Highly Potent Bisphosphonate Ligands for Phosphoglycerate Kinase[†]

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Received December 12, 1997

We have synthesized a series of novel analogs of 1,3-bisphospho-D-glyceric acid, 1,3-BPG,[3] and evaluated their binding to phosphoglycerate kinase, PGK (EC 2.7.2.3). Nonscissile methanephosphonic acids replace the two phosphate monoesters of 1,3-BPG and lead to several stable, tight-binding mimics of this intermediate species in glycolysis. Multiple fluorine substitution for hydrogen in the α-methylene groups of the phosphonic acid 1,3-BPG analogs markedly improves their binding to PGK as determined by NMR analysis. The best ligands bind some 50−100 times more strongly than does the substrate 3-phospho-D-glyceric acid and show a requirement for pK_{a^3} to be generally below 6.0, while the presence of a β-carbonyl group seems to be of secondary importance.

Introduction

Inhibition of the glycolytic enzyme phosphoglyceric phosphokinase, PGK, leads to the conversion of 1,3-bisphosphoglycerate, 1,3-BPG, into 2,3-BPG catalyzed by bisphosphoglycerate mutase. Thus, when red blood cells pass through inadequately oxygenated tissues they produce increased amounts of 2,3-BPG. This increase in 2,3-BPG causes a decrease in hemoglobin affinity for oxygen which enables more oxygen to be extracted from blood as it passes through the tissues. Thus inhibition of PGK is a potential means for treatment of cardiovascular and respiratory disorders.[1]

PGK is the enzyme which equilibrates phosphate transfer between position-1 of 1,3-BPG and the γ-phosphate of ATP (Scheme 1).[2] It is the seventh enzyme in the glycolytic pathway and is also utilized for carbon dioxide fixation in plants. The cofactor for the reaction is Mg^{2+}, and the correct substrate for phosphoryl transfer is $MgATP^{2-}$. In the absence of magnesium, ATP^{4-} binds to the enzyme in a very different manner.[3,4]

PGK has been isolated from a wide variety of sources. All forms are monomeric enzymes with molecular mass around 45 kDa. There is a high degree of conservation of primary protein structure,[5,6] and their modes of catalysis are similar. On the basis of the conformity of the enzymes, Fifis and Scopes[7] advanced the hypothesis that the tertiary structure and active site regions of PGK are conserved. The enzyme has two distinct domains, corresponding to the N-terminal and C-terminal portions of the enzyme, with the last 10 residues of the C-terminus packed into the N-terminal domain.[6]

The N-terminal domain contains a "basic patch"

Scheme 1. Phosphate Transfer Catalyzed by PGK

$$\text{ADP} + 1,3\text{-BPG} \xrightleftharpoons[]{\text{Mg}^{2+}} \text{ATP} + 3\text{-PGA} \quad \Delta G = -4.8 \text{ kcal mol}^{-1}$$

which is made up of several arginine and histidine residues and provides the proposed binding site for 3-PGA or 1,3-BPG. The C-terminal domain binds the nucleotide in a shallow hydrophobic cleft. The distance between these substrates as bound to the enzyme was modeled[6] as approximately 11 Å for the "open form" of PGK, which is too distant for direct phosphoryl transfer between the substrates.[8,9] In view of this bilobal shape of PGK, it has been proposed[10,11] that the two domains swing together about a hinge region, closing down on substrates and excluding water, and thereby effect catalysis. Such a domain movement to generate a "closed form" for the enzyme would support the sequential pathway for phosphoryl transfer demonstrated for PGK.[12] Many attempts to identify this "closed form" of PGK by X-ray analysis have failed, and so it has been sought by many other biophysical techniques. Sedimentation experiments[13] and small-angle scattering experiments[14,15] have given evidence for large-scale structural changes, but the results are not conclusive.[16] More recent X-ray data have still failed to identify the fully closed state of the enzyme[17] though dramatic bending around the hinge region has been seen in a ternary complex of *Trypanosoma brucei* PGK with ADP and 3-PGA, which brings these noncomplementary substrates 6 Å closer together than seen before and well aligned for catalysis.[18] Paramagnetic Cr^{3+} probes have shown the close proximity of the substrates in the ternary complex,[19] while the kinetics of PGK have been recently studied using a rapid quench flow apparatus,[20] confirming the earlier findings that release of 1,3-BPG is the slow, rate-determining step for the reaction.[21]

An early study on PGK using substrate analogs demonstrated that 2-hydroxy-4-phosphono-DL-butyrate is a substrate for PGK with a K_m that is pH-dependent. When the pH is below pK_{a^2} for the phosphonic acid, the analog reacts slowly. At pHs above pK_{a^2}, when the analog is fully ionized, the kinetics observed are much more comparable to those for 3-PGA.[22] Further work

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Scheme 2. Michaelis–Becker Syntheses of Some Simple Bisphosphonate Esters[a]
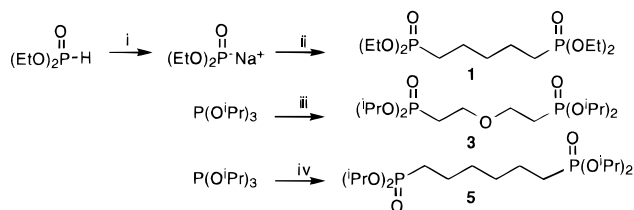
[a] Reagents: (i) sodium, toluene, Δ; (ii) Br(CH$_2$)$_5$Br in ether, Δ, 2 h, 66%; (iii) (BrCH$_2$CH$_2$)$_2$O, 150 °C, 16 h, 86%; (iv) Br(CH$_2$)$_6$Br, 150 °C, 16 h, 85%.

with small analogs of 1,3-BPG has generally failed to identify good inhibitors for PGK.[23] More recently, two keto-bisphosphonates have been studied as inhibitors for the enzyme,[24] and these compounds have been included in the range of bisphosphonates that we have prepared and evaluated as inhibitors of PGK.

In our design of 1,3-BPG analogs, we have focused on the achievement of full ionization of the phosphonic acids, especially as effected by the use of α-fluorophosphonates. Our general analysis of the rectification of the mismatch parameters in the use of alkanephosphonic acids as analogs of biological phosphates for this purpose[25] has been well exemplified by results in many laboratories for analogs of nucleotides, phosphoamino acids, glycerol phosphates, guanosine-5′-P analogs, and the fluorophosphonate analog of 1,6-fructose bisphosphate, etc.[26−34] The presence of a basic patch in PGK at the site of binding 1,3-BPG makes it a suitable enzyme for the evaluation of the contribution of anionic charge to ligand binding. We have prepared a wide range of the bisphosphonates synthesized in the present work and determined their affinities for PGK to enable us to demonstrate the superiority of α-fluoromethylenephosphonic acids over methylenephosphonic acids as 1,3-BPG analogs. This has both generated lead compounds as possible drug candidates for respiratory disorders and also identified symmetrical analogs of 1,3-BPG with micromolar affinity for PGK that are suitable for conversion into bisubstrate analogs for further use in structural studies.

Results

Synthesis of 1,3-BPG Analogs. Chemical syntheses were carried out as described below following Schemes 2–7. Standard procedures were employed for the syntheses of phosphonate and α-difluoromethylenephosphonate esters.[28] A range of methodologies was employed, each being represented in a separate scheme. The final steps in each route were deesterifications performed by hydrolysis in refluxing HCl (for robust products) or by transesterification using bromotrimethylsilane followed by solvolysis with methanol for phosphonates that are sensitive to hot acid conditions.[35] In the case of ester (**44**), silylation required the use of iodotrimethylsilane[36] which was prepared in situ.[37] The free bisphosphonic acids were converted into their sodium salts by titration to pH 7.1 with sodium hydroxide solution followed by lyophilization and used in this form for subsequent enzyme binding studies.

pK_a Determinations. The bisphosphonic acids were subjected to pH titration in the range pH 11 to 3 by the controlled addition of HCl (0.1 M) to a stirred solution of the test sample. The titration curves were plotted in the range 3 < pH < 11 and pK_a values calculated from the experimental data. The pH range analyzed precluded the determination by this method of values for pK_{a^1} and pK_{a^2} for the bisphosphonates but supported evaluation of pK_a values separated by ≥0.3 units. For the symmetrical and unsymmetrical bisphosphonates, values of pK_{a^3} and pK_{a^4} were statistically corrected as appropriate for the number of equivalent ionizable protons and the number of equivalent phosphonate dianions to support quantitative comparisons with ^{31}P chemical shift and IC$_{50}$ data.

A plot of ^{31}P chemical shift versus p$K_a^{3,4}$ is shown (Figure 1). The linear regression curve has a correlation coefficient of 0.92, and an unweighted *linear* fit was found to be the best of a wide range of curve fits attempted.

Protein Dissociation Constants. Titration of the analogs into PGK causes chemical shift changes in the NMR spectrum. These changes are principally to signals previously assigned as belonging to the basic patch and, in particular, histidines-62, -167, and −170. Dissociation constants were obtained from curve fitting, as shown in Figure 2. Dissociation constants stronger than 4 μM are hard to estimate accurately because of lack of curvature in the data. The dissociation constants for all analogs tested along with pK_a data are presented in Table 1. The $\Delta\delta_{max}$ is the average chemical shift change for the histidine residues. The σ values are the standard deviation calculated from the averages. When the shifts of histidine residues were less than 10 ppb, the value of K_d was not calculated because the shifts were not sufficiently precise. As a result, only one set of data was used to determine the K_d for **2**.

The measurement of dissociation constants described here builds on previous studies (reviewed in ref 5). However, it is worth pointing out a significant difference in the composition of the buffer used for the binding studies. Previous work used 100 mM acetate, which made it difficult to compare the results with those from enzyme inhibition studies in which the buffer typically consisted of 30 mM triethanolamine, 50 mM KCl, 40 mM (NH$_4$)$_2$SO$_4$, and 0.2 mM EDTA.[38] Therefore, it was decided to use a buffer composition as close to that used for the enzyme inhibition studies as possible, namely 10 mM triethanolamine and 40 mM KCl. The absence of sulfate from the NMR buffer is significant: sulfate acts as a competitive ligand at both the nucleotide binding site and the phosphoglycerate binding site. At low concentrations (≤40 mM) it helps to displace 3-PGA from the binding site, and hence increases the reaction rate, and also converts the reaction into one that obeys Michaelis–Menten kinetics.[20] At higher concentrations it is a competitive inhibitor of PGK. Addition of sulfate to the buffer results in weaker binding and in many cases makes it impossible to fit a dissociation constant satisfactorily.

Discussion

We have synthesized a number of analogs of 1,3-BPG and measured their dissociation constants against PGK using NMR chemical shifts as a simple and direct measure. It was not possible to measure the dissocia-

Scheme 3. Michaelis–Becker Syntheses of Some *gem*-Difluoroalkanebisphosphonate Esters[a]
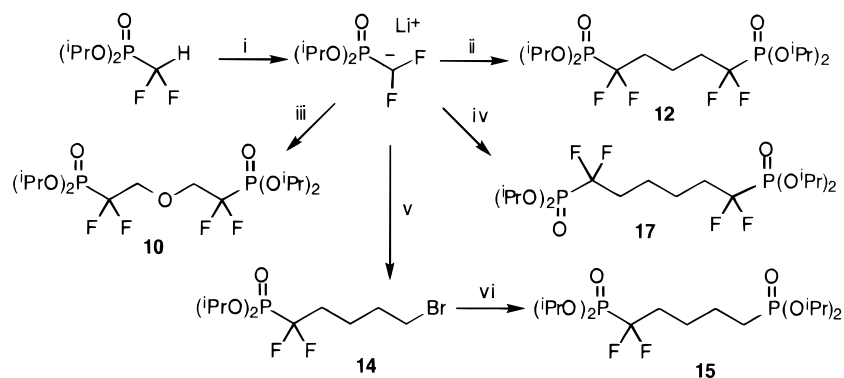
[a] Reagents: (i) LDA, THF, −78 °C; (ii) 1,3-dibromopropane, 63%; (iii) bischloromethyl ether, 16%; (iv) 1,4-dibromobutane, 13%; (v) 1,4-dibromobutane, 26%; (vi) P(OiPr)$_3$, 150 °C, 16 h, 50%.

Scheme 4. Synthesis of 2-Aminoethylphosphonate Ester and Its Amides[a]
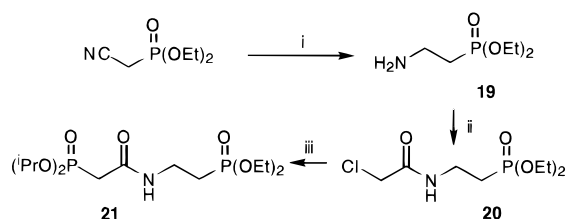
[a] Reagents: (i) PtO$_2$·xH$_2$O, H$_2$, MeOH; (ii) chloroacetic anhydride, Et$_3$N, DCM; (iii) P(O^{i}Pr)$_3$, 130 °C, 73% (3 steps).

Scheme 5. Syntheses of 2-Amino-1,1-difluoroethylphosphonate Esters and Amides[a]
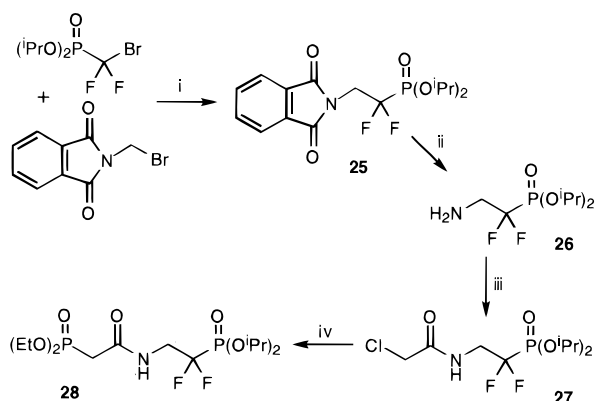
[a] Reagents: (i) Zn, CuBr, monoglyme; (ii) NH$_2$NH$_2$·H$_2$O, *p*-TsOH; (iii) (ClCH$_2$CO)$_2$O, Et$_3$N, DCM; (iv) P(OEt)$_3$, 130 °C, 11% (4 steps).

Scheme 6. Syntheses of Amides of Phosphonodifluoroacetic Acid Esters[a]
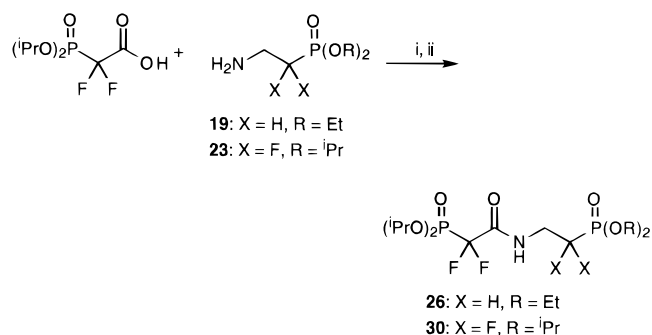
[a] Reagents: (i) oxalyl chloride, DCM, 16 h; (ii) Et$_3$N, DCM, 0 °C, **23**, 50%, **30**, 89%.

Scheme 7. Syntheses of Some 2-Oxoalkanephosphonate Esters and of *gem*-Difluoro-2-oxoalkanephosphonates[a]
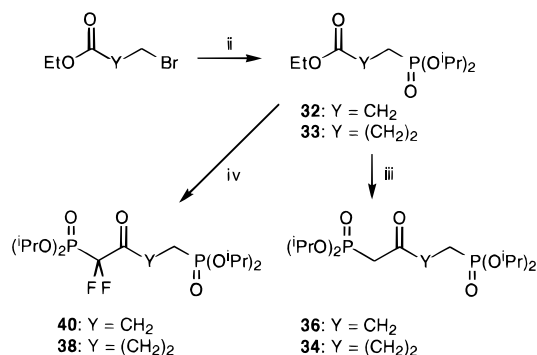
[a] Reagents: (i) P(O^{i}Pr)$_3$, 150 °C, 16 h, **33**, 75%, **32**, 78%; (ii) (^{i}PrO)$_2$P(O)CH$_3$, LDA, −78 °C, **34**, 33%, **36**, 30%; (iii) *n*-BuLi, ^{i}Pr$_2$NH, −60 °C, 15−30 min, (^{i}PrO)$_2$P(O)CF$_2$H, −78 °C, 30 min, **38**, 60%, **40**, 47%.

tion constant of 1,3-BPG itself because of its very short half-life in aqueous solution. However, the dissociation constant of 3-PGA, which is a product of the reaction under physiological conditions (and whose release is normally the rate-limiting step of the reaction catalyzed by PGK), could be measured. Many of the analogs have dissociation constants much stronger than that of 3-PGA and are therefore potential drug candidates for the treatment of respiratory and cardiovascular diseases.

It has recently been proposed[39] that there are two binding sites for 3-PGA on PGK: the first in the basic patch, as has previously been observed, and the second next to the N-terminus of helix-14. It is further proposed that the basic patch site primarily represents the site of anion activation rather than the catalytically active substrate binding site. The results presented

here cast doubt on this view and support the more traditional view that the basic patch is indeed part of the active site in the active conformation because the analogs described here, which were designed as analogs of the substrate, bind well to the enzyme in the basic patch and have no effect on signals in the region of helix-14. Moreover, the better analogs bind more tightly to the enzyme than does the substrate 3-PGA. Studies by McHarg & Littlechild[38] on some of these compounds have shown that the K_i values for these compounds when tested as inhibitors of PGK have the same order of magnitude as the K_d values presented here in the same conditions and that the compounds act approxi-

$$\delta_P = 8.539(pK_a) - 35.438 \quad r = 0.920$$
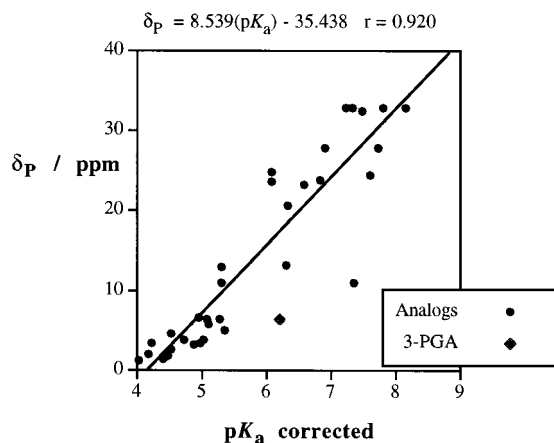
Figure 1. Phosphorus NMR chemical shift vs phosphonate $pK_a^{3,4}$ of 1,3-BPG analogs.
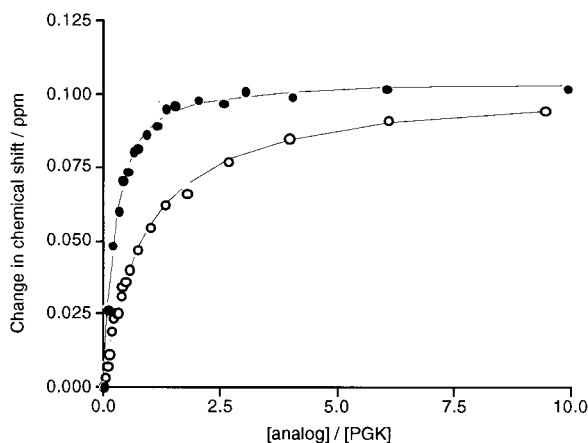
Figure 2. Titration data for His-167: (○) compound **13**, (●) compound **16**. Solid lines represent the dissociation curves calculated from data in Table 1.

mately as competitive inhibitors to 3-PGA but are not competitive to ATP. This is further evidence against binding at helix-14, since this is the binding site for the γ-phosphate of ATP.

From comparisons of the dissociation constants measured for different ligands, it is possible to draw conclusions about the characteristics required for good binding to PGK. These are presented below.

Effect of Chain Length in Bisphosphonate Binding to PGK. There is a clear preference for a five-atom over a six-atom spacer between the two phosphonic acids. 1,5-Pentanebisphosphonic acid **2** is a significantly better inhibitor for PGK than is 1,6-hexanebisphosphonic acid **6**, while the corresponding tetrafluoropentane **13** is some seven times better than the tetrafluorohexane **18**. Unexpectedly, this trend is continued for the butanone-1,4-bisphosphonic acid **37** relative to the pentanone-1,5-bisphosphonic acid **35**, where there is a 10-fold preference for the shorter chain, and also for the corresponding 1,1-difluoro compounds **41** and **39**, where there is a 25-fold advantage for the shorter chain. These data also support the conclusion that the optimization of chain length in the separation of the two phosphonic acids becomes more critical as binding becomes stronger and that the degree of *concentration* of anionic charge may be important for tight binding to PGK.

To evaluate chain flexibility and polarity, the effect of replacing a methylene group by an ether link was

evaluated by the comparison between the 3-oxapentane **4** and pentane **2** and the corresponding tetrafluoro compounds **11** and **13**. The oxa-substitution resulted in a 2-fold and 5-fold weakening of binding to PGK in these cases.

The Effect of a Carbonyl Functionality. To assess the importance of recognition of the carbonyl group in 1,3-BPG, several β-ketophosphonates were examined. The 60-fold increase in affinity for pentanonebisphosphonic acid **35** compared to pentanebisphosphonic acid **2** clearly established the strong recognition of the 2-keto function in analogs of 1,3-BPG. The effect also carries over to the butanonebisphosphonic acid **37**, and we observe values for dissociation constants for **35** and **37** comparable to the K_i values reported by Byers.[24] A similar enhancement for insertion of a C=O group is seen in the corresponding tetrafluoro species **44** compared to **13**. Unexpectedly, the α,α-difluoropentanebisphosphonic acid **16** binds some 20-fold more strongly to PGK than does the difluoropentanone **39**. One possible explanation is that such difluoroketones exist predominantly as covalent hydrates, 2,2-dihydroxy species (as seen in their ^{13}C and ^{31}P NMR spectra), and this may affect their binding. Alternatively, the *gem*-difluoro function may itself provide some of the recognition element of the carbonyl group.

Amide Functionality as a Hydrogen Bond Donor. The functions explored thus far lack the hydrogen bond donor capability of the 3-hydroxyl function of 1,3-BPG. Some isoelectronic mimics of 1,3-BPG are the reversed amides **45** and the related *N*-hydroxylamides **46** (Figure 3), the latter offering a nonchiral equivalent to the D-glycerate 3-phosphate as well as suppressing the possibility of enolization of the ketophosphonates or of hydration of the fluoroketophosphonates. In the event, attempts to prepare the *N*-hydroxylamides were negated by the instability of the functionality, and we were obliged to focus efforts exclusively on the simple amides.

Only a 2-fold improvement in binding results from incorporation of the amide functionality into analog **22** derived from pentanebisphosphonic acid **2**, and it is vastly inferior to the pentanone **35**. Moreover, comparison of **22** with **37** suggests that the ketone functionality gives better binding than the amide. Although at first sight the weak binding of the α,α-difluoroketone **39** compared to that of the amide **24** appears to go against this trend, the weakness of binding is probably a consequence of the preference of **39** to exist in solution as the diol, as discussed above. The diol is too large to fit within the basic patch and consequently binds more weakly because of adverse steric effects. The amide **24** is not hydrated to a diol in the same way and consequently binds more tightly. A similar effect is observed for the tetrafluoroamide **31**, which is better than the tetrafluoropentanone **44** and 35 times better than the tetrafluoropentane **13**.

α-**Fluorination of the Phosphonates.** α-Fluorination is well known to reduce pK_a^2 of a phosphonic acid.[25,27,28] If the binding of analogs of 1,3-BPG is charge-dependent, then fluorinated analogs should show stronger binding than their unfluorinated counterparts. For all of the tetrafluoro analogs described here, there

Table 1. Dissociation Constants for Analogs of 1,3-BPG and 3-PGA Binding to PGK and Values of Phosphonic Acid Second Dissociation Constants, pK_{a3} and pK_{a4} Corrected for Multiplicity[a]

Compound	Structure	Average K_d /μM (s.d.)	Average $\Delta\delta_{max}$ / ppb (s.d.)	Number of determinations	$pK_{a3,4}$ (statistically corrected[†])
3-PGA		110 (10)	300 (285)	3	6.2
2		1300	10	1	7.33, 8.16
4		5000 (880)	115 (40)	2	6.90, 7.72
6		2130 (480)	41 (28)	2	7.24, 7.81
8		72 (6)	71 (36)	3	4.22, 4.97
11		265 (50)	73 (33)	3	4.72, 5.02
13		140 (20)	62 (30)	2	5.09, 5.27
16		4 (2)	69 (35)	2	5.10, 7.48
18		1000 (260)	52 (13)	3	4.95, 4.95
22		675 (80)	110 (60)	3	5.30, 6.32
24		6 (2)	57 (11)	3	4.41, 6.84
29		2 (1)	86 (36)	3	4.52, 6.30
31		4 (2)	58 (18)	3	4.02, 4.88
35		19 (2)	105 (40)	3	6.09, 7.35
37		2 (1)	140 (70)	3	5.30, 6.59
39		76 (11)	57 (17)	3	4.52, 7.60
41		3 (1)	100 (50)	3	4.47, 6.07
44		5 (3)	47 (15)	2	4.17, 5.36

[a] pK_a values were statistically corrected for the dissociation of a proton from two equivalent sites (ΔpK_{a3} + log 2) and for return of a proton to multiple equivalent oxyanions (ΔpK_{a3} − log 2 and ΔpK_{a4} − log 2 or −log 4) as appropriate.

is a uniform trend that overwhelmingly supports this hypothesis. Thus, **18** binds twice as well as hexanebi-

sphosphonic acid **6**; **13** binds 10-fold better than pentanebisphosphonic acid **2**; **44** binds four times more
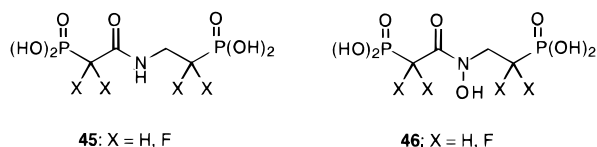
Figure 3. General structures of amide and *N*-hydroxamic acid analogs of 1,3-BPG.

strongly than pentanone **35**; and **31** binds 140-times more tightly than amide **22**. Even the small diol **8** binds strongly to PGK notwithstanding its small separation of the two phosphonic acids, possibly as this gem diol has many opportunities for hydrogen bonding.

For the difluoro analogs, the situation is rather more variable. Thus, the difluoropentane **16** outperforms the tetrafluoropentane **13**. The two difluoroamides **24** and **29** straddle the K_d for the tetrafluoroamide **31**. Yet the difluoroketone **39** is much inferior in binding to PGK than the tetrafluoroketone **44**. These differences may be a consequence of different orientations of the unsymmetrical bisphosphonates in the binding site as discussed below. But with only a single exception, all of the difluoroketonebisphosphonic acids bind more strongly to PGK than do the nonfluorinated counterparts. This is most powerfully exemplified by the difluoroamide **29** where the introduction of two fluorines increases binding affinity to PGK by over 300 times. Clearly, this is primarily a function of the state of ionization of the phosphonic acids.

Effect of Ionic State of Bisphosphonate Analogs. While no attempt has been made to correct the analog binding data to PGK for the state of ionization of the phosphonic acids, it was evident from the outset that the simple methylenephosphonic acids, e.g. **2**, **6**, **16**, and **35**, will be less than completely ionized at pH ~7 while the α,α'-tetrafluorophosphonic acids, e.g. **8**, **13**, **31**, and **44**, will be fully ionized under the binding conditions used.

To discern the significance of the state of ionization on analog binding to PGK, we have explored possible correlations of pK_a with $\log[K_d]$ in a variety of ways. First, we note that there is a good linear correlation for pK_{a^3} and pK_{a^4} with the ^{31}P NMR chemical shifts of the corresponding phosphonate sodium salts (pH* 7.1) over some 4 orders of magnitude (Figure 1, $r = 0.92$). This extends the correlation observed previously for a rather limited range of halogenated methanephosphonic acids.[40] Moreover, the linear fit to these data is superior to any other simple correlation examined. It is perhaps noteworthy that the data for 3-PGA diverge markedly from this correlation.

Next, we have looked at ways of correlating pK_a for these analogs with $\log[K_d]$ for PGK binding. First, a plot of *both* pK_{a^3} *and* pK_{a^4} against $\log[K_d]$ for binding to PGK shows almost random scatter (Figure 4a, $r = 0.38$). Second, a plot of the *average* of pK_{a^3} and pK_{a^4} for each bisphosphonate against $\log[K_d]$ for binding to PGK was created to see whether the enzyme recognizes the state of ionization for both phosphonates. The result gives a poor linear correlation (Figure 4b, $r = 0.47$) which indicates that the enzyme does not recognize the state of ionization of both phosphonates equally strongly. Third, a plot of pK_{a^3} against $\log[K_d]$ shows a modest linear correlation (Figure 4c, $r = 0.66$). However, these

data can be interpreted as describing a hyperbolic curve, strongly suggesting that tight binding of the analogs to PGK requires that at least one of the phosphonates is doubly ionized, with $pK_{a^3} < 6$. By contrast, a plot of pK_{a^4} against $\log[K_d]$ gives a random scatter (not shown) which supports the unexpected conclusion that fourth ionization of the bisphosphonic acid is not a prerequisite for tight binding to PGK under the conditions of pH used in these experiments.
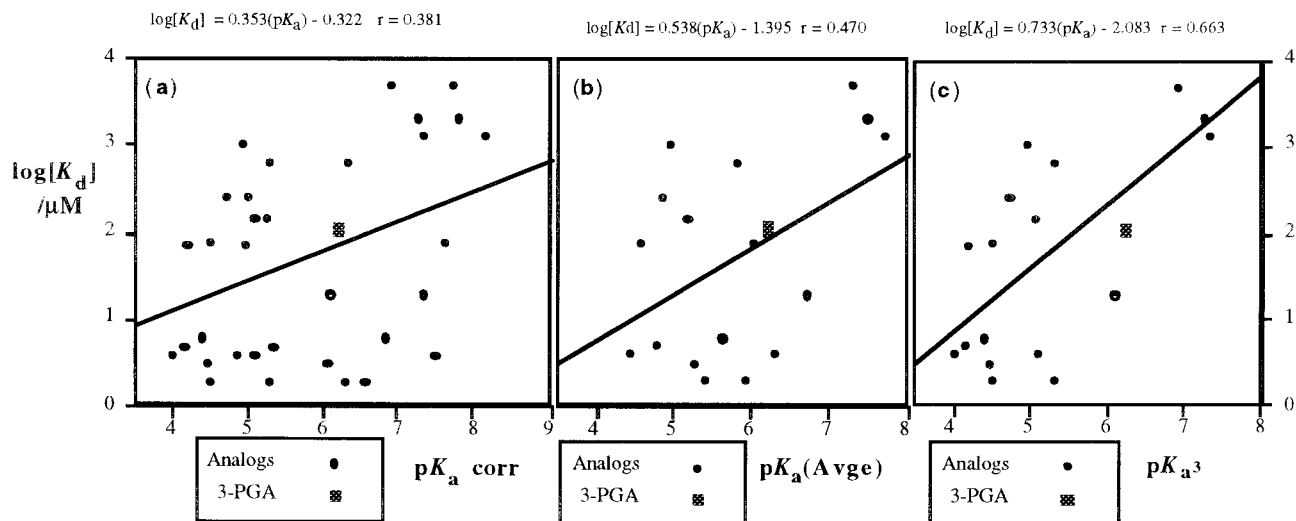
This conclusion is illustrated by the example of change in pK_a in the series of analogs **22**, **24**, **29**, and **31**. The amide bisphosphonic acid **22** binds to PGK rather weakly ($K_d = 0.67$ mM). Bisfluorination at either position adjacent to phosphorus increases binding over 100-fold ($K_d = 6$ μM for **24** and 2 μM for **29**). Further fluorination to the tetrafluoroamide **31** does not improve binding ($K_d = 4$ μM) even though pK_{a^4} falls significantly. It is clear that some additional knowledge of the orientation of the analogs in the "basic patch" binding site is necessary to understand these binding events more completely.

Such orientation of bisphosphonate analogs in this "basic patch" has not hitherto appeared important. However, the amide analogs, keto analogs, and **16** all share the possibility of binding in two alternative orientations. We initially supposed that the carbonyl functionality would determine such orientation by binding in the same locus as the carbonyl group of 1,3-BPG. However, the very tight binding for **29** suggests the possibility that the choice of orientation for a bisphosphonate analog in the "basic patch" may be primarily determined by a high affinity site for a phosph(on)ate dianion and a lower affinity site for the second phosph(on)ate species, which is less critical of complete ionization. We note that the nonfluorinated amide **12** binds weakly while both its difluoromethylene analogs **24** and **29** bind strongly. This further implies that binding affinity is determined primarily by the ionization state of the phosph(on)ate and not by functionality within the chain (i.e. the orientation of the amide has a much smaller effect on binding affinity than the orientation of the difluorophosphonate). From the data presently available, it is impossible to conclude which orientation exists for any particular unsymmetrical analog of 1,3-BPG bound to PGK. Additional experimentation will therefore be necessary to study this phenomenon.

Last, there is clear evidence that lowering of the pK_{a^3} for the bisphosphonic acids below 6 conveys little additional benefit. In this work we have not prepared any of the monofluorophosphonate analogs that might support a more detailed examination of bisphosphonate analogs with a pK_{a^3} in the range 6−7 for the quantitative structure−activity relationship, partly for synthetic reasons and partly to avoid the problems inherent in working with mixtures of stereoisomers. However, the PGK affinities for analogs **37**, **41**, and **44** are very similar, and the latter two appear to derive no advantage from being 10-fold more acidic through fluorination.

Conclusion

Several bisphosphonate analogs of 1,3-BPG have been prepared whose affinity for PGK has been determined by NMR analysis. The resulting K_d data have been used to analyze the structural features of the atomic frame-

$$\log[K_d] = 0.353(pK_a) - 0.322 \quad r = 0.381$$

$$\log[Kd] = 0.538(pK_a) - 1.395 \quad r = 0.470$$

$$\log[K_d] = 0.733(pK_a) - 2.083 \quad r = 0.663$$

Figure 4. Correlations of analog phosphonate pK_a with $-\log[K_d]$ for binding to PGK. (a) Plot of $-\log[K_d]$ vs both pK_{a^3} and pK_{a^4} for all bisphosphonate analogs. (b) Plot of $-\log[K_d]$ vs the average of pK_{a^3} and pK_{a^4} for all bisphosphonate analogs. (c) Plot of $-\log[K_d]$ vs pK_{a^3} for all bisphosphonate analogs.

work linking the two phosphonate functions primarily responsible for optimum binding of these analogs to PGK. The most effective analogs prove to have affinities in the micromolar range. The dominant structural parameters are the separation of the two phosphoryl groups by a four- or five-atom spacer while the presence of a β-carbonyl function appears to be nonessential other than through its contribution to reducing pK_{a^3} in some analogs. In ionization terms, there is a clear requirement for pK_{a^3} to be not greater than 6 and strong indication that complete ionization of the second phosphonate is not essential. These results enable the identification of high affinity, symmetrical bisphosphonate ligands for PGK capable of being incorporated into bisubstrate analogs for PGK in pursuit of the closed conformation of this kinase.

Experimental Section

^{1}H NMR Binding Studies. 3-Phospho-D-glyceric acid (3-PGA) was purchased from Sigma as the trisodium salt, and yeast phosphoglyceric phosphokinase (EC 2.7.2.3) was purchased from Boehringer Mannheim for all experiments. Typically, phosphoglycerate kinase (1 mL, 10 mg) suspension of an ammonium sulfate precipitate) was centrifuged (30 min, 3000g) to form a white pellet. The supernatant was removed, and the pellet resuspended in an extraction buffer (2 mL, 0.1 M TRIS, 0.1 mM EDTA, 6 mM 2-mercaptoethanol, 10 μM PMSF, 5% ammonium sulfate) in a Centricon micro-concentrator with a 30 000 molecular weight cutoff. The protein was then concentrated by centrifuge (30 min, 3000g) to 0.5 mL. The ammonium sulfate was removed and deuterium exchange performed by suspending the protein 4 times in a deuterated buffer (2 mL; 40 mM KCl, 10 mM triethanolamine, in D$_2$O at pH 7.1). It was then centrifuged to a volume of 0.5 mL. The protein solution was split into two, the volume of each half was made up to 500 μL with deuterated buffer, acetone (2 μL, 10% in D$_2$O) was added, and the samples were adjusted to pH 7.1 $\pm$ 0.05 for NMR use. The sample concentration was measured by UV absorbance at 280 nm ($A_{1cm}^{1\%} = 4.9$ mg mL^{-1} and a molar mass of 45 kDa).[41]

One-dimensional proton spectra were recorded at 300 K on a Bruker AMX-500 spectrometer, using a spectral width of 12 500 Hz and 8 k complex data points. Between 320 and 360 scans were run, dependent on the protein concentration. A pulse angle of 90° (8–9 μs) was used with a relaxation delay of 0.6–1.0 s between pulses. A presaturation pulse for suppression of the residual HDO resonance was applied during the delay. The free induction decays were zero-filled to 16 k complex points and the resolution enhanced by Gaussian multiplication (gb = 0.1, lb = −10 Hz). Chemical shifts were determined using acetone as internal reference (2.214 ppm). Aliquots of 1,3-BPG analog solution (1–2 μL, 10 mM, D$_2$O, at pH* 7.1) were added to PGK samples prepared as above. Spectra were recorded up to an analog to enzyme concentration ratio of about 10:1. The pH* determination was repeated after the titration to ensure that pH effects were not involved in shifting the signals.

The change in chemical shifts of H-2 for His-62, His-167, and His-170 versus the volume of analog solution added to a known volume of enzyme solution was fitted using a least squares program written by Dr. Chris Hunter (University of Sheffield) for Apple Macintosh computers. The same results were obtained by using a Microsoft Excel 5.0 worksheet written to perform a least squares solver routine analysis. Both methods gave the same calculated values for K_d and $\Delta\delta_{max}$. Dissociation constants were not determined when the change in chemical shift was less than 10 ppb as data from the spectrometer were insufficiently accurate.

Chemical Synthesis. Melting points were measured on a Koffler hot stage micromelting point apparatus and are uncorrected. Boiling points were recorded from a Kugelröhr distillation apparatus and are uncorrected. Elemental analyses were determined on a Perkin-Elmer 2400 CHN elemental analyzer. Infrared spectra were recorded upon a FT-IR Paragon 1000 spectrometer as thin films or potassium bromide disks.

NMR spectra of analogs were recorded on a Bruker AC-250 spectrometer with a QNP probe, unless indicated otherwise where a Bruker BMX-400 or AMX-500 instrument was used. Spectra were recorded in CDCl$_3$ solution unless otherwise indicated. Coupling constants were measured in Hertz (Hz). Spectra are reported using the δ scale in parts per million, and proton chemical shifts are accurate to ±0.01 ppm. Phosphorus, fluorine, and carbon chemical shifts are accurate to ±0.1 ppm. Proton spectra have been calibrated by either external tetramethylsilane or via internal solvent reference. Phosphorus NMR spectra were recorded on the above machines at 101, 161, or 202 MHz, respectively, and are referenced downfield from external 85% H$_3$PO$_4$. Fluorine NMR spectra were recorded on the Bruker AC-250 or AMX-500 at 235 and 470 MHz, respectively, and are referenced downfield from external CFCl$_3$. Carbon NMR spectra were recorded on the above machines at 62.9, 101, or 126 MHz, respectively, and are referenced to external tetramethylsilane or via internal solvent reference. All D$_2$O spectra were referenced

to external 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Mass spectra were obtained on a Kratos MS80 mass spectrometer in conjunction with a DS90 data station using chemical ionization with ammonia as the reagent gas (CI) or electron impact (EI). Fast atom bombardment (FAB) spectra were obtained on a Kratos MS80RF machine together with the DS90 data station. Electrospray (ES) mass spectra were recorded on a Fisons V.G. Platform instrument.

Solvents were purified as follows. Tetrahydrofuran (THF), diethyl ether (Et$_2$O), and 1,2-dimethoxyethane (monoglyme) were heated under reflux over potassium and benzophenone and distilled immediately prior to use. Toluene was refluxed over calcium hydride and distilled. Dichloromethane (DCM) was heated under reflux with phosphorus pentoxide and distilled. Triethylamine, diisopropylamine, and pyridine were each heated under reflux with potassium hydroxide and distilled. Methanol (MeOH), ethanol (EtOH), and 2-propanol (^{i}PrOH) were heated under reflux over magnesium and iodine; once the iodine color disappeared further alcohol was added, refluxed, and finally distilled. Petroleum ether (petrol, boiling range = 40/60 °C) and ethyl acetate (EtOAc) were distilled from anhydrous potassium carbonate. Hexane was used without distillation. Triisopropyl and triethyl phosphites were stirred over sodium for 16 h and then distilled. Diisopropyl and diethyl phosphites were distilled under nitrogen before use. Diisopropyl difluoromethanephosphonate, diisopropyl phosphonodifluoroethanoic acid, and diisopropyl phosphon- odifluoromethylzinc bromide were prepared using literature methods[42−44] except for the use of toluene as solvent for preparation of diisopropyl difluoromethanephosphonate.

TLC R_f values were obtained using Merck DC-Alufolien 60 F 254 TLC plates. Detection was by phosphomolybdic acid (20% w/v in EtOH) dip developed by heating with a hot air gun. Alternatively, UV or potassium permanganate spray were used where applicable. All primary products were homogeneous by TLC analysis on silica with solvent systems DCM/MeOH (95:5) and hexane/EtOAc/AcOH (54:45:1). Flash chromatography was performed on Kieselgel 60 230−400 mesh (Merck 9385). Dry flash chromatography was performed on Merck 60 GF$_{254}$ mesh silica.

pK_a values were calculated from the pH titration profile of the free phosphonic acids, recorded using a Radiometer TTT 80 titrator, PHM 82 standard pH meter, GK2401B combined glass electrode, ABU 80 autoburet, REC 80 Servograph, and SAM 90 sample station. The data were analyzed using a multiple pK_a analysis program written for Kaleidagraph on an Apple Macintosh Quadra giving an effective resolution of Δp$K_a \geq 0.3$ with an error of ±0.05.

Experimental data, where applicable, are presented in the following order: boiling point; melting point; R_f value; pK_a values; IR spectroscopy data; hydrogen NMR values; phosphorus NMR values; fluorine NMR values; carbon NMR values; mass spectroscopy data; and quantitative analysis results.

Tetraethyl Pentane-1,5-bisphosphonate (1). This was prepared as described[45] to give the product, purified by distillation to yield a colorless mobile oil (22.5 g, 65%): bp 174− 176 °C/0.01 mmHg (lit[45] bp 180−181 °C/0.1 mmHg); δ_H 1.25 (12 H, t, $^3J_{HH}$ 7.5, 4 × CH_3), 1.35−1.76 (10 H, m, 5 × CH_2), 3.95−4.10 (8 H, m, 4 × OCH_2CH$_3$); δ_P 32.5 (1 P, s); m/z (EI+) 344 (M$^+$, 9). Found: M$^+$ 344.1513. C$_{13}$H$_{30}$O$_6$P$_2$ requires M, 344.1514.

Pentane-1,5-bisphosphonic Acid (2). Ester (1) (4.0 g, 12 mmol) was heated under reflux with HCl (30 mL, 6 M) for 10 h. The reaction mixture was cooled to room temperature and coevaporated with methanol (3 × 50 mL). The white solid was dried in a vacuum desiccator over phosphorus pentoxide for 16 h to yield white crystals (2.8 g, 96%): mp 154−156 °C, (lit[45] mp 155 °C); pK_a^3 7.33, pK_a^4 8.76; ν_{max}(KBr)/cm^{-1} 1220 (P=O); δ_H (D$_2$O) 1.30−1.65 (10 H, m, 5 × CH_2); δ_P (D$_2$O) 32.8 (s).

Tetraisopropyl 3-Oxapentane-1,5-bisphosphonate (3). Triisopropyl phosphite (11.2 g, 54 mmol) was heated with bis- (2-bromoethyl) ether (5.0 g, 22 mmol) at 150 °C for 16 h under argon.[46] The reaction mixture was allowed to cool and was

purified by dry column flash chromatography with gradient elution (DCM−MeOH, from 100:0 to 95:5) to yield the title compound as a colorless mobile oil (7.5 g, 86%): bp 190−195 °C/0.2 mmHg; δ_H 1.25 (24 H, d, $^3J_{HH}$ 7.0, 8 × CH$_3$), 2.0 (4 H, ddd, $^2J_{HP}$ 18.9, $^3J_{HH}$ 7.5, 8.1, 2 × CH$_2$P), 3.57 (4 H, dt, $^3J_{HP}$ 9.0, $^3J_{HH}$ 7.5, 2 × CH$_2$CH$_2$P), 4.62 (4 H, octet, $^3J_{HH}$ 7.5, $^3J_{HP}$ 7.5, 4 × CH); δ_P 26.2 (2 P, s); δ_C 24.0, 23.9 (8 C, s, 8 × CH$_3$), 28.1 (2 C, d, $^2J_{PC}$ 140.4, 2 × PCH$_2$) 64.9 (2 C, s, 2 × CH$_2$), 70.1, 70.0 (4 C, s, 4 × CH); m/z (CI+) 403 ([M + H]$^+$, 15); (EI+) 403 ([M + H]$^+$, 50). Found: C, 46.86; H, 8.82. C$_{16}$H$_{36}$O$_7$P$_2$·0.5H$_2$O requires C, 46.71; H, 9.06. Found: M$^+$ 403.2015. C$_{16}$H$_{37}$O$_7$P$_2$ requires M, 403.2016.

3-Oxapentane-1,5-bisphosphonic Acid (4). Ester (3) (4.0 g, 10 mmol) was refluxed for 16 h in HCl (30 mL, 6 M). The volatiles were evaporated in vacuo and the crude bisphospho- nic acid coevaporated with MeOH (5 × 10 mL), triturated with Et$_2$O (20 mL), and finally lyophilized to yield the title compound as a white crystalline solid (2.0 g, 85%): mp 121− 123 °C (lit[46] mp 98−105); pK_a^3 6.90, pK_a^4 8.32; ν_{max}(KBr)/cm^{-1} 1230 (P=O); δ_H (D$_2$O) 1.75 (4 H, dt, $^3J_{HH}$ 6.3, $^3J_{HP}$ 18.8, 2 × PCH$_2$), 3.47 (4 H, dt, $^3J_{HH}$ 6.3, $^3J_{HP}$ 15.6, 2 × CH $_2$CH$_2$P); δ_P (D$_2$O) 27.9 (1 P, s); m/z (FAB+) 235 ([M + H]$^+$, 100%).

Tetraisopropyl Hexane-1,6-bisphosphonate (5). This was prepared as described[45] and purified by Kugelröhr distil- lation to yield the title compound as a colorless oil (6.16 g, 85%): bp 220−240 °C/0.5 mmHg (lit[45] bp 185 °C/0.1 mmHg); R_f (DCM−MeOH, 95:5) 0.2; δ_H 1.00−1.70 (36 H, m, 6 × CH_2, 8 × CH_3), 4.50−4.70 (4 H, m, CH); δ_P 30.7 (2 P, s); m/z (EI+) 414 (M$^+$, 50). Found: M$^+$ 414.2300. C$_{18}$H$_{40}$O$_6$P$_2$ requires M, 414.2297.

Hexane-1,6-bisphosphonic Acid (6). Ester (5) (1.00 g, 2.42 mmol) was refluxed for 16 h in HCl (20 mL, 6 M). Volatiles were evaporated in vacuo, and the crude bisphos- phonic acid was triturated with Et$_2$O (20 mL) and dried to yield the title compound as a white crystalline solid (0.67 g, 99%): mp 191−193 °C, (lit[45] mp 206−208 °C); pK_a^3 7.24; pK_a^4 8.41; ν_{max}(KBr)/cm^{-1} 1245 (P=O); δ_H 1.25−1.80 (12 H, m, 6 × CH_2); δ_P (D$_2$O) 32.9 (2 P,s); m/z (ES+) 247 ([M + H]$^+$, 97). Found: C, 29.35; H, 6.46. C$_6$H$_{16}$O$_6$P$_2$ requires C, 29.23; H, 6.55.

Tetraisopropyl 1,1,3,3-Tetrafluoro-2,2-dihydroxypro- pane-1,3-bisphosphonate (7). The title compound was prepared as described[48] as a colorless liquid (1.2 g, 10%): mp 61−62 °C; δ_H 1.37 (12 H, d, $^3J_{HH}$ 7.5, 4 × CH_3), 1.42 (12 H, d, $^3J_{HH}$ 7.5, 4 × CH$_3$), 4.80 (4 H, octet, $^3J_{HP}$ 7.5, $^3J_{HH}$ 7.5, 4 × CH); δ_P (AMX-500) 2.7−4.7 (m); δ_F −118.5 to −119.5 (m); m/z (CI+) 488 (M$^+$, 60). Found: C, 37.95; H, 6.28. C$_{15}$H$_{30}$F$_4$O$_8$P$_2$ requires C, 37.82; H, 6.35.

1,1,3,3-Tetrafluoro-2,2-dihydroxypropane-1,3-bispho- sphonic Acid (8). Ester 7 (2.0 g, 4.2 mmol) was refluxed for 16 h in HCl (20 mL, 6 M). The volatiles were evaporated in vacuo and the crude bisphosphonic acid coevaporated with MeOH (3 × 20 mL) and dried to yield the title compound as a white hygroscopic glass (1.15 g, 95%): pK_a^3 4.22; pK_a^4 5.57; ν_{max}(KBr)/cm^{-1} 1230 (P=O); δ_P (D$_2$O) 3.4 (2 P, t, $^2J_{FP}$ 91.8); δ_F (D$_2$O) −123.1 (4 F, dt, $^2J_{FP}$ 92.0, $^4J_{FP}$ 12.1).

Tetraisopropyl 1,1,5,5-Tetrafluoro-3-oxapentane-1,5- bisphosphonate (10). LDA (25 mL, 2 M, 50 mmol) was added dropwise to a solution of diisopropyl difluoromethane- phosphonate (10.0 g, 46 mmol) in THF (60 mL) at −60 °C and stirred for 1 h. The anion solution was then introduced by cannula into a solution of bis(2-chloroethyl) ether (9) (2.65 g, 23 mmol) in THF (60 mL) at −60 °C and allowed to warm to room temperature. The reaction mixture was then poured into HCl (200 mL, 1 M) and extracted with DCM (3 × 100 mL). The combined organic extracts were washed with brine (100 mL), dried (Na$_2$SO$_4$), and filtered, and the solvent was evapo- rated in vacuo to yield a thick brown oil. This was purified by dry column flash chromatography with gradient elution (DCM−MeOH, from 100:0 to 95:5) which gave a mixture of three compounds: tetraisopropyl difluoromethylenebisphos- phonate, the desired product, and an unknown which all coeluted. These were separated by repeated Kugelröhr distil- lation taking the fraction boiling in the range 180−200 °C/1 mmHg, to yield the title compound as a pale-yellow oil (1.8 g,

16%): δ_H 1.30 (12 H, d, $^3J_{HH}$ 7.0, 4 × CH_3), 1.32 (12 H, d, $^2J_{HH}$ 7.0, 4 × CH_3), 3.97 (4 H, dt, $^3J_{HH}$ 7.0, $^3J_{HF}$ 16.0, 2 × CF$_2$CH_2), 4.80 (4 H, octet, $^3J_{HP}$ 7.0, $^3J_{HH}$ 7.0, 4 × CH); δ_P 4.0 (2 P, t, $^2J_{FP}$ 100.1); δ_F −120.0 (4 F, d, $^2J_{FP}$ 100.1); δ_C 23.6, 23.7, 24.0, 24.1 (8 C, s, 8 × CH_3), 71.5 (2 C, dt, $^2J_{PC}$ 18.0, $^2J_{FC}$ 24.3, 2 × CH_2), 73.9, 74.0 (4 C, s, 4 × CH), 117.6 (2 C, dt, $^1J_{PC}$ 211.0, $^1J_{FC}$ 262.8, 2 × CF_2); *m/z* (EI+) 474 (M$^+$, 1.4).

1,1,5,5-Tetrafluoro-3-oxapentane-1,5-bisphosphonic Acid (11). Ester **10** (1.79 g, 3.8 mmol) was refluxed for 16 h in HCl (40 mL, 6 M). The volatiles were evaporated in vacuo, and the crude bisphosphonic acid was coevaporated with MeOH (3 × 10 mL) before being dissolved in water (10 mL), extracted with DCM (3 × 10 mL), and lyophilized to yield the title compound as white solid (1.12 g, 97%): mp 50−52 °C; pK_a^3 4.72; pK_a^4 5.62; ν_{max}(KBr)/cm^{-1} 1290 (P=O); δ_H (D$_2$O) 3.90 (4 H, dt, $^3J_{HF}$ 14.4, $^3J_{HP}$ 5.0, 2 × CH_2CF$_2$); δ_P (D$_2$O) 3.2 (2 P, t, $^2J_{FP}$ 91.3); δ_F (D$_2$O) −122.4 (4 F, dt, $^3J_{HF}$ 15.7, $^3J_{FP}$ 91.2, 2 × CH$_2$CF_2).

Tetraisopropyl 1,1,5,5-Tetrafluoropentane-1,5-bisphosphonate (12). Diisopropyl difluoromethanephosphonate (10.0 g, 46 mmol) as a solution in THF (60 mL) was added dropwise to a stirred solution of LDA (25 mL, 50 mmol, 2 M solution in heptane/THF) at −60 °C under argon. After 30 min, the anion solution was introduced by cannula into a stirred solution of 1,3-dibromopropane (4.66 g, 23 mmol) in THF (50 mL) at −60 °C and brought to room temperature. The reaction mixture was then poured into HCl (200 mL, 1 M) and extracted with DCM (3 × 100 mL). The combined organic extracts were washed with brine (100 mL), dried (Na$_2$SO$_4$), and filtered, and the solvent was evaporated in vacuo to yield a thick brown oil. The crude bisphosphonate was further purified by dry column flash chromatography with gradient elution DCM−MeOH, from 100:0 to 98:2, to give the title compound as a yellow oil (6.8 g, 63%): δ_H 1.30 (12 H, d, $^3J_{HH}$ 6.3, 4 × CH$_3$), 1.33 (12 H, d, $^3J_{HH}$ 6.3, 4 × CH$_3$), 1.77−1.90 (2 H, m, CH$_2$CH_2-CH$_2$), 1.92−2.20 (4 H, m, 2 × CF$_2$CH$_2$), 4.77 (2 H, octet, $^3J_{HH}$ 6.3, $^3J_{HP}$ 6.3, 2 × CH); δ_P 5.7 (2 P, t, $^2J_{FP}$ 108.2); δ_F −113.8 (4 F, dt, $^2J_{FP}$ 108.2, $^3J_{HF}$ 19.6, 2 × CF_2); δ_C 12.6 (1 C, t, $^3J_{FC}$ 5.4, CH$_2$CH_2CH$_2$), 23.6, 23.7, 24.0, 24.1 (4 C, s, 4 × CH$_3$), 33.2 (2 C, dt, $^2J_{FC}$ 21.2, $^2J_{PC}$ 14.6, 2 × CH$_2$CF$_2$P), 73.4, 73.5 (4 C, s, 4 × CH), 120.1 (2 C, dt, $^1J_{FC}$ 259.6, $^1J_{PC}$ 217.6, 2 × CF_2); *m/z* (EI+) 473 ([M + H]$^+$, 30). Found: M$^+$ 473.1845. C$_{17}$H$_{35}$F$_4$O$_6$P$_2$ requires M, 473.1848.

1,1,5,5-Tetrafluoropentane-1,5-bisphosphonic Acid (13). Compound **12** (3.6 g, 7.6 mmol) was refluxed for 16 h in HCl (30 mL, 6 M). The volatiles were evaporated in vacuo, and the crude bisphosphonic acid was coevaporated with MeOH (5 × 10 mL) before being triturated with Et$_2$O (20 mL) to give a brown solid. This was taken up in EtOAc, boiled with activated charcoal, filtered, and dried to yield the title compound as an off-white solid (2.0 g, 86%): mp 142−144 °C; pK_a^3 5.09; pK_a^4 5.87; ν_{max}(KBr)/cm^{-1} 1274 (P=O); δ_H (D$_2$O) 1.15−1.35 (2 H, m, CH$_2$CH$_2$CH$_2$), 1.35−1.70 (4 H, m, 2 × CF$_2$CH$_2$); δ_P (D$_2$O) 5.5 (2 P, t, $^2J_{FP}$ 102.4); *m/z* (FAB+) 305 ([M + H]$^+$, 100).

Diisopropyl 1,1-Difluoro-5-bromopentane-1-phosphonate (14). Diisopropyl difluoromethanephosphonate (10 g, 46 mmol) as a solution in THF (60 mL) was added to a stirred solution of LDA (25 mL, 50 mmol, 2 M solution in heptane/THF) at −60 °C under argon. After 30 min, the anion solution was introduced by cannula into a stirred solution of 1,4-dibromobutane (9.76 g, 46 mmol) in THF (50 mL) at −60 °C and slowly brought to room temperature. The reaction mixture was then poured into HCl (200 mL, 1 M) and extracted with DCM (3 × 100 mL). The combined organic extracts were washed with brine (100 mL), dried (Na$_2$SO$_4$), and filtered, and the solvent was evaporated in vacuo to yield a thick brown oil. Kugelröhr distillation (90−100 °C/0.2 mmHg) gave the crude bromophosphonate which was purified by dry column flash chromatography eluting with DCM to yield the title compound as a colorless mobile liquid (4.2 g, 26%): δ_H 1.30 (12 H, d, $^3J_{HH}$ 6.3, 4 × CH_3), 1.55−2.18 (6 H, m, CF$_2$CH_2CH_2CH_2), 3.38 (2 H, t, $^3J_{HH}$ 6.8, CH_2Br), 4.80 (2 H, octet, $^3J_{HH}$ 6.3, $^3J_{HP}$ 6.3, 2 × CH); δ_P 5.6 (1 P, t, $^2J_{FP}$ 108.5); δ_F −113.2 (2 F, dt, $^2J_{FP}$

108.6, $^3J_{HF}$ 19.6, CF_2); *m/z* (EI+) 351 ([M + H]$^+$, 12, ^{79}Br), 353 ([M + H]$^+$, 12, ^{81}Br).

Tetraisopropyl 1,1-Difluoropentane-1,5-bisphosphonate (15). Ester **14** (4.0 g, 11 mmol) was heated at 150 °C with triisopropyl phosphite (2.37 g, 11 mmol) under argon for 16 h. The unreacted starting materials were evaporated by Kugelröhr distillation (125 °C/0.2 mmHg), and the crude bisphosphonate was purified by dry column flash chromatography with gradient elution DCM−MeOH, from 100:0 to 99:1, to give the title compound as a colorless oil (2.4 g, 50%): bp >200 °C/0.2 mmHg (decomp.); δ_H 1.20 (12 H, d, $^3J_{HH}$ 7.5, 4 × CH$_3$), 1.28 (12 H, dd, $^3J_{HH}$ 7.5, $^4J_{HH}$ 3.8, 4 × CH$_3$), 1.45−1.70 (6 H, m, CH$_2$CH$_2$CH$_2$P), 1.80−2.10 (2 H, m, CF$_2$CH$_2$), 4.50−4.67 (2 H, m, 2 × CH), 4.73 (2 H, octet, $^3J_{HH}$ 7.5, $^3J_{HP}$ 7.5, 2 × CH); δ_P 6.1 (1 P, t, $^2J_{FP}$ 108.9, PCF$_2$), 30.1 (1 P, s, PCH$_2$); δ_F −113.5 (2 F, dt, $^2J_{FP}$ 109.0, $^3J_{HF}$ 19.7); δ_C 21.0−21.8 (1 C, m, CF$_2$CH$_2$CH$_2$), 23.6, 23.7, 24.0, 24.1 (8 C, s, 8 × CH$_3$), 26.7 (1 C, d, $^1J_{PC}$ 142.6, CH$_2$P), 33.3 (1 C, dt, $^2J_{FC}$ 21.1, $^2J_{PC}$ 14.4, CF$_2$-CH$_2$), 69.7, 69.8, (2 C, s × CH$_2$P(P)OOCH), 73.3, 73.4 (2 C, s, 2 × CF$_2$P(O)OCH), 122.3 (1 C, dt, $^1J_{FC}$ 259.2, $^1J_{PC}$ 217.3, CF$_2$); *m/z* (EI+) 437 ([M + H]$^+$, 30). Found: C, 21.63; H, 4.84. Requires C, 22.40; H, 4.51. Found: M$^+$ 437.2033. C$_{17}$H$_{37}$F$_2$O$_6$P$_2$ requires M, 437.2039.

1,1-Difluoropentane-1,5-bisphosphonic Acid (16). Ester **15** (1.10 g, 2.6 mmol) was refluxed for 16 h in HCl (20 mL, 6 M). The volatiles were evaporated in vacuo, and the crude bisphosphonic acid was dissolved in water (10 mL) and extracted with DCM (3 × 10 mL) and EtOAc (2 × 10 mL). Lyophilization furnished the title compound as white solid (0.72 g, 99%): mp 158−160 °C; pK_a^3 5.40 (CF$_2$PO$_3$H), pK_a^4 7.78 (CH$_2$PO$_3$H); δ_H (D$_2$O) 1.25−1.65 (6 H, m, PCH$_2$CH$_2$CH$_2$), 1.65−1.94 (2 H, m, CF$_2$CH$_2$); δ_P (D$_2$O) 5.8 (1 P, t, $^2J_{FP}$ 101.3, PCF$_2$), 32.5 (1 P, s, PCH$_2$); δ_C (D$_2$O) 21.2 (1 C, dd, $^3J_{PC}$ 5.0, $^3J_{PC}$ 18.4, PCH$_2$CH$_2$CH_2), 21.6 (1 C, d, $^2J_{PC}$ 4.5, PCH$_2$CH$_2$), 25.6 (1 C, d, $^1J_{PC}$ 134.1, PCH$_2$), 32.5 (1 C, dt, $^2J_{FC}$ 21.2, $^2J_{PC}$ 14.4, CH$_2$CF$_2$), 120.0 (1 C, dt, $^1J_{FC}$ 256.8, $^1J_{PC}$ 201.6, CF$_2$), *m/z* (ES+) 269 ([M + H]$^+$, 100).

Tetraisopropyl 1,1,6,6-Tetrafluorohexane-1,6-bisphosphonate (17). The Kugelröhr distillate from the preparation of (**14**) was purified by dry column flash chromatography eluting with DCM providing a pale-yellow oil which solidified on standing to give the title compound as a waxy white solid (3.0 g, 13%): bp > 200 °C/0.2 mmHg (decomp.); mp 54−56 °C; δ_H 1.30 (12 H, d, $^3J_{HH}$ 6.3, 4 × CH$_3$), 1.33 (12 H, d, $^3J_{HH}$ 6.3, 4 × CH$_3$), 1.50−1.68 (4 H, m, CF$_2$CH_2CH_2CH$_2$), 1.85−2.13 (4 H, m, 2 × CH$_2$CF$_2$), 4.78 (4 H, octet, $^3J_{HH}$ 6.3, $^3J_{HP}$ 6.3, 4 × CH); δ_P 5.8 (1 P, t, $^2J_{FP}$ 109.4); δ_F −113.4 (4 F, dt, $^2J_{FP}$ 109.9, $^3J_{HF}$ 19.7, 2 × CF$_2$); δ_C 20.4, 20.5 (2 C, s, 2 × CF$_2$CH$_2$CH$_2$), 23.6, 23.7, 23.9, 24.0 (8 C, s, 8 × CH$_3$), 33.5 (1 C, dt, $^2J_{FC}$ 20.9, $^2J_{PC}$ 14.4, 2 × CF$_2$CH$_2$), 73.5, 73.6 (4 C, s, 4 × CH), 120.2 (1 C, dt, $^1J_{FC}$ 259.3, $^1J_{PC}$ 217.9, CF$_2$); *m/z* (EI+) 487 ([M + H]$^+$, 35). Found: C, 44.56; H, 7.66. Requires C, 44.45; H, 7.46.

1,1,6,6-Tetrafluorohexane-1,6-bisphosphonic Acid (18). Ester **17** (3.0 g, 6.2 mmol) was refluxed for 16 h in HCl (30 mL, 6 M). The volatiles were evaporated in vacuo and the crude bisphosphonic acid was coevaporated with MeOH (5 × 10 mL) before being triturated with Et$_2$O (20 mL) and dried to yield the title compound as white powder (1.78 g, 91%): mp 198−201 °C; pK_a^3 4.95, pK_a^4 5.55; ν_{max}(KBr)/cm^{-1} 1250 (P=O); δ_H 1.45−1.70 (4 H, m, 2 × CH_2CH$_2$CF$_2$), 1.80−2.15 (4 H, m, 2 × CF$_2$CH$_2$); δ_P (D$_2$O) 6.0 (2 P, t, $^2J_{FP}$ 100.6); *m/z* (FAB+) 319 ([M + H]$^+$, 100). Found C, 21.62; H, 4.24. C$_6$H$_{16}$F$_4$O$_6$P$_2$ requires C, 22.66; H, 3.80.

Diethyl 2-Aminoethyl-1-phosphonate (19). Adams catalyst (PtO$_2$ × H$_2$O) (0.1 g, 10 wt %) was covered with ethanol (20 mL), and diethyl cyanomethylphosphonate (1.0 g, 5.64 mmol) was added as a solution in ethanol (40 mL) followed by HCl (1 mL, concentrated). The reaction was stirred under hydrogen (1 atm) until gas uptake was complete (275 mL). The solution was filtered through Celite and the solvent evaporated in vacuo. The resulting white crystals were dissolved in DCM (30 mL) and washed with NaOH (30 mL, 1 M). The DCM layer was dried (MgSO$_4$), filtered, and evaporated in vacuo to yield the title compound as a colorless mobile oil (1.0 g, 98%): δ_H

1.3 (6H, t, $^3J_{HH}$ 7.5, $2 \times CH_3$), 1.55 (2H, bs, CH$_2$NH_2), 1.9 (2H, dt, $^2J_{HP}$ 18.8, $^3J_{HH}$ 7.5, PCH_2), 2.8 (2H, dt, $^3J_{HP}$ 15.8, $^3J_{HH}$ 7.5, CH_2NH$_2$), 4.05 (4H, qn, $^3J_{HH}$ 7.5, $^3J_{HP}$ 7.5, $2 \times$ CH$_3$CH_2); δ_P 30.8 (1 P, s). HCl salt analysis: Found: C, 31.89; H, 7.98; N, 6.51. C$_6$H$_{17}$ClNO$_3$P0.5H$_2$O requires C, 31.82; H, 7.57; N, 6.12%.

Diethyl 1-Chloro-2-oxo-3-azapentane-5-phosphonate (20). Triethylamine (8.36 mL, 60 mmol) was added dropwise to a solution of chloroacetic anhydride (6.30 g, 36 mmol) and **19** (6.51 g, 30 mmol, as hydrochloride salt) in DCM (150 mL, degassed) at 0 °C under argon. After 4 h the reaction mixture was poured into dilute NaOH (200 mL, 1 M) and extracted with DCM (2 × 100 mL). The combined organic layers were washed with HCl (2 × 50 mL, 1 M), dried (Na$_2$SO$_4$), filtered, and evaporated in vacuo to yield the title compound as an orange oil (3.00 g, 40%): R_f (DCM−MeOH, 95:5) 0.36; δ_H 0.95 (6 H, t, $^3J_{HH}$ 6.3, $2 \times CH_3$), 1.65 (2 H, dt, $^3J_{HH}$ 6.5, $^3J_{HP}$ 18.8, CH_2P), 3.17 (2 H, dq, $^3J_{HH}$ 6.5, $^3J_{HP}$ 18.0, CH_2CH$_2$P), 3.63 (2 H, s, CH_2Cl), 3.60−3.73 (4 H, m, $2 \times$ CH_2CH$_3$), 7.23 (1 H, bt, NH); δ_P 29.4 (1 P, s); δ_C 16.2, 16.3 (2 C, s, $2 \times$ CH$_3$), 25.2 (1 C, d, $^1J_{PC}$ 140.1, CH$_2$P), 33.7 (1 C, s, CH$_2$NH), 42.4 (1 C, s, CH$_2$-Cl), 61.7, 61.8 (2 C, s, $2 \times$ CH$_2$CH$_3$), 166.0 (1 C, s, CO); m/z (EI+) 257 (M+, 15, ^{35}Cl), 259 (M+, 5, ^{37}Cl). The acidic washings were evaporated in vacuo to yield diethyl 2-amino-ethyl-1-phosphonate (**19**) hydrochloride (3.9 g).

P^1-Diisopropyl P^5-Diethyl 2-Oxo-3-azapentane-1,5-bis-phosphonate (21). Ester (**20**) (2.54 g, 10 mmol) was heated for 16 h at 130 °C with triisopropyl phosphite (4.9 mL, 20 mmol) under argon. Volatiles were evaporated under reduced pressure (40 °C/1 mmHg), and the crude bisphosphonate (3.2 g) was purified by dry column flash chromatography with gradient elution DCM−MeOH, from 100:0 to 50:50, to yield the title compound as a yellow oil (2.97 g, 77%); R_f (DCM−MeOH, 95:5) 0.56; δ_H 1.00−1.20 (18 H, m, $6 \times$ CH_3), 1.75 (2 H, dt, $^3J_{HH}$ 6.3, $^3J_{HP}$ 18.8, CH$_2$CH_2P), 2.57 (2 H, d, $^2J_{HP}$ 21.9, COCH_2P), 3.75−3.40 (4 H, m, $2 \times$ CH_2CH$_3$), 4.35−4.55 (2 H, m, $2 \times$ CH), 7.22 (1 H, bt, NH); δ_P 20.8 (1 P, s, PCH$_2$CO), 29.4 (1 P, s, PCH$_2$CH$_2$); m/z (EI+) 387 (M+, 22). Found: M+ 387.1576. C$_{14}$H$_{31}$NO$_7$P$_2$ requires M, 387.1572.

2-Oxo-3-azapentane-1,5-bisphosphonic Acid (22). Bro-motrimethylsilane (1.12 mL, 8.4 mmol) was added dropwise to a solution of ester (**21**) (467 mg, 1.2 mmol) in DCM (20 mL) under argon. After 24 h, ^{31}P NMR indicated the reaction had reached completion; 7.0 (1 P, s, PCH$_2$CO) and 14.0 (1 P, s, PCH$_2$CH$_2$). The volatiles were evaporated in vacuo and the resulting gum solvolyzed with MeOH (3 × 15 mL). The bisphosphonic acid was dissolved in water (15 mL) and titrated to pH* 7.1 with NaOH (1 M). Extraction with DCM (2 × 50 mL) and EtOAc (2 × 50 mL) followed by lyophilization furnished the title compound as a hygroscopic glass (312 mg, 89%); ν_{max}(KBr)/cm^{-1} 1635 (C=O), 1240 (P=O); pK_a^3 5.62 (PCH$_2$COCH$_2$PO$_3$H); pK_a^4 6.60 (CH$_2$PO$_3$H); δ_H (D$_2$O) 1.65−1.83 (2 H, m, PCH_2CH$_2$), 2.55 (2 H, d, $^2J_{HP}$ 18.8, PCH_2O), 3.35 (2 H, q, $^3J_{HH}$ 6.7, $^3J_{HP}$ 6.7, PCH$_2$CH_2); δ_P (D$_2$O) 13.0 (1 P, s, PCH$_2$-CO), 20.7 (1 P, s, PCH$_2$CH$_2$); δ_C 28.3 (1 C, d, $^2J_{PC}$ 7.3, s, PCH$_2$CH$_2$), 36.6 (1 C, d, $^1J_{PC}$ 114.5, PCH$_2$), 117.1 (1 C, s, CO); m/z (ES−) 290 ([M + 2Na − H]+, 5); 268 ([M + Na − H]+, 78), 246 ([M − H]+, 100).

P^1-Diisopropyl P^5-Diethyl 1,1-Difluoro-2-oxo-3-aza-pentane-1,5-bisphosphonate (23). Oxalyl chloride (20.7 g, 165 mmol, freshly distilled) was syringed slowly into a solution of diisopropyl phosphonodifluoroethanoic acid (13.9 g, 54 mmol) in DCM (50 mL) under nitrogen at 0 °C. The reaction mixture was stirred at room temperature for 16 h. The volatiles were evaporated under reduced pressure (0.2 mmHg). Compound **19** (11.4 g, 53 mmol, as its HCl salt) was added at −20 °C (ice/NaCl) followed by dropwise addition of triethyl-amine (10.9 g, 108 mmol). The reaction was stirred 5 h, and the volatiles were evaporated in vacuo. The residue was dissolved in Et$_2$O (40 mL, dry) and filtered, and the organic layer was washed with citric acid (100 mL, 20%), NaHCO$_3$ (100 mL, 1 M), and brine (100 mL) and then dried (Na$_2$SO$_4$). The solvent was evaporated in vacuo, and the crude product (17.9 g) was purified by dry column flash chromatography with a

gradient of DCM−MeOH (from 100:0 to 85:15). This gave the title compound as a clear oil (11.2 g, 50%): R_f (DCM−MeOH, 95:5) 0.55; δ_H 1.30 (6 H, t, $^3J_{HH}$ 7.5, $2 \times$ CH$_2$CH$_3$), 1.35 (12 H, d, $^3J_{HH}$ 7.5, $4 \times$ CHCH$_3$), 2.00 (2 H, dt, $^3J_{HP}$ 18.0, $^3J_{HH}$ 7.5, CH$_2$P), 3.60 (2 H, dq, $^3J_{HP}$ 18.0, $^3J_{HH}$ 7.5, NHCH_2CH$_2$P), 4.00−4.20 (4 H, m, $2 \times$ CH$_2$CH$_3$), 4.85 (2 H, octet, $^3J_{HP}$ 7.5, $^3J_{HH}$ 7.5, $2 \times$ CH), 7.37 (1 H, bt, NH); δ_P 1.9 (1 P, t, $^2J_{FP}$ 96.2, CF$_2$P), 29.0 (1 P, s, PCH$_2$); δ_F −118.1 (2 F, d, $^2J_{FP}$ 96.0, CF$_2$P); m/z (EI+) 423 (M+, 1). Found: C, 39.39; H, 7.1; N, 3.34. C$_{14}$H$_{29}$F$_2$-NO$_7$P$_2$ requires C, 39.72; H, 6.9; N, 3.31. Found: M+ 423.1387. C$_{14}$H$_{29}$F$_2$NO$_7$P$_2$ requires M, 423.1386.

1,1-Difluoro-2-oxo-3-azapentane-1,5-bisphosphonic Acid (24). Bromotrimethylsilane (2.5 mL, 19 mmol) was added dropwise to a solution of ester **23** (1.10 g, 2.6 mmol) in DCM (25 mL) under argon. After 48 h, ^{31}P NMR indicated the reaction had reached completion, −14.7 (1 P, t, PCF$_2$CO, $^2J_{FP}$ 99.7), 12.5 (1 P, s, PCH$_2$CH$_2$). The volatiles were evaporated in vacuo, and the resulting gum was solvolyzed by MeOH (3 × 15 mL). The bisphosphonic acid was then dissolved in water (15 mL) and titrated to pH 7.1 with NaOH (1 M). Extraction with DCM (2 × 50 mL) and EtOAc (2 × 50 mL) followed by lyophilization furnished the title compound as a white solid (1.38 g, 90%): pK_a^3 4.71 (PCF$_2$); pK_a^4 7.14 (PCH$_2$); ν_{max}(KBr disk)/cm^{-1} 1670 (C=O), 1240 (P=O); δ_H (D$_2$O) 1.90−2.07 (2 H, m, $^2J_{HP}$ 17.5, PCH$_2$), 3.40−3.60 (2 H, m, PCH$_2$CH_2); δ_P (D$_2$O) 1.5 (1 P, t, $^2J_{FP}$ 88.0, PCF$_2$), 23.9 (1 P, s, PCH$_2$); δ_F (D$_2$O) −120.0 (2 F, d, $^2J_{FP}$ 88.0, CF$_2$); δ_C (D$_2$O) 26.8 (1 C, d, $^1J_{PC}$ 132, CH$_2$-PO), 34.6 (1 C, s, CH$_2$CH$_2$PO), 113.9 (1 C, dt, $^1J_{PC}$ 182, $^1J_{FC}$ 268, CF$_2$), 165.4 (1 C, dt, $^2J_{PC}$ 14, $^2J_{FC}$ 25, CO); m/z (FAB+) 372 ([M + 4Na + H]+, 6), 350 ([M + 3Na + H]+, 13), 328 ([M + 2Na + H]+, 5); (ES+) 371 ([M + 4Na]+, 25), 350 ([M + 3Na]+, 50), 328 ([M + 2Na]+, 56), 306 ([M + Na]+, 56).

Diisopropyl 1,1-Difluoro-2-N-phthalimidoethane-1-phosphonate (25). Diisopropyl phosphonodifluoromethyl zinc bromide was prepared using diisopropyl difluorobro-momethanephosphonate (10 g, 34 mmol). After addition of CuBr (0.1 g, 0.7 mmol), bromomethylphthalimide (8.4 g, 35 mmol) was added as a powder at room temperature and stirred for 3 d. The reaction mixture was then poured into water (100 mL) and extracted with DCM (3 × 60 mL). The organic layer was dried (Na$_2$SO$_4$), filtered, and evaporated in vacuo to yield a yellow oil. The crude product was purified by dry column flash chromatography with gradient elution of DCM−petrol (from 7:3 to 1:0) to yield the title compound as a soft white solid (6.38 g, 50%): mp 44−46 °C; δ_H 1.37 (12 H, d, $^3J_{HH}$ 6.5, $4 \times$ CH$_3$), 4.25 (2 H, dt, $^2J_{HP}$ 3.0, $^2J_{HF}$ 16.5, CH$_2$), 4.87 (2 H, octet, $^2J_{HH}$ 6.5, $^2J_{HP}$ 6.5, $2 \times$ CH), 7.77 (4 H, m, ArH); δ_P 3.4 (1 P, t, $^3J_{FP}$ 100.0); δ_F −116.3 (2 F, d, $^3J_{FP}$ 100.0); δ_C 23.6, 23.7, 24.1, 24.2 (4 C, s, $4 \times$ CH$_3$), 39.7 (1 C, dt, $^2J_{FC}$, $^2J_{FC}$ 22.8, CH$_2$-CF$_2$), 74.4, 74.5 (2 C, s, $2 \times$ CH), 116.9 (1 C, dt, $^1J_{PC}$ 212.3, $^1J_{FC}$ 264.2, CH$_2$CF$_2$), 123.6, 131.9, 134.3 (6 C, s, $6 \times$ArC), 167.2 (2 C, s, $2 \times$ CO); m/z (EI+) 375 (M, 0.1); (CI+) 375 (M, 25).

Diisopropyl 1,1-Difluoro-2-aminoethane-1-phospho-nate (26). Hydrazine hydrate (1.63 g, 51 mmol) was added dropwise to a solution of **25** (6.38 g, 17 mmol) in DCM (30 mL). After 1 h, water (30 mL) and p-toluenesulfonic acid (until pH 3) were added. The two phases were filtered and then separated, and the organic layer was extracted with water (3 × 30 mL). The aqueous fractions were combined, basified with NaOH (until pH 10), extracted with DCM (3 × 30 mL), dried (Na$_2$SO$_4$), and evaporated in vacuo to yield a colorless oil. The product was further purified by Kugelröhr distillation to give a colorless oil (2.92 g, 70%): bp 120−125 °C/0.2 mmHg; δ_H 1.27 (6 H, d, $^3J_{HH}$ 6.3, $2 \times$ CH$_3$), 1.42 (2 H, bs, NH$_2$), 1.32 (6 H, d, $^2J_{HH}$ 6.3, $2 \times$ CH$_3$), 3.12 (2 H, dt, $^2J_{HP}$ 7.0, $^2J_{HF}$ 16.5, CH$_2$), 4.80 (2 H, octet, $^3J_{HH}$ 6.3, $^3J_{HP}$ 6.3, $2 \times$ CH); δ_P 5.6 (1 P, t, $^3J_{FP}$ 106.3); δ_F −120.7 (2 F, dt, $^2J_{FP}$ 106.3, $^2J_{HF}$ 17.0, CF$_2$); δ_C 23.7, 23.8, 24.1, 24.1 (4 C, s, $4 \times$ CH$_3$) 45.3 (1 C, dt, $^2J_{FC}$ 23.3, $^2J_{PC}$ 16.5, CH$_2$CF$_2$), 73.6, 73.7 (2 C, s, $2 \times$ CH), 118.7 (1 C, dt, $^1J_{PC}$ 210.0, $^1J_{FC}$ 260.0, CH$_2$CF$_2$); m/z (EI+) 245 (M+, 1).

Diisopropyl 1,1-Difluoro-3-aza-4-oxo-5-chloropentane-1-phosphonate (27). Triethylamine (450 μL, 3.2 mmol) was added dropwise to a solution of chloroacetic anhydride (720 mg, 3.2 mmol) and **26** (790 mg, 3.2 mmol) in DCM (10 mL,

degassed) at 0 °C under argon. After 2 h, the reaction mixture was poured into dilute aqueous NaOH (20 mL, 1 M) and extracted with DCM (2 × 10 mL). The combined organic layers were washed with HCl (2 × 5 mL, 1 M), dried (Na_2SO_4), filtered, and evaporated in vacuo to yield the title compound as a white solid (400 mg, 39%): mp 58−60 °C; R_f (DCM−MeOH, 95:5) 0.30; δ_H 1.34 (6 H, d, $^3J_{HH}$ 6.3, 2 × CH_3), 1.35 (6 H, d, $^3J_{HH}$ 6.3, 2 × CH_3), 3.17 (2 H, dq, $^3J_{HP}$ 6.5, $^3J_{HP}$ 18.0, CH_2CH_2P), 3.82 (2 H, tt, $^3J_{HF}$ 15.6, $^3J_{HH}$ 6.3, $^3J_{HP}$ 6.3, CH_2-NH), 4.03 (2 H, s, CH_2Cl), 4.82 (2 H, octet, $^3J_{HH}$ 6.3, $^3J_{HP}$ 6.5, 2 × CH), 7.24 (1 H, bt, NH); δ_P 3.6 (1 P, t, $^2J_{FP}$ 102.3); m/z (EI+) 321 (M^+, 7, ^{35}Cl), 323 (M^+, 3, ^{37}Cl). Found: C, 37.57; H, 6.04; N, 4.23. Requires C, 37.34; H, 5.95; N, 4.35. Found: M^+ 321.0708. $C_{10}H_{19}ClF_2NO_4P$ requires M, 321.0710.

P^1-Diisopropyl P^5-Diethyl 1,1-Difluoro-3-aza-4-oxopentane-1,5-bisphosphonate (28). Ester **27** (400 mg, 1.2 mmol) was heated for 16 h at 130 °C with triethyl phosphite (425 μL, 2.5 mmol) under argon. Volatiles were removed in vacuo (100 °C/20 mmHg, 5 h), and the crude bisphosphonate was purified by dry column flash chromatography with gradient elution of DCM−MeOH, 100:0 to 50:50, to yield the title compound as a yellow oil (412 mg, 81%): R_f (DCM−MeOH, 95:5) 0.20; δ_H 1.25 (6 H, t, $^3J_{HH}$ 6.3, 2 × CH_2CH_3), 1.30 (12 H, d, $^3J_{HH}$ 6.3, 4 × CH_3), 2.58 (2 H, d, $^2J_{HP}$ 18.8, $COCH_2P$), 3.85 (2 H, tt, $^3J_{HH}$ 6.3, $^3J_{HP}$ 6.3, $^3J_{HF}$ 16.9, CH_2CF_2P) 4.10 (4 H, qn, $^3J_{HP}$ 6.3, $^3J_{HH}$ 6.3, 2 × CH_2CH_3), 4.80 (2 H, octet, $^3J_{HP}$ 6.3, $^3J_{HH}$ 6.3, 2 × CH), 7.17 (1 H, bt, NH); δ_P 4.1 (1 P, t, $^2J_{FP}$ 102.3, PCF_2CH_2), 22.5 (1 P, s, PCH_2CO); δ_F −118.6 (2 F, dt, $^3J_{HF}$ 17.2, $^2J_{FP}$ 102.4, CF_2); δ_C 16.2, 16.3, (2 C, s, 2 × CH_2CH_3), 23.6, 23.7, 24.0, 24.1 (4 C, s, 4 × CH), 35.1 (1 C, d, $^1J_{PC}$ 131.3, CH_2P), 41.6 (1 C, $^2J_{FC}$ 23.0, $^2J_{PC}$ 19.0, CH_2CF_2), 62.7, 62.8 (2 C, s, 2 × CH_2CH_3), 74.1, 74.2 (2 C, s, 2 × CH), 117.3 (1 C, dt, $^1J_{FC}$ 261.8, $^1J_{PC}$ 213.1, CF_2), 164.0 (1 C, d, $^2J_{PC}$ 3.9, CO); m/z (EI+) 423 (M^+, 16). Found: M^+ 423.1387. $C_{14}H_{29}F_2NO_7P_2$ requires M, 423.1388.

1,1-Difluoro-4-oxo-3-azapentane-1,5-bisphosphonic Acid (29). Bromotrimethylsilane (850 μL, 6.5 mmol) was added dropwise to a solution of ester **28** (388 mg, 0.9 mmol) in DCM (10 mL) under argon. After 72 h, additional bromotrimethylsilane (400 μL, 3.1 mmol) was syringed into the reaction, and the reaction was warmed to 37 °C. After 5 h the volatiles were evaporated in vacuo, and the resulting gum was solvolyzed by MeOH (3 × 15 mL). The bisphosphonic acid was dissolved in water (10 mL) and titrated to pH 7.1 with NaOH (1 M). Extraction with DCM (2 × 40 mL) and EtOAc (3 × 40 mL) followed by lyophilization furnished the title compound as a white solid (300 mg, 98%): pK_{a^3} 4.82 (CF_2PO_3H); pK_{a^4} 6.70 (CH_2PO_3H); δ_H (D_2O) 2.68 (2 H, d, $^2J_{HP}$ 18.8, $COCH_2P$), 3.80 (2 H, t, $^3J_{HF}$ 18.1, CH_2CF_2P); δ_P 4.6 (1 P, s, $^2J_{FP}$ 79.8, PCF_2), 13.2 (1 P, s, PCH_2CO); δ_F −119.8 (2 F, dt, $^3J_{HF}$ 17.8, $^2J_{FP}$ 80.3, CF_2); m/z (ES+) 372 ([M + 4Na + H]$^+$, 65); 350 ([M + 3Na + H]$^+$, 80).

Tetraisopropyl 1,1,5,5-Tetrafluoro-2-oxo-3-azapentane-1,5-bisphosphonate (30). Oxalyl chloride (2.18 g, 17 mmol, freshly distilled) was added dropwise to diisopropyl phosphonodifluoroethanoic acid (2.1 g, 8.0 mmol) in DCM (25 mL) under an atmosphere of argon and stirred for 16 h. The volatiles were evaporated in vacuo, and the crude acid chloride was dissolved in a solution of ester **26** (1.9 g, 7.7 mmol) in DCM (25 mL). The reaction mixture was cooled to 0 °C, and triethylamine (0.79 g, 7.8 mmol) was slowly added dropwise and stirred for 10 min. The reaction mixture was evaporated in vacuo, the residue dissolved in ether (30 mL) and filtered, and the ether evaporated. The crude amide was dissolved in DCM (50 mL), washed with HCl (50 mL, 1 M), NaOH (50 mL, 1 M), and brine (50 mL), and then dried (Na_2SO_4). The solution was filtered and the solvent evaporated in vacuo to give the title compound as a pale-yellow liquid which solidified on standing at 0 °C (3.5 g, 89%): mp 18−20 °C; bp >190 °C/0.2 mmHg (decomp.); δ_H 1.30 (12 H, d, $^3J_{HH}$ 6.2, 4 × CH_3), 1.32 (12 H, d, $^3J_{HH}$ 6.2, 4 × CH_3), 3.87 (2 H, tt, $^3J_{HF}$ 15.6, $^3J_{HP}$ 6.3, $^3J_{HH}$ 6.3, CH_2CF_2), 4.80 (4 H, octet, $^3J_{HH}$ 6.2, $^3J_{HP}$ 6.2, 4 × CH), 7.15 (1 H, bt, NH); δ_P 3.9 (1 P, t, $^2J_{FP}$ 99.4, PCF_2CH_2), 1.8 (1 P, t, $^2J_{FP}$ 96.8, PCF_2CO); δ_F −118.2 (2 F, d, $^2J_{FP}$ 96.8,

CF_2CO), −118.4 (2 F, dt, $^2J_{FP}$ 99.4, $^3J_{HF}$ 17.0, CF_2CH_2); δ_C 23.4, 23.6, 24.0 (8 C, s, 8 × CH_3), 41.6 (1 C, dt, $^2J_{FC}$ 24.0, $^2J_{PC}$ 19.1, CH_2), 74.4, 74.5, 75.0, 75.1 (4 C, s, 4 × CH), 109.1 (1 C, dt, $^1J_{FC}$ 271.6, $^1J_{PC}$ 203.1, CF_2CH_2), 114.2 (1 C, dt, $^1J_{FC}$ 262.9, $^1J_{PC}$ 212.8, CF_2CO), 159.3 (1 C, dt, $^2J_{FC}$ 25.4, $^2J_{PC}$ 17.8, CO); m/z (EI+) 488 ([M + H]$^+$, 25). Found: M^+ 488.1590. $C_{16}H_{32}F_4$-NO_7P_2 requires M, 488.1582.

1,1,5,5-Tetrafluoro-2-oxo-3-azapentane-1,5-bisphosphonic Acid (31). Bromotrimethylsilane (1.9 mL, 14 mmol) was added dropwise to a solution of ester **30** (1.0 g, 2.0 mmol) in DCM (15 mL) under argon. After 24 h, the volatiles were evaporated in vacuo, and the brown residue was taken up in EtOAc (20 mL) and extracted with water (3 × 50 mL). The combined water extracts were evaporated in vacuo and dissolved in MeOH (10 mL). Cyclohexylamine (407 mg, 4.0 mmol) was added with swirling followed by the dropwise addition of acetone to precipitate the cyclohexylammonium salt which was filtered and dried to give the bis-cyclohexylammonium salt of the title compound as a white crystalline solid (340 mg, 33%): mp 167−169 °C; pK_{a^3} 4.32 ($COCF_2PO_3H$); pK_{a^4} 5.37 (CF_2PO_3H); δ_H 1.00−2.10 (20 H, m, 10 × CH_2(CHA)), 3.05−3.25 (2 H, m, 2 × CH), 3.90 (2 H, t, $^3J_{HF}$ 15.6, CH_2CF_2); δ_P 1.16 (1 P, t, $^2J_{FP}$ 87.1, PCF_2CO), 3.3 (1 P, t, $^2J_{FP}$ 89.4, PCF_2CH_2); δ_F −121.1 (2 F, d, $^2J_{FP}$ 87.2, CF_2CO), −120.4 (2 F, dt, $^2J_{FP}$ 89.4, $^3J_{HF}$ 17.5, CF_2CH_2); δ_C 23.2, 23.7, 29.7 (10 C, s, 10 × CH_2(CHA)), 40.6 (1 C, dd, $^2J_{FC}$ 21.8, $^2J_{PC}$ 20.3, CH_2), 49.7 (2 C, s, 2 × CH), 113.4 (1 C, dt, $^1J_{FC}$ 268.6, $^1J_{PC}$ 181.4, CF_2CH_2), 114.2 (1 C, dt, $^1J_{FC}$ 260.3, $^1J_{PC}$ 192.2, CF_2CO), 165.4 (1 C, dt, $^2J_{FC}$ 25.2, $^2J_{PC}$ 14.4, CO); m/z (ES−) 318 ([M − H]$^+$, 14).

Ethyl Diisopropyl 3-Phosphonopropionate (32). Ethyl 3-bromopropionate (20.0 g, 96 mmol) was heated with triisopropyl phosphite (17.4 g, 96 mmol) at 120 °C for 30 h under argon. The crude phosphonate was purified by Kugelröhr distillation to give the product as a colorless mobile oil (20.0 g, 78%): bp 180−190 °C/2 mmHg;[24] ν_{max}(liquid film)/cm^{-1} 1738 (C=O), 1245 (P=O), 1000 (P−O−^{i}Pr); δ_H 1.25 (3 H, t, $^3J_{HH}$ 6.8, CH_2CH_3), 1.30 (6 H, d, $^3J_{HH}$ 6.3, 2 × CH_3), 1.90−2.17 (2 H, m, CH_2P), 2.48−2.60 (2 H, m, CH_2CH_2P), 4.12 (2 H, q, $^3J_{HH}$ 6.8, CH_2CH_3), 4.45−4.75 (2 H, m, 2 × CH); δ_P 28.4 (1 P, s).

Ethyl Diisopropyl 4-Phosphonobutyrate (33). Ethyl 4-bromobutyrate (20.0 g, 103 mmol) was heated with triisopropyl phosphite (18.8 g, 104 mmol) at 120 °C for 30 h under argon. The crude phosphonate was purified by Kugelröhr distillation to give the title compound as a colorless mobile oil (20.4 g, 75%): bp 180−200 °C/2 mmHg;[24] ν_{max} (liquid film)/cm^{-1} 1739 (C=O), 1244 (P=O), 1011 (P−O−^{i}Pr); δ_H 1.20 (3 H, t, $^3J_{HH}$ 6.8, CH_2CH_3), 1.25 (6 H, d, $^3J_{HH}$ 6.3, 2 × CH_3), 1.58−1.75 (2 H, m, CH_2P), 1.75−1.95 (2 H, m, CH_2CH_2P), 2.83 (2 H, t, CH_2CO), 4.05 (2 H, q, $^3J_{HH}$ 6.8, CH_2CH_3), 4.53−4.70 (2 H, m, 2 × CH); δ_P 29.5 (1 P, s).

Tetraisopropyl 2-Oxopentane-1,5-bisphosphonate (34). LDA (30 mL, 60 mmol, 2 M solution in heptane/THF) was added to a solution of diisopropyl methanephosphonate (11.0 g, 61 mmol) in THF (50 mL) and stirred for 1 h at −75 °C under argon. Ethyl diisopropyl 4-phosphonobutyrate (**33**) (15.7 g, 56 mmol) was added dropwise to the anion solution at −75 °C under argon and maintained at low temperature for 1 h. The reaction mixture was brought to room temperature, then poured into water (150 mL), acidified with HCl (50 mL, 1 M), and extracted with DCM (3 × 125 mL). The combined organic extracts were dried (Na_2SO_4) and filtered, and the solvent evaporated in vacuo to yield a brown gum. The crude bisphosphonate was initially purified by Kugelröhr distillation (bp 220−230 °C/1 mmHg) and isolated by dry column flash chromatography, eluting with a gradient of DCM−MeOH (from 100:0 to 95:5), to yield the title compound[24] as a colorless oil (7.65 g, 33%): δ_H 1.25 (12 H, d, $^3J_{HH}$ 6.3, 4 × CH_3), 1.28 (12 H, d, $^3J_{HH}$ 6.3, 4 × CH_3), 1.55−1.90 (4 H, m, PCH_2CH_2), 2.70 (2 H, t, $^3J_{HH}$ 6.3, CH_2COCH_2P), 2.95 (2 H, d, $^2J_{HP}$ 25.0, PCH_2-CO), 4.50−4.74 (4 H, m, 4 × CH); δ_P 18.1 (1 P, s, PCH_2CH_2), 29.6 (1 P, s, PCH_2CO); m/z (EI+) 414 (M^+, 15). Found: M^+ 414.1936. $C_{17}H_{36}O_7P_2$ requires M, 414.1937.

2-Oxopentane-1,5-bisphosphonic Acid (35). Bromotrimethylsilane (1.4 mL, 11 mmol) was added dropwise to a

solution of ester **34** (640 mg, 1.6 mmol) in DCM (20 mL) under argon. After 24 h, ^{31}P NMR indicated the reaction had reached completion; δ_P 2.0 (1 P, s, PCH$_2$CO) and 20.0 (1 P, s, PCH$_2$CH$_2$). The volatiles were evaporated in vacuo, and the resulting gum was solvolyzed by MeOH (3 × 15 mL). The bisphosphonic acid was then dissolved in water (15 mL) and titrated to pH 7.1 with NaOH (1 M). Extraction with DCM (2 × 50 mL) and EtOAc (2 × 50 mL) followed by lyophilization furnished the title compound[24] as a hygroscopic white solid (400 mg, 89%): pK_{a}[3] 6.39 (CH$_2$COPO$_3$H); pK_a[4] 7.65 (CH$_2$PO$_3$H); δ_H (D$_2$O, AMX-500) 1.27−1.40 (2 H, m, PCH$_2$CH$_2$), 1.50−1.63 (2 H, m, PCH$_2$CH$_2$), 2.60 (2 H, t, $^3J_{HH}$ 8.2, PCH$_2$CH$_2$CH$_2$), 2.78 (2 H, d, $^2J_{HP}$ 21.8, PCH$_2$CO); δ_P (D$_2$O) 11.0 (1 P, s, PCH$_2$CO), 24.8 (1 P, s, PCH$_2$CH$_2$); δ_C (D$_2$O) 18.2 (1 C, s, COCH$_2$CH$_2$), 27.7 (1 C, d, $^1J_{PC}$ 132.1, PCH$_2$CH$_2$), 44.2 (1 C, d, $^2J_{PC}$ 12.6, COCH$_2$CH$_2$), 47.3 (1 C, d, $^1J_{PC}$ 104.5, PCH$_2$CO), 210.1 (1 C, d, $^2J_{PC}$ 16.2, PCH$_2$CO).

Tetraisopropyl 2-Oxobutane-1,4-bisphosphonate (36). LDA (30 mL, 60 mmol, 2 M solution in heptane/THF) was added to a solution of diisopropyl methanephosphonate (11.0 g, 61 mmol) in THF (50 mL) and stirred for 1 h at −75 °C under argon. Compound **32** (15.0 g, 56 mmol) was added dropwise to the anion solution at −75 °C under argon and stirred for 1 h. The reaction mixture was allowed to warm to room temperature, then poured into water (150 mL), acidified with HCl (50 mL, 1 M), and extracted with DCM (3 × 125 mL). The combined organic extracts were dried (Na$_2$SO$_4$) and filtered, and the solvent evaporated in vacuo to yield a brown paste. The crude bisphosphonate was initially purified by Kugelröhr distillation (220 °C/1 mmHg) and isolated by dry column flash chromatography, eluting with a gradient of DCM−MeOH (from 100:0 to 95:5) to yield the title compound[24] as a colorless oil (6.72 g, 30%): δ_H 1.28 (12 H, d, $^3J_{HH}$ 6.3, 4 × CH$_3$), 1.32 (12 H, d, $^3J_{HH}$ 3.8, 4 × CH$_3$), 1.85−2.05 (2 H, m, PCH$_2$CH$_2$), 2.80−2.90 (2 H, m, PCH$_2$CH$_2$), 3.05 (2 H, d, $^2J_{HP}$ 25.0, PCH$_2$CO), 4.55−4.80 (4 H, m, 4 × CH); δ_P 17.8 (1 P, s, PCH$_2$CH$_2$), 29.4 (1 P, s, PCH$_2$CO); m/z (EI+) 400 (M$^+$, 26). Found: M$^+$ 400.1780. C$_{16}$H$_{34}$O$_7$P$_2$ requires M, 400.1776.

2-Oxobutane-1,4-bisphosphonic Acid (37). Bromotrimethylsilane (1.5 mL, 12 mmol) was added dropwise to a solution of ester **36** (664 mg, 1.7 mmol) in DCM (20 mL) under argon. After 24 h, ^{31}P NMR indicated the reaction had reached completion; δ_P 3.2 (1 P, s, PCH$_2$CO) and 20.0 (1 P, s, PCH$_2$CH$_2$). The volatiles were evaporated in vacuo, and the resulting gum was solvolyzed by MeOH (3 × 15 mL). The bisphosphonic acid was then dissolved in water (15 mL) and titrated to pH 7.1 with NaOH (1 M). Extraction with DCM (2 × 50 mL) and EtOAc (2 × 50 mL) followed by lyophilization furnished the title compound[24] as a hygroscopic white solid (400 mg, 87%): pK_a[3] 5.60 (CH$_2$COPO$_3$H), pK_a[4], 6.89 (CH$_2$PO$_3$H); δ_H (D$_2$O) 1.50−1.80 (2 H, m, PCH$_2$CH$_2$), 2.70−2.93 (2 H, m, PCH$_2$CH$_2$), 3.12, (2 H, d, $^2J_{HP}$ 20.0, PCH$_2$CO); δ_P (D$_2$O) 11.1 (1 P, s, PCH$_2$CO), 23.2 (1 P, s, PCH$_2$CH$_2$); δ_C (D$_2$O) 22.7 (1 C, d, $^1J_{PC}$ 132.1, PCH$_2$CH$_2$), 38.3 (1 C, s, COCH$_2$CH$_2$), 48.0 (1 C, d, $^1J_{PC}$ 103.0, PCH$_2$CO), 212.7 (1 C, d, $^2J_{PC}$ 15.5, CO).

Tetraisopropyl 1,1-Difluoro-2-oxopentane-1,5-bisphosphonate (38). n-Butyllithium (37.0 mL, 39 mmol, 1.06 M solution in hexane) was added dropwise to diisopropylamine (3.97 g, 39 mmol) in THF (20 mL) at −70 °C under argon. After 40 min diisopropyl difluoromethanephosphonate (8.48 g, 39 mmol) as a solution in THF (20 mL) at −20 °C was introduced by cannula into the solution of LDA cooled to − 80 °C. After 45 min, ester **33** (10.0 g, 36 mmol) as a solution in THF (20 mL) at −20 °C was added by cannula, and the reaction mixture was maintained at −80 °C for 3 h and then allowed to warm to room temperature before being poured into cold ammonium chloride (200 mL, saturated) and extracted with EtOAc (3 × 200 mL). The combined organic extracts were dried (Na$_2$SO$_4$) and filtered, and the solvent evaporated in vacuo to give a red oil (12.3 g). The crude bisphosphonate was purified by dry column flash chromatography with petrol−EtOAc, 80:20 as eluent, to furnish the title compound as a colorless oil (9.47 g, 60%): R_f (petrol/EtOAc, 80:20) 0.26; bp > 200 °C/1 mmHg (decomp.); ν_{max}(liquid film)/cm^{-1} 1744 (C=O), 1273 (P=O), 1007

(P−O−^{i}Pr); δ_H 1.25 (12 H, t, $^3J_{HH}$ 6.3, 4 × CH$_3$), 1.33 (6 H, d, $^3J_{HH}$ 6.3, 2 × CH$_3$), 1.34 (6 H, d, $^3J_{HH}$ 6.3, 2 × CH$_3$), 1.55−1.75 (2 H, m, CH$_2$P), 1.75−1.95 (2 H, m, CH$_2$CH$_2$), 2.83 (2 H, t, $^2J_{HP}$ 15.0, CH$_2$CO), 4.63−4.78 (2 H, m, 2 × CH), 4.81 (2 H, octet, $^3J_{HH}$ 6.3, $^3J_{HP}$ 6.3, 2 × CH); δ_P 1.6 (1 P, t, $^2J_{FP}$ 97.0, PCF$_2$), 29.2 (1 P, s, PCH$_2$); δ_F −119.4 (2 F, d, $^2J_{FP}$ 96.6, PCF$_2$); m/z (EI+) 450 (M$^+$, 3). Found: C, 45.36; H, 7.61. C$_{17}$H$_{34}$F$_2$O$_7$P$_2$ requires C, 45.34; H, 7.61. Found: M$^+$ 450.1747. C$_{17}$H$_{34}$F$_2$O$_7$P$_2$ requires M, 450.1750.

1,1-Difluoro-2-oxopentane-1,5-bisphosphonic Acid (39). Bromotrimethylsilane (1.0 mL, 7.8 mmol) was added dropwise to a solution of ester **38** (504 mg, 1.1 mmol) in DCM (2 mL) under argon. After 1 week, ^{31}P NMR indicated the reaction had reached completion; δ_P −15.4 (1 P, t, $^2J_{FP}$ 97.1, PCF$_2$CO) and 21.3 (1 P, s, PCH$_2$CH$_2$). The volatiles were evaporated in vacuo, and the resulting gum was triturated with MeOH (5 × 15 mL). The bisphosphonic acid was then dissolved in water (10 mL) and titrated to pH 7.1 with NaOH (1 M). Extraction with DCM (2 × 20 mL) and EtOAc (20 mL) followed by lyophilization furnished the title compound as a white solid (358 mg, 98%): pK_a[3] 4.82 (CF$_2$PO$_3$H); pK_a[4] 7.90 (CH$_2$PO$_3$H); δ_H (D$_2$O) 1.45−1.65 (2 H, m, CH$_2$P), 1.65−1.83 (2 H, m, CH$_2$CH$_2$CO), 2.93 (2 H, q, $^3J_{HH}$ 6.3, $^3J_{HP}$ 6.3, CH$_2$CO); (D$_2$O, BMX-400) 1.40−1.50 (2 H, m, CH$_2$P), 1.60−1.69 (2 H, m, CH$_2$CH$_2$-CO), 2.90 (2 H, q, $^3J_{HH}$ 6.3, $^3J_{HP}$ 6.3, CH$_2$CO); δ_P (D$_2$O) 2.6 (1 P, t, $^2J_{FP}$ 76.5, PCF$_2$, 89%); 5.5 (1 P, t, $^2J_{FP}$ 80.8, PCF$_2$, 11%); 24.5 (1 P, s, PCH$_2$, 89%); 25.2 (1 P, s, PCH$_2$ 11%); δ_F (D$_2$O) −115.9 (2 F, d, $^2J_{FP}$ 76.4, PCF$_2$, 89%), −122.2 (2 F, d, $^2J_{FP}$ 80.5, PCF$_2$, 11%); δ_C (D$_2$O, 400 MHz) 16.2 (1 C, s, PCH$_2$CH$_2$), 26.5 (1 C, d, $^1J_{PC}$ 132.9, PCH$_2$CH$_2$), 37.2−38.2 (1 C, m, CH$_2$CO), 95.5−96.5 (1 C, m, PCF$_2$C(OH)$_2$), 117.5 (1 C, dt, $^1J_{PC}$ 158.3, $^1J_{FC}$ 270.3, PCF$_2$), 206.0 (1 C, dt, $^2J_{PC}$ 12.2, $^2J_{FC}$ 23.2, PCF$_2$-CO). Found: M$^+$ 326.958. C$_5$H$_9$F$_4$O$_7$P$_2$Na$_2$ requires M, 326.9587.

Tetraisopropyl 1,1-Difluoro-2-oxobutane-1,4-bisphosphonate (40). n-Butyllithium (7.8 mL, 8.3 mmol, 1.06 M solution in hexane) was added dropwise to diisopropylamine (0.84 g, 8.3 mmol) in THF (10 mL) at −70 °C under argon. After 15 min, diisopropyl difluoromethanephosphonate (1.79 g, 8.3 mmol) as a solution in THF (5 mL) at −20 °C was transferred by cannula into the solution of LDA at − 80 °C. After 15 min, **32** (10.0 g, 36 mmol) as a solution in THF (5 mL) at −20 °C was introduced by cannula into the reaction mixture which was maintained at −80 °C for 3 h, and then allowed to warm to room temperature before being poured into cold ammonium chloride (100 mL, saturated) and extracted with EtOAc (3 × 100 mL). The combined organic extracts were dried (Na$_2$SO$_4$) and filtered and the solvent evaporated in vacuo to give an orange oil (2.7 g). The crude bisphosphonate was purified by flash chromatography with petrol−EtOAc−acetic acid, 54:45:1 as eluant, to yield the title compound as a colorless oil (1.54 g, 47%): R_f (petrol/EtOAc/acetic acid, 54:45:1) 0.30; bp > 200 °C/1 mmHg (decomp.); ν_{max}(liquid film)/cm^{-1} 1734 (C=O), 1248 (P=O), 1005 (P−O−^{i}Pr); δ_H 1.25 (12 H, t, $^3J_{HH}$ 6.3, 4 × CH$_3$), 1.32 (6 H, d, $^3J_{HH}$ 6.3, 2 × CH$_3$), 1.35 (6 H, d, $^3J_{HH}$ 6.3, 2 × CH$_3$), 1.83−2.03 (2 H, m, CH$_2$P), 3.00 (2 H, q, $^3J_{HH}$ 6.3, $^3J_{HP}$ 6.3, CH$_2$CO), 4.54−4.73 (2 H, m, 2 × CH), 4.82 (2 H, octet, $^3J_{HH}$ 6.3, $^3J_{HP}$ 6.3, 2 × CH); δ_P 1.5 (1 P, t, $^2J_{FP}$ 97.0, PCF$_2$), 28.1 (1 P, s, PCH$_2$); δ_F −119.1 (2 F, d, $^2J_{FP}$ 97.6, PCF$_2$); m/z (EI+) 436 (M$^+$, 0.3). Found: M$^+$ 436.1591. C$_{16}$H$_{32}$F$_2$O$_7$P$_2$ requires M, 436.1590.

1,1-Difluoro-2-oxobutane-1,4-bisphosphonic Acid (41). Bromotrimethylsilane (1.1 mL, 8.1 mmol) was added dropwise to a solution of ester **40** (509 mg, 1.2 mmol) in DCM (2 mL) under argon. After 1 week, ^{31}P NMR indicated the reaction had reached completion; δ_P −15.7 (1 P, t, $^2J_{FP}$ 96.3, PCF$_2$CO) and 19.8 (1 P, s, PCH$_2$CH$_2$). Volatiles were evaporated in vacuo, and the resulting gum was solvolyzed with MeOH (5 × 15 mL). The bisphosphonic acid (348 mg, 1.2 mmol) was then dissolved in water (10 mL) and titrated to pH 7.1 with NaOH (1 M). Extraction with DCM (2 × 20 mL) and EtOAc (20 mL) followed by lyophilization afforded the title compound as a white solid (365 mg, 98%): pK_a[3] 4.77 (CF$_2$PO$_3$H); pK_a[4] 6.37 (CH$_2$PO$_3$H); δ_H (D$_2$O) 1.60−1.85 (2 H, m, CH$_2$P), 3.05 (2 H, q,

$^3J_{HH}$ 6.3, $^3J_{HP}$ 6.3, CH_2CO); δ_P (D_2O) 1.9 (1 P, t, $^2J_{FP}$ 80.0, PCF_2, 85%); 5.5 (1 P, t, $^2J_{FP}$ 80.0, PCF_2, 15%); 23.7 (1 P, s, PCH_2, 85%); 25.7 (1 P, s, PCH_2 15%); m/z (ES−) 311 ([M + 2Na − H]$^+$, 15); 289 ([M + Na − H]$^+$, 100); 267 ([M − H]$^+$, 100). Found: M$^+$ 467.1902. $C_{16}H_{35}F_2N_2O_7P_2$ requires M, 467.1889.

Ethyl Diisopropyl 4,4-Difluoro-4-phosphonobutyrate (42). *n*-Butyllithium (17.5 mL, 18.6 mmol, 1.06 M solution in hexane) was added dropwise to diisopropylamine (1.87 g, 18.6 mmol) in THF (30 mL) at −70 °C under argon. After 15 min the solution was cooled to − 80 °C. Diisopropyl difluoromethanephosphonate (4.0 g, 18.5 mmol) as a solution in THF (5 mL) at −20 °C was transferred by cannula into the LDA. After 15 min, ethyl acrylate (1.20 g, 12 mmol) as a solution in THF (5 mL) at −20 °C was introduced by cannula into the reaction mixture. The reaction mixture was maintained at −80 °C for 3 h and then allowed to warm to room temperature, poured into cold aqueous ammonium chloride (100 mL, saturated), and extracted with EtOAc (1 × 100 mL). The combined organic extracts were dried (Na$_2$SO$_4$) and filtered, and solvent evaporated in vacuo to give an orange oil (3.7 g). The crude phosphonate was purified by dry column flash chromatography with gradient elution of DCM−MeOH (from 0:100 to 2:98) as a solvent to yield the title compound as a colorless oil (800 mg, 21%): R_f (DCM−MeOH, 95:5) 0.34; δ_H 1.15 (3 H, t, $^3J_{HH}$ 6.3, CH_2CH_3), 1.27 (6 H, d, $^3J_{HH}$ 6.3, 2 × CH_3), 1.32 (6 H, d, $^3J_{HH}$ 6.3, 2 × CH_3), 2.16−2.56 (4 H, m, CH_2CH_2CO), 4.04 (2 H, q, $^3J_{HH}$ 6.3, CH_2CH_3), 4.75 (2 H, octet, $^3J_{HH}$ 6.3, $^3J_{HP}$ 6.3, 2 × CH); δ_P 5.2 (1 P, t, $^2J_{FP}$ 107.2, PCF_2); δ_F −114.3 (2 F, dt, $^2J_{FP}$ 107.4, $^3J_{HF}$ 18.8, PCF_2); m/z (EI+) 316 (M$^+$, 0.4).

Tetraisopropyl 1,1,5,5-Tetrafluoro-2-oxo-pentane-1,5-bisphosphonate (43). *n*-Butyllithium (2.3 mL, 2.4 mmol, 1.06 M solution in hexane) was added dropwise to diisopropylamine (245 mg, 2.4 mmol) in THF (6 mL) at −70 °C under argon. After 20 min the solution of LDA was cooled to −80 °C, and diisopropyl difluoromethanephosphonate (525 mg, 2.4 mmol) in THF (5 mL) at −20 °C was transferred into the reaction mixture by cannula. Ester **42** (700 mg, 2.21 mmol) as a solution in THF (10 mL) at −20 °C was added by cannula to the reaction mixture after 15 min, and the reaction mixture was maintained at −80 °C for 4 h and then allowed to warm to room temperature, poured into cold aqueous ammonium chloride (30 mL, sat.), and extracted with EtOAc (3 × 30 mL). The combined organic extracts were dried (Na$_2$SO$_4$) and filtered, and the solvent was evaporated in vacuo to give an orange oil (864 mg). The crude bisphosphonate was purified three times by flash chromatography with petrol/EtOAc (twice at 70:30, and once at 80:20 ratio) as eluant to furnish the title compound as a colorless oil (312 g, 29%): R_f (petrol−EtOAc−acetic acid, 50:49:1) 0.50; bp > 200 °C/1 mmHg (decomp.); ν_{max} (liquid film)/cm^{-1} 1747 (C=O), 1268 (P=O), 1001 (P−O−^{i}Pr); δ_H 1.27−1.46 (24 H, m, 8 × CH$_3$), 2.25−2.55 (2 H, m, CF$_2$-CH$_2$), 3.07 (2 H, t, $^3J_{HH}$ 6.3, CH$_2$CO), 4.85 (4 H, octet, $^3J_{HH}$ 6.3, $^3J_{HP}$ 6.3, 4 × CH); δ_P 1.5 (1 P, t, $^2J_{FP}$ 97.2, PCF$_2$CO), 5.1 (1 P, t, $^2J_{FP}$ 106.8, PCF$_2$CH$_2$); δ_F −113.9 (2 F, d, $^2J_{FP}$ 106.8, PCF$_2$CH$_2$), −119.3 (2 F, d, $^2J_{FP}$ 98.1, PCF$_2$CO); m/z (CI+) 487 ([M + H]$^+$, 100). Found: M$^+$ 487.1638. $C_{17}H_{33}F_4NO_7P_2$ requires M, 487.1636.

1,1,5,5-Tetrafluoro-2-oxopentane-1,5-bisphosphonic Acid (44). Ester **43** (110 mg, 225 µmol) was dissolved in dry acetonitrile-d_3 (1.125 mL). Chlorotrimethylsilane (209 µL, 1.6 mmol) was syringed into the solution under argon followed by solid sodium iodide (236 mg, 1.6 mmol). The reaction was monitored by ^{31}P NMR, and after 8 h the reaction was complete; δ_P −14.7 (1 P, bt, PCF$_2$CO) and −10.5 (1 P, bt, PCF$_2$-CH$_2$). The reaction mixture was filtered through Celite, and the volatiles were evaporated in vacuo. Extraction with MeOH (2 × 20 mL) gave the bisphosphonic acid which was taken up into MeOH (2 mL), and cyclohexylamine (45 mg, 450 µmol) was added. Dropwise addition of acetone (15 mL) with rapid swirling gave the cyclohexylammonium salt (65 mg) as an off-white solid. This was taken up in MeOH and reprecipitated with acetone to yield the bis-cyclohexylammonium salt of the title compound as a white crystalline solid (40 mg, 34%): mp 210−212 °C; pK_{a^3} 4.47; pK_{a^4} 5.10; δ_H (D_2O) 1.00−2.20 (20 H,

m, 10 × CH$_2$(CHA)), 2.20−2.50 (2 H, m, CH$_2$CF$_2$), 3.00−3.25 (2 H, m, 2 × CH$_2$); δ_P (D_2O) 0.9 (1 P, t, $^2J_{FP}$ 85.8, PCF$_2$CO, 60%), 3.8 (1 P, t, $^2J_{FP}$ 90.1, PCF$_2$CO, 40%), 5.0 (1 P, t, $^2J_{FP}$ 95.6, PCF$_2$CH$_2$, 57%), 5.4 (1 P, t, $^2J_{FP}$ 97.0, PCF$_2$CO, 43%); δ_C (D_2O, 400 MHz) 23.2 (2 C, s, 2 × CHA C-4), 23.7 (4 C, s, 2 × CHA C-3,5), 25.1−26.2 (2 C, m, PCF$_2$CH$_2$CH$_2$), 29.7 (4 C, s, 2 × CHA C-2,6), 49.7 (2 C, s, 2 × CHA C-1), 94.3−94.5 (1 C, m, PCF$_2$C(OH)$_2$), 111.8−125.5 (2 C, m, PCF$_2$), 201.5−202.5 (1 C, m, PCF$_2$CO); m/z (ES−) 317 ([M − H]$^+$, 33); (ES+) 100 (CHA$^+$, 100). Found: M$^+$ 517.1825. $C_{17}H_{35}F_4N_2O_7P_2$ requires M, 517.1856.

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