and then TPP (100 μM) was added. After 24 hours at 30 °C enzymic assay still showed no measurable activity. We estimate that the activity must have been less than 0.4% of the initial activity. This upper limit on the recovered activity implies that the rate of dissociation of deazaTPP is less than 2.8×10^{-6} min^{-1}. Combining this with the measured rate of association (k_i = *ca.* 0.2 μM^{-1} min^{-1}), the inhibition constant K_i must be less than 14×10^{-12} M (14 pM).

In order to demonstrate that the effectively irreversible inhibition is not due to any covalent attachment to the protein, we attempted to remove the inhibitor from the enzyme. Initially the inhibited enzyme was subjected to the same treatment as was used for the removal of TPP to form apoPDC but no activity was recovered. As this method did not work, enzyme which had been partially inactivated by treatment of the holoenzyme with deazaTPP was instead denatured by incubation in 6.8 M urea.[14] The deazaTPP in solution was then removed by ultrafiltration. Finally the enzyme was renatured by dilution with buffer containing TPP but no urea. By this procedure the enzyme activity, which had been reduced to 60% of the original

value after the treatment with deazaTPP, was restored to *ca.* 90% of the original activity. Thus it is shown that deazaTPP can be removed if the enzyme is denatured.

The effectively irreversible binding of deazaTPP to apoPDC allowed us to follow the binding by measurement of enzymic activity after addition of excess TPP. However, in order for these measurements to be meaningful it was necessary to show that the enzymic activity is proportional to the concentration of free apoenzyme. PDC from *Z. mobilis* is a tetramer, made up of a dimer of dimers, and there are four active sites per tetramer located at the interface between the subunits in each dimer.[15] It was possible, therefore, that binding of deazaTPP in one active site of the tetramer might affect the activity at one or more of the other active sites. ApoPDC was incubated with various substoichiometric concentrations of deazaTPP for 3 h (long enough for effectively all the deazaTPP to bind) and then the solution was added to the assay mixture containing TPP and the remaining enzyme activity was measured. The resulting graph of residual activity *vs.* [deazaTPP] (Fig. 3) is a straight line with complete inactivation occurring at approximately 1 : 1 molar ratio of deazaTPP to active sites. The linearity of the graph suggests that occupation of one site in the tetramer does not affect the enzymic activity at any of the other sites.

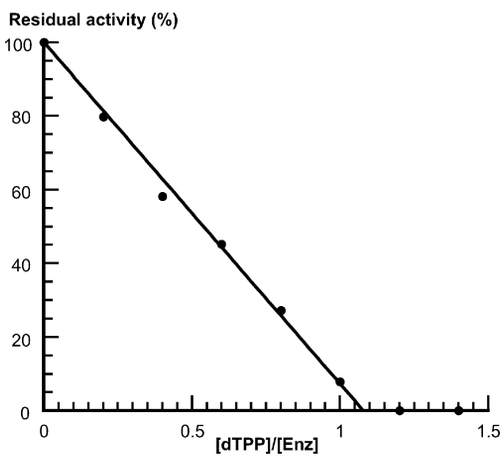


Fig. 3 Graph of residual activity *vs.* molar ratio of deazaTPP to enzyme active sites; the straight line shows that complete inactivation occurs at a 1 : 1 molar ratio and that the enzymic activity at any active site is not affected by occupation of other sites in the tetramer.

The binding of deazaTPP to apoPDC was then followed by measuring enzymic activity in this way, complementing the fluorescence-based method described above. In these experiments apoPDC was incubated with various concentrations of deazaTPP and samples were taken at timed intervals and assayed. For each deazaTPP concentration the enzymic activity decayed exponentially over time (Fig. 4) giving an apparent first order rate constant, k_{app}. Plotting k_{app} against [deazaTPP] (inset to Fig. 4) gave a straight line, whose slope gives the second order rate constant of 0.16 μM^{-1} min^{-1}. This value is close to the value of 0.24 μM^{-1} min^{-1} from the competitive inhibition experiments.

The benzene analogue **17** was also tested for binding to apoPDC. With this compound also, it was found that binding is effectively irreversible. The stoichiometry was also studied and again there was a linear relationship between [inhibitor] and residual activity with complete inactivation occurring at a 1 : 1 molar ratio of inhibitor to active sites. When the time-course for inactivation by **17** was studied, it was found that the points did not fit well onto a simple exponential decay curve. The initial decay was too rapid and the later decay was too slow. This suggested a two-step process in which the faster first step is partially reversible and the slower second step (presumably a conformational change) is irreversible, as in Scheme 5. According to this scheme the residual activity that is measured

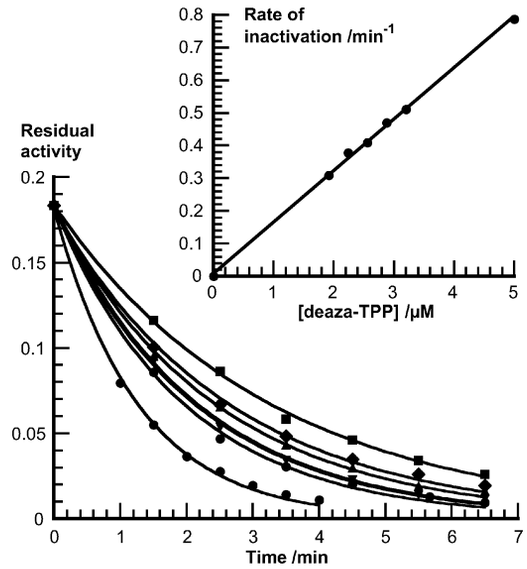


Fig. 4 Binding of deazaTPP to apo-PDC followed by measuring the loss of enzymic activity after reconstitution of the residual apoenzyme with TPP; deazaTPP concentrations were from top curve to bottom curve, 1.92 μM, 2.24 μM, 2.56 μM, 2.88 μM, 3.2 μM and 5 μM. Inset: plot of k_{app} for the loss of activity *vs.* [deazaTPP]; the slope of the graph gives k_{on} for deazaTPP (k_i).

$$E + \mathbf{17} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.\mathbf{17} \xrightarrow{k_2} E.\mathbf{17}^*$$

Scheme 5 Possible two-step inactivation of the holoenzyme by benzene analogue **17**.

after addition of excess TPP will be due to the free enzyme (E) present when the TPP is added plus a certain fraction of the initial complex (E.**17**) which reverts to free enzyme rather than going on to the irreversibly inhibited complex (E.**17***). This fraction is given by $k_{-1}/(k_{-1} + k_2)$.

The kinetics of Scheme 5 were simulated using a spreadsheet program and the parameters k_1, k_{-1} and k_2 were adjusted to get the best fit to the experimental data. As shown in Fig. 5, a very good fit was obtained when $k_1 = 0.264$ μM^{-1} min^{-1}, $k_{-1} = 0.06$ min^{-1} and $k_2 = 0.36$ min^{-1}. This two-step kinetic scheme is in contrast to the one-step process suggested for deazaTPP. Presumably the first step of binding (k_1), which is partially reversible for the benzene analogue **17**, is effectively an irreversible step for deazaTPP **15** due to its better fit in the active site. If there is a subsequent step in deazaTPP binding, corresponding

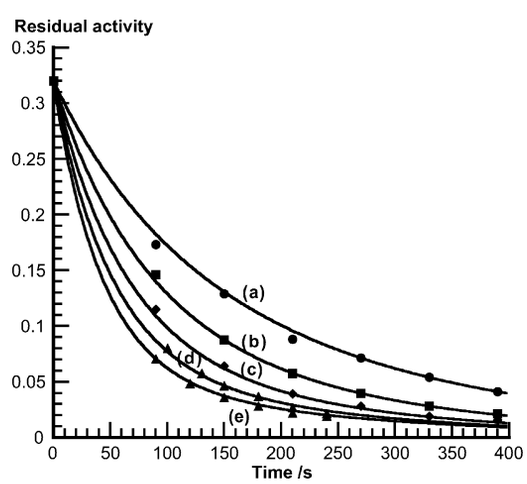
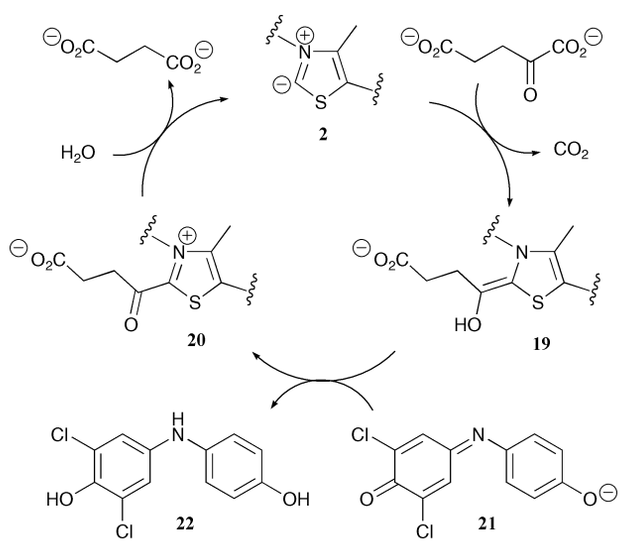


Fig. 5 Binding of the benzene analogue **17** to apo-PDC followed by measuring the loss of enzymic activity after reconstitution of the residual apoenzyme with TPP. The curves are the best fit obtained in a simulation of the two-step kinetic model shown in Scheme 5 with $k_1 = 0.26$ μM^{-1} min^{-1}, $k_{-1} = 0.06$ min^{-1} and $k_2 = 0.36$ min^{-1}.

to k_2 for binding of **17**, it is not observed in the inhibition kinetics because the enzyme is already irreversibly inhibited before it occurs.

α-Ketoglutarate dehydrogenase

α-Ketoglutarate dehydrogenase is a multienzyme complex which catalyses the NAD^+-dependent conversion of α-ketoglutarate and coenzyme A into succinyl CoA. The E1 subunit (known as E1o) is the TPP-dependent α-ketoglutarate decarboxylase. In the multienzyme complex, the active aldehyde intermediate **19** reacts with a lipoyl group attached to a lipoyl carrier domain of the E2o subunit instead of being protonated as the active aldehyde intermediate **4** is in Scheme 1. In this work we used purified E1o from *E. coli* free of E2o (gift of R. Frank, Department of Biochemistry). To assay the activity of this subunit, therefore, an alternative oxidising agent must be present in place of the lipoyl group. We employed dichlorophenolindophenol (DCPIP) **21** for this purpose, which has previously been used to assay the activity of the E1 subunit of the pyruvate dehydrogenase complex (E1p).[16] The active aldehyde intermediate **19** formed upon decarboxylation is oxidised by the DCPIP to give the acyl thiazolium salt **20**, which rapidly hydrolyses to regenerate the TPP ylid **2** (Scheme 6). The activity is followed by monitoring the decrease in the absorbance due to DCPIP at 600 nm.



Scheme 6 Reaction catalysed by the E1 subunit of α-ketoglutarate dehydrogenase in the presence of DCPIP **21**.

In the planned competitive inhibition experiments we did not want to use any higher concentration of TPP than necessary as this would only slow down the inhibition, still further. We therefore preincubated the enzyme with various concentrations of TPP (5 to 100 μM) and in each case samples were taken for assay after various times of preincubation at 30 °C. For TPP concentrations of 25, 50 and 100 μM the enzymic activity remains constant from the first sample time (10 min) onwards, but for 5 and 10 μM [TPP] the enzymic activity was highest after 10 min and decreased gradually thereafter. It would seem that the binding of TPP to any apoenzyme present in the purified enzyme is complete within the first 10 min but that at a TPP concentration of 10 μM or below there is some apoenzyme in equilibrium with the holoenzyme and the apoenzyme is not stable at 30 °C resulting in a fall of activity with time. Plotting the initial enzyme activity measurements against concentration and fitting a Michaelis–Menten equation (not shown) gave an apparent K_M value for TPP of about 2 to 3 μM. However this figure is only approximate because of the instability of the enzyme at lower [TPP] values. As a result of these experiments, all subsequent assays were performed in the presence of 25 μM TPP, the lowest concentration at which the enzyme appeared stable.

In the inhibition experiments, the enzyme was preincubated with TPP to ensure that it was all in the holoenzyme form and then deazaTPP was added. Samples were removed at timed intervals and assayed for remaining enzymic activity. At all deazaTPP concentrations the activity decayed exponentially as shown in Fig. 6. As with PDC, the higher concentrations of deazaTPP all gave effectively the same rate of inhibition, which would be the rate of unbinding of the already bound TPP. Only when the concentration of deazaTPP was lowered to 0.25 μM or below was a significant retardation of the inhibition observed. Fitting a Michaelis–Menten-type curve to the plot of k_{app} *vs.* [deazaTPP] (inset to Fig. 6), as before, gave values of k_{off} for TPP of about 0.07 min^{-1} and of k_i/k_{on} of 74. Comparing these values with those obtained for PDC one finds that the TPP complex is more labile for E1o (unbinding is more than 10-fold faster) and the ratio of the rates of deazaTPP binding to TPP binding is more than ten times greater for E1o than PDC.

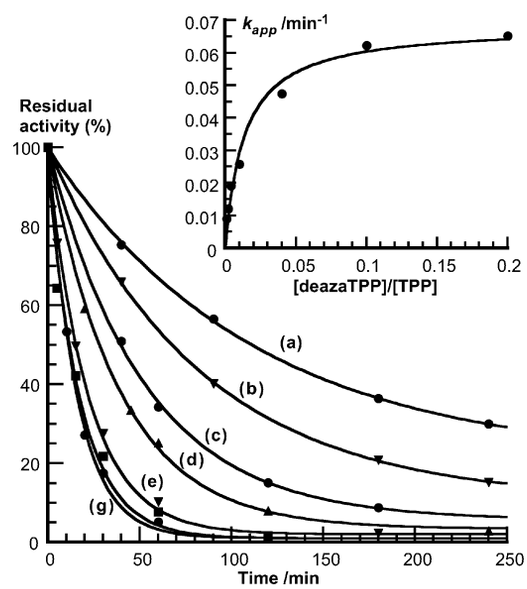


Fig. 6 Time-dependent inactivation of holo-E1o by deazaTPP; deazaTPP concentrations used were (a) 0.025 μM, (b) 0.05 μM, (c) 0.1 μM, (d) 0.25 μM, (e) 1 μM, (f) 2.5 μM, (g) 5 μM. Inset: plot of k_{app} for the inactivation *vs.* [deazaTPP] shows the expected saturation kinetics and allows k_{off} for TPP and the ratio of k_{on} for TPP to k_{on} for deazaTPP (k_i) to be measured.

The reversibility of the inhibition of E1o by deazaTPP was then explored by incubating E1o with deazaTPP and TPP (50 μM) for 30 min and then raising the concentration of TPP to 1 mM. After a further 30 min the enzymic activity had risen from 23% to 34% of the original activity. This system would not have reached equilibrium in the second 30-minute period as the unbinding of deazaTPP is slow. Nevertheless this value along with the previously estimated values of K_M and k_{off} for TPP and of k_i/k_{on} allow a rough estimate of all the rate constants to be made as follows. For TPP a value for k_{on} of *ca.* 0.03 μM^{-1} min^{-1} can be calculated from the measured k_{off} value of 0.07 min^{-1} and the observed apparent K_M value of 2 to 3 μM; for deazaTPP the k_{on} value (*i.e.* k_i) can be calculated as 2.22 μM^{-1} min^{-1} from the measured k_i/k_{on} value of 74. Finally a k_{off} value for deazaTPP can be derived by trial and error because the expected amount of recovered activity after 30 min in the presence of excess TPP can be calculated from any particular estimated value of k_{off}. A k_{off} value for deazaTPP of *ca.* 0.01 min^{-1} would give the observed 34% activity after 30 min, whereas a lower k_{off} value would give less recovered activity and a higher k_{off} value would give more. A K_i value

for deazaTPP of about 5 nM can then be derived from the k_{on} and k_{off} values. Thus for this enzyme deazaTPP **15** binds approximately 500 times more tightly than TPP **1**.

Discussion

DeazaTPP **15** has been shown to be an exceptionally strong inhibitor of both TPP-dependent enzymes studied here. For the E1 subunit of α-ketoglutarate dehydrogenase a dissociation constant of about 5 nM could be determined, indicating binding 500 times tighter than that of TPP. For PDC the binding of deazaTPP is so tight as to be effectively irreversible under normal conditions and all that we could deduce is that the dissociation constant must be in the low picomolar region or less. This is equivalent to binding that is at least 25,000 times tighter than that of TPP, based on the reported value of 0.35 μM for the K_d of TPP.[13] For both enzymes deazaTPP also binds more rapidly than TPP (6.9-fold and 74-fold for PDC and E1o respectively).

The benzene analogue **17** is also an exceptionally strong inhibitor of PDC. It binds at almost the same rate as deazaTPP ($0.23\ \mu M^{-1}\ min^{-1}$ compared with $0.16\ \mu M^{-1}\ min^{-1}$) and, as with deazaTPP, no reversal of the binding could be detected.

It is remarkable that the change of charge from +1 for the thiazolium ring of TPP to zero for the equivalent thiophene ring of deazaTPP can make such a difference to the binding affinity. This tight binding can be ascribed to the increased hydrophobicity of deazaTPP compared to TPP and it is clear that the enzyme active site forms stronger interactions with the neutral thiophene ring than the positively charged thiazolium ring. It makes sense that the enzyme should bind compounds with no net charge on this ring better than ones with a positively charged ring. In this way the enzyme would promote formation of the ylid **2** and the enamine **4** from their precursors **1**, **3** and **5**. This stabilisation of a neutral *vs.* a charged species could be achieved simply by lowering the local dielectric constant in the active site and it has been estimated[17] from the emission wavelength of a fluorescent TPP analogue bound to yeast PDC that the active site has a dielectric constant of about 14, close to that of *n*-hexanol.

Crystal structures of PDC[15,18] show a glutamate residue (Glu-477 in yeast and Glu-473 in *Z. mobilis*) which is in apparent contact with the thiazolium salt (Fig. 7). As there is no other nearby ion-pair for Glu-477, it is reasonable to assume that the carboxylate ion and the thiazolium ring of TPP form an intimate ion pair. The distance from N-3 of TPP to the nearest oxygen of the glutamate residue is only 3.4 Å in the yeast crystal structure. The presence of a counterion to the positively charged thiazolium salt makes it somewhat surprising that neutral species bind so well in place of the thiazolium salt. One can only assume that this glutamate residue is in its protonated form when neutral species are bound. This conclusion would equally apply to the neutral species in the reaction mechanism, *i.e.* ylid **2** and enamine **4**. This is in accord with the conclusions drawn from their molecular mechanics calculations by Lobell and Crout.[19] These authors showed this glutamate as being protonated when the ylid **2** attacks the pyruvate molecule and staying protonated until the protonation of the enamine **4**, at which point the glutamate provides the required proton *via* an intervening water molecule.

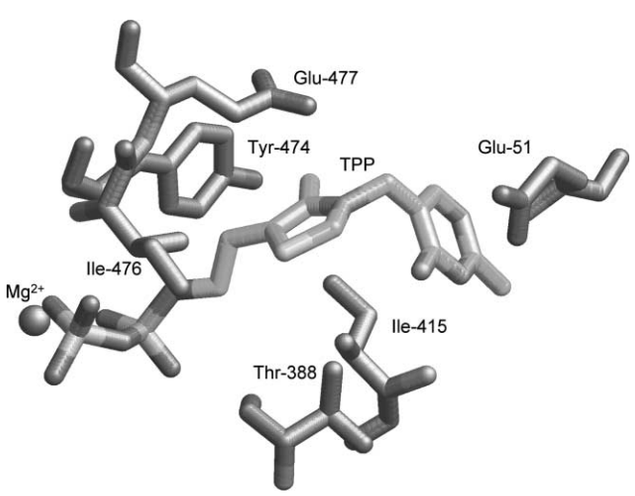
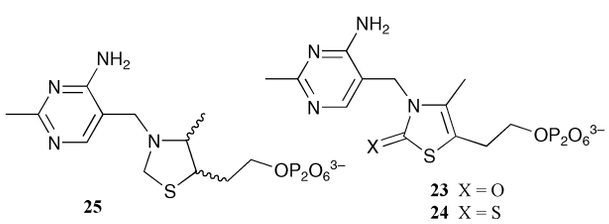


Fig. 7 Structure of TPP and some of the amino acids residues surrounding it from the crystal structure of yeast PDC[18] (PDB accession code 1PYD). Glu-477 is just above the thiazolium ring and Ile-415 just below (in the "V" formed by the thiazolium and pyrimidine rings).

It should be noted that there are other TPP analogues having a neutral ring in place of the thiazolium ring which have been studied before, notably thiamin thiazolone and thiazolethione diphosphates[9,20–23] **23** and **24** and tetrahydrothiamin diphosphate[24] **25**. The results reported with these inhibitors are less complete but, as far as they go, broadly similar to those reported here, *i.e.* the inhibitors are competitive with TPP and bind much more strongly. With the E1 subunit of pyruvate dehydrogenase from *E. coli*, inhibition by **23** does show slow reversal upon dialysis and a K_i value in the region of 3 to 13 nM was calculated.[23] Inhibition by **25** was also found to be reversible over a period of 2–3 hours and the K_i value for the most inhibitory isomer (one of the enantiomers of the *cis* diastereoisomer) was thought to be about 20 nM.[24] For pyruvate decarboxylase from wheat germ, inhibition by **23** shows two-step kinetics with the second step claimed to be irreversible[22] (though no experiment was described that tested whether slow reversal of the inhibition could be observed).



Thus the inhibition observed with deazaTPP is very much in line with previous studies on TPP analogues **23** to **25**. The big advantage that deazaTPP has over these other inhibitors, however, is that it should be possible to attach a variety of groups to C-2 of deazaTPP that match the intermediates in the various reaction mechanisms. We have already synthesised the 2-(1-hydroxyethyl) derivative of deazaTPP (the deaza analogue of intermediate **5** in Scheme 1) and studies of the effect of this compound on PDC are in progress and will be reported in a future publication.

Experimental

General directions for synthesis

Melting points were determined on a Reichert melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 297 FTIR spectrophotometer, using sodium chloride plates for thin film spectra and Nujol mulls. Proton NMR spectra were recorded on a Bruker AM/DPX 250 (250 MHz), a Bruker AM/DPX 400 (400 MHz) or a Bruker DPX 500 (500 MHz) spectrometer. All coupling constants (*J*) are given in Hz. Carbon NMR spectra were recorded with proton decoupling on either a Bruker AC/DPX 250 (62 MHz), a Bruker AC/DPX 400 (100 MHz) or a Bruker DPX 500 (125 MHz) spectrometer. APT spectra (*J*-resolved spin echo) were also run to assist in the assignments. Chemical shifts (δ) are reported in ppm downfield from tetramethylsilane. Phosphorus NMR spectra were recorded with proton decoupling on a Bruker DPX 400 spectrometer at 162 MHz. Samples for phos-

phorus NMR were prepared by dissolving the phosphate in milliQ purified water and adjusting the pH to 7.0 with solid carbon dioxide and then concentrating by lyophilisation prior to dissolving in D_2O. Mass spectra were run on either a Micromass Q-Tof or a Micromass Concept spectrometer using electron impact (EI) or electrospray ionization (ESI) in positive ion mode. Anion exchange was performed on a Pharmacia Acta Prime chromatography system using a column packed with DEAE-Sephacel (from Sigma). Analytical TLC was performed on commercial Merck glass plates, coated to a thickness of 0.25 mm with Kieselgel 60 F_{254} silica. Column chromatography was performed using Merck Kieselgel 60 (230–400 mesh) silica using a small positive pressure of air. All solvents were redistilled before use. Solvents and reagents for anhydrous reactions were dried by conventional methods[25] and such reactions were performed under a small positive pressure of argon.

Methyl 3-bromomethylbenzoate 8

m-Toluoyl chloride **7** (46.35 g, 0.30 mol) was heated to 180 °C with vigorous stirring in a three necked round bottomed flask fitted with a dry ice condenser. Bromine (52 g, 0.32 mol) was added dropwise over 1 h. The reaction mixture was stirred for a further 30 min at 180 °C and then cooled to room temperature and added, with stirring, to methanol (300 ml) pre-cooled to −78 °C. The mixture was left in the freezer overnight and then the precipitate was filtered off and recrystallised from petroleum ether (bp 40–60 °C) to give the bromo ester **8** as needles (44.5 g, 65%), mp 46–47 °C (lit.[8] mp 46–47 °C); δ_H(400 MHz; $CDCl_3$) 3.92 (3 H, s, C*H*$_3$), 4.51 (2 H, s, C*H*$_2$), 7.42 (1 H, t, *J* 7.7, 5-*H*), 7.58 (1 H, dt, *J* 7.7 and 1.3, 4-*H*), 7.96 (1H, dt, *J* 7.7 and 1.3, 6-*H*) and 8.06 (1 H, t, *J* 1.3, 2-*H*); δ_C(100 MHz; $CDCl_3$) 32.4 (*C*H$_2$), 52.2 (*C*H$_3$), 128.9, 129.5 and 130.0 (aryl *C*H), 130.7 (aryl *C*), 133.4 (aryl *C*H), 138.2 (aryl *C*) and 166.4 (*C*=O).

Methyl 3-cyanomethylbenzoate 9

A mixture of bromo ester **8** (26 g, 113 mmol), potassium cyanide (14.7 g, 226 mmol) and 18-crown-6 (2.5 g, 9.4 mmol) in acetonitrile (280 ml) was stirred for 40 h at room temperature, then filtered and evaporated under reduced pressure. A solution of the residue in dichloromethane (100 ml) was washed with water and then with brine, dried ($MgSO_4$) and evaporated under reduced pressure. Purification by silica gel chromatography (petroleum ether–ethyl acetate, 8 : 2, R_f 0.15) gave the nitrile **9** as an oil (18 g, 91%), with spectroscopic data consistent with those previously reported;[26] δ_H(400 MHz; $CDCl_3$) 3.78 (2 H, s, C*H*$_2$), 3.90 (3 H, s, C*H*$_3$), 7.44 (1 H, t, *J* 8.0, Ar*H*), 7.52 (1 H, m, Ar*H*), 7.97 (1 H, m, Ar*H*) and 7.98 (1 H, m, Ar*H*); δ_C(100 MHz; $CDCl_3$) 23.4 (*C*H$_2$), 52.2 (*C*H$_3$), 117.3 (*C*N), 129.0, 129.1 and 129.2 (3 × aryl *C*H), 130.3 and 131.0 (2 × aryl *C*), 132.2 (aryl *C*H) and 166.2 (*C*=O); MS (+EI) *m*/*z* 175.1 (M^+).

Methyl 3-(methoxycarbonylmethyl)benzoate 10

Methanol (150 ml) was saturated with hydrogen chloride gas at −10 °C. Nitrile **9** (17 g, 97 mmol) was added dropwise with stirring and the reaction mixture was then allowed to warm to room temperature, stirred for 6 h, then cooled to 0 °C and quenched by the addition of ice-water (150 ml), ensuring the temperature remained below 10 °C. The solution was neutralised by the addition of sodium hydrogen carbonate, filtered and extracted with ethyl acetate (3 × 100 ml). The combined organic layers were washed with brine, dried ($MgSO_4$) and evaporated under reduced pressure to give the diester **10** as an oil (17.5 g, 87%); ν_{max}(Nujol)/cm^{-1} 2953, 1722, 1608 and 1590; δ_H(400 MHz; $CDCl_3$) 3.66 (2 H, s, C*H*$_2$), 3.68 (3 H, s, C*H*$_3$), 3.89 (3 H, s, C*H*$_3$) and 7.38, 7.46, 7.93 and 7.94 (each 1 H, m, Ar*H*); δ_C(100 MHz; $CDCl_3$) 40.7 (*C*H$_2$), 52.0 (2 × *C*H$_3$), 128.3, 128.5, 130.4 and 133.7 (4 × aryl *C*H), 134.2 and 134.3 (2 × aryl *C*) and 166.8 and 171.5 (2 × *C*=O); *m*/*z* (EI) 208.0716 (M^+. $C_{11}H_{12}O_4$ requires 208.0735).

3-(2-Hydroxyethyl)benzyl alcohol

Lithium aluminium hydride (5.9 g, 156 mmol) was added to dry diethyl ether (125 ml), under an atmosphere of argon, and the mixture was stirred and heated at reflux for 30 min. A solution of diester **10** (16 g, 78 mmol) in dry diethyl ether (125 ml) was then added dropwise such that the ether maintained a gentle reflux. The reaction mixture was heated at reflux for a further 3 h, then cooled, poured into ice water (200 ml) and extracted with ethyl acetate (3 × 100 ml). The combined organic layers were washed with water and then with brine, dried ($MgSO_4$) and evaporated under reduced pressure. Purification by chromatography on silica gel using ethyl acetate–hexane (1 : 1, R_f 0.15) as eluent gave the diol as an oil (7.1 g, 60%); δ_H(400 MHz; $CDCl_3$) 2.80 (2 H, t, *J* 6.5, C*H*$_2$CH$_2$OH), 3.77 (2 H, t, *J* 6.5, CH$_2$C*H*$_2$OH), 4.58 (2 H, s, C*H*$_2$), 7.11 and 7.16 (each 1 H, d, *J* 7.5, 4-*H* and 6-*H*), 7.18 (1 H, s, 2-*H*) and 7.27 (1H, t, *J* 7.5, 5-*H*); δ_C(100 MHz; $CDCl_3$) 38.9 (Ar*C*H$_2$CH$_2$), 63.3 and 64.9 (2 × *C*H$_2$OH), 125.0, 127.6, 128.1 and 128.6 (4 × aryl *C*H) and 138.9 and 141.0 (2 × aryl *C*); *m*/*z* (EI) 152.0843 (M^+. $C_9H_{12}O_2$ requires 152.0837).

3-(2-Hydroxyethyl)benzaldehyde 11

A solution of the above diol (5.0 g, 33 mmol) in dry chloroform (70 ml) at room temperature was stirred vigorously with activated manganese dioxide (20.0 g, 230 mmol) for 3 h, then filtered through Celite and evaporated under reduced pressure to give aldehyde **11** as an oil (4.55 g, 91%) which was used without purification in the next step; δ_H(500 MHz; $CDCl_3$) 2.94 (2 H, t, *J* 6.5, ArC*H*$_2$CH$_2$O), 3.91 (2 H, t, *J* 6.5, CH$_2$C*H*$_2$OH), 7.47 (1 H, t, *J* 7.5, 5-*H*), 7.51 (1 H, dt, *J* 7.5 and 1.6, 4-*H*), 7.75 (1 H, dt, *J* 7.5 and 1.6, 6-*H*), 7.79 (1 H, t, *J* 1.6, 2-*H*) and 9.98 (1 H, s, C*H*O); δ_C(125 MHz; $CDCl_3$) 38.5 (Ar*C*H$_2$CH$_2$O), 63.0 (ArCH$_2$*C*H$_2$O), 127.9, 128.9, 129.7 and 135.0 (4 × aryl *C*H), 136.4 and 139.6 (2 × aryl *C*) and 192.1 (*C*HO); *m*/*z* (+ESI) 173.0581 (M+Na^+. $C_9H_{10}O_2Na$ requires 173.0578).

2-[3-(2-Hydroxyethyl)benzyl]-3-(phenylamino)acrylonitrile 12

A mixture of aldehyde **11** (4.55 g, 30 mmol) and 3-anilinopropionitrile (4.5 g, 33 mmol) was stirred in dry dimethyl sulfoxide (60 ml) at 40 °C under an atmosphere of argon. A solution of sodium methoxide (1.66 g, 33 mmol) in methanol (7 ml) was added dropwise and the reaction mixture was stirred at 40 °C for a further 2 h, then cooled and added to vigorously stirred ice-water. The sticky precipitate was filtered off and recrystallised to give one isomer of the acrylonitrile **12** as needles (4.0 g, 48%), mp 110–112 °C (from toluene) [Found: C, 77.65; H, 6.6; N, 9.9%; M+Na^+ (+ESI), 301.1304. $C_{18}H_{18}N_2O$ requires C, 77.7; H, 6.5; N, 10.1%; M+Na, 301.1317]; ν_{max}(Nujol)/cm^{-1} 3434 (OH), 2186 (CN) and 1601, 1593 and 1497 (Ph); δ_H(400 MHz; $CDCl_3$) 2.86 (2 H, t, *J* 6.5, ArC*H*$_2$-CH$_2$O), 3.55 (2 H, s, C*H*$_2$), 3.85 (2 H, t, *J* 6.5, ArCH$_2$C*H*$_2$O), 6.26 (1 H, br d, *J* 13.1, N*H*), 6.77 (2 H, d, *J* 8.5, 2 × Ar*H*), 7.00 (1 H, t, *J* 7.4, Ar*H*), 7.17 (3 H, m, Ar*H*), 7.28 (3 H, m, Ar*H*) and 7.35 (1 H, d, *J* 13.1, C*H*NH); δ_C(125 MHz; $CDCl_3$) 32.6 and 38.8 (2 × Ar*C*H$_2$), 63.3 (*C*H$_2$O), 82.5 (*C*CN), 115.0 (2 × anilino *o*-*C*H), 122.2 (*C*N), 122.7, 126.0, 127.7, 128.7 and 129.0 (4 × aryl *C*H and anilino *p*-*C*H), 129.5 (2 × anilino *m*-*C*H), 136.8, 139.4 and 139.8 (3 × aryl *C*) and 140.3 (*C*HNH).

The filtrate was extracted with ethyl acetate (3 × 50 ml), and combined organic layers were washed with brine, dried ($MgSO_4$) and concentrated under reduced pressure to give an

oil which was a mixture of both isomers of acrylonitrile **12** (2.6 g, 31%). No attempt was made to separate the isomers.

4-Amino-5-[3-(2-hydroxyethyl)benzyl]-2-methylpyrimidine 13

To a stirred suspension of acetamidine hydrochloride (0.68 g, 7.2 mmol) in dry ethanol (3 ml) was added dropwise a solution of sodium methoxide (0.78 g, 14.4 mmol) in ethanol (4 ml). A solution of **12** (1 g, 3.6 mmol) in ethanol (3 ml) was then added dropwise and the reaction mixture was heated at reflux for 48 h and then cooled. Addition of ice-water produced a precipitate which was filtered off, dried and recrystallised to give the pyrimidine **13** as colourless needles (0.71 g, 81%), mp 152–153 °C (from EtOH) [Found: C, 69.1; H, 7.0; N, 17.1%; MH^+ (+ESI), 244.1454. $C_{14}H_{17}N_3O$ requires C, 69.1; H, 7.0; N, 17.3%; MH, 244.1450]; ν_{max}(Nujol)/cm^{-1} 3343 (OH), 3159 (NH_2), 1731 (C=O) and 1671 (NH_2); δ_H(400 MHz; $CDCl_3$) 2.46 (3 H, s, CH_3), 2.80 (2 H, t, *J* 6.6, ArCH_2CH$_2$O), 3.72 (2 H, s, CH_2), 3.81 (2 H, t, *J* 6.6, ArCH$_2$CH_2O), 4.88 (2 H, br s, NH_2), 6.99 (1 H, d, *J* 7.7, 4-*H*), 7.00 (1 H, s, 2-*H*), 7.10 (1 H, d, *J* 7.7, 6-*H*), 7.23 (1 H, t, *J* 7.7, 5-*H*) and 7.97 (1 H, s, pyrimidine*H*); δ_C(100 MHz; CD_3OD) 24.7 (*C*H$_3$), 34.6 and 40.1 (2 × Ar*C*H$_2$), 64.1 (*C*H$_2$O), 115.3 (*C*=CNH$_2$), 127.5, 128.4, 129.8 and 130.5 (4 × aryl *C*H), 139.0 and 141.0 (2 × aryl *C*), 154.7 (pyrimidine*C*H) and 163.7 and 166.3 (*C*N*C*NH$_2$).

4-Amino-5-{3-[2-(*p*-toluenesulfonyloxy)ethyl]benzyl}-2-methylpyrimidine 16

To a solution of **13** (100 mg, 0.41 mmol) in pyridine (1.5 ml) at −5 °C was added portionwise *p*-toluenesulfonyl chloride (313 mg, 1.64 mmol). The reaction mixture was stirred at −5 °C for 2 h and then quenched with cold hydrochloric acid (1 M; 5 ml) and neutralised by the addition of solid sodium hydrogen carbonate. The mixture was extracted with ethyl acetate (3 × 5 ml) and the combined organic layers were washed with brine, dried ($MgSO_4$) and evaporated under reduced pressure to give tosylate **16** as a yellow solid (140 mg, 86%), mp 106–107 °C (from EtOH–H_2O); δ_H(400 MHz; $CDCl_3$) 2.42 and 2.48 (each 3 H, s, CH_3), 2.90 (2 H, t, *J* 6.8, ArCH_2CH$_2$O), 3.71 (2 H, s, CH_2), 4.17 (2 H, t, *J* 6.8, ArCH$_2$CH_2O), 4.85 (2 H, br s, NH_2), 6.94 (1 H, s, 2-*H*), 7.00 (1 H, d, *J* 7.6, 4-*H*), 7.01 (1 H, d, *J* 7.6, 6-*H*), 7.20 (1 H, t, *J* 7.6, 5-*H*), 7.28 (2 H, d, *J* 8.4, 2 × tosyl*H*), 7.77 (2 H, d, *J* 8.4, 2 × tosyl*H*) and 8.00 (1 H, s, pyrimidine*H*); δ_C(100 MHz; $CDCl_3$) 21.4 and 25.1 (2 × *C*H$_3$), 34.3 and 35.0 (2 × Ar*C*H$_2$), 70.1 (*C*H$_2$O), 112.6 (*C*=CNH$_2$), 126.6, 127.4, 127.6, 128.7, 129.1 and 129.6 (8 × aryl *C*H), 132.8, 137.0, 137.3 and 144.5 (4 × aryl *C*), 155.4 (pyrimidine*C*H) and 161.4 and 166.1 (*C*N*C*NH$_2$); *m*/*z* (EI) 397.1450 (M^+. $C_{21}H_{23}N_3O_3S$ requires 397.1460).

4-Amino-5-[3-(2-hydroxyethyl)benzyl]-2-methylpyrimidine diphosphate 17

To a stirred solution of tosylate **16** (60 mg, 0.15 mmol) in dry acetonitrile (0.3 ml) under an atmosphere of argon was added portionwise tris(tetrabutylammonium) hydrogen pyrophosphate (272 mg, 0.30 mmol). The reaction mixture was stirred at room temperature for 16 h, then diluted with water (0.5 ml), filtered through a 0.22 μm filter under positive pressure and purified by ion-exchange chromatography on a DEAE-Sephacel column eluting with a gradient of 0.1 to 1 M aqueous ammonium bicarbonate. Lyophilisation of appropriate fractions gave the pyrophosphate ester **17** as a powder (15 mg, 24%); δ_H(400 MHz; D_2O) 2.47 (3 H, s, CH_3), 2.90 (2 H, t, *J* 6.4, ArCH_2CH$_2$), 3.78 (2 H, s, CH_2), 4.09 (2 H, dt, *J* 6.9 and 6.4, ArCH$_2$CH_2O), 7.09 (1 H, d, *J* 7.5, 4-*H*), 7.19 (1 H, s, 2-*H*), 7.23 (1 H, d, *J* 7.5, 6-*H*), 7.31 (1 H, t, *J* 7.5, 5-*H*) and 7.56 (1 H, s, pyrimidine*H*); δ_C(125 MHz; D_2O) 20.4 (*C*H$_3$), 32.4 and 35.8 (2 × Ar*C*H$_2$), 66.9 (*C*H$_2$O), 115.7 (*C*=CNH$_2$), 126.9, 128.0, 129.2 and 129.7 (4 × aryl *C*H), 135.3 and 139.3 (2 × aryl *C*), 140.8 (pyrimidine*C*H) and 160.7 and 163.9 (*C*N*C*NH$_2$); δ_P(162 MHz; D_2O) −9.76 and −5.83 (each 1 P, d, *J* 21.3, O*POP*O); *m*/*z* (+ESI) 426.0605 ($M+Na^+$. $C_{14}H_{19}N_3O_7P_2Na$ requires 426.0596).

3-(4-Amino-2-methylpyrimidin-5-ylmethyl)-4-methyl-5-[2-(*p*-toluenesulfonyloxy)ethyl]thiophene 14

To a solution of deazathiamin **6** (280 mg, 1.1 mmol) in pyridine (4.0 ml) at −5 °C was added portionwise *p*-toluenesulfonyl chloride (1.0 g, 5.2 mmol). The reaction mixture was stirred at −5 °C for 1 h, then quenched with cold hydrochloric acid (1 M; 10 ml) and neutralised by the addition of solid sodium hydrogen carbonate. The mixture was extracted with ethyl acetate (2 × 10 ml) and the combined organic layers were washed with saturated aqueous copper sulfate, then water and then brine, dried ($MgSO_4$) and concentrated under reduced pressure to give the tosylate **14** as a pale yellow solid (360 mg, 80%), mp 123–124 °C (from EtOH) [Found: C, 57.71; H, 5.56; N, 9.91%; $M+Na^+$ (+ESI), 440.1076. $C_{20}H_{23}N_3O_3S_2$ requires C, 57.53; H, 5.55; N, 10.06%; M+Na, 440.1079]; δ_H(400 MHz; $CDCl_3$) 1.99 (3 H, s, CH_3), 2.43 (3 H, s, CH_3), 2.49 (3 H, s, CH_3), 3.08 (2 H, t, *J* 7.0, ArCH_2CH$_2$O), 3.56 (2 H, s, ArCH_2), 4.13 (2 H, t, *J* 7.0, ArCH$_2$CH_2O), 4.82 (2 H, br s, NH_2), 6.64 (1 H, s, thiophene*H*), 7.31 (2 H, d, *J* 8.2, 2 × Ar*H*), 7.73 (2 H, d, *J* 8.2, 2 × Ar*H*) and 7.92 (1 H, s, pyrimidine*H*); δ_C(125 MHz; $CDCl_3$) 12.0, 21.4 and 25.2 (3 × *C*H$_3$), 27.9 and 28.9 (2 × Ar*C*H$_2$), 69.3 (*C*H$_2$O), 111.3 (*C*=CNH$_2$), 119.4 (thiophene*C*H), 127.6 and 129.6 (2 × *o*-*C*H and 2 × *m*-*C*H), 132.8, 132.9, 133.5, 136.7 and 144.6 (3 × thiophene*C* and 2 × aryl *C*), 155.5 (pyrimidine*C*H) and 161.3 and 166.1 (*C*N*C*NH$_2$).

3-(4-Amino-2-methylpyrimidin-5-ylmethyl)-4-methyl-5-(2-hydroxyethyl)thiophene pyrophosphate (deazaTPP) 15

A solution of tosylate **14** (71 mg, 0.17 mmol) and tris(tetrabutylammonium) hydrogen pyrophosphate (317 mg, 0.34 mmol) in dry acetonitrile (0.34 ml) under an inert atmosphere was stirred at 4 °C for 10 h and then diluted with water (1 ml) and purified by ion-exchange chromatography using a DEAE-Sephacel column eluting with a gradient of 0 to 0.25 M aqueous ammonium bicarbonate. Lyophilisation of appropriate fractions gave the alkene **18** (5 mg, 12%) and the pyrophosphate ester **15** as a powder (58 mg, 72%).

For the pyrophosphate **15**: δ_H(400 MHz; D_2O) 1.94 and 2.42 (each 3 H, s, CH_3), 3.04 (2 H, t, *J* 6.4, ArCH_2CH$_2$), 3.62 (2 H, s, ArCH_2), 4.02 (2 H, dt, *J* 6.8 and 6.4, CH$_2$CH_2OP), 6.89 (1 H, s, thiophene-H) and 7.37 (1 H, pyrimidine-H); δ_C(125 MHz; D_2O) 11.3 and 22.6 (2 × *C*H$_3$), 27.4 and 28.9 (2 × Ar*C*H$_2$), 65.8 (CH$_2$O), 113.5 (*C*=CNH$_2$), 119.4 (thiophene-CH), 133.6, 135.1 and 136.6 (3 × thiophene-C), 149.9 (pyrimidine-CH) and 162.4 and 163.8 (*C*N*C*NH$_2$); δ_P(162 MHz; D_2O) −5.74 and −9.91 (each 1 P, d, *J* 19.8, OPOPO); *m*/*z* (+ESI) 446.0309 ($M+Na^+$. $C_{13}H_{19}N_3O_7P_2SNa$ requires 446.0317).

For alkene **18**: δ_H(400 MHz, $CDCl_3$) 2.12 (3 H, s, CH_3), 2.51 (3 H, s, CH_3), 3.60 (2 H, s, CH_2), 4.75 (2 H, br s, NH_2), 5.15 (1 H, d, *J* 10.9, CH=CH_AH$_B$), 5.52 (1 H, d, *J* 17.2, CH=CH$_A$$H_B$), 6.65 (1 H, s, thiophene-H), 6.83 (1 H, dd, *J* 10.9 and 17.2, C*H*=CH$_2$), 7.99 (1 H, br s, pyrimidine-H). δ_C(100 MHz, $CDCl_3$) 12.4 (CH_3), 25.5 (CH_3), 29.0 (CH_2), 107.0 (*C*=CNH$_2$), 113.5 (CH=*C*H$_2$), 119.8 (*C*H=CH$_2$), 128.2 (thiophene-CH), 133.8, 137.4, 138.5 (3 × thiophene-C), 155.5 (pyrimidine-CH) and 162.0 and 166.0 (*C*N*C*NH$_2$); *m*/*z* (EI) 245.0992 (M^+. $C_{13}H_{15}N_3S$ requires 245.0987).

General enzymic methods

All assays were carried out on a Cary 100 Bio UV-visible spectrophotometer. Protein concentrations were determined by the Bradford assay.[27] Buffers were autoclaved prior to use and kept on ice unless otherwise stated. E1o was donated by

R. Frank as an aqueous solution (60 mg ml^{-1}) and stored at 4 °C. Solutions containing cofactors and substrates were prepared freshly on the day of use.

Assay for PDC activity

PDC activity was measured by a coupled enzyme assay using the NADH-dependent reduction of the product, acetaldehyde, by alcohol dehydrogenase (ADH). PDC was dissolved in MES–KOH buffer (50 mM; pH 6.5) containing $MgCl_2$ (5 mM), TPP (0.1 mM), NADH (0.15 mM) and ADH (10 units per ml) at 30 °C and the assay was started by addition of aqueous sodium pyruvate (to give 5 mM). The oxidation of NADH was monitored at 340 nm.

Inactivation of holoPDC

A solution of holoPDC (0.029 μM), $MgCl_2$ (5 mM) and TPP (from 1.38 μM to 15 μM) in MES–KOH buffer (50 mM; pH 6.5) was preincubated at 30 °C for 60 min. After addition of inhibitor (1 μM to 10 μM) the mixture was kept at 30 °C and samples (50 μl) were taken at intervals and assayed for PDC activity. In each case a control experiment was run without inhibitor present to ensure that the enzyme is stable. The residual activity given is a percentage of the value for the control.

In order to show that the effect of deazaTPP was not due to inhibition of alcohol dehydrogenase, the same experiment was run using a deazaTPP concentration of 30 μM and no PDC. A sample (50 μl) was taken and assayed as above except that acetaldehyde (5 mM) was used as substrate instead of pyruvate. Consumption of NADH was very rapid, showing that the ADH retains a high level of activity that is easily sufficient for its role in this assay.

Preparation of apoPDC

All buffers used for preparation of the apoenzyme were previously stirred with Chelex-100 cation exchange resin (Na^+ form) to remove bivalent metal. HoloPDC was incubated in HEPES–KOH buffer (50 mM; pH 8.2) containing dipicolinic acid (1 mM) for 20 min at 4 °C and concentrated by ultrafiltration. This treatment was performed three times. The buffer was then replaced by MES–KOH buffer (50 mM; pH 6.5) by ultrafiltration and dilution (three times). The apoenzyme was stored at 4 °C under N_2.

Change of fluorescence of ApoPDC

Experiments were performed in an Amico Bowman Series 2 spectrofluorimeter at 30 °C. Fluorescence decay was measured at 340 nm with excitation wavelength at 300 nm. Bandwidth was 4 nm for both excitation and emission. The solution contained 0.5 μM apoPDC (2 μM of active sites), 5 mM $MgCl_2$ and different concentrations of deazaTPP **15** in MES–KOH buffer (50 mM; pH 6.5).

Denaturation–renaturation of partially inactivated PDC

HoloPDC was partially inactivated by incubation with deazaTPP **15** (5 μM) in the presence of TPP (16.5 μM) and the residual enzymic activity was assayed as above. A solution (0.5 ml) containing this partially inactivated holoPDC (0.25 μM, 1 mM monomer), TPP (100 μM), $MgCl_2$ (5 mM), DTT (100 mM), and urea (6.8 M) in MES–KOH buffer (50 mM; pH 6.5) was incubated at 25 °C for 2 min. The inhibitor was then removed by several rounds of ultrafiltration and dilution with the same urea-containing buffer. Renaturation was initiated by a rapid twenty-fold dilution of the sample (to 10 ml) with the same buffer without urea. The volume was then reduced to 0.5 ml by ultrafiltration and the activity of PDC was measured by the standard enzymatic assay.

Stoichiometry of binding to apoPDC

ApoPDC (4.1 μM of active sites) was incubated in MES–KOH buffer (50 mM; pH 6.5; 15.5 μl) containing $MgCl_2$ (32 mM) and inhibitor (0.8 μM to 4.92 μM) for 3 hours at 30 °C. After dilution of the solution to 200 μl with the same buffer, samples were withdrawn (20 μl) and the enzymic activity was measured.

Measurement of the rate of inactivation of apoPDC

A solution of apoPDC (0.32 μM of active sites) in MES–KOH buffer (50 mM; pH 6.5; 200 μl) containing $MgCl_2$ (5 mM) and inhibitor (1.92 μM to 5 μM for deazaTPP **15** or 2 μM to 6 μM for benzene analogue **17**) was incubated at 30 °C. At timed intervals samples (20 μl) were taken and added to the assay mixture without pyruvate (780 μl). After preincubation for 3 min at 30 °C, aqueous sodium pyruvate (2 μl; 2 M) was added to initiate the assay.

Assay for E1o component activity

The enzyme was preincubated at 30 °C for 15 min in potassium phosphate buffer (106 mM; pH 7.0; 960 μl) containing $MgCl_2$ (2.1 mM) and TTP (25 μM). Aqueous DCPIP (2.5 mM; 20 μl, final concentration 50 μM) was then added and the reaction was started by adding α-ketoglutarate (20 mM; 20 μl, final concentration 400 μM). The decrease in absorbance at 600 nm was monitored between 0.1 and 2 minutes. Enzyme activity is expressed in terms of ΔA_{600} min^{-1}.

A range of different enzyme concentrations was tested and it was concluded that a final concentration of 6.7 μg ml^{-1} (32 nM of dimeric enzyme assuming it is homogeneous) was the least that could be used in this assay while maintaining a satisfactory level of accuracy when the enzyme was inhibited by up to 95%. With no inhibition this concentration of enzyme gave a rate of *ca.* 0.025 ΔA_{600} min^{-1}.

Time-dependent inhibition of E1o by deazaTTP

A stock solution (51.84 ml) of enzyme (6.7 μg ml^{-1}) in assay buffer containing TPP (25 μM) was divided into 9 equal portions and kept at 3 °C. Each portion in turn was pre-incubated at 30 °C for 15 min and then an aliquot (960 μl) was taken and assayed. A solution of deazaTPP (125, 50, 25, 12.5 or 5 μl of 1 mM or 125, 50, 25 or 12.5 μl of 10 μM) was added to the remaining enzyme solution and pre-incubation was continued at 30 °C. Aliquots (960 μl) were removed at timed intervals and mixed with DCPIP and α-ketoglutarate and assayed as above.

The addition of the deazaTPP solution represents a small dilution of the enzyme compared to the $t = 0$ sample taken before addition of deazaTPP and so the measured enzyme activities have all been adjusted to compensate for this dilution. The results are shown in Fig. 6.

Demonstration that binding of deazaTPP to E1o is reversible

A solution (4.8 ml) containing enzyme (13.3 μg ml^{-1}), TPP (52 μM) and buffer (as above) was incubated for 15 min at 30 °C. An aliquot (960 μl) was taken and assayed. A solution of deazaTTP (1 mM; 5 μl giving 1.3 μM) was added to the remaining enzyme solution and further aliquots were assayed after 5, 10 and 30 min. At this point 10 mM TPP (100 μl) was added to the remaining 960 μl of enzyme solution. After incubation for a further 30 min, a final 960 μl aliquot was assayed. The enzyme activity of this final aliquot was adjusted to compensate for the dilution caused by the addition of the TTP solution.

Acknowledgements

We thank the BBSRC and Syngenta for financial support, Dr D. A. Griffin (Syngenta) for his advice and encouragement and Prof. R. N. Perham and R. Frank (Department of Biochemistry) for the gift of E1o.

References

1 F. Jordan, *Nat. Prod. Rep.*, 2003, **20**, 184.
2 E. Chabriere, M. H. Charon, A. Volbeda, L. Pieulle, E. C. Hatchikian and J. C. Fontecilla-Camps, *Nat. Struct. Biol.*, 1999, **6**, 182.
3 U. Nilsson, L. Meshalkina, Y. Lindqvist and G. Schneider, *J. Biol. Chem.*, 1997, **272**, 1864.
4 E. Fiedler, S. Thorell, T. Sandalova, R. Golbik, S. Konig and G. Schneider, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 591.
5 E. Chabriere, C. Vernede, B. Guigliarelli, M. H. Charon, E. C. Hatchikian and J. C. Fontecilla-Camps, *Science*, 2001, **294**, 2559.
6 S. S. Pang, R. G. Duggleby, R. L. Schowen and L. W. Guddat, *J. Biol. Chem.*, 2004, **279**, 2242.
7 D. Hawksley, D. A. Griffin and F. J. Leeper, *J. Chem. Soc., Perkin Trans. 1*, 2001, 144.
8 W. Davies and W. H. Perkin, *J. Chem. Soc.*, 1922, **121**, 2202.
9 S. Bearne, K. Karimian and R. Kluger, *Biochemistry*, 1988, **27**, 3084.
10 H. Koike and T. Yusa, *Methods Enzymol.*, 1970, **18**, 105.
11 V. J. Davisson, D. R. Davis, V. M. Dixit and C. D. Poulter, *J. Org. Chem.*, 1987, **52**, 1794.
12 G. Schenk, F. J. Leeper, R. England, P. F. Nixon and R. G. Duggleby, *Eur. J. Biochem.*, 1997, **248**, 63.
13 R. J. Diefenbach and R. G. Duggleby, *Biochem. J.*, 1991, **276**, 439.
14 M. Pohl, J. Groetzinger, A. Wollmer and M. R. Kula, *Eur. J. Biochem.*, 1994, **224**, 651.
15 D. Dobritzsch, S. Koenig, G. Schneider and G. Lu, *J. Biol. Chem.*, 1998, **273**, 20196.
16 L. S. Khailova, R. Bernhardt and G. Hübner, *Biokhimiya (Moscow)*, 1977, **42**, 113.
17 F. Jordan, H. J. Li and A. Brown, *Biochemistry*, 1999, **38**, 6369.
18 F. Dyda, W. Furey, S. Swaminathan, M. Sax, B. Farrenkopf and F. Jordan, *Biochemistry*, 1993, **32**, 6165.
19 M. Lobell and D. H. G. Crout, *J. Am. Chem. Soc.*, 1996, **118**, 1867.
20 J. A. Gutowski and G. E. Lienhard, *J. Biol. Chem.*, 1976, **251**, 2863.
21 U. Nilsson, Y. Lindqvist, R. Kluger and G. Schneider, *FEBS Lett.*, 1993, **326**, 145.
22 R. Kluger, G. Gish and G. Kauffman, *J. Biol. Chem.*, 1984, **259**, 8960.
23 N. Nemeria, Y. Yan, Z. Zhang, A. M. Brown, P. Arjunan, W. Furey, J. R. Guest and F. Jordan, *J. Biol. Chem.*, 2001, **276**, 45969.
24 P. N. Lowe, F. J. Leeper and R. N. Perham, *Biochemistry*, 1983, **22**, 150.
25 D. D. Perrin, W. L. F. Armarego and D. R. Perrin, *Purification of Laboratory Chemicals*, Pergamon, Oxford, 1980.
26 P. Chand, Y. S. Babu, S. Bantia, N. Chu, L. B. Cole, P. L. Kotian, W. G. Laver, J. A. Montgomery, V. P. Pathak, S. L. Petty, D. P. Shrout, D. A. Walsh and G. M. Walsh, *J. Med. Chem.*, 1997, **40**, 4030.
27 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248.