Inhibition of pyruvate decarboxylase from *Z. mobilis* by novel analogues of thiamine pyrophosphate: investigating pyrophosphate mimics†

Karl M. Erixon, Chester L. Dabalos and Finian J. Leeper*

Received (in Cambridge, UK) 1st November 2006, Accepted 27th November 2006
First published as an Advance Article on the web 8th December 2006
DOI: 10.1039/b615861g

Replacement of the thiazolium ring of thiamine pyrophosphate with a triazole gives extremely potent inhibitors of pyruvate decarboxylase from *Z. mobilis*, with K_I values down to 20 pM; this system was used to explore pyrophosphate mimics and several effective analogues were discovered.

The coenzyme thiamine pyrophosphate (TPP) **2** is the active form of vitamin B_1 **1** (Fig. 1). TPP cooperates in a number of enzymes, which all catalyse the cleavage and formation of bonds adjacent to the carbon of a carbonyl group. Examples include α-keto acid (*e.g.* pyruvate) decarboxylases, dehydrogenases and oxidases, transketolase, benzaldehyde lyase and acetohydroxyacid, acetolactate and deoxyxylulose 5-phosphate synthases.[1] As TPP-dependent enzymes catalyse key steps in many metabolic pathways they are also interesting targets for inhibition in the pharmaceutical and agrochemical industries.

We have previously reported the synthesis of the isoelectronic thiophene analogue of TPP, 3-deazathiamine pyrophosphate **3**, which proved to be an extremely potent inhibitor of several TPP-dependent enzymes with $K_I < 14$ pM for *Zymomonas mobilis* pyruvate decarboxylase (ZmPDC), compared to a K_D value of approximately 0.35 μM for TPP.[2]

The pyrophosphate is of great importance in the binding of TPP to enzymes, where it is coordinated to a magnesium ion in the active site.[1] However, in terms of drug properties, this group is unsuitable, since it is highly charged and analogues possessing it will suffer from poor bioavailability and cellular uptake. We, therefore wanted to explore whether the pyrophosphate group could be replaced by other analogues without losing too much binding affinity for the enzyme. The synthesis of deazaTPP involves 12 steps, however, so we also sought to use an analogue of TPP which was easier to make for this investigation. In this communication, we report the synthesis of eight novel triazole-based analogues of TPP, **9–16**, and inhibition studies with ZmPDC (Scheme 1).

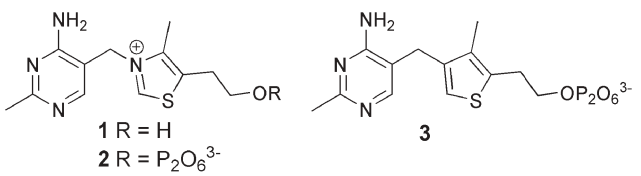
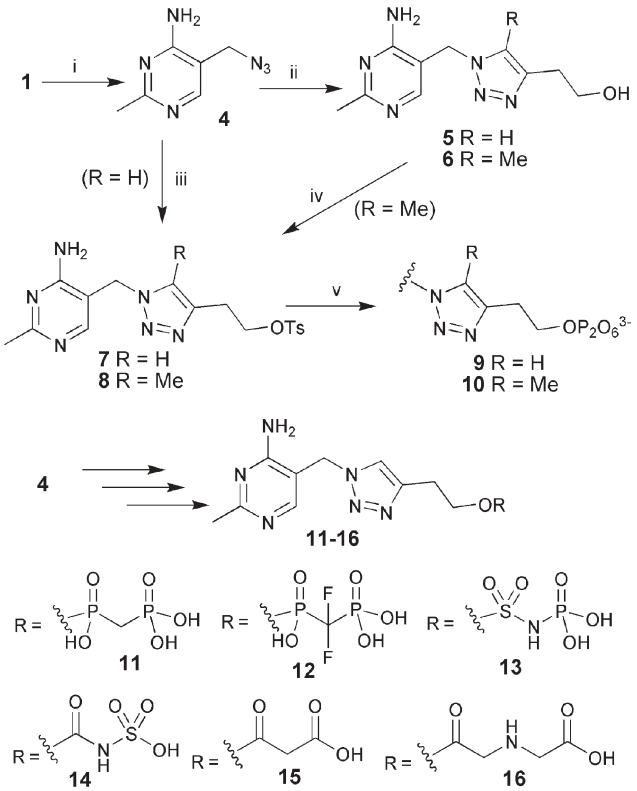


Fig. 1 Thiamine, TPP and deaza-TPP analogue.

Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, UK CB2 1EW. E-mail: fjl1@cam.ac.uk; Fax: +44 1233 336362; Tel: +44 1223 336493

† Electronic supplementary information (ESI) available: Assay details, double exponential curves and reactivation graphs. See DOI: 10.1039/b615861g

The analogues we planned to make contained a triazole ring in place of the thiazolium ring of TPP. Thiamine itself is a convenient starting material and nucleophilic substitution by azide, catalysed by sodium sulfite,[3] gave the azide **4**. Cu(I)-catalysed cycloaddition of **4** with 3-butynol or its tosylate ('click' chemistry[4]) gave the triazole analogues **5** and **7** in 47 to 68% yield over the two steps from thiamine. Conversion of tosylate **7** to its pyrophosphate **9**



Scheme 1 Synthesis of TPP analogues. *Reagents and conditions*: (i) NaN_3 (2.5 eq.), Na_2SO_3 (0.1 eq.), H_2O, 60–65 °C, 6 h, 83%; (ii) for **5**: 3-butynol (1.0 eq.), sodium ascorbate (0.1 eq.), $CuSO_4 \cdot 5H_2O$ (0.01 eq.), *t*-BuOH–H_2O (2 : 1), 25 °C, 16 h, 81%; for **6**: 3-pentynol (1.0 eq.), *n*-BuOH, reflux at 130 °C, 96 h, 80% (isomeric mixture); (iii) 3-butynyl tosylate (1.0 eq.), sodium ascorbate (0.1 eq.), $CuSO_4 \cdot 5H_2O$ (0.01 eq.), *t*-BuOH–H_2O (2 : 1), 25 °C, 24 h, 56%; (iv) TsCl (2.5 eq.), pyridine, −5 to 25 °C, 3 h, 30% of desired isomer; (v) $(Bu_4N)_3HP_2O_7$ (2.0 eq.), MeCN, −5 to 25 °C, 50–60%.

was effected by displacement with pyrophosphate trianion as in the synthesis of deazaTPP **3**.[2]

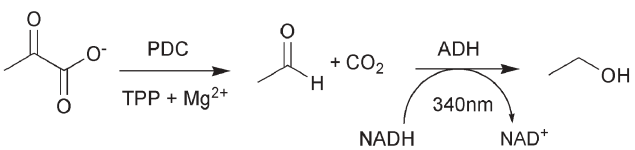
Triazole **5** lacks the 4-methyl group of TPP but unfortunately cycloaddition of azide **4** with 3-pentynol gives an approximately 1 : 1 mixture of the two possible regioisomers. We were only able to separate this mixture, with difficulty, after tosylation to give **8**. Nevertheless this permitted access to the methyl triazole analogue of TPP, **10**.

Triazole-based analogues of TPP **9–16**, in which the pyrophosphate has been replaced by other groups, were all made *via* similar 'click' chemistry starting from azide **4**; details will be given in our full paper. Most of these analogues are designed to be ligands to the Mg^{2+} ion in the active site but **16** contains an amine group which would be protonated at neutral pH and it was hoped this would replace the Mg^{2+} ion.

Pyruvate decarboxylase (PDC) catalyses the conversion of pyruvate to acetaldehyde and carbon dioxide. In the inhibition studies, the activity of PDC was measured by a coupled enzyme assay using the NADH-dependent reduction of acetaldehyde by alcohol dehydrogenase (ADH) (Scheme 2). Because the TPP analogues can only bind to the holo-enzyme once TPP has dissociated and the dissociation of TPP is known to be very slow,[5] we instead studied binding of the analogues to the apo-enzyme, prepared as described previously.[2] In the assay, the analogues (typically 2–10 μM) were incubated with apo-PDC in a Mg^{2+}-containing buffer and small samples were taken out at timed intervals (1–15 min) and added to the assay solution containing ADH, Mg^{2+}, NADH and an excess of TPP (100 μM). The assay was then started by the addition of pyruvate. Under these conditions any apo-PDC that does not already have the TPP analogue bound will bind TPP when it is added and thus show activity. However any TPP analogues that are only loosely bound will be displaced by the excess TPP and so will also show activity.

Incubation of apo-PDC with analogues **9** to **13** gave a decrease of activity to close to zero over a 15 min time-scale (see Fig. 2 and supplementary material) but no decrease was observed for analogues **14–16** under these conditions. The time-points obtained did not fit well onto simple exponential curves, the initial decay being too fast and the subsequent decay too slow. This suggests a two-step process and indeed a much better fit was obtained using a double exponential curve. When the faster of the two rate constants obtained in each case was plotted against inhibitor concentrations a straight line was obtained, which gave a value of the second-order rate constant for the initial binding, k_{on} (Fig. 2, inset). The values of k_{on} for the different analogues are collected in Table 1.

The apparent two-step inhibition of TPP-dependent enzymes has been seen before, most recently in our studies of a benzene analogue of TPP[2] but also with thiamine thiazolone pyrophosphate with wheat germ PDC.[6] There are two possible explanations: one is the normal explanation for slow-binding inhibition, that



Scheme 2 Coupled assay of PDC.

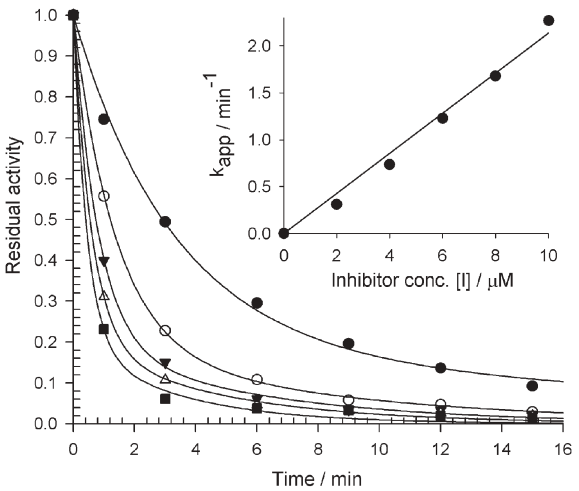


Fig. 2 Inactivation of ZmPDC by various concentrations of TPP analogue **10**; from top to bottom curve: (●) 2, (○) 4, (▼) 6, (△) 8, (■)10 μM. The curves are the best fits of the equation $y = (1 - x)\exp(-k_1 t) + x\exp(-k_2 t)$, with $x = 0.2$ for each curve. Inset: Apparent first order rate constants, k_1, plotted *vs.* inhibitor concentrations; the slope of the graph gives k_{on}. the second order rate constant for the initial binding.

Table 1 Inhibition parameters for analogues **9–16**

Analogue	$k_{on}/\mu M^{-1}\ min^{-1}$	K_I
9	0.05 ± 0.02	30 ± 3 pM
10	0.21 ± 0.03	20 ± 2 pM
11	0.17 ± 0.03	1.2 ± 0.1 nM
12	0.09 ± 0.01	0.95 ± 0.1 nM
13	0.02 ± 0.004	0.14 ± 0.02 μM
14–16	NI[a]	NI[a]

[a] NI, no inhibition observed.

initial reversible binding is followed by a slower step, presumably a conformational change of the enzyme, which effectively makes the binding irreversible on the time-scale of the assay. A second possible explanation arises from the fact that ZmPDC is a homotetramer consisting of a dimer of dimers and there is considerable evidence for communication between the two active sites of each dimer in TPP-dependent enzymes.[7] Thus binding of the TPP analogue in the first active site of each dimer could be faster than in the second active site. TPP itself has been reported to show negative cooperativity in its binding to transketolase.[8] In support of the second explanation, we note that the apparent rate constant for the slower of the two stages also seems to be dependent on concentration of inhibitor.

In order to determine whether binding of the TPP analogues is reversible and, if so, to obtain a K_I value, a large excess of TPP (1 or 10 mM) was added to the enzyme which had been fully inactivated (<1% activity) by inhibitor (10 or 20 μM). The recovery of activity was followed over a period of 8 days. In each case recovery of some activity was observed and the activity reached a plateau after up to *ca.* 2 days. In a control experiment, uninhibited enzyme retained full activity, so this plateau of activity is presumably due to establishment of the equilibrium between binding of TPP and binding of the analogue. Using the reported K_D value for TPP (0.35 μM)[5] and the relative concentrations of

TPP and analogue, the K_I values for the analogues can be calculated and are listed in Table 1.

Not surprisingly, the two analogues possessing the important pyrophosphate group (**9** and **10**) were the most potent inhibitors, with 8.0 and 5.5%, respectively, of the original activity recovered upon reactivation with 10 mM TPP, corresponding to K_I values of 30 and 20 pM, which are in the same range as we previously reported for deazaTPP.[2] The 5-methyl group of **10**, which TPP possesses also, improves the strength of binding, as might be expected, but interestingly it also speeds up the binding, perhaps because of the greater hydrophobicity. The reason why analogues such as **9**, **10** and deazaTPP **3**, bind so much more tightly than TPP itself is thought to be because the enzyme stabilises neutral rings at this position better than the positively charged thiazolium ring.[2,9] In this way the enzyme would promote both the initial formation of the TPP ylide and the later decarboxylation step.

Some of the analogues containing pyrophosphate mimics also show strong inhibitory activity. In **11** and **12**, the bridging oxygen in the pyrophosphate has been replaced by a CH_2 or CF_2 group. These have recently been reported to be effective non-hydrolysable mimics of pyrophosphate, used in *e.g.* antiviral nucleotide triphosphate mimics.[10] We expected these to be effective replacements for the pyrophosphate because the crystal structures of PDC complexed with TPP show no hydrogen bonds to the bridging oxygen of the pyrophosphate.[11] However, in our case, it appears that the bridging oxygen is actually of some importance for the binding. Although they bind faster than the corresponding pyrophosphate **9**, the K_I values for both these analogues (**11** and **12**) were estimated to be 40-fold greater. Methylenediphosphonate esters such as **11** are known to have higher pK_a values than pyrophosphates,[12] which might explain why **11** binds faster, being less hydrophilic, but less tightly than **9**. Difluoromethylenediphosphonate esters such as **12**, however, have a similar pK_a to pyrophosphates.[13] Presumably the greater size of the CF_2 group compared to O reduces the binding affinity in this instance.

The results with the other pyrophosphate mimics suggest that the charge on the group is of vital importance. Thus a marked decrease of inhibition is seen in going from analogues **9–12** (charge = −3) to phosphoramidic acid **13** (−2), to carbamate **14** and malonate **15** (−1). Hence, if the magnesium is present in the active site, the more negatively charged analogues will bind best. One could try to displace the Mg^{2+}, which was the idea of the iminodiacetic acid analogue **16**, but unfortunately this did not turn out to be an effective inhibitor.

In summary, eight new analogues of TPP based on a triazole scaffold have been synthesised. These analogues were prepared in just a few steps using 'click' chemistry and are shown to be very effective at binding in the TPP binding site. Six of the analogues contain mimics of the pyrophosphate group and several of these, particularly the methylenediphosphonates **11** and **12**, still bind with high affinity. These results are likely to be relevant not only to studies of TPP-dependent enzymic reactions but also to the wide range of other proteins that bind pyrophosphate esters.

Notes and references

1 (*a*) F. Jordan, *Nat. Prod. Rep.*, 2003, **20**, 184; (*b*) A. Schellenberger, *Biochim. Biophys. Acta*, 1998, **1385**, 177.
2 S. Mann, C. Perez Melero, D. Hawksley and F. J. Leeper, *Org. Biomol. Chem.*, 2004, **2**, 1732.
3 (*a*) J. A. Zoltewicz and G. M. Kauffman, *J. Am. Chem. Soc.*, 1977, **99**, 3134; (*b*) G. Uray, I. Kriessmann and J. A. Zoltewicz, *Bioorg. Chem.*, 1993, **21**, 294; (*c*) J. A. Zoltewicz, G. Uray and G. M. Kauffman, *J. Am. Chem. Soc.*, 1980, **102**, 3653.
4 F. Himo, T. Lovell, R. Hilgraf, V. V. Rostovtsev, L. Noodleman, K. B. Sharpless and V. V. Fokin, *J. Am. Chem. Soc.*, 2005, **127**, 210.
5 R. J. Diefenbach and R. G. Duggleby, *Biochem. J.*, 1991, **276**, 3439.
6 R. Kluger, G. Gish and G. Kauffman, *J. Biol. Chem.*, 1984, **259**, 8960.
7 (*a*) E. M. Ciszak, L. G. Korotchkina, P. M. Dominiak, S. Sidhu and M. S. Patel, *J. Biol. Chem.*, 2003, **278**, 21240; (*b*) F. Jordan, N. S. Nemeria and E. Sergienko, *Acc. Chem. Res.*, 2005, **38**, 755; (*c*) R. A. W. Frank, C. M. Titman, J. V. Pratap, B. F. Luisi and R. N. Perham, *Science*, 2004, **306**, 872; (*d*) F. Jordan, *Science*, 2004, **306**, 818.
8 R. M. Egan and H. Z. Sable, *J. Biol. Chem.*, 1981, **256**, 4877.
9 F. Jordan, H. J. Li and A. Brown, *Biochemistry*, 1999, **38**, 6369.
10 (*a*) N. A. Boyle, *Org. Lett.*, 2006, **8**, 187; (*b*) G. Wang, N. A. Boyle, F. Chen, V. Rajappan, P. Fagan, J. L. Brooks, T. Hurd, J. M. Leeds, V. K. Rajwanshi, Y. Jin, M. Prhavc, T. W. Bruice and P. D. Cook, *J. Med. Chem.*, 2004, **47**, 6902; (*c*) L. J. Jennings, M. Macchia and A. Parkin, *J. Chem. Soc., Perkin Trans. 1*, 1992, 2197.
11 (*a*) D. Dobritzsch, S. König, G. Schneider and G. Lu, *J. Biol. Chem.*, 1998, **273**, 20196; (*b*) F. Dyda, W. Furey, S. Swaminathan, M. Sax, B. Farrenkopf and F. Jordan, *Biochemistry*, 1993, **32**, 6165.
12 G. M. Blackburn, D. E. Kent and F. Kolkmann, *J. Chem. Soc., Chem. Commun.*, 1981, 1188.
13 (*a*) G. R. J. Thatcher and A. S. Campbell, *J. Org. Chem.*, 1993, **58**, 2272; (*b*) G. M. Blackburn, D. A. England and F. Kolkmann, *J. Chem. Soc., Chem. Commun.*, 1981, 3523.

