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Aminophosphonic Acids and Aminobis(phosphonic acids) as Potential Inhibitors of Penicillin-Binding Proteins

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Aminophosphonic acids and aminobis(phosphonic acids) have been prepared by the alkylation of Schiff bases with methyl bromoacetate or ethyl acrylate. Other pathways, like the modified Pudovik reaction and Kabachnik–Fields reaction, have been considered for the synthesis of the α-phosphonic bioisoster of aminocitrate. Partial or complete deprotection of the phosphonate ester have been realised by either acidic hydrolysis or by treatment with trimethylsilyl bromide. Evaluation against penicillin-binding proteins has shown that our compounds are modest inhibitors of class A β-lactamases, but have an interesting activity against R39 (D,D-peptidase/carboxypeptidase).

Introduction

Resistance to antibiotics is currently a major health concern.[1] The production of β-lactamases is the most common mechanism of resistance to β-lactam antibiotics.[2] Several strategies have been considered to overcome the action of these enzymes. Among them, the use of combination therapy, which involves the treatment with a β-lactam antibiotic and a β-lactamase inhibitor, has been successfully used for two decades for the treatment of infections.[3] Clavunic acid, sulbactam and tazobactam are the three β-lactamase inhibitors currently marketed.[4] However, although these β-lactam inhibitors are active against class A β-lactamases, they are almost inactive against the other classes. The emergence of novel β-lactamases belonging to classes B, C and D amplified the problem. The need to discover novel non-β-lactam inhibitors is now urgent because the β-lactam cycle is a structure well "known" by bacteria, which are able to evolve quickly to fight against the new β-lactam drugs. Numerous non-β-lactam inhibitors or substrates of β-lactamases have been described in the literature, for example, depsipeptides,[5] azadepsipeptides,[6] α-oxo heterocycles,[7] phenaceturates,[8] boronic acids[9] and phosph(on)ates.[10] Pratt and co-workers have studied phosphonic acids as potential inhibitors that act as transition-state analogues. The reaction involves phosphonylation of the active site Ser-OH and formation of a stable tetrahedral phosphonyl–enzyme intermediate. They have described the preparation and biochemical evaluation of phosphonic analogues of depsipeptides,[11] cyclic phosphates,[12] diaroyl phosph(on)ates[13] and oxo phosph(on)ates.[14]

For several years, our laboratory has been involved in the discovery of novel non-β-lactam inhibitors of bacterial enzymes, β-lactamases and D,D-peptidases. Our work is based on a fortuitous observation made by Fonze et al. during the X-ray diffraction study of *Bacillus licheniformis* BS3 β-lactamase (class A enzyme) crystallised from a citrate buffer: this revealed the unexpected role of this compound, which was perfectly located in the enzyme's cavity.[15] Citrate is an inhibitor of all class A β-lactamases at the micromolar level, with a K_i value of 490 μM at pH 5 against BS3. These observations led us to consider citrate as a "hit" for the design of new affinity inhibitors of class A β-lactamases. Recently, we described the synthesis and biochemical evaluation of a series of amino analogues and homologues of citrate and isocitrate.[16] The activity against class A β-lactamases was confirmed. Moreover, three compounds protected as lipophilic esters were good inhibitors of OXA-10, a class D β-lactamase. In this work, the complex formed between aminocitrate and BS3 was analysed by X-ray diffraction. The results of this study have allowed us to explain the slightly better but still modest activity of aminocitrate compared with citrate (K_i value of 250 μM).[17]

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As a continuation of this work, we have studied the replacement of the α-carboxylic function of aminocitrate by the bioisoster phosphonic acid and the lengthening of the two other acidic chains to possibly create novel interactions. We were also interested in the biochemistry of aminobis(phosphonic) compounds structurally related to citrate. The target molecules are presented in Figure 1.

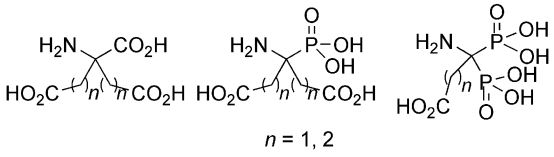


Figure 1. Aminocitrate and phosphonic bioisosters.

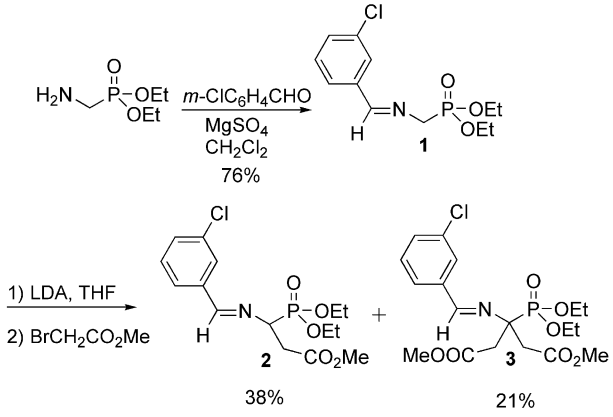
The key role of amino acids in life chemistry has led to intense research into various mimetics. In particular, aminophosphonic acids very efficiently mimic amino acids and compete with them for the active sites of enzymes. They exhibit numerous activities of interest in medicinal chemistry, namely as inhibitors of various proteolytic enzymes, agonists or antagonists of receptors involved in the central nervous system (CNS), natural product analogues and metal complexing agents.[18,19] Geminal bis(phosphonic acids) are metabolically stable analogues of naturally occurring inorganic pyrophosphates. Fleisch et al. have shown that they are able to impair the formation and dissolution of calcium phosphate in vitro.[20] Bis(phosphonic acids) are currently used for the treatment of various diseases of bone mineral metabolism like osteoporosis.[20,21] To the best of our knowledge, aminophosphonic compounds have never been considered as potential inhibitors of bacterial enzymes implicated in cell wall biosynthesis and resistance to penicillin antibiotics.

Results and Discussion

Synthesis of the α-Phosphonic Analogue of Aminocitrate

Several strategies have been reported in the literature for the synthesis of α-aminophosphonic derivatives.[22] Among them are the Kabachnik–Fields and Pudovik reactions, and the alkylation of iminophosphonates or related compounds. The Kabachnik–Fields condensation reaction is a three-component coupling reaction of carbonyl, amine and hydrophosphoryl or phosphite compounds that can be carried out with or without catalyst in a solvent or a solvent-free medium.[23] The Pudovik reaction is the addition of hydrophosphoryl or phosphite compounds to imines.[24] Numerous Lewis acid catalysts have been used to improve the efficacy of the reaction, for example, $ZnCl_2$, $AlCl_3$ and CdI_2.[25] The last approach involves the preliminarily creation of a protected α-aminophosphonate and its subsequent reaction with a base and appropriate electrophilic reagents.[26]

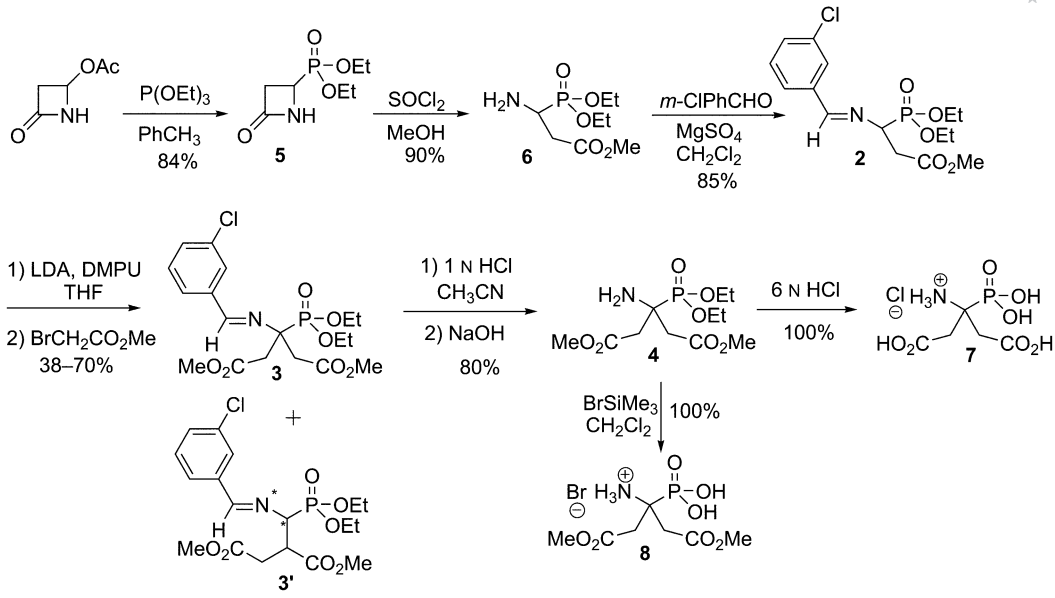
The first strategy considered for the synthesis of an α-phosphonic analogue of aminocitrate was the alkylation of an α-phosphonic imine. The Schiff base **1** was prepared by the reaction of diethyl (aminomethyl)phosphonate[27] and 3-chlorobenzaldehyde in the presence of magnesium sulfate in dichloromethane in 76% yield after purification by chromatography (Scheme 1). Thus, imine **1** was treated with 2 equiv. of lithium diisopropylamide (LDA) and 2 equiv. of methyl bromoacetate in tetrahydrofuran at –78 °C.[28] These conditions, previously used for the synthesis of aminocitrate,[17] led to monoalkylated product **2** in 51% isolated yield with no detectable dialkylated product **3**.[17] The best results were obtained by treatment of imine **1** with two successive fractions of 1 equiv. each of LDA and bromoacetate. A mixture of mono- and dialkylated products **2** and **3** was obtained, separable by chromatography to give 38 and 21% yields, respectively. The addition of hexamethylphosphoramide (HMPA) did not improve the yield of the desired product **3**.



Scheme 1. Alkylation of imine **1**.

Therefore an alternative method was applied that produced high yields of imine **2** as the key intermediate (Scheme 2). This was prepared in three steps from 4-acetoxyazetidinone by an Arbuzov reaction. The acetoxy group was substituted by triethyl phosphite in toluene in 84% yield.[29] Treatment of the azetidinone **5** with thionyl chloride in methanol led to opening of the β-lactam ring and formation of methyl ester **6** in 90% yield.[30] Finally, the amine **6** was treated with 3-chlorobenzaldehyde in the presence of magnesium sulfate in dichloromethane to furnish the imine **2** in 85% yield after purification by chromatography.

Several conditions have been tested for the deprotonation and alkylation of intermediate **2**, and the yields of compound **3** have been determined on the crude mixture by ^1H NMR spectroscopy, as summarised in Table 1 and Scheme 2. In the first tests, the imine solution was added to the base in solution (Entries 1–6, addition mode A). The conditions tested led either to the deterioration of the starting material or to the formation of at least four products (Entries 1–4). The best result was 56% of **3**, obtained by deprotonation with sodium hydride in tetrahydrofuran (Entry 5), but this reaction was carried out on a small scale (200 mg). In the next tests, the base was added to imine **2** in solution (Entries 7–9; addition mode B). When using



Scheme 2. Synthesis of target molecule **7**.

Table 1. Conditions and bases used for the alkylation of imine **2**.

Entry	Base (equiv.)	Conditions[a]	Results; ratio **2**/**3**[b]
1	*t*BuOK (1.2)	THF, TA (A)	degradation
2	*t*BuOK (1.2)	THF, 0 °C (A)	**2**, **3** and unidentified products
3	*t*BuOK (1.2)	THF, –60 °C (A)	**2**, **3** and unidentified products
4	NaH (2)	DMF, 0 °C (A)	degradation
5	NaH (2)	THF, 0 °C (A)	**2**/**3**; 44:56
6	KHMDS (1.4)	THF, –78 °C (A)	**2**, **3** and unidentified products
7	KHMDS (1.2)	THF, –78 °C (B)	**2**/**3**; 62:38
8	K_2CO_3 (2)	CH_3CN, 50 °C (B)	**2**/**3**; 94:6
9	EtONa (1.5)	EtOH, 80 °C (B)	transesterification of **2**
10	LDA (1.4)	THF, –78 °C (A)	**2**/**3**; 58:42
11	LDA (1.4) + DMPU (1.6)	THF, –78 °C (A)	**2**/**3**; 30:70

[a] Addition mode A or B, see text. [b] Ratio determined by ^{1}H NMR spectroscopy.

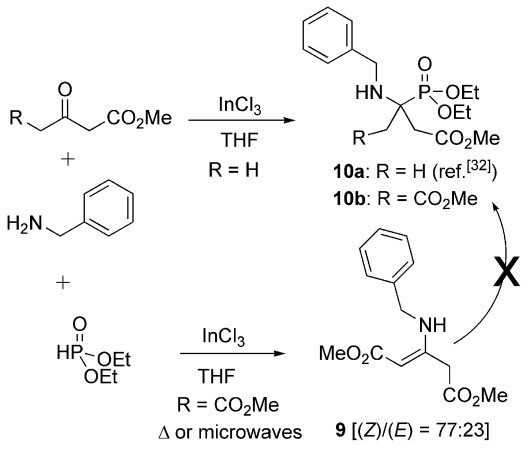
potassium hexamethyldisilazide (KHMDS) as the base (Entry 7), 38% of **3** was formed, whereas phase-transfer conditions (Entry 8) led to just traces of **3**. The reaction with sodium ethoxide gave mainly the transesterification product of the starting material (Entry 9). Finally, the Schiff base **2** was added to a solution of LDA in tetrahydrofuran, and methyl bromoacetate was then added after 1 h at low temperature (Entry 10) to furnish 42% of **3**; similar results were achieved on the 0.2- and 1-g scales. Treatment with two fractions of base successively did not improve the yields of **3**. However, addition of 1,3-dimethyl-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one (DMPU) allowed to reach 70% of the desired product (Entry 11). This product was purified by chromatography with some difficulties and isolated with 24% yield.

When the quantity of LDA was increased, a secondary product **3′**, resulting from deprotonation and alkylation of the β-carbon atom, was isolated with 5% yield. This result may be explained by the steric bulk of the phosphonate group.

Amine **4** was easily obtained in 80% yield by acidic hydrolysis of imine **3**. Further treatment of **4** with 6 N HCl produced the target molecule **7**, the phosphonic analogue of aminocitrate, isolated as the hydrochloride salt. Selective deprotection of the phosphonate ester was carried out with trimethylsilyl bromide in dichloromethane and led quantitatively to compound **8** as the hydrobromide salt.[31] Although successful, this method is handicapped by the modest yields of the alkylation step, combined with the arduous purification of the protected precursor **3**.

Thus, a third strategy based on the Kabachnik–Fields reaction was envisaged. There are many examples of this reaction in the literature, but one particular case held our attention in the work of Ranu et al.[32] They reported the one-pot synthesis of a disubstituted α-aminophosphonate **10a** catalysed by indium chloride (Scheme 3 with R = H). Indium chloride was added to a solution of dimethyl 1,3-acetonedicarboxylate (Scheme 3 with R = CO_2Me), benzylamine and diethyl phosphite in tetrahydrofuran, and the mixture was heated at reflux for several hours. Instead of the condensation product **10b**, the enamine **9** was isolated in 65% yield. Prolonged treatment of pure **9** with diethyl phosphite and indium chloride in tetrahydrofuran did not furnish **10b**. Kabachnik et al. showed that microwave acti-

vation in the case of hindered ketones gave better results than conventional heating,[33] but in our case it was not helpful.



Scheme 3. Kabachnik–Fields reaction described by Ranu et al. (**10a**) and applied to our substrate (**10b**).

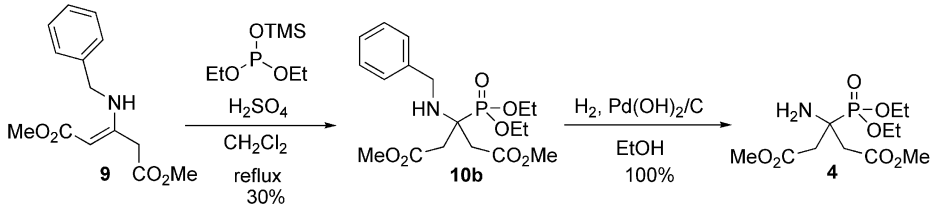
Finally, a Pudovik-type reaction was investigated by using **9** as the starting material. Indeed, enamines can be used in such a reaction, similarly to imines. For instance, the phosphonic analogue of aspartic acid, in which the α-carboxy group is replaced by a phosphonic group, was obtained by the reaction of diethyl phosphite with (acetamidomethylene)malonate followed by hydrolysis and decarboxylation.[34] Diethyl phosphite is generally considered as a poor nucleophile.[35] However, its nucleophilicity can be improved in three ways: (1) The use of strong bases permits the isolation of the lithium, sodium or potassium salts, which are then used in the Pudovik reaction.[36] (2) The use of sodium ethoxide allows the tautomer equilibrium to be moved towards the more nucleophilic $\sigma^3\lambda^3$ form.[35] (3) The addition of an acid catalyst to activate diethyl phosphite. Numerous Lewis acids have been used for the synthesis of α-aminophosphonates, for example, BF_3, $AlCl_3$, Me_2AlCl, $ZnCl_4$, $TiCl_4$, $SnCl_4$ and SmI_2.[37] Finally, the last way to increase the reactivity of diethyl phosphite has been described by Afarinkia et al.[38] who reported its transformation to diethyl trimethylsilyl phosphite. This last method has been successfully applied to our substrate. Benzylamine was treated with dimethyl 1,3-acetonedicarboxylate in the presence of molecular sieves in methanol to give **9**, as described by Prugh and Deana.[39] The enamine **9** [used as a (*Z*)/(*E*) mixture of stereoisomers in variable ratios after chromatography] was added to a solution of diethyl trimethylsilyl phosphite prepared in situ in dichloromethane in the presence of triethylammonium chloride (Scheme 4). After stirring the mixture at room temperature or heating at reflux for several days, the starting material **9** was recovered. In fact, a proton source is necessary to allow a tautomeric equilibrium between enamine and imine. Triethylammonium chloride (using the conditions of Afarinkia et al.) did not seem to be a sufficiently strong proton source in our case.

Moonen et al. recently reported a one-pot tandem 1,4/1,2-addition of diethyl trimethylsilyl phosphite to α,β-unsaturated imines in the presence of triethylammonium chloride, ammonium sulfate or sulfuric acid.[40] Surprisingly, the reaction was very efficient when using sulfuric acid as the catalyst. This result prompted us to replace triethylammonium chloride by sulfuric acid. The thus-modified Pudovik reaction of **9** allowed the aminophosphonate **10b** to be obtained in 30% yield. Attempts to improve the yield by increasing the quantity of acid and/or nucleophile were unsuccessful. The amine **4** was obtained quantitatively by the reaction of aminophosphonate **10b** with hydrogen and palladium hydroxide. This novel route to **4** (and **7**, see Scheme 2) is a valuable alternative to the alkylation of α-aminophosphonic derivatives. Overall yields are quite similar, but the number of steps is reduced.

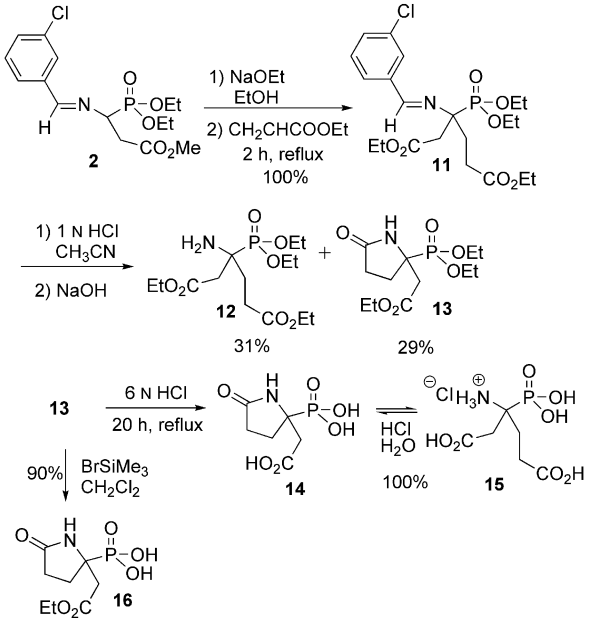
Synthesis of Homologues of α-Aminophosphonocitrate

Our strategy for the synthesis of products possessing longer acidic chains was based on the alkylation of the imine intermediates **1** and **2** by Michael addition. Thus, the Schiff base **2** was treated with sodium ethoxide[41] and ethyl acrylate to furnish quantitatively the adduct **11**. Direct hydrolysis of imine **11** led to the formation of a mixture of amine **12** and pyrrolidinone **13**, which can be separated by chromatography and isolated in 31 and 29% yields, respectively. However, pure amine **12** cyclised spontaneously to furnish pyrrolidinone **13**, even when the compound was stored at –20 °C (Scheme 5).

Treatment of pyrrolidinone **13** with 6 N HCl at reflux was expected to give the target product **15**. Actually, a mixture of cyclic **14** and open-chain **15** was obtained, even after prolonged heating of **13**, as confirmed by ^{1}H NMR spectroscopy. Oleksyszyn et al. encountered the same problem during the synthesis of phosphonic analogues of pyroglutamic acid: in acidic media, the α-methylpyroglutamic acid analogue was in equilibrium with the open-chain compound.[42] Selective deprotection of the phosphonate group



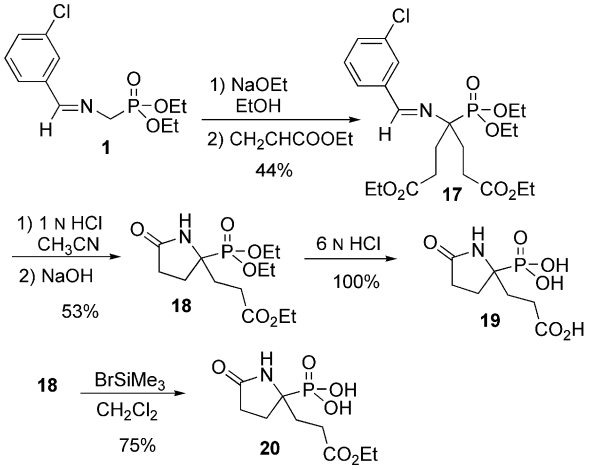
Scheme 4. Addition of diethyl trimethylsilyl phosphite to enamine **9**.



Scheme 5. Synthesis of compound **15**.

of **13** was carried out with trimethylsilyl bromide in dichloromethane followed by treatment with methanol to give compound **16** as a white hygroscopic solid in 90% yield.

The same method was applied to the synthesis of the bis(homologue) (Scheme 6). The reaction of imine **1** with sodium ethoxide and excess ethyl acrylate at 80 °C in ethanol for 24 h led to the product **17**, isolated in 44% yield after purification by chromatography. The monoalkylated product was obtained exclusively at 20 °C with 2 equiv. of acrylate. The starting material **1** was recovered when using potassium carbonate as base. The acidic hydrolysis of imine **17** led to the pyrrolidinone **18** in 53% yield. After heating **18** in 6 N HCl, the cyclic acid **19** was obtained quantitatively without a trace of the open-chain product (confirmed by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy and mass spectrometry). Thus, the bis(homologue) of **7** could not be obtained.



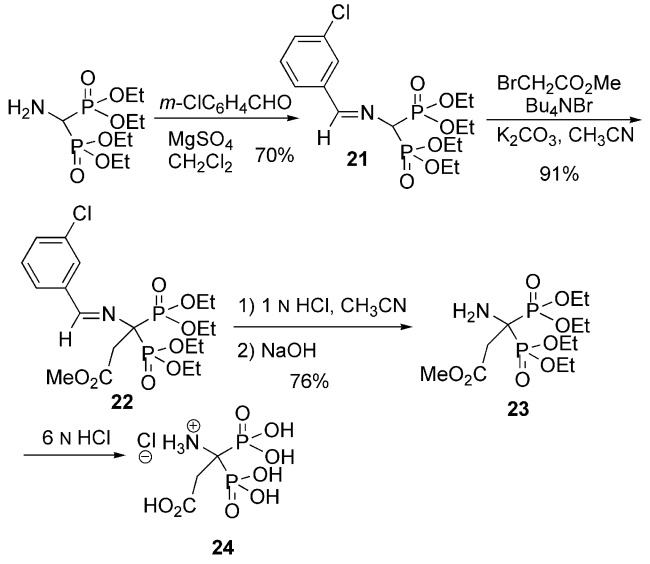
Scheme 6. Synthesis of compound **19**.

Treatment of pyrrolidinone **18** with trimethylsilyl bromide in dichloromethane gave the phosphonic acid **20** in 75% yield (determined by ^{1}H NMR spectroscopy).

Synthesis of α-Aminobis(phosphonic) Derivatives

Several methods have been described for the synthesis of 1,1-bis(phosphonates).[43] A simple way to prepare such compounds is a multicomponent reaction between carboxylic acids with phosphorous acid and phosphorus trichloride, phosphorus pentachloride or phosphorus oxychloride.[44] Another recent route exploits Michael-type additions to vinylidenebis(phosphonates).[45] Mizrahi et al. have reported the preparation of aminobis(phosphonates) derived from amino acids: the key step is the reaction of an α-(aminoacyl)phosphonate with a dialkyl phosphite in the presence of triethylamine.[46] The synthesis of iminobis(phosphonic) derivatives and their subsequent reduction has been described by Palacios et al.[47] Lastly, phosphonoazadienes have been used as precursors of substituted α-aminobis(phosphonates).[48]

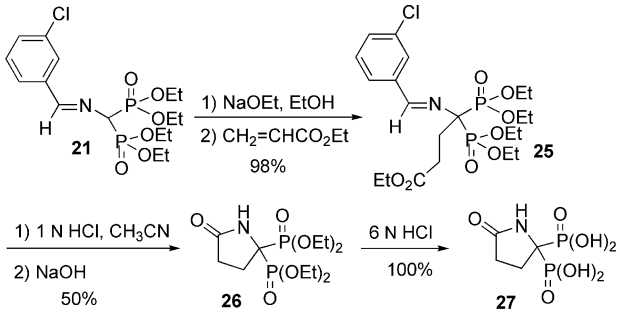
Derivatives bearing two phosphonic groups were synthesised by alkylation of the imine **21**. The amine precursor, which was synthesised beforehand,[49] was condensed with 3-chlorobenzaldehyde to furnish **21** in 70% yield after purification (Scheme 7). The Schiff base **21** was treated with potassium carbonate, triethylammonium bromide and methyl bromoacetate to give the crude imine **22** in 91% yield.[50] The amine **23** was isolated after the smooth acidic hydrolysis of imine **22** in 76% yield after purification by chromatography. The target molecule **24** (hydrochloride salt) was obtained quantitatively after treatment with 6 N HCl.



Scheme 7. Synthesis of α-aminobis(phosphonic acid) **24**.

To prepare the homologue, imine **21** was treated with sodium ethoxide and ethyl acrylate in ethanol at 80 °C for 2 h to furnish the crude imine **25** in 98% yield (Scheme 8). The pyrrolidinone **26**, obtained by acidic hydrolysis at room

temperature, was heated with 6 N HCl to give quantitatively the pyrrolidinone **27**. The open-chain product (homologue of **24**) was not obtained.



Scheme 8. Synthesis of pyrrolidinone **27**.

Purification/Characterisation

A major difficulty of the chemistry of phosphonate and phosphonic derivatives lies in their purification by standard chromatography owing to their high polarity. Usually, pure phosphonates can be obtained after arduous column chromatography on silica gel, but with loss of yield. The deprotected compounds, phosphonic/carboxylic derivatives, were extracted with water and recovered by lyophilisation. The deprotections were thus preferably performed on pure phosphonate precursors.

The NMR characterisation of phosphonates is also complicated due to supplementary short- and long-range couplings with phosphorus atoms ($J_{H,P}$ and $J_{C,P}$). The NMR data for the series of Schiff bases are collected in Table 2. We have summarised some typical ^1H, ^{13}C and ^{31}P NMR chemical shifts of structural features common to the compounds described above. The imine proton is deshielded (signal shifted by 0.3–0.7 ppm) after dialkylation of the α-carbon atom (compare **1** and **21** with **3**, **11**, **17**, **22** and **25**), and the signal appears in the region of δ = 8.2–8.7 ppm as a doublet or triplet due to long-range H–P coupling. The signals of the corresponding carbon atoms are found at $\delta \approx$ 160–165 ppm. The α-carbon atoms ($\delta \approx$ 65 ppm) are easily identified due to their large C–P coupling constant of 140–155 Hz.

Table 3 shows the NMR spectroscopic data for the aminophosphonates and pyrrolidinones. Relative to their imine precursors, the α-carbon atom of the aminophosphonates is shielded (signal shifted by about 10 ppm; compare **4**, **12** and **23** with **3**, **11** and **22**) and the signal appears at δ = 53–54 ppm as a large doublet or triplet. In the cyclic structures (**13**, **18** and **26**), the corresponding quaternary carbon atom is more deshielded (signal shifted by 58–59 ppm). For all structures the ^{31}P NMR spectra show the signal typical of diethyl phosphonates at δ = 16–26 ppm in $CDCl_3$. For the corresponding phosphonic acids the ^{31}P atom was shielded (signal shifted by about 4 ppm), and the signal is found at δ = 12–22 ppm in D_2O.

Table 3. NMR spectroscopic data for the aminophosphonates and pyrrolidinones.[a]

Compound	C-α: δ_C, mult., $^1J_{C,P}$	δ_P
4	53.18, d, 158.3	26.43
10	57.90, d, 150.8	25.34
12	53.74, d, 158.6	28.30
23	55.81, t, 145.5	21.61
13	57.84, d, 164.7	24.60
18	59.27, d, 162.2	26.20
26	58.75, t, 152.5	19.82; 19.84

[a] δ given in ppm and J given in Hz.

Biochemical Evaluation

The aminophosphonic and aminobis(phosphonic) derivatives were evaluated for their activity against a series of clinically representative β-lactamases of class A (TEM-1,[51] BS3,[15] NMCA[52]), class C (P99[53]), class D (Oxa-10[54]) and class B (BcII,[55] VIM-4[56]). The enzymes were incubated with the tested compounds at 37 °C and pH 7 or 5 for 30 min. Then nitrocefine (a chromogenic substrate) was added, and the hydrolysis rate of this substrate was monitored by spectrophotometry at 482 nm. In this way, the residual activity of the β-lactamases could be determined. The results shown in Table 4 are expressed as a percentage of the β-lactamase initial activity. The compounds were tested at a concentration of 100 μM; low % values indicate very active compounds. The limit for considering a compound as a possible "hit" in this screening has been fixed at 80%.

The biochemical results are disappointing because none of the tested compounds inhibited BcII and VIM-4 (class B, zinc β-lactamase) nor P99 (class C) and Oxa-10 (class D). Like the amino analogues of citrate, the aminophosphonic derivatives are modestly active against class A β-lactamases.

Table 2. NMR spectroscopic data for the Schiff bases.[a]

Cmpound	HC=N: δ_H, mult., $^4J_{H,P}$	HC=N and C-α: δ_C, mult., $^3J_{C,P}$; δ_C, mult., $^1J_{C,P}$	δ_P
1	8.23, d, 4.9	165.24, d, 16.4; 57.55, d, 152.9	22.48
2	8.33, d, 4.8	164.44, d, 15.5; 64.93, d, 157.7	22.92
3	8.93, d, 5.0	160.22, d, 12.9; 64.06, d, 155.3	22.81
11	8.49, d, 4.7	160.98, d, 12.3; 64.91, d, 150.1	25.62
17	8.49, d, 4.2	161.04, d, 12.6; 64.82, d, 147.4	24.31
21	8.29, t, 4.3	165.92, t, 15.2; 67.97, t, 149.0	15.9; 15.92
22	8.74, t, 3.7	164.35, t, 13.3; 68.98, t, 150.1	17.38
25	8.76, t, 3.3	164.63, t, 12.0; 68.02, t, 136.8	19.25

[a] δ given in ppm and J given in Hz.

Table 4. Inhibition of β-lactamases by aminophosphonic and aminobis(phosphonic) derivatives at pH 5 (pH 7).[a]

Compound[b]	TEM-1	BS3	NMCA	P99	Oxa-10	Bc-II	VIM-4
4	83 (96)	97 (89)	83 (100)	98 (100)	100 (92)	100 (X)	X (97)
7	89 (100)	97 (89)	83 (100)	98 (100)	100 (92)	100 (X)	X (97)
8	92 (100)	98 (100)	76 (100)	97 (100)	91 (88)	100 (X)	X (100)
10	88 (80)	100 (100)	95 (100)	97 (93)	97 (100)	95 (100)	X (100)
12	87 (100)	100 (100)	99 (100)	93 (100)	96 (85)	87 (X)	X (99)
13	84 (100)	100 (100)	83 (91)	97 (100)	98 (93)	92 (X)	X (100)
14/15	94 (100)	98 (99)	64 (98)	100 (92)	92 (87)	100 (X)	X (95)
16	100 (95)	100 (100)	100 (100)	100 (100)	87 (99)	85 (97)	X (100)
18	92 (100)	100 (99)	97 (100)	95 (100)	92 (85)	100 (X)	X (100)
19	97 (100)	97 (100)	92 (100)	100 (95)	92 (88)	88 (X)	X (96)
20	100 (90)	99 (100)	100 (100)	100 (98)	96 (100)	95 (100)	X (91)
23	100 (95)	100 (100)	90 (99)	100 (100)	91 (97)	99 (100)	X (96)
24	93 (89)	97 (100)	100 (100)	100 (98)	100 (100)	99 (100)	X (96)
26	91 (98)	100 (100)	97 (100)	91 (100)	96 (84)	100 (X)	X (100)
27	90 (100)	89 (100)	88 (95)	100 (86)	100 (96)	96 (100)	X (89)

[a] Results are expressed as a percentage of the initial activity. [b] Compounds were tested in 50 mM phosphate buffer at pH 7 and in 50 mM acetate buffer at pH 5 at a concentration of 100 μM; X = not tested.

Compound **8** and the mixture **14** and **15** are inhibitors of NMCA (class A). The inhibition constants (K_i) measured at pH 5 are 800 and 360 μM, respectively.

Some of these molecules were tested against R39,[57] a low molecular weight D,D-transpeptidase/carboxypeptidase. The enzyme was incubated with the test compounds for 16 h, and then fluorescent ampicillin was added. After 45 min, the protein was denatured and the fluorescence intensity was measured to determine the residual activity of the peptidase. The results shown in Table 5 are expressed as percentages of the R39 initial activity. The compounds were tested at a concentration of 500 μM; low % values indicate a very active compound. The limit for considering a compound as a possible "hit" in this screening was fixed at 70%. The compounds selected in the initial run (first column) were tested again under the same conditions (second column) and in the presence of bovin serum albumin (BSA) to control the selectivity of the inhibition.

Table 5. Inhibition of R39 by aminophosphonic and aminobis(phosphonic) derivatives.[a,b]

Compounds	R39	R39	R39 + 500 μM BSA
4	97 ± 2	n.d.	n.d.
7	36 ± 3	47 ± 5	52 ± 4
8	71 ± 5	n.d.	n.d.
12	98 ± 7	n.d.	n.d.
13	91 ± 5	n.d.	n.d.
14/15	56 ± 5	66 ± 4	73 ± 5
18	100 ± 15	n.d.	n.d.
19	69 ± 6	67 ± 8	72 ± 9
26	95 ± 9	n.d.	n.d.
27	94 ± 10	n.d.	n.d.

[a] Results are expressed as a percentage of the initial activity. [b] n.d. = not determined.

Compounds **7**, **14/15** and **19** were active against R39. They are selective, interacting with the active site that recognises penicillins. The phosphonic bioisoster of aminocitrate **7** can be considered as a good inhibitor of R39 because the activity of the enzyme decreased by more than half. The sample **14/15** is a mixture of pyrrolidinone and the open-chain compound and gives a residual activity of about 60%. The enzyme activity was similarly diminished in the presence of pyrrolidinone **19**. These results seem to indicate that the cyclic form in the mixture **14/15** is active against R39.

Conclusion

Novel aminophosphonic acids and aminobis(phosphonic acids), analogues or homologues of aminocitrate, have been synthesised in acceptable yields. The alkylation of α-aminophosphonates and -bis(phosphonates) remains a valuable synthetic strategy. However, our modified Pudovik reaction is a straightforward original route towards the phosphonic bioisoster of aminocitrate **7**. This compound and its homologues **14/15** and **19** showed good activity against R39 D,D-peptidase. The mixture of **14/15** and the related monophosphonic derivative **8** were modestly active against NMCA β-lactamase. The mode of action of these novel "hits" is under investigation by co-crystallisation experiments with the target enzymes.

The library of (bis)phosphonic derivatives that we have prepared in our search for bacterial enzyme inhibitors could be of great interest in other applications, for example, as agonists/antagonists of glutamate receptors,[19] antiproliferation agents of parasites[45] and inhibitors of tumour cell proliferation.[58]

Experimental Section

General: Reagents and solvents were purchased from Acros, Aldrich, Fluka, Sigma and Balsen. Column chromatography was carried out with silica gel 60 (70–230 or 230–400 mesh ASTM) supplied by Merck. The IR spectra were recorded with a Shimadzu FTIR-8400S instrument; only the most significant adsorption bands are reported; samples analyzed as thin films deposited on Zn/Se crystal. The ^{1}H and ^{13}C NMR spectra were recorded with Bruker spectrometer models 300 UltraShield and AM-500 (at 300 or 500 MHz for ^{1}H and at 75 or 125 MHz for ^{13}C). The ^{31}P NMR was recorded with a Bruker spectrometer (121 MHz). Chemical shifts (δ) were measured relative to chloroform or D_2O and are expressed in ppm. Coupling constants (J) are expressed in Hz. The

mass spectra were obtained with a Finnigan MAT TSQ-70 instrument. The high-resolution mass spectra (HRMS) were performed by Prof. Flammang at the University of Mons, Belgium.

Diethyl {[(3-Chlorobenzylidene)amino]methyl}phosphonate (1): Diethyl (aminomethyl)phosphonate (1 g, 5.98 mmol), $MgSO_4$ (1 g) and 3-chlorobenzaldehyde (610 μL, 5.39 mmol, 0.85 equiv.) were dissolved in dichloromethane (10 mL) and stirred for 16 h. After filtration, the organic solution was concentrated under vacuum. The residue was purified by chromatography on silica gel (elution: hexane/EtOAc, 8:2) to give **1** as a yellow oil (1.32 g, 76%). R_F(hexane/EtOAc, 8:2) = 0.30. IR: ṽ = 2981 (CHAr), 1639 (C=N), 1242 [P(O)(OEt)$_2$], 1026 [P(O)(OEt)$_2$] cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.31 (t, J = 7.0 Hz, 6 H, CH_3), 4.08 (d, $J_{H,P}$ = 17.8 Hz, 2 H, CH_2), 4.14 (q, J = 7.0 Hz, 4 H, CH_2), 7.31 (t, J = 7.7 Hz, 1 H, CHAr), 7.37 (d, J = 8.0 Hz, 1 H, CHAr), 7.55 (d, J = 7.6 Hz, 1 H, CHAr), 7.73 (s, 1 H, CHAr), 8.23 (d, $J_{H,P}$ = 4.9 Hz, 1 H, N=CH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 16.56 (d, $J_{C,P}$ = 5.8 Hz, CH_3), 57.55 (d, $J_{C,P}$ = 152.9 Hz, CH_2), 62.71 (d, $J_{C,P}$ = 6.6 Hz, CH_2), 126.80 (CHAr), 127.98 (CHAr), 129.98 (CHAr), 131.17 (CHAr), 134.88 (CAr), 137.49 (d, $J_{C,P}$ = 3.5 Hz, CAr), 165.24 (d, $J_{C,P}$ = 16.4 Hz, N=CH) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 22.48 ppm. MS (ESI): m/z = 290.11 [M + H]$^+$, 312.09 [M + Na]$^+$, 600.75 [2 M + Na]$^+$. HRMS (ESI): calcd. for $C_{12}H_{17}ClNO_3P$ + Na 312.0532; found 312.0526.

Methyl 3-Amino-3-(diethoxyphosphoryl)propanoate (6): Thionyl chloride (850 μL, 11.61 mmol, 3 equiv.) was added dropwise to a stirred and cooled (0 °C) solution of azetidinone **5** (800 mg, 3.87 mmol) in methanol (10 mL). The mixture was stirred for 24 h and concentrated under vacuum. The residue was taken up in dichloromethane, and $NaHCO_3$ (5.6 g) was added. The mixture was stirred for 30 min, filtered and concentrated under vacuum to obtain **5** as an orange oil (824 mg, 90%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.36 (t, J = 6.9 Hz, 6 H, CH_3), 2.59 (m, 1 H, CH_2), 2.86 (m, 1 H, CH_2), 3.60 (m, 1 H, CH), 3.74 (s, 3 H, CH_3), 4.19 (q, J = 6.9 Hz, 4 H, CH_2) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 16.6 ($J_{C,P}$ = 5.5 Hz, CH_3), 36.87 ($J_{C,P}$ = 2.3 Hz, CH_2), 45.82 (d, $J_{C,P}$ = 154.8 Hz, CH), 52.1 (CH_3), 62.60 ($J_{C,P}$ = 6.4 Hz, CH_2), 171.82 ($J_{C,P}$ = 20.5 Hz, CO_2Me) ppm. MS (APCI): m/z = 240.31 [M + H]$^+$, 102.22 [M – P(O)(OEt)$_2$]$^+$.

Methyl 3-[(3-Chlorobenzylidene)amino]-3-(diethoxyphosphoryl)propanoate (2): Methyl 3-amino-3-(diethoxyphosphoryl)propanoate **6** (814 mg, 3.41 mmol), $MgSO_4$ (1 g) and 3-chlorobenzaldehyde (328 μL, 2.89 mmol, 0.85 equiv.) were dissolved in dichloromethane (10 mL) and stirred for 16 h. After filtration, the solution was concentrated under vacuum. The residue was purified by chromatography on silica gel with EtOAc as eluent to obtain **2** as a white solid (1.06 g, 85%). R_F(EtOAc) = 0.54. IR: ṽ = 2986 (CHAr), 1740 (CO_2Me), 1639 (C=N), 1250 [P(O)(OEt)$_2$], 1049 {[P(O)(OEt)$_2$]} cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.30 (t, J = 7.0 Hz, 3 H, CH_3), 1.2 (t, J = 7.0 Hz, 3 H, CH_3), 3.00 (m, 2 H, CH_2), 3.62 (s, 3 H, CH_3), 4.13 (m, 5 H, CH_2, CH), 7.32 (dd, J = 8, J = 7.6 Hz, 1 H, CHAr), 7.40 (d, J = 8.05 Hz, 1 H, CHAr), 7.57 (d, J = 7.6 Hz, 1 H, CHAr), 7.76 (s, 1 H, CHAr), 8.33 (d, $J_{H,P}$ = 4.8 Hz, 1 H, N=CH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 16.64 ($J_{C,P}$ = 5.7 Hz, CH_3), 35.41 ($J_{C,P}$ = 2.4 Hz, CH_2), 53.02 (CH_3), 63.17 ($J_{C,P}$ = 6.9 Hz, CH_2), 64.93 (d, $J_{C,P}$ = 157.7 Hz, CH), 127.21 (CHAr), 128.15 (CHAr), 129.99 (CHAr), 131.31 (CHAr), 134.94 (CAr), 137.53 (CAr), 164.44 ($J_{C,P}$ = 15.5 Hz, N=CH), 171.26 ($J_{C,P}$ = 21.1 Hz, CO_2Me) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 22.92 ppm. MS (ESI): m/z = 362.19 [M + H]$^+$, 384.14 [M + Na]$^+$, 744.66 [2 M + Na]$^+$, 224.12 [M – P(O)(OEt)$_2$]$^+$. HRMS (ESI): calcd. for $C_{15}H_{21}ClNO_5P$ + Na 384.0744; found 384.0731.

Dimethyl 3-[(3-Chlorobenzylidene)amino]-3-(diethoxyphosphoryl)-pentane-1,5-dioate (3): The crude imine **2** (383 mg, 1.06 mmol) dissolved in dry THF (3 mL) and cooled to –78 °C was added under argon to a solution of LDA (commercial solution in THF, 750 μL, 1.48 mmol, 1.4 equiv.) and DMPU (205 μL, 1.69 mmol, 1.6 equiv.) in THF (2 mL). After 1 h at –78 °C, methyl bromoacetate (110 μL, 1.11 mmol, 1.05 equiv.) was added, and the mixture was allowed to slowly warm to 20 °C overnight whilst being stirred. After addition of water, the solution was concentrated under vacuum. The oily residue was dissolved in dichloromethane, and the organic layer was washed successively with NH_4Cl and brine. After drying with $MgSO_4$ and concentration under vacuum, the residue was purified by chromatography on silica gel (elution: EtOAc/hexane, 8:2), which was neutralised with Et_3N before use. The imine **3** was isolated as a yellow oil in 24% yield (79 mg). R_F(EtOAc/hexane, 8:2) = 0.55. IR: ṽ = 2985 (CHAr), 1732 (CO_2Me), 1643 (C=N), 1242 [P(O)(OEt)$_2$], 1095 [P(O)(OEt)$_2$] cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.30 (t, J = 7.0 Hz, 6 H, CH_3), 3.36 (m, 4 H, CH_2), 3.66 (s, 6 H, CH_3), 4.14 (m, 4 H, CH_2), 7.34 (dd, J = 8, J = 7.5 Hz, 1 H, CHAr), 7.38 (d, J = 8.1 Hz 1 H, CHAr), 7.60 (d, J = 7.6 Hz, 1 H, CHAr), 7.78 (s, 1 H, CHAr), 8.39 (d, $J_{H,P}$ = 5.0 Hz, 1 H, N=CH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 16.59 ($J_{C,P}$ = 5.6 Hz, CH_3), 36.41 (CH_2), 51.84 (CH_3), 64.06 (d, $J_{C,P}$ = 155.3 Hz, 1 C), 63.72 ($J_{C,P}$ = 7.1 Hz, CH_2), 127.22 (CHAr), 128.13 (CHAr), 130.03 (CHAr), 131.28 (CHAr), 134.98 (CAr), 137.93 (CAr), 160.22 ($J_{C,P}$ = 12.9 Hz, N=CH), 170.80 ($J_{C,P}$ = 12.8 Hz, CO_2Me) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 22.81 ppm. MS (ESI): m/z = 434.16 [M + H]$^+$, 456.20 [M + Na]$^+$, 888.68 [2 M + Na]$^+$, 296.10 [M – P(O)(OEt)$_2$]$^+$. HRMS (ESI): calcd. for $C_{18}H_{25}ClNO_7P$ + Na 456.0955; found 456.0947.

Characterisation of the Side-Product Dimethyl 2-{[(3-Chlorobenzylidene)amino](diethoxyphosphoryl)methyl}succinate (3′): This product was obtained as above from imine **2** (415 mg, 1.14 mmol), LDA (1.1 mL, 2.28 mmol, 2 equiv.), DMPU (220 μL, 1.82 mmol, 1.6 equiv.) and methyl bromoacetate (115 μL, 1.19 mmol, 1.05 equiv.) in THF (6 mL). After workup, the brown residue was purified by chromatography on silica gel (elution: EtOAc/hexane, 8:2) and isolated as a yellow oil in 5% yield (10 mg). R_F(EtOAc/hexane, 8:2) = 0.50. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.28 (t, J = 7 Hz, 3 H, CH_3), 1.34 (t, J = 7 Hz, 3 H, CH_3), 3.01 (dd, J = 17.1, J = 9.5 Hz, 1 H, CH_{2A}), 3.11 (dd, J = 17, J = 4 Hz, 1 H, CH_{2A}), 3.34–3.38 (m, 1 H, CH), 3.67 (s, 3 H, CH_3), 3.68 (s, 3 H, CH_3), 4.05–4.21 (m, 4 H, CH_2), 4.27 (dd, $J_{H,P}$ = 16, J = 3.9 Hz, 1 H, CH), 7.35 (dd, J = 8, J = 7 Hz, 1 H, CHAr), 7.42 (d, J = 8 Hz, 1 H, CHAr), 7.58 (d, J = 7 Hz, 1 H, CHAr), 7.76 (s, 1 H, CHAr), 8.28 (d, $J_{H,P}$ = 5.0 Hz, 1 H, N=CH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 16.67 (t, $J_{C,P}$ = 2.61 Hz, CH_3), 32.45 (d, $J_{C,P}$ = 4.19 Hz, CH_2), 42.74 (d, $J_{C,P}$ = 5.74 Hz, CH), 51.96 (CH_3), 52.48 (CH_3), 63.27 (d, $J_{C,P}$ = 6.98 Hz, CH_2), 63.37 (d, $J_{C,P}$ = 6.92 Hz, CH_2), 68.36 (d, $J_{C,P}$ = 151.52 Hz, CH), 127.24 (CHAr), 128.14 (CHAr), 130.05 (CHAr), 132.17 (CHAr), 135.05 (CAr), 137.36 (d, $J_{C,P}$ = 3.34 Hz, CAr), 160.77 ($J_{C,P}$ = 13.36 Hz, N=CH), 172.81 (CO_2Me), 172.91 (d, $J_{C,P}$ = 17.21 Hz, CO_2Me) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 21.56 ppm. MS (ESI): m/z = 434.10 [M + H]$^+$, 456.15 [M + Na]$^+$, 296.08 [M – P(O)(OEt)$_2$]$^+$.

Dimethyl 3-Amino-3-(diethoxyphosphoryl)pentanedioate (4): A 1 N HCl solution (1 mL, 1 mmol, 2.5 equiv.) was added to imine **3** (158 mg, 0.37 mmol) dissolved in acetonitrile (0.5 mL). After 10–15 min at 20 °C, the solution was concentrated under vacuum, and the aqueous phase was extracted three times with diethyl ether. The aqueous phase was then neutralised with $NaHCO_3$ and basified with 1 N NaOH to pH 10. This was extracted five times with CH_2Cl_2. The organic layers were collected and washed with brine.

After drying ($MgSO_4$) and concentration under vacuum, the residue was purified by column chromatography on silica gel (elution: EtOAc/*i*PrOH, 9:1). Amine **4** was recovered as a colourless oil (82 mg, 80%). R_F(EtOAc/*i*PrOH, 9:1) = 0.44. IR: $\tilde{\nu}$ = 3442 (NH_2), 1735 (CO_2Me), 1236 [P(O)(OEt)$_2$], 1020 [P(O)(OEt)$_2$] cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): δ = 1.31 (t, J = 7.0 Hz, 6 H, CH_3), 2.19 (br. s, 2 H, NH_2), 2.81 (d, J = 12.0 Hz, 4 H, CH_2), 3.67 (s, 6 H, CH_3), 4.15 (q, J = 7.0 Hz, 4 H, CH_2) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 16.62 ($J_{C,P}$ = 5.5 Hz, CH_3), 39.85 ($J_{C,P}$ = 1.7 Hz, CH_2), 51.92 (CH_3), 53.18 (d, $J_{C,P}$ = 158.3 Hz, 1 C), 63.36 ($J_{C,P}$ = 7.5 Hz, CH_2), 171.08 ($J_{C,P}$ = 12.7 Hz, CO_2Me) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 26.43 ppm. MS (ESI): *m*/*z* = 312.02 [M + H]$^+$, 334.14 [M + Na]$^+$, 174.07 [M – P(O)(OEt)$_2$]$^+$. HRMS (ESI): calcd. for $C_{11}H_{22}NO_7P$ + Na 334.1032; found 334.1038.

3-Amino-3-phosphonopentane-1,5-dioic Acid Hydrochloride (7): Amine **4** (47 mg) was treated with 6 N HCl (10 mL) at reflux overnight. After extraction with CH_2Cl_2, the aqueous phase was concentrated under vacuum, and the residue was dried under high vacuum to furnish **7** as a white solid (40 mg, quantitative yield). IR: $\tilde{\nu}$ = 3221 (CO_2H, C–OH), 2781 (PO_3H, P–OH), 2318 (NH_3^+), 1651 (NH_3^+), 1141 (PO_3H) cm^{-1}. ^1H NMR (D_2O, 500 MHz): δ = 2.94 (br. s, 2 H, CH_2), 2.97 (br. s, 2 H, CH_2) ppm. ^{13}C NMR (D_2O, 125 MHz): δ = 36.91 (CH_2), 53.73 (d, $J_{C,P}$ = 141.7 Hz, 1 C), 173.67 ($J_{C,P}$ = 10.1 Hz, CO_2H) ppm. ^{31}P NMR (D_2O, 121 MHz): δ = 12.45 ppm. MS (ESI): *m*/*z* = 226.08 [M – H]$^-$, 208.02 [M – H_2O]$^-$, 147.10 [M – PO_3H_2]$^-$. HRMS (ESI): calcd. for $C_5H_{10}NO_7P$ – H 226.0117; found 226.0118.

[3-Amino-1,5-bis(methoxycarbonyl)pent-3-yl]phosphonic Acid Hydrobromide (8): $BrSiMe_3$ (103 μL, 0.78 mmol, 4 equiv.) was added dropwise to amine **4** (61 mg, 0.19 mmol) dissolved in dichloromethane (3 mL). The mixture was stirred at room temperature for 24 h. After concentration under vacuum, the residue was taken up in methanol (10 mL). The solution was heated at 50 °C for 2 h and concentrated under high vacuum to obtain **8** as a yellow solid (64 mg, quantitative yield). IR: $\tilde{\nu}$ = 2792 (PO_3H, P–OH), 2360 (NH_3^+), 1713 (CO_2Me), 1608 (NH_3^+), 1180 (PO_3H) cm^{-1}. ^1H NMR (D_2O, 500 MHz): δ = 2.96 (d, J = 8.0 Hz, 4 H, CH_2), 3.67 (s, 6 H, CH_3) ppm. ^{13}C NMR (D_2O, 125 MHz): δ = 37.07 (CH_2), 53.11 (CH_3), 53.89 (d, $J_{C,P}$ = 141.4 Hz, C), 172.33 ($J_{C,P}$ = 15.1 Hz, CO_2Me) ppm. ^{31}P NMR (D_2O, 121 MHz): δ = 12.38 ppm. MS (ESI): *m*/*z* = 254.08 [M – 1]$^-$. HRMS (ESI): calcd. for $C_7H_{14}NO_7P$ + H 256.0586; found 256.0589.

Dimethyl 3-(Benzylamino)pent-2-enedioate (9): Dimethyl acetonedicarboxylate (1 mL, 6.77 mmol) and benzylamine (740 μL, 6.77 mmol, 1 equiv.) were allowed to react in the presence of molecular sieves (3 Å) in methanol (15 mL). The mixture was stirred at room temperature overnight and filtered through Celite. The product was purified by chromatography on silica gel (elution: hexane/EtOAc, 7:3) to give **9** as a white solid (1.78 g, 67%). R_F(hexane/EtOAc, 7:3) = 0.47. IR: $\tilde{\nu}$ = 3375 (NH), 2950 (CHAr), 1740 (CO_2Me), 1654 (C=C), 1601 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): δ = 3.22 (s, 2 H, CH_2, *Z*), 3.62 (s, 3 H, CH_3, *E*), 3.65 (s, 3 H, CH_3, *Z*), 3.68 (s, 3 H, CH_3, *Z*), 3.74 (s, 3 H, CH_3, *E*), 3.96 (s, 2 H, CH_2, *E*), 4.24 (d, J = 5.4 Hz, 2 H, CH_2-Ph, *E*), 4.45 (d, J = 6.4 Hz, 2 H, CH_2-Ph, *Z*), 4.62 (s, 1 H, CH, *Z*), 4.79 (s, 1 H, CH, *E*), 5.17 (br. s, 1 H, NH, *E*), 7.32 (m, 5 H, CHAr), 8.92 (br. s, 1 H, NH, *Z*) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 36.90 (CH_2, *Z*), 38.82 (CH_2, *E*), 47.24 (CH_2-Ph, *Z*), 47.98 (CH_2-Ph, *E*), 50.46 (CH_3, *Z*), 50.57 (CH_3, *E*), 52.47 (CH_3, *E*), 52.69 (CH_3, *Z*), 85.35 (CH, *E*), 85.37 (CH, *Z*), 127.01 (CHAr, *E*), 127.03 (CHAr, *E*), 127.73 (CHAr, *Z*), 127.84 (CHAr, *Z*), 129.07 (CHAr, *E*), 129.08 (CHAr, *Z*), 136.94 (CAr, *E*), 138.45 (CAr, *Z*), 154.32 (CAr, *E*), 156.94 (CAr, *Z*), 169.14 (CO_2Me, *E*), 169.31 (CO_2Me, *Z*), 170.94 (CO_2Me, *E* + *Z*) ppm. MS (ESI): *m*/*z* = 264.24 [M + H]$^+$, 286.12 [M + Na]$^+$.

Dimethyl 3-(Benzylamino)-3-(diethoxyphosphoryl)pentanedioate (10b): Diethyl trimethylsilyl phosphite (90 mg, 0.205 mmol, 1.05 equiv.) and sulfuric acid (11 μL, 0.21 mmol, 0.5 equiv.) were added to a stirred solution of enamine **9** (108 mg, 0.41 mmol) in dichloromethane (2 mL). The mixture was stirred for 3 h and then heated for 24 h. The organic phase was washed with $NaHCO_3$ and then brine, and dried with $MgSO_4$. After concentration under vacuum, the product was purified by chromatography on silica gel (elution: hexane/EtOAc, 7:3) to give **10** as a colourless oil (49.6 mg, 30%). R_F(hexane/EtOAc, 7:3) = 0.47. IR: $\tilde{\nu}$ = 3471 (NH), 2985 (CHAr), 1736 (CO_2Me), 1207 [P(O)(OEt)$_2$], 1018 [P(O)(OEt)$_2$] cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): δ = 1.34 (t, J = 7.0 Hz, 6 H, CH_3), 2.97 (d, J = 15.0 Hz, 2 H, CH_2), 3.00 (d, J = 15.0 Hz, 2 H, CH_2), 3.68 (s, 6 H, CH_3), 4.04 (s, 2 H, CH_2), 4.19 (q, J = 7.1 Hz, 4 H, CH_2), 7.28 (m, 5 H, CHAr) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 16.71 (d, $J_{C,P}$ = 5.7 Hz, CH_3), 37.63 (d, $J_{C,P}$ = 6 Hz, CH_2), 47.89 (d, $J_{C,P}$ = 1.7 Hz, CH_2), 51.90 (CH_3), 57.90 (d, $J_{C,P}$ = 150.8 Hz, 1 C), 62.89 ($J_{C,P}$ = 8.1 Hz, CH_2), 127.08 (CHAr), 128.33 (CHAr), 128.53 (CHAr), 140.68 (CHAr), 171.08 ($J_{C,P}$ = 12.6 Hz, CO_2Me) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 25.34 ppm. MS (ESI): *m*/*z* = 424.05 [M + Na]$^+$, 264.12 [M – P(O)(OEt)$_2$]$^+$. HRMS (ESI): calcd. for $C_{18}H_{28}NO_7P$ + Na 424.1501; found 424.1516.

Treatment of **10b** (25 mg, 0.062 mmol) by catalytic hydrogenation [Pd(OH)$_2$/C, 1 atm, 25 °C, 2 h] in EtOAc solution (1 mL) furnished **4** (19 mg, 100% yield), identical to the sample previously prepared.

Diethyl 3-[(3-Chlorobenzylidene)amino]-3-(diethoxyphosphoryl)-hexanedioate (11): A solution of sodium ethoxide in ethanol (*c* = 2.68 mol/L, 104 μL, 0.28 mmol, 0.2 equiv.) was added to a stirred solution of imine **2** (504 mg, 1.4 mmol) in ethanol (2 mL), and the mixture was stirred for 10 min. Then ethyl acrylate (300 μL, 2.78 mmol, 2 equiv.) was added, and the mixture was heated at reflux for 2 h. After concentration under vacuum, the residue was taken up in dichloromethane, washed with a saturated solution of NH_4Cl and dried with $MgSO_4$. After concentration under vacuum, imine **11** was obtained as a purple oil (613 mg, 92%). R_F(EtOAc) = 0.68. IR: $\tilde{\nu}$ = 2981 (CHAr), 2931–2908 (CH_2), 1732 (CO_2Me), 1639 (C=N), 1242 [P(O)(OEt)$_2$], 1022 [P(O)(OEt)$_2$] cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): δ = 1.20 (t, J = 7 Hz, 3 H, CH_3), 1.21 (t, J = 7.1 Hz, 3 H, CH_3), 1.32 (t, J = 7.0 Hz, 6 H, CH_3), 2.52 (m, 3 H, CH_2), 2.74 (d, J = 11 Hz, 1 H, CH_2), 2.93 (dd, J = 14.6, J = 7.7 Hz, 1 H, CH_2), 3.12 (dd, J = 14.6, J = 13.6 Hz, 1 H, CH_2), 4.14 (m, 8 H, CH_2), 7.35 (dd, J = 8, J = 7.6 Hz, 1 H, CHAr), 7.41 (d, J = 8.0 Hz, 1 H, CHAr), 7.61 (d, J = 7.5 Hz, 1 H, CHAr), 7.79 (s, 1 H, CHAr), 8.49 (d, $J_{H,P}$ = 4.6 Hz, 1 H, N=CH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 14.33 (CH_3), 14.36 (CH_3), 16.61 (d, $J_{C,P}$ = 5.0 Hz, CH_3), 29.11 (CH_2), 29.59 (d, $J_{C,P}$ = 4.5 Hz, CH_2), 38.03 (CH_2), 60.57 (CH_2), 60.80 (CH_2), 63.44 (d, $J_{C,P}$ = 7.3 Hz, CH_2), 63.46 (d, $J_{C,P}$ = 7.1 Hz, CH_2), 64.91 (d, $J_{C,P}$ = 150.1 Hz, 1 C), 127.19 (CHAr), 128.11 (CHAr), 130.00 (CHAr), 131.28 (CHAr), 134.94 (CAr), 138.04 (d, $J_{C,P}$ = 3.4 Hz, CAr), 160.98 ($J_{C,P}$ = 12.3 Hz, N=CH), 169.69 ($J_{C,P}$ = 16.1 Hz, CO_2Et), 173.41 (CO_2Et) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 24.31 ppm. MS (ESI): *m*/*z* = 476.16 [M + H]$^+$, 498.19 [M + Na]$^+$, 972.81 [2 M + Na]$^+$, 338.16 [M – P(O)(OEt)$_2$]$^+$. HRMS (ESI): calcd. for $C_{21}H_{31}ClNO_7P$ + Na 498.1424; found 498.1439.

Diethyl 3-Amino-3-(diethoxyphosphoryl)hexanedioate (12) and 5-(Diethoxyphosphoryl)-5-[(ethoxycarbonyl)methyl]pyrrolidin-2-one (13): Imine **11** (676 mg, 1.42 mmol) was dissolved in acetonitrile (1.5 mL), and 1 N HCl (2.2 mL, 2.2 mmol, 1.5 equiv.) was added (1 mL, 1 mmol, 2.5 equiv.). After 30 min at room temperature, the

solution was concentrated under vacuum, and the aqueous phase was extracted three times with diethyl ether. The aqueous phase was then neutralised with $NaHCO_3$ and basified with 1 N NaOH to pH 10. This was extracted five times with CH_2Cl_2. The organic layers were collected and washed with brine. After drying ($MgSO_4$) and concentration under vacuum, the oily residue was purified by column chromatography on silica gel (elution: EtOAc/*i*PrOH, 14:1). Two products were recovered as a colourless oil: amine **12** (154 mg, 31%) and pyrrolidinone **13** (125 mg, 29%). **12:** R_F(EtOAc/*i*PrOH, 20:1) = 0.47. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.27 (t, J = 7.1 Hz, 6 H, CH_3), 1.35 (t, J = 7.1 Hz, 6 H, CH_3), 1.85 (br. s, 2 H, NH_2), 2.09 (td, J = 16.3, J = 8.2 Hz, 2 H, CH_2), 2.61 (m, 4 H, CH_2), 4.16 (m, 8 H, CH_2) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 14.29 (CH_3), 14.36 (CH_3), 16.61 ($J_{C,P}$ = 1.0 Hz, CH_3), 29.06 (CH_2), 31.53 (CH_2), 39.7 (CH_2), 53.74 (d, $J_{C,P}$ = 158.6 Hz, C), 60.66 (CH_2), 60.91 (CH_2), 62.85 (d, $J_{C,P}$ = 9.1 Hz, CH_2), 63.24 (d, $J_{C,P}$ = 7.8 Hz, CH_2), 170.64 ($J_{C,P}$ = 13 Hz, CO_2Et), 173.54 (CO_2Et) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 28.30 ppm. MS (ESI): m/z = 354.06 $[M + H]^+$, 376.14 $[M + Na]^+$, 216.18 $[M – P(O)(OEt)_2]^+$. HRMS (ESI): calcd. for $C_{14}H_{28}NO_7P$ + Na 376.1501; found 376.1508. **13:** R_F(EtOAc/*i*PrOH, 20:1) = 0.15. IR: $\tilde{\nu}$ = 3213 (NH), 2981 (CH_2), 1732 (CO_2Et), 1701 (NHCO), 1238 [P(O)(OEt)$_2$], 1022 [P(O)(OEt)$_2$] cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.28 (t, J = 7.1 Hz, 3 H, CH_3), 1.35 (td, J = 7.1, $J_{H,P}$ = 2.7 Hz, 6 H, CH_3), 2.22 (m, 1 H, CH_2), 2.36 (m, 1 H, CH_2), 2.57 (m, 3 H, CH_2), 2.90 (dd, J = 15.1, 7.3 Hz, 1 H, CH_2), 4.18 (m, 6 H, CH_2), 6.21 (br. s, NH) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 14.24 (CH_3), 16.66 ($J_{C,P}$ = 5.62 Hz, CH_3), 28.47 (CH_2), 29.71 (CH_2), 40.74 ($J_{C,P}$ = 9.4 Hz, CH_2), 57.84 (d, $J_{C,P}$ = 164.7 Hz, C), 61.26 (CH_2), 63.33 (d, $J_{C,P}$ = 7.6 Hz, CH_2), 63.76 (d, $J_{C,P}$ = 7.4 Hz, CH_2), 169.61 ($J_{C,P}$ = 11 Hz, CO_2Et), 177.35 ($J_{C,P}$ = 3.7 Hz, NHCO) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 24.60 ppm. MS (ESI): m/z = 308.18 $[M + H]^+$, 330.20 $[M + Na]^+$, 636.90 $[2 M + Na]^+$, 170.16 $[M – P(O)(OEt)_2]^+$. HRMS (ESI): calcd. for $C_{12}H_{22}NO_6P$ + Na 330.1082; found 330.1087.

Mixture of 2-(5-Oxo-2-phosphonopyrrolidin-2-yl)acetic Acid (14) and 3-Amino-3-phosphonohexanedioic Acid Hydrochloride (15): Pyrrolidone **13** (100 mg) was treated with 6 N HCl (10 mL) at reflux for 20 h. After extraction with CH_2Cl_2, the aqueous phase was concentrated under vacuum, and the residue was dried under high vacuum to furnish a white solid (74 mg, quantitative yield), which was a mixture in equilibrium of cyclic **14** and the noncyclic product **15**. **14:** IR: $\tilde{\nu}$ = 3333 (NH), 2923 (CO_2H, PO_3H, C–OH, P–OH), 1647 (CO_2H, NHCO), 1207 (PO_3H) cm^{-1}. ^{1}H NMR (D_2O, 500 MHz): δ = 2.25–2.28 (m, 1 H, CH_2), 2.45–2.47 (m, 3 H, CH_2), 2.74 (dd, J = 15.5, 7.5 Hz, 1 H, CH_2), 2.84 (dd, J = 15.5, 8.1 Hz, 1 H, CH_2) ppm. ^{13}C NMR (D_2O, 125 MHz): δ = 26.91 (CH_2), 30.51 (CH_2), 39.74 ($J_{C,P}$ = 8.5 Hz, CH_2), 59.28 (d, $J_{C,P}$ = 156.5 Hz, 1 C), 174.53 ($J_{C,P}$ = 14.8 Hz, CO_2H), 181.68 ($J_{C,P}$ = 4.9 Hz, NHCO) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 20.81 ppm. MS (ESI): m/z = 224.04 $[M + H]^+$, 447.00 $[2 M + H]^+$. HRMS (ESI): calcd. for $C_6H_{10}NO_6P$ + H 224.0324; found 224.0316. **15:** ^{1}H NMR (D_2O, 500 MHz): δ = 2.12 (m, 2 H, CH_2), 2.53 (m, 1 H, CH_2), 2.58 (m, 1 H, CH_2), 2.76 (m, 1 H, CH_2), 2.87 (dd, J = 17.0, 7.3 Hz, 1 H, CH_2) ppm. ^{13}C NMR (D_2O, 125 MHz): δ = 28.53 (CH_2), 29.10 ($J_{C,P}$ = 2.8 Hz, CH_2), 35.93 (CH_2), 55.29 (d, $J_{C,P}$ = 143.6 Hz, 1 C), 173.80 (d, $J_{C,P}$ = 13.5 Hz, CO_2H), 176.91 (CO_2H) ppm. MS (ESI): m/z = 242.05 $[M + H]^+$. HRMS (ESI): calcd. for $C_6H_{12}NO_7P$ + H 242.0430; found 242.0441.

[2-(Ethoxycarbonylmethyl)-5-oxopyrrolidin-2-yl]phosphonic Acid (16): $BrSiMe_3$ (77 μL, 0.58 mmol, 4 equiv.) was added dropwise to the pyrrolidone **13** (45 mg, 0.15 mmol) dissolved in dichloromethane (3 mL). The mixture was stirred at room temperature for 24 h. After concentration under vacuum, the residue was taken up in water and freeze-dried to obtain **16** as a white hygroscopic solid (34 mg, 90%). IR: $\tilde{\nu}$ = 3282 (NH, PO_3H, P–OH), 1712 (CO_2Et), 1651 (NHCO), 1207 (PO_3H) cm^{-1}. ^{1}H NMR (D_2O, 500 MHz): δ = 1.25 (t, J = 7.1 Hz, 3 H, CH_3), 2.42 (m, 4 H, CH_2), 2.78 (ddd, J = 14.9, J = 6.9, J = 5.1 Hz, 1 H, CH_2), 2.91 (ddd, J = 15.4, J = 8, J = 3.1 Hz, 1 H, CH_2), 4.19 (q, J = 7.1 Hz, 2 H, CH_2) ppm. ^{13}C NMR (D_2O, 125 MHz): δ = 14.80 (CH_3), 27.83 (CH_2), 31.53 (CH_2), 40.97 (d, $J_{C,P}$ = 8.7 Hz, CH_2), 60.27 (d, $J_{C,P}$ = 161 Hz, C), 63.58 (CH_2), 173.50 (d, $J_{C,P}$ = 15.6 Hz, CO_2Et), 182.65 (d, $J_{C,P}$ = 5 Hz, NHCO) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 22.3 ppm. MS (ESI): m/z = 252.06 $[M + H]^+$, 170.13 $[M – PO_3H_2]$. HRMS (ESI): calcd. for $C_8H_{14}NO_6P$ + H 252.0637; found 252.0632.

Diethyl 4-[(3-Chlorobenzylidene)amino]-4-(diethoxyphosphoryl)heptanedioate (17): A solution of sodium ethoxide in ethanol (c = 2.68 mol/L, 94 μL, 0.25 mmol, 0.25 equiv.) was added to a stirred solution of imine **1** (290 mg, 0.97 mmol) in ethanol (3 mL), and the mixture was stirred for 15 min. Then ethyl acrylate (422 μL, 3.9 mmol, 4 equiv.) was added, and the mixture was heated at reflux for 24 h. After concentration under vacuum, the residue was taken up in ethyl acetate, washed with a saturated solution of NH_4Cl and dried with $MgSO_4$. After concentration under vacuum, the crude brown oil was purified by chromatography on silica gel (elution: CH_2Cl_2/EtOAc, 7:3). Imine **17** was recovered as a yellow oil (206 mg, 44% yield). R_F(CH_2Cl_2/EtOAc, 7:3) = 0.49. IR: $\tilde{\nu}$ = 2981 (CHAr), 2931–2908 (CH_2), 1732 (CO_2Me), 1639 (C=N), 1242 [P(O)(OEt)$_2$], 1022 [P(O)(OEt)$_2$] cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.26 (t, J = 7.0 Hz, 6 H, CH_3), 1.32 (t, J = 7.1 Hz, 6 H, CH_3), 2.29 (m, 4 H, CH_2), 2.45 (m, 2 H, CH_2), 2.58 (m, 2 H, CH_2), 4.2 (m, 8 H, CH_2), 7.36 (dd, J = 8.1, J = 7.5 Hz, 1 H, CHAr), 7.42 (d, J = 8.1 Hz, 1 H, CHAr), 7.61 (d, J = 7.5 Hz, 1 H, CHAr), 7.79 (s, 1 H, CHAr), 8.49 (d, $J_{H,P}$ = 4.2 Hz, 1 H, N=CH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 14.37 (CH_3), 16.68 (d, $J_{C,P}$ = 7.2 Hz, CH_3), 29.04 (d, $J_{C,P}$ = 6.5 Hz, CH_2), 29.42 (CH_2), 64.82 (d, $J_{C,P}$ = 147.4 Hz, 1 C), 60.71 (CH_2), 63.12 ($J_{C,P}$ = 7.4 Hz, CH_2), 127.15 (CHAr), 127.85 (CHAr), 130.04 (CHAr), 131.25 (CHAr), 135.01 (CAr), 138.15 (d, $J_{C,P}$ = 4.8 Hz, CAr), 161.04 ($J_{C,P}$ = 12.5 Hz, N=CH), 173.50 (CO_2Et) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 25.62 ppm. MS (ESI): m/z = 490.03 $[M + H]^+$, 512.08 $[M + Na]^+$, 352.09 $[M – P(O)(OEt)_2]^+$. HRMS (ESI): calcd. for $C_{22}H_{33}ClNO_7P$ + Na 512.1581; found 512.1595.

5-(Diethoxyphosphoryl)-5-[2-(ethoxycarbonyl)ethyl]pyrrolidin-2-one (18): Imine **17** (206 mg, 0.42 mmol) was dissolved in acetonitrile (0.5 mL), and 1 N HCl (1 mL, 1 mmol, 2.5 equiv.) was added. After 30 min at room temperature, the solution was concentrated under vacuum, and the aqueous phase was extracted three times with diethyl ether. The aqueous phase was then neutralised with $NaHCO_3$ and basified with 1 N NaOH to pH 10. This was extracted five times with CH_2Cl_2. The organic layers were collected and washed with brine. After drying ($MgSO_4$) and concentration under vacuum, the oily residue was purified by column chromatography on silica gel (elution: CH_2Cl_2/EtOAc, 5:5). Pyrrolidinone **18** was isolated as a pale-yellow oil (70 mg, 53% yield). R_F(CH_2Cl_2/EtOAc, 5:5) = 0.18. IR: $\tilde{\nu}$ = 3321 (NH), 2982–2847 (CH_2), 1704 (NHCO, CO_2Et), 1242 [P(O)(OEt)$_2$], 1022 [P(O)(OEt)$_2$] cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.25 (t, J = 7.1 Hz, 3 H, CH_3), 1.34 (td, J = 7.1, $J_{H,P}$ = 2.3 Hz, 6 H, CH_3), 1.97 (m, 2 H, CH_2), 2.36 (m, 6 H, CH_2), 4.16 (m, 6 H, CH_2), 6.24 (br. s, 1 H, NH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 14.35 (CH_3), 16.73 (t, $J_{C,P}$ = 5.6 Hz, CH_3), 27.32 (CH_2), 28.54 (d, $J_{C,P}$ = 7.5 Hz, CH_2), 30.02 (CH_2), 30.74 (d, $J_{C,P}$ = 9.2 Hz, CH_2), 59.27 (d, $J_{C,P}$ = 162.2 Hz, 1 C), 61.01 (CH_2), 63.08 (d, $J_{C,P}$ = 7.8 Hz, CH_2), 63.68 (d, $J_{C,P}$ = 7.4 Hz, CH_2), 172.97 (CO_2Et), 177.97 (d, $J_{C,P}$ = 7.4 Hz, NHCO) ppm. ^{31}P NMR

($CDCl_3$, 121 MHz): δ = 26.20 ppm. MS (ESI): *m/z* = 322.06 [M + H]$^+$, 344.18 [M + Na]$^+$, 184.05 [M – P(O)(OEt)$_2$]$^+$. HRMS (ESI): calcd. for $C_{13}H_{24}NO_6P$ + Na 344.1239; found 344.1236.

3-(5-Oxo-2-phosphonopyrrolidin-2-yl)propanoic Acid (19): Pyrrolidone **18** (70 mg) was treated with 6 N HCl (10 mL) at reflux for 48 h. After extraction with CH_2Cl_2, the aqueous phase was freeze-dried to furnish **19** as a white hygroscopic solid (56 mg, quantitative yield). IR: $\tilde{\nu}$ = 3198 (NH), 3313 (CO_2H, C–OH), 2955 (CH_2), 2851 (PO_3H, P–OH), 1732 (CO_2H), 1701 (NHCO), 1207 (PO_3H_2) cm^{-1}. ^{1}H NMR (D_2O, 300 MHz): δ = 1.98 (m, 4 H, CH_2), 2.35 (m, 4 H, CH_2) ppm. ^{13}C NMR (D_2O, 75 MHz): δ = 25.89 (CH_2), 28.23 ($J_{C,P}$ = 9 Hz, CH_2), 29.68 ($J_{C,P}$ = 7.7 Hz, CH_2), 30.42 (CH_2), 60.35 (d, $J_{C,P}$ = 156 Hz, C), 177.77 (CO_2H), 181.44 (NHCO) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 22.46 ppm. MS (ESI): *m/z* = 236.16 [M + H]$^+$, 218.01 [M – H_2O]$^+$. HRMS (ESI): calcd. for $C_7H_{12}NO_6P$ + Na 238.0481; found 238.0483.

2-[2-(Ethoxycarbonyl)ethyl]-5-oxopyrrolidin-2-ylphosphonic Acid (20): $BrSiMe_3$ (78 μL, 0.59 mmol, 4 equiv.) was added dropwise to the pyrrolidinone **18** (48 mg, 0.15 mmol) dissolved in dichloromethane (2 mL). The mixture was stirred at room temperature for 24 h. After concentration under vacuum, the residue was taken up in water and freeze-dried to obtain **20** as a white solid (38 mg, 75% yield determined by ^{1}H NMR). IR: $\tilde{\nu}$ = 3282 (NH), 2935 (PO_3H, P–OH), 2981 (CH_2), 1716 (CO_2Et), 1666 (NHCO), 1196 (PO_3H_2) cm^{-1}. ^{1}H NMR (D_2O, 500 MHz): δ = 1.09 (t, *J* = 7.1 Hz, 3 H, CH_3), 1.98 (m, 3 H, CH_2), 2.33 (m, 5 H, CH_2), 4.00 (q, *J* = 7.1 Hz, 2 H, CH_2) ppm. ^{13}C NMR (D_2O, 125 MHz): δ = 13.62 (CH_3), 26.08 (CH_2), 28.59 ($J_{C,P}$ = 8.9 Hz, CH_2), 29.92 ($J_{C,P}$ = 8.4 Hz, CH_2), 30.50 (CH_2), 60.67 (d, $J_{C,P}$ = 158 Hz, 1 C), 62.37 (CH_2), 176.23 (CO_2Et), 181.52 (NHCO) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 24.63 ppm. MS (ESI): *m/z* = 288.10 [M + Na]$^+$, 530.99 [2 M + H]$^+$, 552.96 [2 M + Na]$^+$, 184.04 [M – PO_3H_2]$^+$. HRMS (ESI): calcd. for $C_9H_{16}NO_6P$ + Na 288.0613; found 287.9703.

Tetraethyl {[(3-Chlorobenzylidene)amino]methylene}diphosphonate (21): Tetraethyl (aminomethylene)diphosphonate (546 mg, 1.8 mmol), $MgSO_4$ (1 g) and 3-chlorobenzaldehyde (183 μL, 1.62 mmol, 0.9 equiv.) were stirred in dichloromethane (7 mL) for 16 h. After filtration, the organic solution was concentrated under vacuum. The oily residue was purified by chromatography on silica gel (elution: EtOAc/CH_2Cl_2, 7:3) to give **21** as a yellow oil (566 mg, 70% yield). R_F(EtOAc/CH_2Cl_2, 7:3) = 0.34. IR: $\tilde{\nu}$ = 2981 (CHAr), 1631 (C=N), 1253 [P(O)(OEt)$_2$], 1026 [P(O)(OEt)$_2$] cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.33 (t, *J* = 7.1 Hz, 6 H, CH_3), 1.35 (t, *J* = 7.1 Hz, 6 H, CH_3), 4.25 (m, 8 H, CH_2), 4.38 (t, $J_{H,P}$ = 18.3 Hz, 1 H, CH), 7.37 (dd, *J* = 8.3, *J* = 7.4 Hz, 1 H, CHAr), 7.44 (d, *J* = 8.3 Hz, 1 H, CHAr), 7.63 (d, *J* = 7.4 Hz, 1 H, CHAr), 7.81 (s, 1 H, CHAr), 8.29 (t, $J_{H,P}$ = 4.3 Hz, 1 H, N=CH) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 16.30 (CH_3), 16.35 (CH_3), 63.37 (d, $J_{C,P}$ = 7.2 Hz, CH_2), 63.50 (d, $J_{C,P}$ = 7.1 Hz, CH_2), 67.97 (t, $J_{C,P}$ = 149 Hz, CH), 126.98 (CHAr), 127.92 (CHAr), 129.90 (CHAr), 131.39 (CHAr), 134.75 (CAr), 137.10 ($J_{C,P}$ = 2.8 Hz, CAr), 166.00 ($J_{C,P}$ = 15.3 Hz, N=CH) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 15.90–15.92 ppm. MS (ESI): *m/z* = 426.13 [M + H]$^+$. HRMS (ESI): calcd. for $C_{16}H_{26}ClNO_6P_2$ + Na 448.0822; found 448.0809.

Methyl 3-[(Chlorobenzylidene)amino]-3,3-bis(diethoxyphosphoryl)-propanoate (22): Potassium carbonate (44 mg, 0.33 mmol, 2.0 equiv.), tetrabutylammonium bromide (5 mg, 0.016 mmol, 0.1 equiv.) and methyl bromoacetate (16 μL, 0.17 mmol, 1.05 equiv.) were added successively to imine **21** (70 mg, 0.16 mmol) dissolved in acetonitrile (1 mL). The heterogeneous mixture was heated at 50 °C for 18 h. The mixture was filtered and concentrated under vacuum. The residue was taken up in dichloromethane, washed with brine and dried with $MgSO_4$. Crude **22** was recovered as a yellow oil after concentration under vacuum (73 mg, 91%). R_F(EtOAc/*i*PrOH, 9:1) = 0.48. IR: $\tilde{\nu}$ = 2981 (CHAr), 1740 (CO_2Me), 1639 (C=N), 1250 [P(O)(OEt)$_2$], 1022 [P(O)(OEt)$_2$] cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.29 (t, *J* = 7.1 Hz, 6 H, CH_3), 1.30 (t, *J* = 7.1 Hz, 6 H, CH_3), 3.31 (dd, *J* = 14.4, *J* = 11.1 Hz, 2 H, CH_2), 3.61 (s, 3 H, CH_3), 4.18 (m, 8 H, CH_2), 7.33 (dd, *J* = 7.5, *J* = 8 Hz, 1 H, CHAr), 7.39 (d, *J* = 8 Hz, 1 H, CHAr), 7.64 (d, *J* = 7.5 Hz, 1 H, CHAr), 7.79 (s, 1 H, CHAr), 8.74 (t, $J_{H,P}$ = 3.7 Hz, 1 H, N=CH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 16.55 (d, $J_{C,P}$ = 6 Hz, CH_3), 35.66 (CH_2), 51.95 (CH_3), 64.02 (d, $J_{C,P}$ = 7.5 Hz, CH_2), 64.14 (d, $J_{C,P}$ = 6.9 Hz, CH_2), 68.98 (t, $J_{C,P}$ = 144 Hz, 1 C), 127.11 (CHAr), 128.20 (CHAr), 130.05 (CHAr), 131.37 (CHAr), 134.95 (CAr), 137.90 (t, $J_{C,P}$ = 3 Hz, CAr), 164.35 (t, $J_{C,P}$ = 13.3 Hz, N=CH), 169.65 (t, $J_{C,P}$ = 11.6 Hz, CO_2Me) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 17.38 ppm. MS (ESI): *m/z* = 498.12 [M + H]$^+$, 520.16 [M + Na]$^+$, 1016.68 [2 M + Na]$^+$. HRMS (ESI): calcd. for $C_{19}H_{30}ClNO_8P_2$ + H 498.1213; found 498.1221.

Methyl 3-Amino-3,3-bis(diethoxyphosphoryl)propanoate (23): Imine **22** (152 mg, 0.31 mmol) was dissolved in acetonitrile (0.5 mL), and 1 N HCl (1 mL, 1 mmol, 2.5 equiv.) was added. After 30 min at room temperature, the solution was concentrated under vacuum, and the aqueous phase was extracted three times with diethyl ether. The aqueous phase was then neutralised with $NaHCO_3$ and basified with 1 N NaOH to pH 10. This was extracted five times with CH_2Cl_2. The organic layers were collected and washed with brine. After drying ($MgSO_4$) and concentration under vacuum, the oily residue was purified by column chromatography on silica gel (elution: EtOAc/*i*PrOH, 9:1). Amine **23** was isolated as a colourless oil (87 mg, 76%). R_F(EtOAc/*i*PrOH, 9:1) = 0.21. IR: $\tilde{\nu}$ = 3464 (NH_2), 1736 (CO_2Me), 1242 [P(O)(OEt)$_2$], 1022 [P(O)(OEt)$_2$] cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.33 (t, *J* = 7.1 Hz, 12 H, CH_3), 2.30 (br. s, 2 H, NH_2), 2.86 (d, *J* = 14.1 Hz, 1 H, CH_2), 2.91 (d, *J* = 14.1 Hz, 1 H, CH_2), 3.69 (s, 3 H, CH_3), 4.18 (m, 8 H, CH_2) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 16.58 (d, $J_{C,P}$ = 2.9 Hz, CH_3), 16.66 (d, $J_{C,P}$ = 3 Hz, CH_3), 36.85 (t, $J_{C,P}$ = 2.4 Hz, CH_2), 52.07 (CH_3), 55.81 (t, $J_{C,P}$ = 145.5 Hz, 1 C), 63.68 (t, $J_{C,P}$ = 3.6 Hz, CH_2), 64.09 (t, $J_{C,P}$ = 3.4 Hz, CH_2), 170.81 (t, $J_{C,P}$ = 12.9 Hz, CO_2Me) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 21.61 ppm. MS (ESI): *m/z* = 376.00 [M + H]$^+$, 398.16 [M + Na]$^+$, 750.79 [2 M + H]$^+$, 772.74 [2 M + Na]$^+$, 238.11 [M – P(O)(OEt)$_2$]$^+$. HRMS (ESI): calcd. for $C_{12}H_{27}NO_8P_2$ + Na 398.1110; found 398.1126.

3-Amino-3,3-diphosphonopropanoic Acid Hydrochloride (24): Amine **23** (54 mg) was treated with 6 N HCl (10 mL) at reflux overnight. After extraction with CH_2Cl_2, the aqueous phase was freeze-dried to furnish **24** as a white solid (39 mg, quantitative yield). IR: $\tilde{\nu}$ = 3055 (CO_2H, C–OH), 2762 (PO_3H, P–OH), 2360 (NH_3^+), 1716 (CO_2H), 1508 (NH_3^+), 1141(PO_3H) cm^{-1}. ^{1}H NMR (D_2O, 500 MHz): δ = 3.00 (t, $J_{H,P}$ = 11.5 Hz, 2 H, CH_2) ppm. ^{13}C NMR (D_2O, 125 MHz): δ = 35.14 (CH_2), 55.19 (t, $J_{C,P}$ = 127 Hz, 1 C), 174.50 ($J_{C,P}$ = 10.3 Hz, CO_2H) ppm. ^{31}P NMR (D_2O, 121 MHz): δ = 11.59 ppm. MS (ESI): *m/z* = 248.05 [M – H]$^-$. HRMS (ESI): calcd. for $C_3H_9NO_8P_2$ + H 249.9882; found 249.9889.

Ethyl 4-[(3-Chlorobenzylidene)amino]-4,4-bis(diethoxyphosphoryl)-butanoate (25): A solution of sodium ethoxide in ethanol (*c* = 2.68 mol/L, 240 μL, 0.64 mmol, 0.25 equiv.) was added to a stirred solution of imine **21** (1 g, 2.5 mmol) in ethanol (8 mL), and the mixture was stirred for 15 min. Then ethyl acrylate (555 μL, 5.12 mmol, 2 equiv.) was added, and the mixture was heated at reflux for 2 h. After concentration under vacuum, the residue was taken up in ethyl acetate, washed with a saturated solution of NH_4Cl and dried with $MgSO_4$. After concentration under vacuum,

crude imine **25** was recovered as a brown oil (1.33 g, 98%). R_F(EtOAc) = 0.35. IR: $\tilde{\nu}$ = 2981 (CHAr), 1740 (CO_2Me), 1639 (C=N), 1250 [P(O)(OEt)$_2$], 1022 [P(O)(OEt)$_2$] cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.25 (t, J = 7 Hz, 3 H, CH_3), 1.29 (t, J = 7 Hz, 6 H, CH_3), 1.32 (t, J = 7 Hz, 6 H, CH_3), 2.68 (m, 4 H, CH_2), 4.14 (q, J = 7 Hz, 2 H, CH_2), 4.26 (m, 8 H, CH_2), 7.36 (t, J = 7.5 Hz, 1 H, CHAr), 7.41 (d, J = 7.5 Hz, 1 H, CHAr), 7.62 (d, J = 7.5 Hz, 1 H, CHAr), 7.78 (s, 1 H, CHAr), 8.76 (t, $J_{H,P}$ = 3 Hz, 1 H, N=CH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 14.41 (CH_3), 16.66 (CH_3), 29.68 (t, $J_{C,P}$ = 6.2 Hz, CH_2), 32.22 (CH_2), 60.68 (CH_2), 63.34 ($J_{C,P}$ = 7.2 Hz, CH_2), 63.49 ($J_{C,P}$ = 7 Hz, CH_2), 68.02 (d, $J_{C,P}$ = 136.8 Hz, 1 C), 127.17 (CHAr), 127.98 (CHAr), 130.10 (CHAr), 131.46 (CHAr), 135.05 (CAr), 138.12 (CAr), 164.63 ($J_{C,P}$ = 12 Hz, N=CH), 173.53 (CO_2Et) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 19.25 ppm. MS (ESI): m/z = 525.94 [M + H]$^+$, 548.06 [M + Na]$^+$, 1072.78 [2 M + Na]$^+$, 388. [M – P(O)(OEt)$_2$]$^+$. HRMS (ESI): calcd. $C_{21}H_{34}ClNO_8P_2$ + Na 548.1346, found 548.1342.

2,2-Bis(diethoxyphosphoryl)pyrrolidin-5-one (26): Imine **25** (758 mg, 1.6 mmol) was dissolved in acetonitrile (1.5 mL), and 1 N HCl (4.8 mL, 4.8 mmol, 1.5 equiv.) was added. After 30 min at room temperature, the solution was concentrated under vacuum, and the aqueous phase was extracted three times with diethyl ether. The aqueous phase was then neutralised with $NaHCO_3$ and basified with 1 N NaOH to pH 10. This was extracted five times with CH_2Cl_2. The organic layers were collected and washed with brine. After drying ($MgSO_4$) and concentration under vacuum, pyrrolidinone **26** was recovered as a white solid (225 mg, 50% yield). IR: $\tilde{\nu}$ = 3321 (NH), 2981–2847 (CH_2), 1701 (NHCO), 1246 [P(O)-(OEt)$_2$], 1161 [P(O)(OEt)$_2$], 1018 [P(O)(OEt)$_2$] cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.36 (t, J = 7.1 Hz, 12 H, CH_3), 2.55 (m, 4 H, CH_2), 4.24 (m, 8 H, CH_2), 5.84 (br. s, 1 H, NHCO) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 16.55 (CH_3), 24.76 (CH_2), 29.20 (CH_2), 58.76 (t, $J_{C,P}$ = 152.5 Hz, 1 C), 177.70 (NHCO) ppm. ^{31}P NMR ($CDCl_3$, 121 MHz): δ = 19.82–19.94 ppm. MS (ESI): m/z = 358 [M + H]$^+$, 380.14 [M + Na]$^+$. HRMS (ESI): calcd. for $C_{12}H_{25}NO_7P_2$ + Na 380.1004; found 380.0996.

5-Oxopyrrolidine-2,2-diyldiphosphonic Acid (27): Pyrrolidinone **26** (91 mg) was treated with 6 N HCl (10 mL) at reflux for 48 h. After extraction with CH_2Cl_2, the aqueous phase was concentrated under vacuum, and the residue was dried under high vacuum to furnish **27** as a white solid (66 mg, quantitative yield). IR: $\tilde{\nu}$ = 3128 (NH), 3043 (CH_2), 2808 (PO_3H, P–OH), 1662 (NHCO), 1153 (PO_3H_2) cm^{-1}. ^{1}H NMR (D_2O, 300 MHz): δ = 2.41 (m, 4 H, CH_2) ppm. ^{13}C NMR (D_2O, 125 MHz): δ = 23.47 ($J_{C,P}$ = 2.7 Hz, CH_2), 28.56 (CH_2), 58.37 (t, $J_{C,P}$ = 144.7 Hz, 1 C), 180.56 (NHCO) ppm. ^{31}P NMR (D_2O, 121 MHz): δ = 18.86 ppm. MS (ESI): m/z = 244.07 [M + H]$^+$. HRMS (ESI): calcd. for $C_{12}H_{25}NO_7P_2$ + H 245.9933; found 245.9941.

Determination of Biochemical Activity

Assay with β-Lactamases: The enzymes were produced and purified as described previously.[15,51–56] The enzymes (1–100 nM) were incubated with the tested compounds (100 μM) and the chromogenic substrate (nitrocefine, 100 μM) in phosphate (50 mM, pH 7) or acetate buffer (50 mM, pH 5). In the case of metallo-enzymes (class B), $ZnCl_2$ (50 μM) added to HEPES buffer (10 mM, pH 7) was used. The tested compounds were dissolved in DMSO at 10–100 mM and then diluted with the buffer (final concentration of DMSO in the test solution ≤2%); ≤2% DMSO had no effect on the enzyme activity. The hydrolysis rate of nitrocefine was followed by spectrophotometry at 482 nm with UVIKON 860, 940 and XL instruments connected to a computer through an RS232 line. The residual activity was determined by comparison with the variation of the absorbance of the reference (sample without inhibitor) and is shown in Table 4. Results are expressed as a percentage of the initial activities; variations of results are within an error of ±3%. A plot of V/V_I (ratio of hydrolysis in the absence and in the presence of inhibitors) versus inhibitor concentration gave the inhibition constant indicated in the text. All experiments were performed three times.

Assay with R39: The tested enzyme was produced and purified at the University of Liège (Centre d'Ingénierie des Proteins, Prof. B. Joris). Inhibition studies were performed at 30 °C in sodium phosphate buffer (50 mM, pH 7) containing 5% DMF to ensure product solubility. Compounds were tested at a concentration of 500 μM; experiments were performed in triplicate. Inhibitor (500 μM) and protein (0.8 μM) were mixed and incubated for 16 h. The residual free protein was counterlabelled with fluorescein-labelled ampicillin (25 μM) for 45 min. Then the reaction was stopped by the addition of SDS-PAGE loading buffer and the mixture was heated at 100 °C for 4 min. The reaction mixture was then submitted to SDS-PAGE. The fluorescent complexes (formed by reaction of residual PBPs and ampicillin) were visualised by using a Molecular Imager FX and quantified with the Quantity One Software of BIORAD.[59] The background fluorescence was subtracted in each assay.

Supporting Information (see footnote on the first page of this article): ^{13}C NMR spectra of the described compounds (Figures S1–S23).

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