Synthesis and biological evaluation of pyrophosphate mimics of thiamine pyrophosphate based on a triazole scaffold

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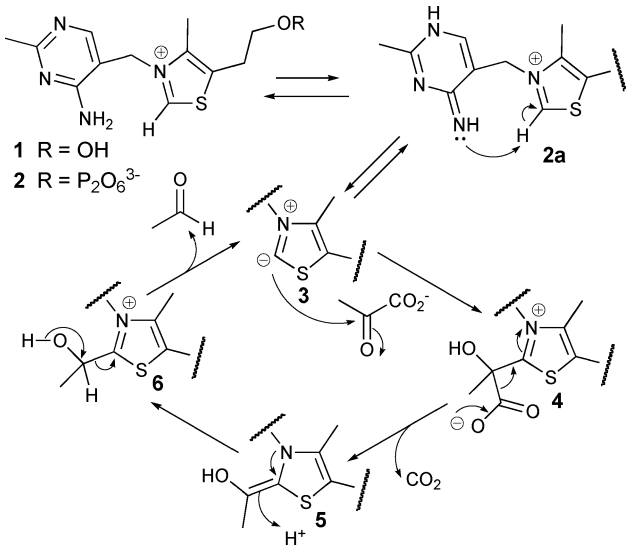
Novel triazole-based pyrophosphate analogues of thiamine pyrophosphate (TPP) have been synthesised and tested for inhibition of pyruvate decarboxylase (PDC) from *Zymomonas mobilis*. The thiazolium ring of thiamine was replaced by a triazole in an efficient two-step procedure. Pyrophosphorylation then gave extremely potent triazole inhibitors with K_I values down to 20 pM, compared to a K_D value of 0.35 μM for TPP. This triazole scaffold was used for further investigation and six analogues containing mimics of the pyrophosphate group were synthesised and tested for inhibition of PDC. Several effective analogues were found with K_I values down to around 1 nM.

Introduction

Thiamine pyrophosphate **2** (TPP) is an important coenzyme, made in animals by pyrophosphorylation of thiamine **1** (vitamin B_1). TPP-dependent enzymes serve crucial roles in many metabolic processes of carbohydrates and branched-chain amino acids. Examples include α-keto acid decarboxylases, dehydrogenases and oxidases, transketolase, acetohydroxyacid synthase and deoxyxylulose 5-phosphate synthase.[1] All TPP-dependent enzymes have the common feature that they catalyse the cleavage and formation of bonds adjacent to the carbon of a carbonyl group. The catalysis occurs at the thiazolium ring of TPP. However, the pyrimidine ring of TPP also plays an important role in the mechanism, as it is involved in the initial activation step (as shown in Scheme 1). The enzyme promotes the conversion of TPP **2** to its normally less stable tautomer **2a**, and it is this imino form of the amino group of TPP that effects the deprotonation of C-2 of the thiazolium ring to give ylid **3**.[2] In the case of pyruvate decarboxylase (PDC) the ylid **3** attacks the substrate (pyruvate) to give 2-lactylTPP **4**. The next step is decarboxylation to give enamine **5**, facilitated by the positively charged thiazolium ring of TPP, which acts as an electron sink. Similar bond cleavage and formation of enamine (also called the "activated aldehyde") intermediate, is seen in all TPP catalysis, whatever the substrate. The fate of this enamine then depends on the particular enzyme. In PDC **5** is protonated to give hydroxyethylTPP **6**, followed by release of acetaldehyde product and regeneration of ylid **3**. In other examples the electrophile could be an aldehyde or a ketone of another substrate molecule, a lipoyl group or an oxidant.[1]

There are crystal structures of many TPP-dependent enzymes available. However, due to fast reaction of substrate bound to the active site and instability of reaction intermediates, it has proven, with a few exceptions,[3] to be difficult to trap these natural intermediates. Hence, much information regarding substrate binding and important catalytic groups in the active site still remains to be revealed. In light of this, we have recently synthesised deazaTPP **7**



Scheme 1 Mechanism of pyruvate decarboxylase.

(Fig. 1), which is an isoelectronic inactive analogue of TPP. This analogue **7** has been studied in detail for inhibition of several TPP-dependent enzymes, and it has been shown that **7** is an extremely potent inhibitor, with K_I <15 pM for *Zymomonas mobilis* PDC (ZmPDC) compared to a K_D value of approximately 0.35 μM for TPP.[4,5]

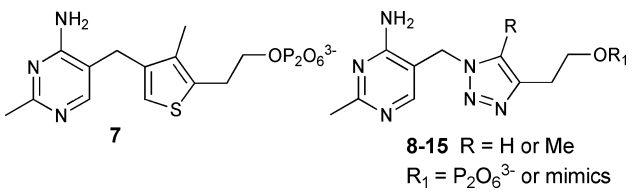


Fig. 1 DeazaTPP **7** and the triazole analogues of TPP.

All TPP-dependent enzymes are either dimers or tetramers (dimers of dimers), *e.g.* PDC is a homotetramer. The two active sites in each dimer are located at the interface of the two subunits. The TPP **2** is anchored by the pyrimidine moiety on one side and by the pyrophosphate group of TPP on the other side. The most

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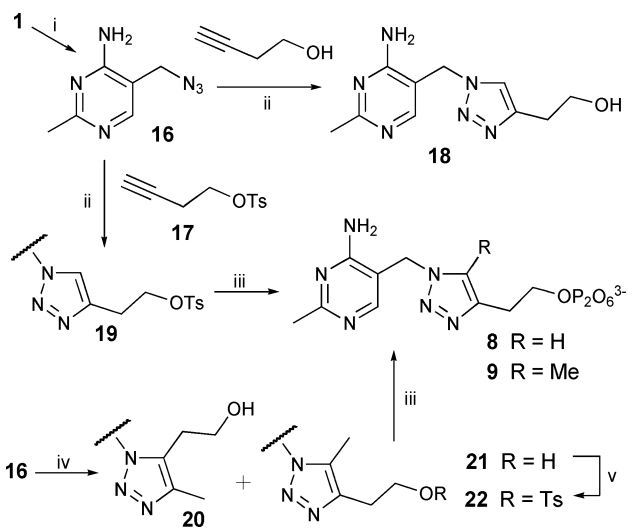
important binding interaction originates from the pyrophosphate which is coordinated to a bivalent metal ion (usually Mg^{2+}) in the active site.[1] However, the pyrophosphate is unsuitable for whole cell studies or pharmaceutical development, since it is highly charged and compounds possessing it will suffer from poor bioavailability and cellular uptake. We therefore wanted to explore whether the pyrophosphate group could be replaced by isosteres without losing too much binding affinity for the enzyme. The synthesis of deazaTPP **7** was quite difficult, however, involving 12 steps,[5,6] so in this study we also sought to use a TPP analogue which was easier to make.

In this paper we report the synthesis of eight novel triazole-based analogues of TPP, **8–15**, and inhibition studies with ZmPDC. Preliminary results have recently been reported in a communication[7] but here we report full details of the syntheses of all the TPP analogues and a more extensive evaluation of their interaction with ZmPDC.

Results and discussion

Synthesis of analogues

Synthesis of triazole pyrophosphates 8 and 9. Triazole analogues **8** and **9** were synthesised conveniently from thiamine **1** itself (Scheme 2). In the first step the thiazolium moiety of **1** was displaced by azide in a bisulfite-catalysed process[8] to give the azidomethylpyrimidine **16** in 83% yield. Cu(I)-catalysed 1,3-dipolar cycloaddition[9] of **16** with 1-butynol or its tosylate **17** then gave triazoles **18** and **19** respectively, in good to moderate yields (56–81%) after recrystallisation. 5-Methyltriazole **22** was synthesised by heating **16** with 3-pentynol at reflux in butanol for 3 days to afford the isomeric mixture of **20** and **21** (approx. 1 : 1) in 80% total yield. This mixture was partially separated by silica gel chromatography and then tosylated. The desired isomer

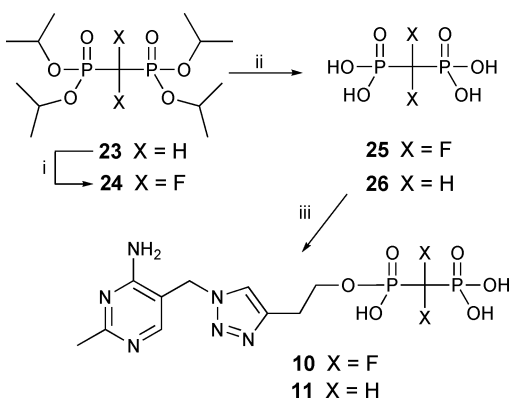


Scheme 2 Synthesis of pyrophosphates **8** and **9**. *Reagents and conditions*: (i) NaN_3 (2.5 eq.), Na_2SO_3 (0.1 eq.), H_2O, 60–65 °C, 6 h, 83%; (ii) sodium ascorbate (0.1 eq.), $CuSO_4 \cdot 5\ H_2O$ (0.01 eq.), *t*BuOH–H_2O (2 : 1), 25 °C, 16–24 h, 81% for **18** and 56% for **19**; (iii) $(Bu_4N)_3HP_2O_7$ (2.0 eq.), MeCN, −5 to 25 °C, 16 h, 50–60%; (iv) 3-pentynol (1.0 eq.), *n*BuOH, reflux at 130 °C, 96 h, 80% (isomeric mixture); (v) TsCl (2.5 eq.), pyr, −5 to 25 °C, 3 h, 34% (desired isomer).

22 was identified by its NOE between the triazole methyl group and the bridging CH_2 group in a NOESY spectrum. The mixture of isomeric tosylates was separated with difficulty to give a pure sample of **22** (>95% isomeric purity).

Each tosylate (**19** and **22**) was converted to its corresponding pyrophosphate (**8** and **9**) by nucleophilic displacement with tris(tetrabutylammonium) pyrophosphate.[5] Purification by anion exchange and then cation exchange chromatography gave products **8** and **9** in moderate yields (50–60%). A cation exchange resin was used to separate the product from the tosylate anion that was formed in the reaction and co-eluted on the anion exchange resin. Although successful in separating these two compounds, the strongly acidic resin did cause a small amount (<10%) of cleavage of the pyrophosphates, **8** and **9**, to their corresponding monophosphates.

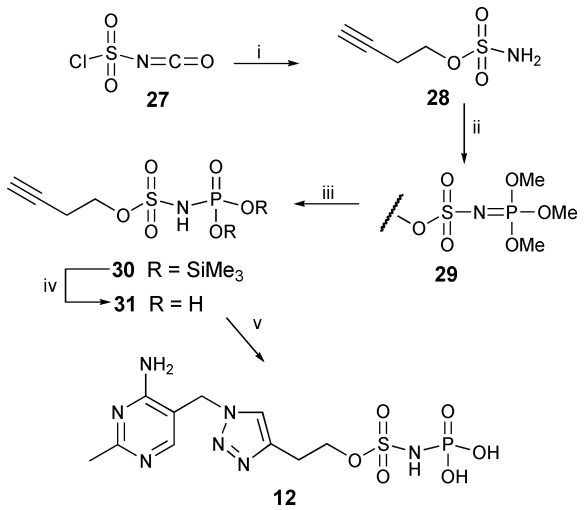
Synthesis of methylenediphosphonate esters 10 and 11. Difluoromethylene-diphosphonic acid **25** (Scheme 3) was synthesised by a published method[10] in which tetraisopropyl methylenediphosphonate **23** was treated alternately with 10 portions each of sodium hexamethyldisilazide (NaHMDS) and *N*-fluorobenzenesulfonimide (NFSi). Acid **25** was then prepared in approximately 90% yield by deprotection of the isopropyl groups using bromotrimethylsilane and subsequent treatment with methanol.[10] Both commercially available methylenediphosphonic acid **26** and its difluoro derivative **25** were treated with three equivalents of tetrabutylammonium hydroxide to form the corresponding trianions, which were then reacted with tosylate **19** to give pyrophosphate mimics **10** and **11**. The purification and yields were similar to those for pyrophosphates **8** and **9** described above.



Scheme 3 Synthesis of methylenediphosphonates **10** and **11**. *Reagents and conditions*: (i) sodium hexamethyldisilazide (NaHMDS) (3.0 eq.), *N*-fluorobenzenesulfonimide (NFSI) (3.3 eq.), THF, 25 °C to reflux, 20 min, 48%; (ii) Me_3SiBr (6.1 eq.), DCM, reflux (~33 °C), 24 h, 90%; (iii) $(Bu_4N)OH \cdot 30H_2O$ (6.0 eq.), then **19** (1.0 eq.), MeCN, 0 to 25 °C, 12 h, 58% for **10** and 66% for **11**.

Synthesis of *N*-(alkoxysulfonyl)phosphoramidic acid 12. Bonnac *et al.* have recently described a method to synthesise *N*-(alkoxysulfonyl)phosphoramidic acids as new mimics of pyrophosphate esters.[11] In their paper they report the cleavage of a number of trimethyl phosphorimidate derivatives (as in **29**) to give trimethylsilyl esters using bromotrimethylsilane and subsequent hydrolysis in water for seven days to give the corresponding phosphoramidic acids. In our synthesis of phosphoramidic acid **12**

(Scheme 4), sulfamoyl chloride was prepared from chlorosulfonyl isocyanate **27** and formic acid and then coupled *in situ* with 3-butynol to give the sulfamate **28** in 67% yield (over two steps).[12] Then reaction with di-*tert*-butyl azodicarboxylate and trimethyl phosphite afforded trimethyl phosphorimidate **29** in 80% yield after chromatography.



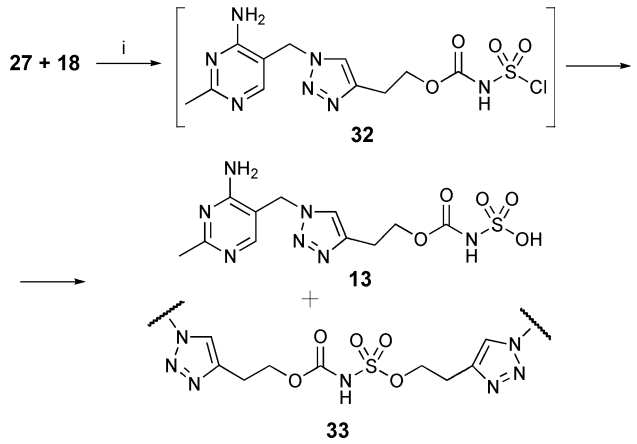
Scheme 4 Synthesis of *N*-(alkoxysulfonyl)phosphoramidic acid **12**. *Reagents and conditions*: (i) formic acid (1.0 eq.), then 3-butynol (0.67 eq.), pyridine (1.0 eq.), DCM, 0 to 25 °C, 17 h, 67%; (ii) $(MeO)_3P$ (2.0 eq.), di-*tert*-butyl azodicarboxylate (2.0 eq.), 25 °C, 2 h, 80%; (iii) Me_3SiBr (6.2 eq.), 25 °C, 3 h, 100%; (iv) MeOH, 25 °C, 5 min, 100%; (v) **16** (1.0 eq.), CuI (0.6 eq.), DMF, 25 °C, 24 h, 57%.

Following the published procedure,[11] **29** was treated with bromotrimethylsilane. However, rather than forming the tris(trimethylsilyl) ester, as described in the paper, the bis(trimethylsilyl) ester **30** was obtained quantitatively. Hydrolysis of **30** in water (pH 7, for seven days) was monitored *in situ* by ^{31}P NMR spectroscopy and peaks were observed at similar shifts to those reported by Bonnac *et al.* (*i.e.* −25.5, −10.5, −4.8 and +2.5 ppm, corresponding to the starting material, different hydrolysis intermediates and the final product respectively). However isolation of the organic product of this hydrolysis gave back the undesired sulfamate **28**, product of cleavage of the N–P bond. The conclusion that the ^{31}P NMR peak at +2.5 ppm must be due to inorganic phosphoric acid, rather than the phosphoramidic acid **31**, was confirmed by spiking the NMR sample with commercial phosphoric acid. We suggest that the same is probably true of the peak at +2.5 ppm observed by Bonnac *et al.*

Fortunately, it was found that bis(trimethylsilyl) ester **30** could be cleaved to give phosphoramidic acid **31** by treatment with methanol for 5 min at room temperature. The acid **31** gave a single peak at −5.4 ppm in its ^{31}P NMR spectrum. Our experience suggests that the four chemical shifts described by Bonnac *et al.* are more likely due to the bis(trimethylsilyl) ester, the mono(trimethylsilyl) ester, the phosphoramidic acid, and inorganic phosphate, respectively.

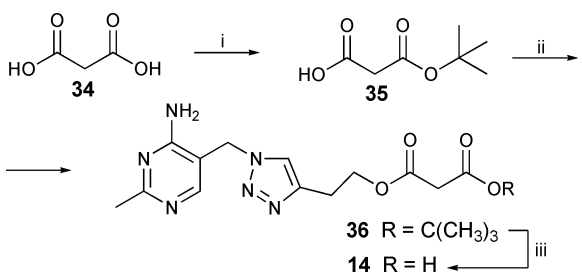
Application of 'click' chemistry – cycloaddition of **31** and azide **16** in DMF with Cu(I) as catalyst – gave the triazole **12** in 57% yield after anion exchange chromatography.

Synthesis of *N*-(alkoxycarbonyl)sulfamic acid 13. Analogue **13** was synthesised in a facile one-pot reaction in which alcohol **18** was added to a slight excess of chlorosulfonyl isocyanate **27** to form intermediate **32**, which was subsequently hydrolysed with water to give carbamate **13** (Scheme 5). The mixture was purified by ion exchange chromatography, which gave a 9 : 1 mixture of desired analogue **13** and double reaction product **33** in approximately 55% total yield. Attempts to avoid dimer formation, *e.g.* by adding more equivalents of isocyanate, or to improve the separation, *e.g.* by varying the eluant gradient, were unsuccessful. Nevertheless, the purity (by ^1H NMR spectroscopic analysis) was considered to be good enough for biological testing, especially as **33** was not expected to bind to the enzyme.



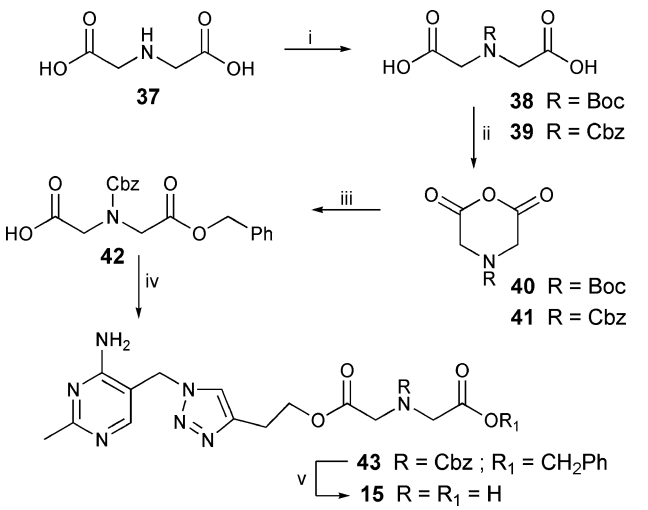
Scheme 5 Synthesis of *N*-(alkoxycabonyl)sulfamic acid **13**. *Reagents and conditions*: (i) pyridine, then H_2O, 0 to 25 °C, 2.5 h, 55% (9 : 1 molar ratio of **13** and **33**).

Synthesis of malonate ester 14. In our first approach, attempts were made to simply couple malonic acid **34** with the triazole alcohol **18** using DCC as a coupling reagent. Even though the desired product **14** was identified, the purification of the reaction mixture turned out to be very troublesome, since both starting alcohol **18** and malonate **14** are highly water-soluble and **14** is unstable on silica gel. Because of these difficulties, malonic acid **34** was first coupled with *tert*-butanol using DCC which gave mono-*tert*-butyl malonate **35** in 66% yield (Scheme 6).[13] Subsequent coupling of **35** with alcohol **18** afforded the *tert*-butyl-protected product **36** in 51% yield. Finally, deprotection of **36** using TFA gave the malonate analogue **14** in 80% yield.



Scheme 6 Synthesis of the malonate ester **14**. *Reagents and conditions*: (i) *t*BuOH (2.0 eq.), DCC (2.2 eq.), MeCN, 25 °C, 30 min, 66%; (ii) **18** (1.0 eq.), DCC (1.0 eq.), THF–pyridine, 25 °C, 18 h, 51%; (iii) neat TFA, 25 °C, 3 h, 80%.

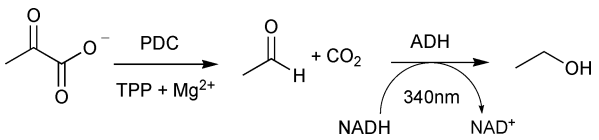
Synthesis of iminodiacetic acid monoester 15. In our first approach to monoester **15**, iminodiacetic **37** was Boc-protected to give **38** (Scheme 7) and then coupled with alcohol **18** *via* anhydride **40** using DCC.[14] Unfortunately, as in the synthesis of malonate **14**, it was extremely troublesome to purify the resulting mixture. Consequently, attempts were made to couple the anhydride **40** with *tert*-butanol instead to give the Boc-protected mono-*tert*-butyl ester before coupling with the triazole alcohol **18**. Unfortunately, both *tert*-butanol and potassium *tert*-butoxide were completely unreactive in this reaction. As a result, it was decided to use the less hindered benzyl alcohol instead and, to avoid the need for two separate deprotection steps later in the synthesis, the amine was first protected with a Cbz-group to give **39** in 71% yield.[15] In the next step, the solid anhydride **41** was first formed (not isolated) and then benzyl alcohol and dicyclohexylamine, as a base, were added. The use of this base was very convenient, since it not only deprotonated the alcohol but also formed a solid salt with the carboxylic acid **42**, which precipitated during the reaction.[16] The pure product, in its salt form, was collected and recrystallised and the base was then removed by extraction into aqueous acid, giving **42** as an oil in 74% yield. Acid **42** was coupled with the alcohol **18** using DCC and DMAP (no reaction occurred without DMAP) to give diester **43** in 70% yield. Final deprotection by hydrogenation over palladium-on-carbon gave monoester **15** in 76% yield.



Scheme 7 Synthesis of iminodiacetic acid monoester **15**. *Reagents and conditions*: (i) BnOCOCl (2.0 eq.), aq. NaOH (2 M), 25 °C, 2 h, 71%; (ii) DCC (1.0 eq.), THF, 25 °C, 12 h; (iii) BnOH (3.1 eq.), dicyclohexylamine (1.1 eq.), Et_2O, 0 to 25 °C, 12 h, 74%; (iv) **18** (1.0 eq.), DCC (1.1 eq.), DMAP (1.1 eq.), 25 °C, 24 h, 70%; (v) Pd/C (10%), H_2 (1 atm), 25 °C, 20 min, 76%.

Inhibition studies with pyruvate decarboxylase

Pyruvate decarboxylase (PDC) catalyses the conversion of pyruvate to acetaldehyde and carbon dioxide. Its activity is measured by a coupled enzyme assay using the NADH-dependent reduction of acetaldehyde by alcohol dehydrogenase (ADH) (Scheme 8).[4,5] 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,[4] we instead studied binding of the analogues to the apo-enzyme, prepared as described previously.[5]



Scheme 8 Coupled assay of PDC.

In the binding experiments, 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, TPP analogues that are only loosely bound may be displaced by the excess TPP and so could also show activity.

Using this assay, the eight TPP analogues (**8–15**) were incubated with apo-PDC and the decrease of activity was measured. All analogues except for **13**, **14** and **15** showed strong binding affinity under these conditions and completely inactivated the enzyme within 15–20 min. The iminodiacetic acid moiety of analogue **15** was intended to not only occupy the pyrophosphate binding site but also replace the Mg^{2+} ion, with the protonated amine binding in its place, and so for this analogue the binding was also performed in the absence of Mg^{2+}. However no inhibition was observed either in the presence or absence of Mg^{2+}.

In all cases of inhibition the data-points fitted poorly onto a simple exponential curve, the initial decrease of activity being too fast and the later decay too slow, but fitted reasonably well onto a double exponential curve of equation:

$$y = (1 - x)\exp(-k_1 t) + x\exp(-k_2 t).$$

The value of x was fixed at 0.35, which gave a good fit in all cases.

Fig. 2 shows the binding of analogue **9**. The faster of the two apparent first-order rate constants obtained from each curve was plotted against concentration to give a straight line, which gave the second order rate constant for the initial binding event, k_{on}. Similar curves were seen for the other analogues (*i.e.* **8** and **10–12**), and k_{on} values are presented in Table 1.

The apparent two-stage inhibition has been seen in previous studies,[5,17] and two possible explanations are (i) normal slow-binding inhibition, in which a more rapid initial binding is followed by a slower irreversible step, presumably a conformational change of the enzyme, or (ii) the two rates observed in our inhibition experiments are for binding of the first molecule of inhibitor to the enzyme dimer and for binding of the second molecule of inhibitor. Many TPP-dependent enzymes show so-called half-of-sites reactivity (see recently published review[18]), due to some form of communication between the two active sites of each dimer, so it is quite possible that the rate of binding of a TPP analogue to one active site is dependent on whether or not the other active site of the dimer is occupied. If this second explanation is correct, the rate constant for the second slower step would also be dependent on inhibitor concentration. Although the k_2 values did appear to increase with increasing inhibitor concentration, the errors in these values were much larger than in the k_1 values and, as a result, we were not able to show a linear dependence.

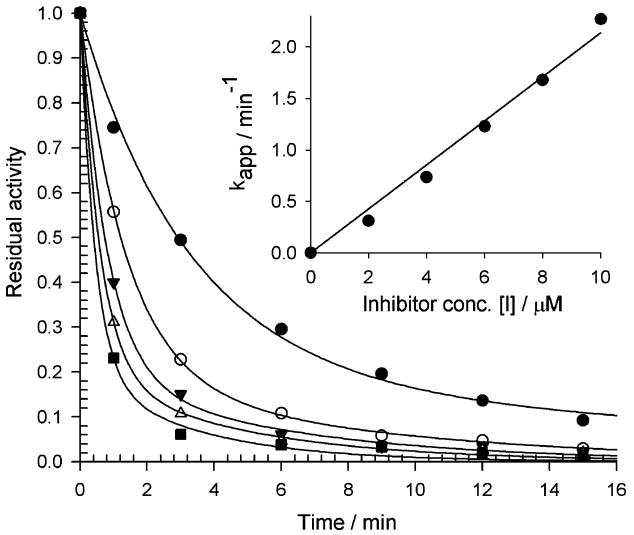


Fig. 2 Inactivation of ZmPDC by various concentrations of TPP analogue **9**, from top to bottom: ● 2 μM, ○ 4 μM, ▼ 6 μM, △ 8 μM, ■ 10 μM; the residual activity is given as a fraction of the initial activity of uninhibited enzyme. Inset: Apparent first-order rate constants plotted *versus* inhibitor concentrations; the slope of the graph gives k_{on}.

Table 1 Inhibition parameters for analogues **8–15** and **18**

Analogue	k_{on}/μM^{-1} min^{-1}	K_I
8	0.05 ± 0.02	30 ± 3 pM
9	0.21 ± 0.03	20 ± 2 pM
10	0.09 ± 0.01	0.95 ± 0.1 nM
11	0.17 ± 0.03	1.2 ± 0.1 nM
12	0.02 ± 0.004	0.14 ± 0.02 μM
13	n.o.[a]	8 ± 2 μM
14	n.o.[a]	0.4 ± 0.1 mM
15	n.o.[a]	0.4 ± 0.1 mM
18	n.o.[a]	0.3 ± 0.1 mM

[a] Not observable.

By adding a large excess of TPP (100 μM to 10 mM) to inactivated PDC (generally <1% activity) the reversiblity of the binding of each analogue was investigated. The recovery of activity was followed over a period of 7–8 days and in each case some activity was regained to reach a plateau within *ca.* 2 days (*e.g.* Fig. 3 depicts the reactivation of PDC pre-inhibited by analogue **9**). As full activity was retained in a control experiment with uninhibited enzyme, even after 7–8 days, the plateau of activity reached with inhibitor present can be regarded as due to establishment of the equilibrium between binding of TPP and binding of the inhibitor. Using the relative concentrations of TPP and inhibitor and the previously reported K_D value for TPP (0.35 μM),[4] the K_I values for the analogues were calculated and are listed in Table 1.

Not surprisingly, the two analogues possessing the important pyrophosphate group (**8** and **9**) were the most potent inhibitors: only 8.0% and 5.5% respectively of the original activity was recovered when enzyme inhibited with 10 μM of inhibitor was reactivated with 10 mM TPP. This corresponds to K_I values of 30 pM and 20 pM. The 5-methyl group of **9** slightly improves the strength of binding compared with **8**, as might be expected because TPP also possesses a methyl group in this position, but

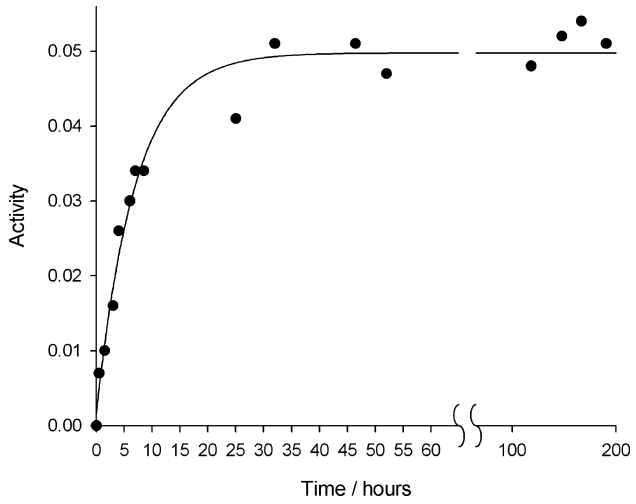


Fig. 3 Recovery of activity for ZmPDC fully inhibited by TPP analogue **9** (10 μM) and then incubated with TPP (10 mM). The activity is given as a fraction of the initial activity of uninhibited enzyme.

interestingly it also speeds up the rate of binding quite markedly, perhaps because of the greater hydrophobicity.

Previously we reported that we were unable to observe any reactivation of enzyme inhibited with deazaTPP **7**.[5] However, in this work, by using higher concentrations of TPP and longer time periods, we were able to observe some reactivation in a similar way as for **8** and **9** (3.5% activity in the presence of 10 μM of **7** and 10 mM of TPP). The K_I value obtained from this experiment was 13 (±3) pM.

The reason why analogues such as **7**, **8** and **9**, 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.[5,19] In this way the enzyme promotes both the formation of the ylide and the decarboxylation reaction.

Some of the analogues containing pyrophosphate mimics also show strong inhibitory activity. In **10** and **11**, 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 nucleoside analogues.[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 group.[20] 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 **8**, the K_I values for both these analogues (**10** and **11**) were estimated to be more than 10-fold higher. Methylenediphosphonate esters such as **11** are known to have higher pK_a values than pyrophosphates,[21] which might explain why **11** binds faster, being less hydrophilic, but less tightly than **8**. Difluoromethylene-diphosphonate esters such as **10**, however, have a similar pK_a to pyrophosphates.[22] Presumably the greater size of the CF_2 group compared to O reduces the binding affinity in this instance.

The assay described above is only useful for TPP analogues which bind very tightly. This is because analogues that bind weakly will be displaced by TPP when the enzyme activity assay is started (TPP is added in a large excess) and consequently,

any binding that might have occurred will not be seen. Hence, analogues **13–15** required a different type of experiment in order to measure their binding. In this second type of experiment, the same coupled assay shown in Scheme 8 was used, but instead of studying the binding of the TPP analogue to apo-PDC, the effect of analogues **13–15** on the binding of TPP to apo-PDC was studied. Accordingly, TPP **2** (4 μM) and analogue (**13–15**; various concentrations) were incubated with apo-PDC (*ca.* 0.4 μM active sites) and the activity was measured at timed intervals. As expected, the increase of activity followed a saturation-like curve and under these conditions the activity reached a maximum after *ca.* 45 min of incubation when no analogue was present (see Fig. 4). For comparison, alcohol **18** was also included in this study. In all cases the presence of analogue did not have much effect on the initial rate of activation but it did affect the maximum activity reached. By analogy with the reactivation experiment with analogues **8–12**, it was believed that this lower level of activity was due to an equilibrium between TPP binding and analogue binding. Hence the corresponding K_I values could be calculated. The K_I values listed in Table 1 for **13–15** and **18** are the average values obtained from all concentrations tested for each analogue.

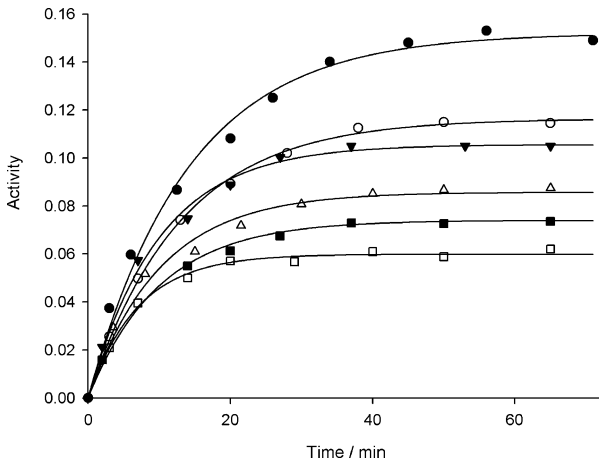


Fig. 4 Inhibition of binding of TPP (4 μM) to PDC by carbamate **13**, from top to bottom: ● no inhibitor, ○ 20 μM, ▼ 40 μM, △ 80 μM, ■ 100 μM, □ 160 μM; the activity is given in arbitrary units.

As the carbamate **13** had shown some activity in the first assay for strong inhibitors, it was not surprising that **13** was most potent among these weak binders. In fact, the K_I value of 8 μM for **13** is only about 23-fold greater than the K_D for TPP. Analogues **14** and **15**, however, showed almost the same affinity as alcohol **18**, and thus it is likely that their interaction with the active site comes mostly from the pyrimidine part. The iminodiacetic acid moiety of **15** is presumably too large to fit into the pyrophosphate pocket in the active site.

Conclusion

In conclusion, we report here the synthesis and biological evaluation of eight new triazole-based analogues of TPP. The analogues were prepared in relatively few steps using 'click' chemistry. The pyrophosphate esters **8** and **9** proved to be low picomolar inhibitors of PDC, almost as potent as deazaTPP **7**.

Using the most readily synthesised triazole **18**, we set out to find less charged and more stable mimics of the pyrophosphate group that would still be potent inhibitors. Six pyrophosphate analogues were synthesised and tested. The results provide a striking example of the great importance of the pyrophosphate group, with *ca.* 1.0×10^7-fold higher affinity of pyrophosphate **8** for the active site compared to its corresponding alcohol **18**. However, it was also shown that several of the analogues containing mimics of the pyrophosphate group, particularly the methylene-diphosphonates **10** and **11** (with K_I values just 30- to 40-fold greater than that of **8**), still bind with high affinity.

Marked decreases of inhibition are seen in going from trianionic analogues **8–11** to the dianionic phosphoramidic acid **12**, to the monoanionic carbamate **13** and malonate **14**. Hence, with a magnesium ion in the binding site, the more negatively charged analogues clearly bind tightest. With the iminodiacetic acid analogue **15** the intention was that the protonated amino group would replace the magnesium ion, but unfortunately this compound did not turn out to be an effective inhibitor at all.

This study is relevant not only to TPP-dependent enzymes but also a wide range of other enzymes and proteins that bind pyrophosphate esters (*e.g.* ADP and GDP).

Experimental

General synthesis methods

Proton NMR spectra were recorded on a Bruker AM/DPX 400 (400 MHz) or a Bruker DPX 500 (500 MHz) spectrometer. The chemical shifts (δ) and coupling constants (J) are given in ppm (downfield of TMS) and Hz, respectively. Carbon NMR spectra were recorded on either a Bruker AC/DPX 400 (100 MHz) or a Bruker DPX 500 (126 MHz) spectrometer. To assist in the assignments, the numbers of attached protons were determined using either APT (Attached Proton Test, J-resolved spin echo) or DEPT (Distortionless Enhancement by Polarisation Transfer) spectra. Phosphorus NMR spectra were recorded on a Bruker DPX 400 (162 MHz) with broadband proton decoupling. IR spectra were recorded on a Perkin-Elmer FTIR spectrometer and only significant bands are reported. Melting points were determined on a Reichert melting point apparatus. Mass spectra were run on a Bruker BioApex II 4.7e FTICR or Waters LCT Premier using electrospray ionisation (ESI). Analytical TLC was performed using commercial Merck glass plates, coated to thickness of 0.25 mm with Kieselgel 60 F_{254} silica and visualised under UV light or by staining with vanillin, ninhydrin or $KMnO_4$ dip solutions. Flash chromatography was performed using Merck Kieselgel 60 (230–400 mesh) silica under a slight positive pressure of air. Anion exchange was performed on a Pharmacia Acta Prime chromatography system using a column packed with DEAE-Sephacel and eluting with a gradient of 0 to 0.25 M aqueous ammonium bicarbonate. Solvents and reagents for anhydrous reactions were dried prior to use by conventional methods.[23]

5-Azidomethyl-2-methylpyrimidin-4-ylamine 16

To a solution of thiamine chloride (10.3 g, 30.6 mmol) and sodium azide (4.9 g, 75.4 mmol) in water (100 ml) was added sodium sulfite (0.38 g, 3.0 mmol) and the mixture was stirred at 65 °C for 5 h.

Citric acid (21.0 g, 100 mmol, to pH ≈ 4) was added and the aqueous solution was washed with dichloromethane. Potassium carbonate (to pH ≈ 8) was added, upon which some precipitation of the product occurred. The suspension was filtered and the filtrate was extracted with ethyl acetate and the combined organic layers were washed with brine, dried ($MgSO_4$) and evaporated under reduced pressure. The solid residue was pooled with the precipitate and recrystallised from ethyl acetate–hexane to give the *azide* **16** as fine needles (3.15 g, 63%), m.p. 150–153 °C [Found: C, 43.6; H, 4.9; N, 50.7; M + H^+ (+ESI), 165.0881 $C_6H_8N_6$ requires C, 43.9; H, 4.9; N, 51.2; M + H 165.0889]; ν_{max}/cm^{-1} 3300 and 3094 (NH_2), 2086 and 2107 (N_3), 1668, 1587 and 1561 (pyrimidine ring); δ_H (400 MHz, $CDCl_3$) 2.50 (3 H, s, CH_3), 4.19 (2 H, s, CH_2), 5.47 (2 H, broad, NH_2), 8.05 (1 H, s, C*H*); δ_C (100 MHz, DMSO-d_6) 24.5 (*C*H_3), 46.7 (*C*H_2), 106.9 (*C*CNH$_2$), 155.1 (*C*H), 161.1 and 166.2 (*C*N*C*NH$_2$).

But-3-ynyl toluene-4-sulfonate 17

To a stirred solution of 3-butynol (0.11 ml, 1.46 mmol) in anhydrous pyridine (15 ml) at-5 °C was added in portions *p*-toluenesulfonyl chloride (697 mg, 3.65 mmol). The reaction mixture was stirred at room temperature for 3 h, then quenched with hydrochloric acid (1 M) and extracted with ethyl acetate. The combined organic layers were washed with aqueous sodium bicarbonate, aqueous $CuSO_4$ and then with brine, dried ($MgSO_4$) and evaporated under reduced pressure to yield the tosylate **17**[24] as an oil (261 mg, 80%) [Found: M + Na^+ (+ESI), 247.0406 $C_{11}H_{12}O_3S$ requires M + Na, 247.0399]; ν_{max}/cm^{-1} 3287 (H–CC), 1357 and 1173 (SO_2O), 978 (aromatic C–H); δ_H (400 MHz, $CDCl_3$) 1.95 (1 H, t, *J* 2.7, C*H*), 2.42 (3 H, s, CH_3), 2.53 (2 H, dt, *J* 2.7 and 7.0, CH_2CH$_2$O), 4.08 (2 H, t, *J* 7.0, CH$_2$CH_2O), 7.33 (2 H, d, *J* 8.2, 2 × ArC*H*), 7.77 (2 H, d, *J* 8.2, 2 × ArC*H*); δ_C (100 MHz, $CDCl_3$) 17.9 (*C*H$_2$CH$_2$O), 20.1 (*C*H$_3$), 66.0 (CH$_2$*C*H$_2$O), 69.3 (C≡*C*H), 76.9 (C≡*C*–C), 126.5 and 128.4 (4 × Ar*C*H), 131.3 and 143.6 (2 × Ar*C*).

2-[1-(4-Amino-2-methylpyrimidin-5-ylmethyl)-1*H*-[1,2,3]triazol-4-yl]ethanol 18

To a stirred solution of 3-butynol (907 µl, 12.0 mmol) and aminopyrimidine azide **16** (1.968 g, 12.0 mmol) in *tert*-butanol/water (12 ml; 2 : 1) were added sodium ascorbate (238 mg, 1.2 mmol) and $CuSO_4 \cdot 5H_2O$ (30 mg, 0.12 mmol). The reaction mixture was stirred at room temperature for 16 h. The crude mixture was evaporated under reduced pressure and the solid residue was dissolved in 1-butanol. The organic layer was washed with aqueous potassium carbonate (0.1 M) and then with brine, dried ($MgSO_4$) and evaporated under reduced pressure to give a white solid. Recrystallisation from 2-propanol/hexane gave the *triazole* **18** as fine needles (2.27 g, 81%), m.p. 164–165 °C [Found: M + H^+ (+ESI), 235.1305 $C_{10}H_{14}N_6O$ requires M + H, 235.1307]; ν_{max}/cm^{-1} 3500–3000 (broad OH), 3340 and 3140 (NH_2), 1657 and 1566 (pyrimidine ring) and 1043 (CO); δ_H (400 MHz, DMSO-d_6) 2.28 (3 H, s, CH_3), 2.74 (2 H, t, *J* 6.8, CH_2CH$_2$O), 3.58 (2 H, t, *J* 6.8, CH$_2$CH_2O), 4.65 (1 H, s, O*H*), 5.35 (2 H, s, CH_2 bridge), 6.85 (2 H, s, NH_2), 7.83 (1 H, s, triazole C*H*), 7.93 (1 H, s, pyrimidineC*H*); δ_C (100 MHz, DMSO-d_6) 26.0 (*C*H$_3$), 29.9 (*C*H$_2$CH$_2$O), 47.3 (*C*H$_2$ bridge), 61.0 (CH$_2$*C*H$_2$O), 109.3 (*C*CNH$_2$), 123.3 (triazole *C*H), 145.4 (triazole *C*), 158.8 (*C*H), 162.2 and 167.6 (*C*N*C*NH$_2$).

2-[1-(4-Amino-2-methylpyrimidin-5-ylmethyl)-1*H*-[1,2,3]triazol-4-yl]ethyl toluene-4-sulfonate 19

To a stirred solution of tosylate **17** (297 mg, 1.32 mmol) and azide **16** (217 mg, 1.32 mmol) in *tert*-butanol–water (6 ml, 2 : 1) were added sodium ascorbate (26 mg, 0.13 mmol) and $CuSO_4 \cdot 5H_2O$ (3 mg, 0.12 mmol). The reaction mixture was stirred at room temperature for 16 h and then evaporated under reduced pressure. The oily residue was dissolved in 1-butanol and washed with aqueous potassium carbonate (0.1 M) and then with brine, dried ($MgSO_4$) and evaporated under reduced pressure to give a white solid. Recrystallisation from 2-propanol–petroleum ether (b.p. 60–80 °C) gave the *triazole* **19** as fine yellow crystals (289 mg, 56%), m.p. 85–88 °C [Found: M + H^+ (+ESI), 389.1400 $C_{17}H_{20}N_6O_3S$ requires M + H, 389.1396]; ν_{max}/cm^{-1} 3335 and 3130 (NH_2), 1661 and 1568 (pyrimidine ring), 1348 and 1174 (SO_2O), 905 (aromatic C–H); δ_H (400 MHz, DMSO-d_6) 2.28 (3 H, s, CH_3), 2.38 (3 H, s, CH_3), 2.92 (2 H, t, *J* 6.4, CH_2CH$_2$O), 4.18 (2 H, t, *J* 6.4, CH$_2$CH_2O), 5.35 (2 H, s, CH_2 bridge), 6.86 (2 H, br s, NH_2), 7.40 (2 H, d, *J* 8.1, 2 × Ar-*H*), 7.67 (2 H, d, *J* 8.1, 2 × Ar-*H*) 7.83 (1 H, s, triazole C*H*), 7.93 (1 H, s, pyrimidineC*H*); δ_C (126 MHz, DMSO-d_6) 21.2 (CH$_3$), 25.3 (*C*H$_3$), 25.6 (*C*H$_2$CH$_2$O), 46.6 (*C*H$_2$ bridge), 69.6 (CH$_2$*C*H$_2$O), 108.5 (*C*CNH$_2$), 123.0 (triazole *C*H), 127.6 and 130.2 (4 × Ar*C*H), 132.2 and 142.2 (2 × Ar*C*), 145.0 (triazole *C*), 156.0 (pyrimidine *C*H), 161.6 and 166.9 (*C*N*C*NH$_2$).

2-[1-(4-Amino-2-methylpyrimidin-5-ylmethyl)-5-methyl-1*H*-[1,2,3]triazol-4-yl]ethanol 21 and 2-[1-(4-amino-2-methyl-pyrimidin-5-ylmethyl)-4-methyl-1*H*-[1,2,3]triazol-5-yl]ethanol 20

A solution of azide **16** (328 mg, 2.0 mmol) and 3-pentynol (185 µl, 2.0 mmol) in 1-butanol (2 ml) was heated at reflux (120 °C) for 72 h. The crude mixture was concentrated under reduced pressure and purified by silica gel chromatography eluting with DCM–MeOH (3 : 1) to give an approximately 1 : 1 mixture of **20** and **21** (399 mg, 80%) as a light yellow solid. The two regioisomers were partially separated by further chromatography (as above) to give a mixture of **20** and **21** (1 : 3; 250 mg) [Found: M + H^+ (+ESI), 249.1460 $C_{11}H_{16}N_6O$ requires M + H, 249.1464]; ν_{max}/cm^{-1} 3000–3450 (broad), 3395 and 3112 (NH_2), 1649, 1595 and 1559 (pyrimidine ring) and 1045 (CO); δ_H for **21** (500 MHz, DMSO-d_6) 2.19 (3 H, s, CH_3), 2.29 (3 H, s, CH_3), 2.68 (2 H, t, *J* 7.0, CH_2CH$_2$OH), 3.57 (2 H, q, *J* 5.2 and 7.0, CH$_2$CH_2OH) 4.69 (1 H, t, *J* 5.2, CH$_2$CH$_2$O*H*), 5.31 (2 H, s, CH_2 bridge), 6.82 (2 H, broad, NH_2), 7.73 (1 H, s, pyrimidine C*H*); δ_C for **21** (126 MHz, DMSO-d_6) 10.6 (triazole *C*H$_3$), 25.6 (*C*H$_3$), 29.0 (*C*H$_2$CH$_2$O), 45.3 (*C*H$_2$ bridge), 61.0 (CH$_2$*C*H$_2$O), 108.5 (*C*CNH$_2$), 131.5 and 142.5 (triazole *C*), 155.2 (pyrimidine *C*H), 161.9 and 167.0 (*C*N*C*NH$_2$); δ_H for **20** (500 MHz, DMSO-d_6) 2.17 (3 H, s, CH_3), 2.29 (3 H, s, CH_3), 2.79 (2 H, t, *J* 6.4, CH_2CH$_2$OH), 3.48 (2 H, q, *J* 6.4, CH$_2$CH_2OH), 5.0 (1 H, b, CH$_2$CH$_2$O*H*), 5.34 (2 H, s, CH_2 bridge), 6.82 (2 H, br s, NH_2), 7.73 (1 H, s, pyrimidine C*H*); δ_C for **20** (126 MHz, DMSO-d_6) 18.6 (*C*H$_3$), 25.6 (*C*H$_3$), 26.0 (*C*H$_2$CH$_2$O), 45.6 (*C*H$_2$ bridge) 59.8 (CH$_2$*C*H$_2$O),108.8 (*C*CNH$_2$),

132.5 and 141.0 (triazole *C*), 155.2 (pyrimidine *C*H), 161.8 and 166.9 (*C*N*C*NH$_2$).

2-[1-(4-Amino-2-methylpyrimidin-5-ylmethyl)-5-methyl-1H-[1,2,3]triazol-4-yl]ethyl toluene-4-sulfonate 22

To a stirred solution of the mixture of alcohols **20** and **21** (230 mg, 0.93 mmol, 1 : 3 mixture) in anhydrous pyridine (10 ml) at 0 °C was added in portions *p*-toluenesulfonyl chloride (886 mg, 4.64 mmol). The reaction mixture was stirred at room temperature for 3 h, then quenched with hydrochloric acid (0.1 M) and extracted with ethyl acetate. The combined organic layers were washed with brine, dried ($MgSO_4$) and evaporated under reduced pressure to yield a mixture (340 mg, 91%) of the two tosylates. This mixture was partially separated by silica gel chromatography eluting with DCM–MeOH (9 : 1) to give *tosylate* **22** (>95% isomeric purity) as an oil (95 mg, 34%). [Found: M + H$^+$ (+ESI), 403.1544 $C_{18}H_{22}N_6O_3S$ requires M + H, 403.1547]; ν_{max}/cm^{-1} 3361 and 3139 (NH_2), 1669 and 1571 (pyrimidine ring) 1358 and 1185 (SO_2O), 902 (aromatic C–H); δ_H (400 MHz, $CDCl_3$) 2.15 (3 H, s, CH_3), 2.37 (3 H, s, CH_3), 2.42 (3 H, s, CH_3), 2.90 (2 H, t, *J* 6.7, CH_2CH$_2$O), 4.19 (2 H, t, *J* 6.7, CH$_2$CH_2O), 5.15 (2 H, s, CH_2 bridge), 5.70 (2 H, br s, NH_2), 7.23 (2 H, d, *J* 8, 2 × Ar-*H*), 7.63 (2 H, d, *J* 8, 2 × Ar-*H*), 8.08 (1 H, s, pyrimidine C*H*); δ_C (100 MHz, $CDCl_3$) 6.7 (triazole *C*H$_3$), 21.2 (*C*H$_3$), 25.5 (*C*H$_3$), 27.1 (*C*H$_2$CH$_2$O), 46.9 (*C*H$_2$ bridge), 69.2 (CH$_2$*C*H$_2$O), 107.9 (*C*CNH$_2$), 127.2 and 130.6 (4 × Ar*C*H), 131.2 (triazole *C*) 132.8 and 142.7 (2 × Ar*C*), 145.4 (triazole *C*), 155.6 (pyrimidine *C*H), 161.7 and 167.1 (*C*N*C*NH$_2$).

2-[1-(4-Amino-2-methylpyrimidin-5-ylmethyl)-1*H*-[1,2,3]triazol-4-yl]ethyl pyrophosphate 8

To a stirred solution of tosylate **19** (20 mg, 0.05 mmol) in anhydrous acetonitrile (150 µl) at −10 °C under an atmosphere of argon was added in portions tris(tetra-*n*-butylammonium) hydrogen pyrophosphate (93 mg, 0.10 mmol). The reaction mixture was allowed to warm to room temperature, stirred for 16 h under argon, then diluted with water (1 ml) and purified first by anion-exchange chromatography and then by absorption onto Dowex 50 H$^+$-form and elution with aqueous NH_3 (1 M). Lyophilisation gave the *pyrophosphate* **8** as a white ammonium salt (12 mg, 60%), m.p. 209–211 °C [Found: M + H$^+$ (+ESI), 395.0645 $C_{10}H_{16}N_6O_7$ requires M + H, 395.0628]; ν_{max}/cm^{-1} 3130 and 3048 (broad NH_2), 1654 and 1547 (pyrimidine ring), 1194 (P=O), 1057 (P–OR), 905 (P–O–P); δ_H (400 MHz, D_2O) 2.31 (3 H, s, CH_3), 2.94 (2 H, t, *J* 6.6, CH_2CH$_2$O), 4.02 (2 H, dt, *J* 6.7 and *J* 6.6, CH$_2$CH_2OP), 5.37 (2 H, s, CH_2 bridge), 7.85 (1 H, s, triazole C*H*), 7.97 (1 H, s, pyrimidine C*H*); δ_C (126 MHz, D_2O) 23.5 (*C*H$_3$), 26.3 (*C*H$_2$CH$_2$O, d, *J* 7.6), 47.5 (*C*H$_2$ bridge), 64.3 (CH$_2$*C*H$_2$O, d, *J* 5.5), 108.4 (*C*CNH$_2$), 124.2 (triazole *C*H), 145.3 (triazole *C*), 154.6 (pyrimidine *C*H), 161.8 and 167.3 (*C*N*C*NH$_2$); δ_P (162 MHz, D_2O) −6.54 and −9.95 (each 1 P, br s, O*P*O*P*O).

2-[1-(4-Amino-2-methylpyrimidin-5-ylmethyl)-5-methyl-1*H*-[1,2,3]triazol-4-yl]ethyl pyrophosphate 9

To a stirred solution of tosylate **22** (60 mg, 0.15 mmol) in acetonitrile (anhydrous, 350 µl) at −10 °C under argon was added in portions tris(tetra-*n*-butylammonium) hydrogen pyrophosphate (271 mg, 0.30 mmol). The reaction mixture was allowed to warm to room temperature, stirred for 16 h under an atmosphere of argon, then diluted with water (1 ml) and purified first by anion-exchange chromatography and then by absorption onto Dowex 50 H$^+$-form and elution with aqueous NH_3 (1 M). Lyophilisation gave the *pyrophosphate* **9** as a white ammonium salt (30 mg, 49%). [Found: M + H$^+$ (+ESI), 409.0794 $C_{10}H_{16}N_6O_7$ requires M + H, 409.0785]; δ_H (400 MHz, D_2O) 2.22 (3 H, s, CH_3), 2.34 (3 H, s, CH_3), 2.92 (2 H, t, *J* 6.5, CH_2CH$_2$O), 4.03 (2 H, dt, *J* 6.9 and *J* 6.5, CH$_2$CH_2OP), 5.30 (2H, s, CH_2 bridge), 7.78 (1H, s, pyrimidine C*H*); δ_C (100 MHz, D_2O) 6.5 (triazole-*C*H$_3$), 21.5 (pyrimidine-*C*H$_3$), 25.1 (*C*H$_2$CH$_2$O, d, *J* 7.6), 44.5 (*C*H$_2$-bridge), 64.4 (CH$_2$*C*H$_2$O, d, *J* 5.7), 108.5 (*C*CNH$_2$), 133.0 and 142.0 (triazole *C*), 146.9 (pyrimidine *C*H), and 161.6 and 164.1 (*C*N*C*NH$_2$); δ_P (162 MHz, D_2O) −8.8 (1P, d, *J* 20.4) and −9.7 (1P, d, *J* 20.4).

Tetraisopropyl difluoromethylenediphosphonate 24[10]

To stirred neat tetraisopropyl methylenediphosphonate **23** (359 µl, 1.12 mmol) at room temperature were added, alternately in 10 portions each, a solution of sodium hexamethyldisilazide (NaHMDS) in THF (1.67 M; 2 ml, 3.35 mmol) and a solution of *N*-fluorobenzenesulfonimide (NFSi) (1.16 g, 3.68 mmol) in THF (2 ml). The sequence of additions was started with NaHMDS, and as the reaction was exothermic the temperature rose to near reflux after each addition. After the additions, the reaction mixture was allowed to cool to room temperature, then cooled to −78 °C, quenched with saturated aqueous ammonium chloride and diluted with diethyl ether. The mixture was warmed to room temperature, the organic layer was separated and the aqueous layer was extracted with diethyl ether. The combined organic layers were washed with 5% aqueous citric acid, then saturated aqueous sodium bicarbonate, and then brine, dried ($MgSO_4$) and concentrated under reduced pressure. The residual syrup was purified by silica gel chromatography eluting with diethyl ether to give difluoromethylenediphosphonate **24** as an oil (203 mg, 48%) δ_H (400 MHz, $CDCl_3$) 1.38 (12 H, d, *J* 4.5, 4 × CH_3), 1.40 (12 H, d, *J* 4.5, 4 × CH_3), 4.91 (4 H, m, 4 × C*H*); δ_F (376 MHz, $CDCl_3$) −122.3 (2 F, t, *J* 87.1); all analytical data are consistent with those previously reported.[10]

({2-[1-(4-Amino-2-methylpyrimidin-5-ylmethyl)-1*H*-[1,2,3]triazol-4-yl]ethoxy}hydroxyphosphoryldifluoromethyl)phosphonic acid 10

To a stirred solution of **24** (66 mg, 0.17 mmol) in anhydrous CH_2Cl_2 (2 ml) under an atmosphere of argon was added dropwise bromotrimethylsilane (137 µl, 1.04 mmol). The reaction mixture was stirred and heated at gentle reflux (~33 °C) for 24 h and then evaporated under reduced pressure. CH_2Cl_2 (5 ml) was twice added and evaporated again. The residual oil was cooled on ice and methanol (5 ml) was added and then evaporated under reduced pressure. Methanol (5 ml) was twice more added and evaporated again. The residual oil was dissolved in water (8 ml) and the aqueous solution was washed with ethyl acetate. Lyophilisation gave acid **25** as a thick oil (33 mg, 90%); ^{1}H NMR spectroscopy showed no peaks, consistent with the desired product. The product was used in the next step without further purification.

A solution of tetrabutylammonium hydroxide·30H$_2$O (376 mg, 0.47 mmol) and difluoromethylenediphosphonic acid **25** (33 mg,

0.154 mmol) in water (5 ml) was stirred at room temperature for 15 min and then lyophilised to give the tris(tetrabutylammonium) salt. The salt was dissolved in anhydrous acetonitrile (300 µl), and tosylate **19** (31 mg, 0.08 mmol) was added at 0 °C under an atmosphere of argon. The reaction mixture was allowed to warm to room temperature and stirred for 12 h. The mixture was evaporated under reduced pressure, dissolved in water (1 ml) and purified by anion-exchange chromatography. Lyophilisation gave a mixture of **10** and tosylate anion. This mixture was dissolved in water (0.5 ml), separated by absorption onto Dowex 50 H^+-form and elution with aqueous NH_3 (1 M), and then lyophilised to give *phosphonic acid* **10** as a white ammonium salt (20 mg, 58%) [Found: M + H^+ (+ESI), 429.0645; $C_{11}H_{18}N_6O_6P_2$ requires M + H, 429.0647]; δ_H (400 MHz, D_2O) 2.34 (3 H, s, CH_3), 2.96 (2 H, t, *J* 6.5, CH_2CH$_2$O), 4.14 (2 H, dt, *J* 7 and 6.5, CH$_2$CH_2OP), 5.40 (2 H, s, CH_2 bridge), 7.90 (1 H, s, triazole C*H*), 8.02 (1 H, s, pyrimidine C*H*); δ_C (126 MHz, D_2O) 22.8 (*C*H$_3$), 27.5 (*C*H$_2$CH$_2$O, d, *J* 6.0), 49.8 (*C*H$_2$-bridge), 66.1 (CH$_2$*C*H$_2$O, d, *J* 5.3), 110.1 (*C*CNH$_2$), 128.4 (triazole *C*H), 141.3 (triazole *C*), 145.2 (pyrimidine *C*H), 164.7 and 165.2 (*C*N*C*NH$_2$); δ_P (162 MHz, D_2O) 5.0 (1 P, dt, *J* 51.5 and *J* 73.0) and 7.9 (1 P, dt, *J* 51.5 and *J* 87.0); δ_F (376 MHz, D_2O) −117.3 (2 F, dd, *J* 87.0 and 73.0).

({2-[1-(4-Amino-2-methylpyrimidin-5-ylmethyl)-1*H*-[1,2,3]triazol-4-yl]ethoxy}hydroxyphosphorylmethyl) phosphonic acid 11

A solution of tetrabutylammonium hydroxide·30H_2O (370 mg, 0.463 mmol) and methylenediphosphonic acid **26** (27 mg, 0.154 mmol) in anhydrous acetonitrile (5 ml) was stirred at room temperature for 1 h. The mixture was concentrated under high vacuum to give the tris(tetrabutylammonium) salt. The salt was dissolved in anhydrous acetonitrile (300 µl) and tosylate **19** (30 mg, 0.077 mmol) was added at 0 °C under an atmosphere of argon. The reaction mixture was allowed to warm to room temperature and stirred for 12 h. The mixture was evaporated under reduced pressure, dissolved in water (1 ml) and purified by anion-exchange chromatography and then by absorption onto Dowex 50 H^+-form and elution with aqueous NH_3 (1 M) to give *phosphonic acid* **11** as a white ammonium salt (20 mg, 66%), m.p. 160–162 °C; [Found: M − H^+ (−ESI), 391.0685; $C_{11}H_{18}N_6O_6P_2$ requires M − H, 391.0685]; ν_{max}/cm^{-1} 3044 (broad NH_2), 1652, 1601 and 1563 (pyrimidine ring), 1167 (P=O), 1020 (P–OR); δ_H (400 MHz, D_2O) 2.00 (2 H, t, *J* 19.8, PCH_2P), 2.36 (3 H, s, CH_3), 2.95 (2 H, t, *J* 6.4, CH_2CH$_2$O), 4.00 (2 H, dt, *J* 6.5 and *J* 6.7, CH$_2$CH_2OP), 5.41 (2 H, s, CH_2 bridge), 7.88 (1 H, s, triazole C*H*), 8.01 (1 H, s, pyrimidine C*H*); δ_C (126 MHz, D_2O) 23.4 (*C*H$_3$), 26.6 (*C*H$_2$CH$_2$O, d, *J* 6.8), 27.3 (P*C*H$_2$P, dd, *J* 123 and 125), 47.4 (*C*H$_2$ bridge), 63.1 (CH$_2$*C*H$_2$O, d, *J* 5.5), 108.5 (*C*CNH$_2$), 124.1 (triazole *C*H), 145.3 (triazole *C*), 153.9 (pyrimidine *C*H), 161.8 and 167.0 (*C*N*C*NH$_2$); δ_P (162 MHz, D_2O) 14.8 (1 P, d, *J* 9.3) and 18.4 (1 P, d, *J* 9.3).

O-But-3-ynyl sulfamate 28

Formic acid (180 µl, 4.6 mmol) was added dropwise to neat chlorosulfonyl isocyanate **27** (400 µl, 4.6 mmol) at 0 °C, whereupon vigorous gas evolution was observed. The resulting viscous suspension was stirred at 0 °C for 5 min. Dichloromethane (5 ml) was added and the solution was stirred at 0 °C for 1 h and then at 25 °C for 4 h. To the stirred solution was added slowly a mixture of 3-butynol (227 µl, 3.0 mmol) and pyridine (371 µl, 4.6 mmol) at 0 °C. The reaction mixture was allowed to warm to room temperature, stirred for 12 h and then was evaporated under reduced pressure. The residue was dissolved in ethyl acetate, washed with hydrochloric acid (1 M) and then with brine, dried ($MgSO_4$) and concentrated under reduced pressure to give the sulfamate **28** as an oil (300 mg, 67% over two steps) [Found: M + Na^+ (+ESI), 172.0042; $C_4H_7NO_3S$ requires M + Na, 172.0039]; ν_{max}/cm^{-1} 3287 (H–C≡C), 1357 and 1173 (SO_2O); δ_H (400 MHz, $CDCl_3$) 2.06 (1 H, t, *J* 2.7, C≡C*H*), 2.66 (2 H, dt, *J* 2.7 and 6.8, CH_2CH$_2$O), 4.29 (2 H, t, *J* 6.8, CH$_2$CH_2O), 4.80 (2 H, s, NH_2); δ_C (100 MHz, $CDCl_3$) 19.5 (*C*H$_2$CH$_2$O), 68.5 (CH$_2$*C*H$_2$O), 71.1 (C≡*C*H), 79.0 (*C*≡CH); all analytical data are consistent with those previously reported.[25]

N-(But-3-ynyloxysulfonyl)trimethoxyphosphazene 29

To a stirred solution of sulfamate **28** (300 mg, 2.01 mmol) and trimethyl phosphite (475 µl, 4.03 mmol) in anhydrous THF (2 ml) was added in portions di-*tert*-butyl azodicarboxylate (928 mg, 4.03 mmol). The reaction mixture was stirred at room temperature for 2 h and then concentrated under reduced pressure. The residue was purified by silica gel chromatography eluting with dichloromethane–methanol (98 : 2) to give the *phosphazene* **29** as an oil (436 mg, 80%). [Found: M + H^+ (+ESI), 272.0362; $C_7H_{14}NO_6PS$ requires M + H, 272.0358]; ν_{max}/cm^{-1} 3287 (H–C≡C), 1379 and 1183 (SO_2O), 1033 (P–O–C); δ_H (400 MHz, $CDCl_3$) 1.99 (1 H, t, *J* 2.7, C≡C*H*), 2.64 (2 H, dt, *J* 2.7 and 7.1, CH_2CH$_2$O), 3.93 (9 H, d, *J* 11.9, 3 × POCH_3), 4.24 (2 H, t, *J* 7.1, CH$_2$CH_2O); δ_C (100 MHz, $CDCl_3$) 18.0 (*C*H$_2$CH$_2$O), 55.0 (PO*C*H$_3$, d, *J* 6.0), 69.7 (CH$_2$*C*H$_2$O), 71.3 (C≡*C*H), 77.2 (*C*≡CH); δ_P (162 MHz, $CDCl_3$) 3.7 (s).

O,*O*-Bis(trimethylsilyl) *N*-(but-3-ynyloxysulfonyl)phosphoramidate 30

Bromotrimethylsilane (950 µl, 7.2 mmol) was added to **29** (314 mg, 1.16 mmol) at room temperature with stirring under an argon atmosphere. The reaction mixture was stirred at room temperature for 3 h and then concentrated under reduced pressure to give the *phosphoramidate ester* **30** as a thick oil (432 mg, 100%). ν_{max}/cm^{-1} 3287 (H–C≡C), 1358 and 1173 (SO_2O); δ_H (400 MHz, $CDCl_3$) 0.35 (18 H, s, 2 × OSiMe_3), 2.03 (1 H, t, *J* 2.7, C≡C*H*), 2.65 (2 H, dt, *J* 2.7 and *J* 7.1, CH_2CH$_2$O), 4.30 (2 H, t, *J* 7.1, CH$_2$CH_2O); δ_C (100 MHz, $CDCl_3$) 1.4 (SiMe_3), 18.9 (*C*H$_2$CH$_2$O), 67.9 (CH$_2$*C*H$_2$O), 70.4 (C≡*C*H), 78.4 (*C*≡CH); δ_P (162 MHz, $CDCl_3$) −23.5 (s).

N-(But-3-ynyloxysulfonyl)phosphoramidic acid 31

A mixture of anhydrous methanol (1 ml) and phosphoramidate ester **30** (163 mg, 0.44 mmol) was stirred at room temperature for 5 min and then evaporated under reduced pressure to give the *acid* **31** as an oil (101 mg, 100%) [Found: M − H^+ (−ESI), 227.9737 $C_6H_8NO_6PS$ requires M − H, 227.9732]; ν_{max}/cm^{-1} 3290 (H–C≡C), 2587 (PO–H), 1358 and 1173 (SO_2O); δ_H (400 MHz, MeOD) 2.35 (1 H, t, *J* 2.7, C≡C*H*), 2.64 (2 H, dt, *J* 2.7 and *J* 6.9, CH_2CH$_2$O), 4.28 (2H, t, *J* 6.9, CH$_2$CH_2O); δ_C (100 MHz, CD_3OD) 20.2 (*C*H$_2$CH$_2$O), 68.7 (CH$_2$*C*H$_2$O), 71.8 (C≡*C*H), 80.5 (*C*≡CH); δ_P (162 MHz, $CDCl_3$) −5.4 (s).

N-({2-[1-(4-Amino-2-methylpyrimidin-5-ylmethyl)-1*H*-[1,2,3]triazol-4-yl]ethoxy}sulfonyl)phosphoramidic acid 12

To a stirred solution of phoshoramidic acid **31** (46 mg, 0.20 mmol) and azide **16** (33 mg, 0.20 mmol) in anhydrous DMF (0.5 ml) was added CuI (6 mg, 0.12 mmol). The reaction mixture was stirred at room temperature for 24 h and then evaporated under reduced pressure, dissolved in water (1 ml), filtered and purified by anion-exchange chromatography. Lyophilisation gave the *triazole* **12** as a white ammonium salt (45 mg, 57%) m.p. 138–140 °C [Found: M + H^+ (+ESI), 394.0702 $C_{10}H_{16}N_7O_6PS$ requires M + H, 394.0699]; ν_{max}/cm^{-1} 3055 (NH_2), 1651 and 1566 (pyrimidine ring), 1347 and 1170 (SO_2O); δ_H (400 MHz, D_2O) 2.32 (3 H, s, CH_3), 3.04 (2 H, t, *J* 6.2, CH_2CH$_2$O), 4.28 (2 H, t, *J* 6.2, CH$_2$CH_2O), 5.38 (2 H, s, CH_2 bridge), 7.85 (1 H, s, triazole C*H*), 7.98 (1 H, s, pyrimidine C*H*); δ_C (126 MHz, D_2O) 23.3 (*C*H$_3$), 24.4 (*C*H$_2$CH$_2$O), 47.1 (*C*H$_2$ bridge), 67.7 (CH$_2$*C*H$_2$O), 107.9 (*C*CNH$_2$), 123.7 (triazole *C*H), 144.1 (triazole *C*), 155.0 (pyrimidine *C*H), 161.2 and 167.3 (*C*N*C*NH$_2$); δ_P (162 MHz, D_2O) −3.5 (s).

***O*-2-[1-(4-Amino-2-methylpyrimidin-5-ylmethyl)-1*H*-[1,2,3]triazol-4-yl]ethyl sulfamate 13**

To a solution of chlorosulfonyl isocyanate **27** (32.7 μl, 0.38 mmol) in anhydrous dioxane (4 ml) at 0 °C was added dropwise a solution of alcohol **18** (58 mg, 0.25 mmol) in anhydrous pyridine (1 ml). The reaction mixture was stirred at 0 °C for 30 min. Water (1 ml) was added slowly at 0 °C and the mixture was stirred at room temperature for 2 h and then evaporated under reduced pressure. The residue was diluted with water (to 1 ml) and purified by anion-exchange chromatography. Lyophilisation gave a mixture of the *carbamate* **13** and the *dimer* **33** (9 : 1 molecular ratio) as their white ammonium salts (50 mg, 55%), m.p. 178–180 °C [Found: M + H^+ (+ESI), 358.0920 $C_{11}H_{15}N_7O_5S$ requires M + H, 358.0934]; ν_{max}/cm^{-1} 3137 (NH_2), 1714 (C=O), 1651 and 1601 (pyrimidine ring); δ_H for **13** (400 MHz, D_2O) 2.34 (3 H, s, CH_3), 2.98 (2 H, t, *J* 6.1, CH_2CH$_2$O), 4.27 (2 H, t, *J* 6.1, CH$_2$CH_2O), 5.39 (2 H, s, CH_2 bridge), 7.79 (1 H, s, triazole C*H*), 8.00 (1 H, s, pyrimidine C*H*); δ_C (100 MHz, D_2O) 22.3 (*C*H$_3$), 23.8 (*C*H$_2$CH$_2$O), 46.4 (*C*H$_2$ bridge), 63.5 (CH$_2$*C*H$_2$O), 107.6 (*C*CNH$_2$), 123.1 (triazole *C*H), 143.8 (triazole *C*), 152.2 (pyrimidine *C*H), 160.9 and 165.8 (*C*N*C*NH$_2$).

Mono-*tert*-butyl malonate 35[13]

To a stirred solution of malonic acid **34** (208 mg, 2.0 mmol) and *tert*-butanol (296 mg, 4.0 mmol) in anhydrous acetonitrile (10 ml) was added a solution of DCC (454 mg, 2.2 mmol) in anhydrous acetonitrile (2 ml). The reaction mixture was stirred at room temperature for 30 min, then filtered and evaporated under reduced pressure. The residue was dissolved in diethyl ether and extracted with saturated aqueous sodium bicarbonate. The aqueous phase was acidified to pH ≈ 1 by addition of hydrochloric acid (1 M) and extracted with ethyl acetate. The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure to give the malonate **35** as an oil (219 mg, 66%). [Found: M − H^+ (−ESI), 159.0651 $C_7H_{12}O_4$ requires M–H, 159.0657]; ν_{max}/cm^{-1} 1713 (C=O), 1142 (C–O); δ_H (400 MHz, $CDCl_3$) 1.50 (9 H, s, 3 × CH_3), 3.34 (2 H, s, CH_2); δ_C (100 MHz, $CDCl_3$) 28.3 (3 × *C*H$_3$), 42.1 (*C*H$_2$) 83.4 (*C*), 167.0 and 171.4 (2 × *C*=O); all analytical data are identical to those previously reported.[13]

2-[1-(4-Amino-2-methylpyrimidin-5-ylmethyl)-1*H*-[1,2,3]triazol-4-yl]ethyl *tert*-butyl malonate 36

To a stirred solution of *tert*-butyl malonate **35** (150 mg, 0.94 mmol) and DCC (194 mg, 0.94 mmol) in anhydrous THF (6 ml) was added a solution of alcohol **18** (136 mg, 0.94 mmol) in anhydrous pyridine (2 ml). The reaction mixture was stirred at room temperature for 18 h, then filtered and evaporated under reduced pressure. The residue was dissolved in ethyl acetate, washed with saturated aqueous sodium bicarbonate, dried ($MgSO_4$) and evaporated under reduced pressure to give the *diester* **36** as an oil (180 mg, 51%) [Found: M + H^+ (+ESI), 377.1936 $C_{17}H_{24}N_6O_4$ requires M + H, 377.1937]; ν_{max}/cm^{-1} 3329 and 3120 (NH_2), 1723 (C=O), 1661, 1596 and 1568 (pyrimidine ring), 1141 (C–O); δ_H (400 MHz, DMSO-d_6) 1.34 (9 H, s, ^{t}Bu), 2.27 (3 H, s, CH_3), 2.92 (2 H, t, *J* 6.8 CH_2CH$_2$O), 3.33 (2 H, s, CH_2), 4.26 (2 H, t, *J* 6.8, CH$_2$CH_2O), 5.36 (2 H, s, CH_2 bridge), 6.84 (2 H, s, NH_2), 7.90 (1 H, s, triazole C*H*), 7.92 (1 H, s, pyrimidine C*H*); δ_C (100 MHz, CD_3OD) 24.5 (*C*H$_3$), 25.5 (*C*H$_2$CH$_2$O), 27.7 (C*Me*$_3$), 34.2 (*C*H$_2$), 49.0 (*C*H$_2$ bridge), 64.4 (CH$_2$*C*H$_2$O), 82.5 (*C*Me$_3$), 109.4 (*C*CNH$_2$), 123.6 (triazole *C*H), 145.0 (triazole *C*), 155.9 (pyrimidine *C*H), 163.0, 167.1, 167.9 and 168.4 (2 × C=O and *C*N*C*NH$_2$).

Mono{2-[1-(4-amino-2-methylpyrimidin-5-ylmethyl)-1*H*-[1,2,3]triazol-4-yl]ethyl} malonate 14

A mixture of trifluoroacetic acid (4 ml) and diester **36** (173 mg, 0.46 mmol) was stirred at room temperature for 3 h, then evaporated under high vacuum, dissolved in water (5 ml) and washed with dichloromethane. One equivalent of sodium bicarbonate (39 mg, 0.46 mmol) was added and the aqueous solution was lyophilised to give the *malonate* **14** as its sodium salt as an oil (126 mg, 80%) [Found: M + H^+ (+ESI), 321.1297 $C_{13}H_{16}N_6O_4$ requires M + H, 321.1311]; ν_{max}/cm^{-1} 3342 and 3143 (NH_2), 1723 and 1651 (C=O), 1543 (pyrimidine ring), 1183 and 1132 (C–O); δ_H (400 MHz, CD_3OD) 2.54 (3 H, s, CH_3), 3.07 (2 H, t, *J* 6.4, CH_2CH$_2$O), 3.35 (2 H, s, CH_2), 4.38 (2 H, t, *J* 6.4, CH$_2$CH_2O), 5.53 (2 H, s, CH_2 bridge), 7.92 (1 H, s, triazole C*H*), 8.01 (1 H, s, pyrimidine C*H*); δ_C (100 MHz, CD_3OD) 24.4 (*C*H$_3$), 28.8 (*C*H$_2$CH$_2$O), 32.5 (*C*H$_2$), 50.2 (*C*H$_2$ bridge), 67.6 (CH$_2$*C*H$_2$O), 113.8 (*C*CNH$_2$), 127.6 (triazole *C*H), 147.4 (pyrimidine *C*H), 148.6 (triazole *C*), 166.1, 167.8, 171.2 and 172.9 (2 × C=O and *C*N*C*NH$_2$).

***N*-Benzyloxycarbonyliminodiacetic acid 39[15]**

To a stirred solution of iminodiacetic acid **37** (878 mg, 6.6 mmol) in aqueous sodium hydroxide (2 M; 15 ml) was added dropwise at 5 °C benzyl chloroformate (2.25 g, 13.2 mmol). A further volume of aqueous sodium hydroxide (2 M; 8 ml) was added and the mixture was stirred at room temperature for 2 h. The reaction mixture was washed with diethyl ether and acidified to pH ≈ 2 with hydrochloric acid (1 M). The aqueous layer was extracted with diethyl ether and the combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure to give the Cbz-protected amine **39** as an oil (1.25 g, 71%) [Found: M − H^+ (−ESI), 266.0675 $C_{12}H_{13}NO_6$ requires M − H, 266.0665]; ν_{max}/cm^{-1} 3035

(C–H), 1689 (C=O); δ_H (400 MHz, $CDCl_3$) 4.12 (2 H, s, CH_2), 4.19 (2 H, s, CH_2), 5.17 (2 H, s, OCH_2), 7.30–7.37 (5 H, m, Ar*H*); δ_C (100 MHz, $CDCl_3$) 48.4 and 48.5 (2 × *C*H_2), 66.8 (O*C*H_2), 126.0 (Ar*C*H), 126.2 (2 × Ar*C*H), 126.9 (2 × Ar*C*H), 135.4 (Ar*C*), 154.4, 171.4 and 172.9 (3 × *C*=O); all analytical data are consistent with those previously reported.[15]

Monobenzyl *N*-benzyloxycarbonyliminodiacetate 42

A solution of **39** (1.25 g, 4.68 mmol) and DCC (964 mg, 4.68 mmol) in anhydrous THF (10 ml) was stirred at room temperature for 12 h. The mixture was filtered and evaporated under reduced pressure to give the solid anhydride **41**, which was used in the next step without further purification. To a stirred suspension of **41** (944 mg, 3.79 mmol) and benzyl alcohol (1.2 ml, 11.6 mmol) in anhydrous diethyl ether (8 ml) was added at 0 °C dicyclohexylamine (829 μl, 4.17 mmol) and the reaction mixture was allowed to warm to room temperature and stirred for 12 h. The solid was collected, washed several times with diethyl ether and recrystallised from ethyl acetate–hexane to yield the dicyclohexylammonium salt. The solid was dissolved in ethyl acetate, washed with hydrochloric acid (1 M) and concentrated under reduced pressure to give the ester **42**[16] as an oil (1.24 g, 74%). [Found: M − H^+ (−ESI), 356.1146; $C_{19}H_{19}NO_6$ requires M − H, 356.1134]; ν_{max}/cm^{-1} 3034 (C–H), 1706 (C=O); δ_H (400 MHz, $CDCl_3$; many of the protons show two sets of signals due to slowly interconverting rotamers about the N–C=O bond) 4.12 (2 H, 2 × s due to rotamers, CH_2), 4.20 (2 H, 2 × s due to rotamers, CH_2), 5.12 (2H, s, OCH_2), 5.19 (2 H, 2 × s due to rotamers, OCH_2), 7.26–7.37 (10 H, m, Ar*H*); δ_C (100 MHz, $CDCl_3$, many carbons show two peaks due to the rotamers) 49.9, 50.13, 50.29 and 50.32 (2 × *C*H_2), 67.61, 67.96, 68.48 and 68.57 (2 × O*C*H_2), 128.09, 128.47, 128.59, 128.67, 128.76, 128.83 and 128.88 (Ar*C*H), 135.00, 135.14, 135.86 and 135.92 (2 × Ar*C*), 155.98, 156.16, 169.93, 170.92, 172.91 and 173.48 (3 × *C*=O).

2-[1-(4-Amino-2-methylpyrimidin-5-ylmethyl)-1*H*-[1,2,3]triazol-4-yl]ethyl benzyl *N*-(benzyloxycarbonyl)iminodiacetate 43

To a suspension of **42** (715 mg, 2.0 mmol), alcohol **18** (468 mg, 2.0 mmol) and DCC (453 mg, 2.2 mmol) in anhydrous THF (15 ml) was added DMAP (269 mg, 2.2 mmol). The reaction mixture was stirred at room temperature for 24 h, then filtered and evaporated under reduced pressure. The residual oil was purified by silica gel chromatography eluting with DCM–MeOH (9 : 1) to give the *diester* **43** as an oil (800 mg, 70%) [Found: M + H^+ (+ESI), 574.2390 $C_{29}H_{31}N_7O_6$ requires M + H, 574.2414]; ν_{max}/cm^{-1} 3341 and 3200 (NH_2), 1744 and 1703 (C=O), 1631, 1594 and 1561 (pyrimidine ring); δ_H (400 MHz, $CDCl_3$, many of the protons show two sets of signals due to slowly interconverting rotamers about the N–C=O bond) 2.47 (3 H, s, CH_3), 2.93 and 3.04 (2 H, 2 × t, *J* 6.3 and 6.5, CH_2CH_2O), 4.07, 4.11, 4.12 and 4.14 (4 H, 4 × s, 2 × CH_2), 4.31 and 4.38 (2 H, t, *J* 6.3 and 6.5, CH$_2$CH_2O), 5.09, 5.16, 5.23 and 5.26 (4 H, 4 × s, 2 × OCH_2), 5.52 and 5.54 (2 H, 2 × s, CH_2 bridge), 7.22 and 7.35 (1 H, s, triazole C*H*), 7.26–7.34 (10 H, m, Ar*H*), 8.16 and 8.18 (1 H, 2 × s, pyrimidine C*H*); δ_C (100 MHz, $CDCl_3$, many carbons show two peaks due to the rotamers) 26.46 and 26.59 (*C*H_2CH$_2$O), 26.70 (*C*H_3), 49.70 (*C*H_2 bridge), 50.70, 50.81 and 50.90 (2 × *C*H_2), 64.90 (CH$_2$*C*H$_2$O), 68.29, 68.35, 69.17 and 69.23 (2 × O*C*H_2), 109.19 and 109.28 (*C*CNH$_2$), 123.05 and 123.33 (triazole *C*H), 128.85, 129.02, 129.38, 129.45, 129.50, 129.54, 129.73 and 129.85 (Ar*C*H), 136.35, 136.37, 137.10 and 137.13 (Ar*C*), 145.90 and 145.94 (triazole *C*), 157.19 and 157.23 (pyrimidine *C*H), 163.19, 169.91 and 170.00 (*C*N*C*NH$_2$), 170.21, 170.37 and 170.46 (2 × *C*=O).

Mono({2-[1-(4-Amino-2-methylpyrimidin-5-ylmethyl)-1*H*-[1,2,3]triazol-4-yl]ethyl) iminodiacetate 15

A mixture of **43** (150 mg, 0.26 mmol) and palladium-on-carbon (10%; 40 mg) in methanol (10 ml) was stirred under hydrogen (1 atm) at room temperature for 20 min. The suspension was filtered through Celite and the residue was washed several times with methanol. The filtrate was evaporated under reduced pressure to give the *acid* **15** as a white solid (69 mg, 76%), m.p. 195–200 °C; [Found: M + H^+ (+ESI), 350.1582; $C_{14}H_{19}N_7O_4$ requires M + H, 350.1571]; ν_{max}/cm^{-1} 3134 (NH_2), 1749 (C=O), 1681 and 1577 (pyrimidine ring); δ_H (400 MHz, D_2O) 2.33 (3 H, s, CH_3), 3.00 (2 H, t, *J* 6.2, CH_2CH$_2$O), 3.49 (2 H, s, CH_2), 3.83 (2 H, s, CH_2), 4.40 (2 H, t, *J* 6.2, CH$_2$CH_2O), 5.39 (2 H, s, CH_2 bridge), 7.78 (1 H, s, triazole C*H*), 7.95 (1 H, s, pyrimidine C*H*); δ_C (100 MHz, D_2O) 23.9 (*C*H_3), 24.7 (*C*H$_2$CH$_2$O), 47.6 and 47.9 (2 × CH_2), 49.7 (*C*H_2 bridge), 65.5 (CH$_2$*C*H$_2$O), 108.9 (*C*CNH$_2$), 124.2 (triazole *C*H), 145.0 (triazole *C*), 154.8 (pyrimidine *C*H), 162.2 and 167.8 (*C*N*C*NH$_2$), 168.2 and 172.0 (2 × *C*=O).

General methods for enzymic assays

All assays were performed on a Cary 100 Bio UV-visible spectrophotometer, using disposable plastic cuvettes (Fisherbrand® semi-micro polystyrene). Buffer and assay solutions were prepared on the day of use. Pyruvate decarboxylase from *Zymomonas mobilis* was overexpressed in *E. coli* and purified as described previously.[26] Protein concentrations were determined by the Bradford assay.[27]

Assay for PDC activity[4,5]

The assay solution used in all experiments contained alcohol dehydrogenase (ADH; 10 units per ml), NADH (0.15 mM), TPP (0.1 mM) and $MgCl_2$ (5 mM) in MES–KOH buffer (50 mM; pH 6.5). Pyruvate decarboxylase (*ca.* 8 nM of active sites) was incubated in the assay solution for 3 min at 25 °C and the assay was then started by addition of the substrate pyruvate to give 5 mM. The consumption of NADH was followed spectrophotometrically at 340 nm ($\varepsilon_{340} = 5700\ M^{-1}\ cm^{-1}$).

Preparation of apoPDC[5]

The holoPDC was dissolved in HEPES-KOH buffer (50 mM; pH 8.2) containing dipicolinic acid (1 mM). After incubation at 5 °C for 30 min the apoPDC was separated from cofactors by repeated ultrafiltration. The buffer was finally replaced by MES–KOH buffer (50 mM; pH 6.5). The preparation of the apoenzyme was confirmed by assays of activity with and without TPP present.

Time-dependent inactivation and reactivation of PDC

ApoPDC (*ca.* 0.3 μM of active sites) was incubated at 25 °C with $MgCl_2$ (5 mM) and inhibitor (various concentrations, *e.g.* from 2 μM to 10 μM) in MES–KOH buffer (50 mM; pH 6.5). At timed intervals, aliquots (20 μl) were added to the assay solution (778 μl) and after a further incubation at 25 °C for 3 min a solution of sodium pyruvate (2 M; 2 μl, to give 5 mM) in water was added and the decrease of A_{340} with time was followed.

In order to ensure the stability of the enzyme, control experiments were carried out without inhibitor present. In every case the percentage activity is expressed as the ratio of the activity with inhibitor to the corresponding activity obtained without inhibitor.

In order to study the rate of unbinding, apoenzyme was first incubated with inhibitor (10–20 μM) until low (or no) activity was observed. An excess of TPP (to give 100 μM to 10 mM) was then added and the recovery of activity was followed for several (7–8) days.

Time-dependent reactivation/inactivation of PDC

ApoPDC (*ca.* 0.4 μM of active sites) was incubated at 25 °C with TPP (4 μM), $MgCl_2$ (10 mM) and analogue (concentrations ranging from 20 μM to 6 mM) in MES–KOH buffer (50 mM; pH 6.5). At timed intervals, aliquots (20 μl) were added to the assay solution containing NADH (0.15 mM), ADH (10 units ml^{-1}) and pyruvate (5 mM) in MES–KOH buffer (50 mM; pH 6.5) and the decrease of A_{340} with time was followed.

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References

1 (*a*) F. Jordan, *Nat. Prod. Rep.*, 2003, **20**, 184; (*b*) A. Schellenberger, *Biochim. Biophys. Acta*, 1998, **1385**, 177.
2 (*a*) D. Kern, G. Kern, H. Neef, K. Tittman, M. Killenberg-Jabs, C. Wikner, G. Schneider and G. Hübner, *Science*, 1997, **275**, 67; (*b*) N. Nemeria, L. Korotchkina, M. J. McLeish, G. L. Kenyon, M. S. Patel and F. Jordan, *Biochemistry*, 2007, **46**, 10739.
3 (*a*) E. Fiedler, S. Thorell, T. Sandalova, R. Golbik, S. König and G. Schneider, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 591; (*b*) U. Nilsson, L. Meshalkina, Y. Lindqvist and G. Schneider, *J. Biol. Chem.*, 1997, **272**, 1864.
4 R. J. Diefenbach and R. G. Duggleby, *Biochem. J.*, 1991, **276**, 439.
5 S. Mann, C. Perez Melero, D. Hawksley and F. J. Leeper, *Org. Biomol. Chem.*, 2004, **2**, 1732.
6 D. Hawksley, D. A. Griffin and F. J. Leeper, *J. Chem. Soc., Perkin Trans. 1*, 2001, 44.
7 K. M. Erixon, C. L. Dabalos and F. J. Leeper, *Chem. Commun.*, 2007, 960.
8 (*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.
9 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.
10 (*a*) N. A. Boyle, *Org. Lett.*, 2006, **8**, 187; (*b*) G. Wang, N. 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; (*d*) E. Klein, H.-O. Nghiêm, A. Valleix, C. Mioskowski and L. Lebeau, *Chem. Eur. J.*, 2002, **8**, 4649.
11 L. Bonnac, V. Barragan, J.-Y. Winum and J.-L. Montero, *Tetrahedron*, 2004, **60**, 2187.
12 J. D. Park and D. H. Kim, *Bioorg. Med. Chem.*, 2004, **12**, 2349.
13 R. Shelkov, M. Nahmany and A. Melman, *J. Org. Chem.*, 2002, **67**, 8975.
14 S. Girault, E. Davioud-Charvet, L. Maes, J.-F. Dubremetz, M.-A. Debreu, V. Landry and C. Sergheraert, *Bioorg. Med. Chem.*, 2001, **9**, 837.
15 A. C. Benniston, P. Gunnung and R. D. Peacock, *J. Org. Chem.*, 2005, **70**, 115.
16 H. Gregory, J. S. Morley, J. M. Smith and M. J. Smithers, *J. Chem. Soc. C*, 1968, 715.
17 R. Kluger, G. Gish and G. Kauffman, *J. Biol. Chem.*, 1984, **259**, 8960.
18 R. A. W. Frank, F. J. Leeper and B. F. Luisi, *Cell. Mol. Life Sci.*, 2007, **64**, 892.
19 F. Jordan, H. J. Li and A. Brown, *Biochemistry*, 1999, **38**, 6369.
20 (*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.
21 G. M. Blackburn, D. E. Kent and F. Kolkmann, *J. Chem. Soc., Chem. Commun.*, 1981, 1188.
22 (*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.
23 D. D. Perrin, W. L. F. Armarego and D. R. Perrin, *Purification of Laboratory Chemicals*, 2nd edn, Pergamon Press, Oxford, 1980.
24 G. Eglinton and M. C. Whiting, *J. Chem. Soc.*, 1950, 3650.
25 J.-Y. Winum, D. Vullo, A. Casini, J.-L. Montero, A. Scozzafava and C. T. Supuran, *J. Med. Chem.*, 2003, **46**, 5471.
26 G. Schenk, F. J. Leeper, R. England, P. F. Nixon and R. G. Duggleby, *Eur. J. Biochem.*, 1997, **248**, 63.
27 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248.

