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Original article

Design, synthesis and activity of bisubstrate, transition-state analogues and competitive inhibitors of aspartate transcarbamylase

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Abstract

Aspartate transcarbamylase initiates the *de novo* biosynthetic pathway for the production of the pyrimidine nucleotides, precursors of nucleic acids. This pathway is particularly active in rapidly growing cells and tissues. Thus, this enzyme has been tested as a potential target for antiproliferative drugs. In the present work, on the basis of its structural and mechanistic properties, a series of substrate analogues, including potential suicide-pseudosubstrates was synthesized and their putative inhibitory effects were tested using *E. coli* aspartate transcarbamylase as a model. Two of these compounds appear to be very efficient inhibitors of this enzyme.

Keywords: Phosphonopeptides; Fluorinated phosphonates; Carbamylphosphate analogues; ATCase inhibitors

1. Introduction

Aspartate transcarbamylase (ATCase, EC 2.1.3.2) catalyses the first unique step of the pyrimidine pathway, that is the carbamylation of the amino group of L-aspartate by carbamylphosphate to produce *N*-carbamyl-L-aspartate [1]. A high activity of this enzyme is a marker of rapidly dividing cells [2]. As such, this enzyme is an interesting target for the development of antiproliferative drugs and it is not surprising that numerous approaches to the design of new antitumoral agents were based on the search for ATCase inhibitors [3]. *N*-(Phosphonoacetyl)-L-aspartate (PALA), a bisubstrate analogue of this enzyme, is a powerful inhibitor of its activity [4]. This compound was shown to inhibit proliferative growth of mammalian cancer cells and to exhibit considerable activity against certain transplantable solid tumours in mice [5]. It has been used in clinical investigation, but a notable decrease of effectiveness was observed, possibly due both to difficult transportation of PALA to the enzyme active site [6] and development of resistance by gene amplification [7]. Numerous analogues of PALA were synthesized, but they are often poor inhibitors of ATCase [8]. These approaches were based on structural rather than mechanistic considerations.

In this work, we describe an investigation to the rational design of ATCase inhibitors based on the understanding of the mechanism of the catalytic reaction. This approach consisted in the synthesis and assay towards ATCase enzymatic activity of the bisubstrate analogues **1** and **2a** and **2b** [8e], a transition-state analogue **3**, and the competitive inhibitors **4a–b** (Fig. 1).

From archae to mammals the structure of the ATCase catalytic site and the aminoacids involved in substrate binding and catalysis are fully conserved [9]. Consequently, in this study, the *E. coli* enzyme was used as a model. This enzyme is made from the association of two trimeric catalytic subunits with three dimeric regulatory subunits [1]. Its activity is feedback inhibited by the endproducts CTP and UTP, and activated by ATP. These three nucleotides bind competitively to the same allosteric sites on the regulatory subunits. *E. coli* ATCase can be dissociated into its catalytic and regulatory subunits. The isolated catalytic subunits are active but, in contrast with the native enzyme, they show neither

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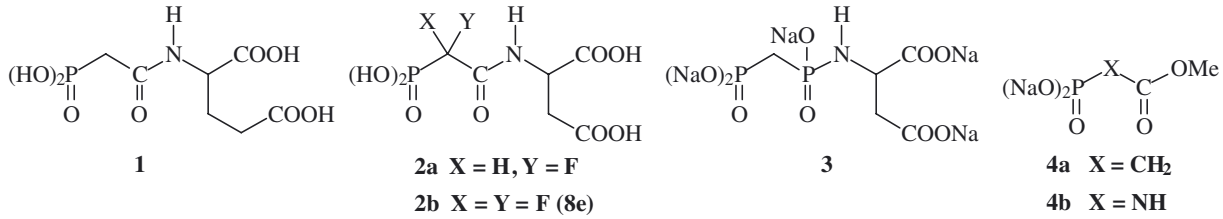


Fig. 1. Structure of the putative inhibitors synthesized and used in this study.

sensitivity to the allosteric effectors nor homotropic cooperative interactions between the catalytic sites for the binding of the substrate aspartate.

2. Chemistry

The rationales for the devise of these putative inhibitors and the procedures for their syntheses were as follows.

2.1. Synthesis of bisubstrate analogues of ATCase

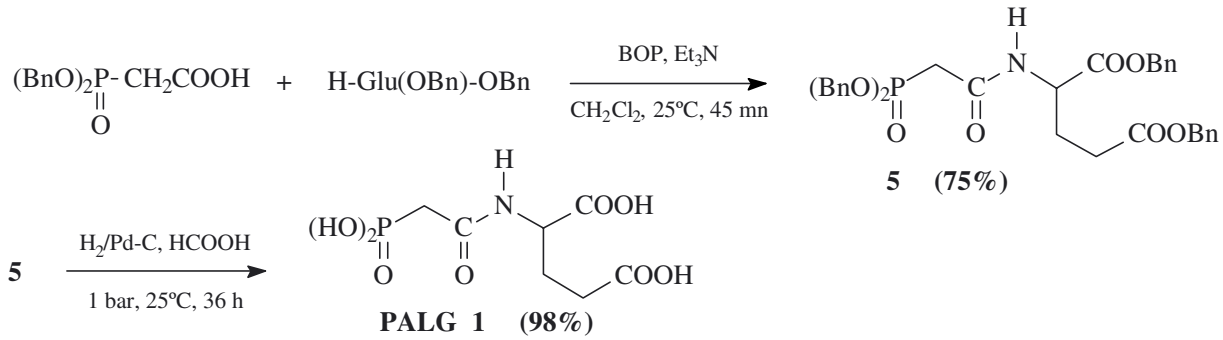
2.1.1. Synthesis of N*-phosphonacetyl*-L*-glutamate (PALG) **1***

N-phosphonoacetyl-L-glutamate (PALG) was considered as potentially able to stabilize the T conformation of ATCase. In this enzyme, the homotropic cooperative interactions between the catalytic sites for aspartate binding are explained by a transition from an enzyme conformation which has a low affinity for aspartate (T state) to a conformation which has a high affinity for this substrate (R state). The binding of the two substrates or of PALA to the active site of a catalytic chain induces a domain closure in which the carbamyl phosphate-binding domain and the aspartate-binding domain come closer [1]. This intramolecular movement is supposed to induce the T–R transition. Consequently, in the T state, the amino acid side chains of the catalytic site that bind to the phosphate group and the carboxyl groups of aspartate or PALA are more distant than in the R state. It was, thus, postulated that PALG in which these groups are more distant than in PALA, would bind to the T state and fail to promote the domain closure, stabilizing, and at the same time, inhibiting this low affinity conformation.

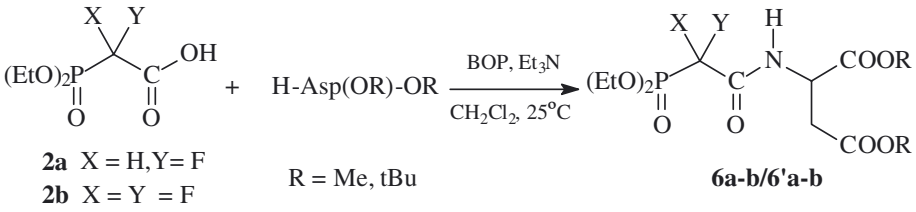
In a previous paper, we described a general route to phosphopeptides with a *N*-acylphosphonate terminal group [10]. The method allows the preparation of various phosphonopeptides from the easily available dialkylphosphonoalkanoic acids as starting materials and thus could be used for the preparation of PALG **1** (Scheme 1). Accordingly, the synthesis was based on the coupling between dibenzylphosphonoacetic acid and H-Glu(OBn)-OBn using BOP as the coupling reagent in the presence of triethylamine. The expected product **5** was obtained after usual treatment and purification by chromatography on silica gel with 75% yield. It was completely debenzylated by catalytic hydrogenation under 1 bar in the presence of palladium on charcoal 10%, in formic acid for 36 h. The resulting PALG 1 was obtained after separation of the catalyst on celite. This quadruple deprotection was quantitative (Scheme 1).

2.1.2. Synthesis of N*-phosphonofluoroacetyl- and* N*-phosphonodifluoroacetyl*-L*-aspartate (PALA(F) **2a** and PALA(FF) **2b***

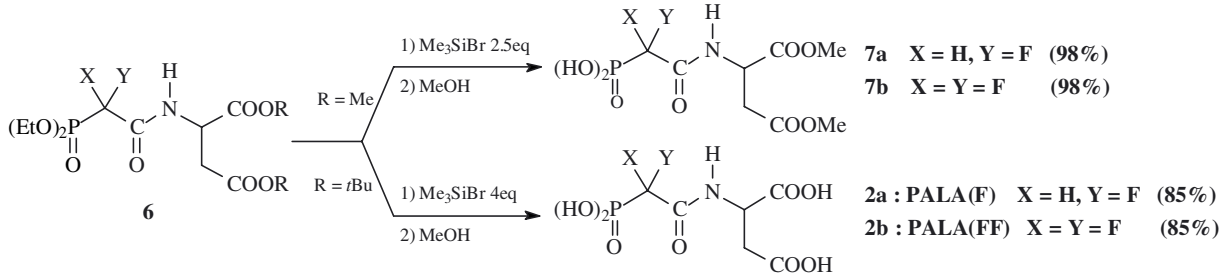
Because of its small size (close to the size of a hydrogen atom) and the high energy of the carbon-fluorine bond, fluorine atom has proven to be a good choice to design enzyme inhibitors. In the case of ATCase inhibition, the introduction of one or two fluor atoms in α-position of the phosphorus atom could provide information on the mechanism of the uptake of PALA in cytoplasm. It is hypothesized that PALA enters the cells *via* endocytosis, but this supposition is always debated. A best knowledge of the uptake of PALA could help to devise new analogues. With one or two fluor atoms, the second dissociation of the phosphonic acid moiety of **2a** and **2b** (Fig. 1) should occur more readily than with PALA, because of the great electronegativity of the fluor [11]. If PALA enters the cells by endocytosis, fluorinated PALA would be partially protonated at the lysosomal acid pH. An increase in the net charge of the inhibitor should decrease the rate of diffusion across the lysosomal membrane. Conse-



Scheme 1.



Scheme 2.



Scheme 3.

quently, PALA(F) **2a** and PALA(FF) **2b** could provide new information on the mechanism of PALA uptake[1].

The phosphonopeptides **6a, 6a′** and **6b, 6b′** were synthesized by coupling L-aspartic acid dialkylester hydrochloride with fluoro- or difluorophophonoacetic acid [12,13] (Scheme 2). In the presence of BOP as coupling reagent, the desired peptides were obtained in good yields (Table 1). The reaction occurred at room temperature with two equivalents of triethylamine in dichloromethane and was complete after stirring for 45 min when X = H, Y = F and 2 h when X= Y = F. The formed HMPT was easily removed by chromatography on silica gel. The chemioselective deprotection of the diethylphosphonic ester of **6a** and **6b** was then achieved with a small excess of bromotrimethylsilane (2.5 equivalents) followed by methanolysis of the disilylated phosphonate. The resulting crude products were washed with ether and afforded the compounds **7a** and **7b** [14]. Simultaneous removal of the diethylphosphonic ester and *tert*-butyl carboxylic ester groups was carried out in a one-step sequence involving the reaction between **6′a** or **6′b** and a large excess of bromotrimethylsilane (four equivalents, 6 h in dichloromethane) followed by methanolysis. After *vacuum* evaporation, the phosphonic acids were obtained as a brown pasty solid, which was purified by washing with acetone. In these conditions, **6′a** and **6′b** afforded directly the entirely deprotected target molecules PALA(F) **2a** and PALA(FF) **2b** (Scheme 3). If the phosphonic acids are suspected to being contaminated with siloxanes they may be purified by treatment with cyclohexylamine. Cyclohexylammonium salts were isolated by precipitation from acetone.

The pK_a[2] values of **7a, 7b** and PALA were evaluated with an automatic titrator (Tacussel TT Processor 2) using a 0.1 N sodium hydroxide solution (Table 2). As expected, the effect of fluor substitution into PALA was very pronounced. It could be calculated (Fig. 2) that, at pH 5, 39% of **7b** was a tetraanion, whereas 99% of PALA was trianionic. As a consequence, PALA, **7a** and **7b** should have a different behaviour in vitro towards the target enzyme in the hypothesis of a membrane uptake by endocytosis. PALA (F) **2a**, and PALA (FF) **2b** should have still more difficulties to cross the lysosomal membrane.

2.2. Synthesis of the transition-state analogue PALA(P), 3

A potential mechanism-based inhibitor was designed. If we consider the enzymatic ATCase reaction and the mechanism of carboxamide bond formation, the reactive centre is a tetrahedral site. As a consequence, PALA is not a real transition state mimic as previously claimed, but a bisubstrate analogue. Thus, we postulated that incorporation of a phosphorus tetrahedral atom, instead of the carbonyl carbon atom, would provide a compound which would resemble more closely the transition state and could improve notably the affinity for the catalytic site of ATCase (Fig. 3).

2.2.1. Synthesis of PALA(P) 3

In a previous paper, we described the one-pot synthesis of phosphonophosphonamide **8** [15]; we wish to report herein the complete deprotection of **8** to give the target compound **3**. The choice of the protective groups in **8** was directed by the fragility of the P–C–P–N chain. Actually, it is well known that unsymmetrical alkylidene diphosphorylated derivatives are unstable in the presence of nucleophiles since these later attack the most electrophilic phosphorus atom, leading to a P–C cleavage. In other hand, it is also well documented that P–N linkage are acid sensitive [16].

The best deprotection conditions were the tandem reaction silylation/hydrolysis with careful control of pH. The use of a large excess of triethylamine (13 equivalents) during the

[1] *N*-Phosphonodifluoroacetyl-L-aspartate **2b** has been described by Lindell and Turner [**8e**] but ATCase-inhibitory properties has been evaluated with mung bean ATCase.

	X	Y	pK_a^2
PALA	H	H	7.70
7a	H	F	6.20
7b	F	F	5.20

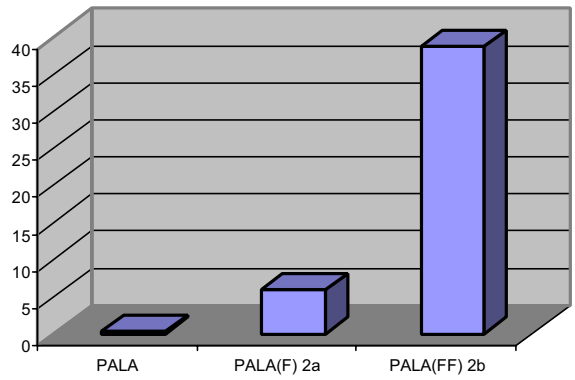


Fig. 2. Degree of ionisation of the fluorinated derivatives of PALA.

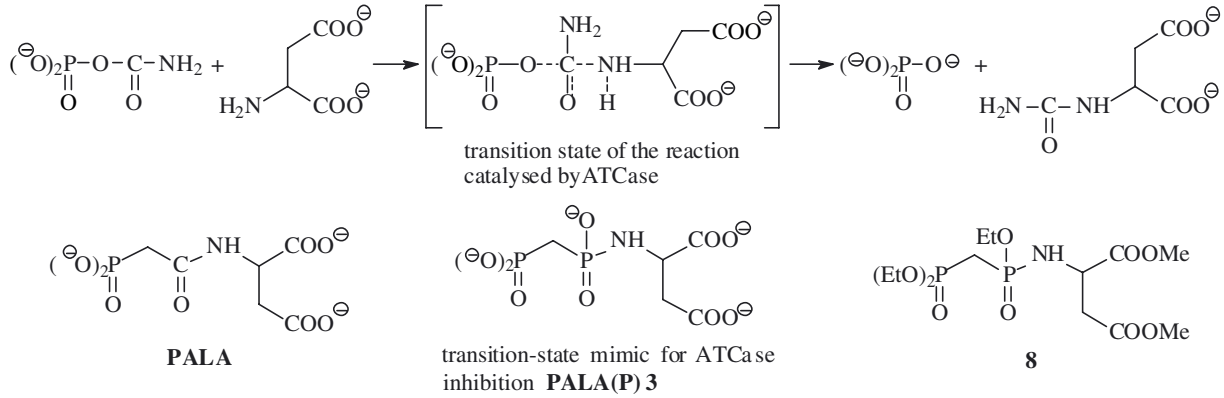


Fig. 3. Structure of the transition state analogues PALA(P).

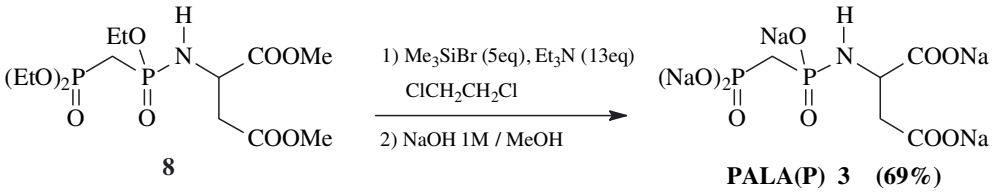
silylation was a good solution in response to the need for smooth phosphonic ester-deprotection in the presence of acid-sensitive P–N bond (Scheme 4). Addition of triethylamine as scavenger of the in situ generated bromhydric acid efficiently avoided the P–N linkage degradation. The silylation was slowed down and needed 3 days to complete the reaction. The treatment of the resulting trimethylsiloxyphosphoryl moieties with sodium hydroxide in methanol led to the expected phosphonophosphonamide **3** in interesting yield (69%).

Observation of a triplet (2.17 ppm, $^2J_{HP}$ = 18 Hz) in ^{1}H-NMR, a triplet (32.0 ppm, $^1J_{CP}$ = 114 Hz) in ^{13}C-NMR revealed the presence of the P–C–P linkage. The AM system (two doublets, 22.6 ppm and 14.1 ppm, $^2J_{PP}$ = 9 Hz) showed the non-equivalence of the two phosphorus atoms and was in accordance with the P–C–P–N chain. Multiplicities observed in non ^{1}H-decouplated ^{31}P-NMR showed the connexion of the amino acid residue on the P–C–P moiety: the phosphorus atom linked to the nitrogen atom (PN) resonated as a ddt, while the other phosphorus atom resonated as dt only. This result was confirmed by the detection of a correlation between the CH proton of aspartate and the phosphorus atom PN in a bidimensional ^{31}P/^{1}H-NMR experiment. Positive electron spray mass spectrum was in good agreement with complete deprotected structure.

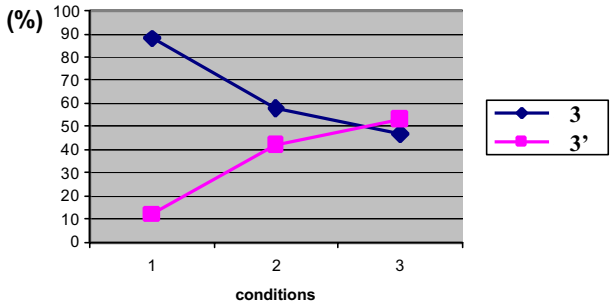
2.2.2. *Stability of PALA(P) **3***

The lability of the P–C–P chain required a study of the stability of the transition-state analogue PALA(P) under conditions where the inhibition of ATCase was measured.

The stability of this compound was studied in ^{31}P-NMR under the followed conditions: 8.10^{-5} mole of phosphonophosphonamide **3** (the sensitivity of the NMR technique needed to concentrate the phosphorylated compound eight times as compared to the conditions of the inhibition assay) was added to an aqueous solution of tris-acetate (20 ml,



Scheme 4.



conditions 1: 20°C, without substrates of ATCase
conditions 2: 37°C, without substrates of ATCase
conditions 3: 37°C, with substrates of ATCase

Fig. 4. Degradation of PALA(P**3**) .

40.10^{-3} M, pH = 8.0). Different experiments were realized at 20 °C or 37 °C with or without the two substrates of ATCase, carbamylphosphate and aspartate. After stirring for 10 min, an analytical sample was cut off reaction solution and analysed rapidly in ^{31}P-NMR in sweep-off mode. Results are reported in Fig. 4.

The ^{31}P-NMR spectrum displayed two compounds, or four if carbamylphosphate was present (Scheme 5):

- carbamylphosphate (–4.6 ppm),
- phosphate (0.0 ppm) produced by degradation of carbamylphosphate,
- signal of **3** (17.3 ppm, 12.3 ppm) that decreased in intensity when temperature increased,
- singulet at 13.7 ppm that increased to the detriment of **3**. This signal was assigned to the tetrasodium salt **3′** derived from the methylene bisphosphonic acid. This formed side-product evidenced the sensitivity of the phosphorus atom in the diphosphorylated compound **3** to nucleophilic attack of water.

As carbamylphosphate, the natural substrate of ATCase, PALA(P) was thermally unstable. Nevertheless, the decomposition was partial and easily quantified. Twenty-five percent of carbamylphosphate and 43% of the transition state analogues were degraded after 10 min at 37 °C. It was important to notice that the effect of the tetrasodium salt **3′** derived from the methylene bisphosphonic acid on the ATCase was known and did not interfere with the measure of the ATCase activity (see paragraph 3).

*2.3. Synthesis of the competitive inhibitors CP(C), **4a** and CP(N), **4b***

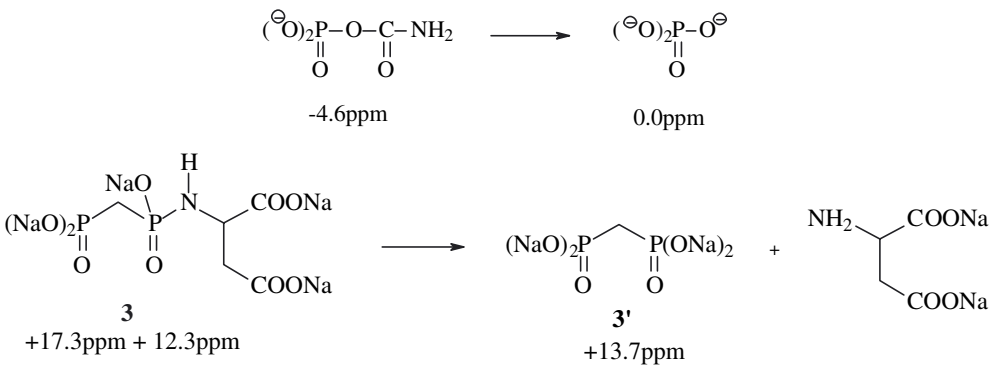
We propose here a new concept of inhibition based on the synthesis of a chemically reactive substrate analogue of ATCase able to react with the other substrate of the enzyme to generate in situ an inhibitor of ATCase. The principle is the use of the enzyme as a catalyst for the synthesis of its self-inhibitor.

This new approach is illustrated by the synthesis of two mimics of carbamylphosphate, CP(C) (**4a**) and CP(N) (**4b**) where the carboxylic acid methyl ester **4a** or the carbamic acid methyl ester **4b** could act as an electrophile towards aspartate to afford respectively, in situ, PALA or a new PALA analogue.

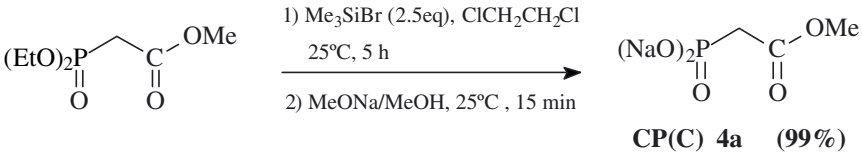
*2.3.1. Synthesis of methoxycarbonylmethylphosphonic acid disodium salt **4a***

Methyl diethylphosphonoacetate is commercially available and can be used without purification as starting material.

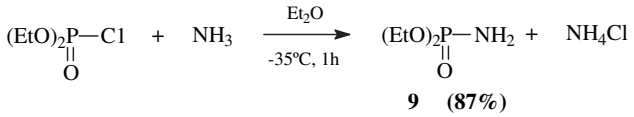
Chemioselective deprotection of the diethylphosphonic ester was achieved with a small excess of bromotrimethylsilane (2.5 equivalents) followed by a methanolysis to keep the methoxycarbonyl group. Washing of the resulting brown solid with methanol afforded quantitatively the target compound **4a** (Scheme 6).



Scheme 5.



Scheme 6.



Scheme 7.

*2.3.2. Synthesis of methoxycarbonylamidophosphonic acid disodium salt **4b***

Several methods are reported in literature for the synthesis of the methyl (diethylphosphono)carbamate precursor **10** (Scheme 8).

The decomposition of dialkylphosphonourea into dialkylphosphonoisocyanate under acidic conditions, followed by alcoholysis has been described for the preparation of carbamate **10** [17].

A different approach was reported by Kirsanov and Marenets [18] who built the urethanphosphoric ester by thermal decomposition of trichlorophosphazocarbonic ester. These later can also be transformed into urethanphosphoric ester by solvolysis. The two ways use the phosphazocarbonic intermediate, an explosive reagent.

These preparations are rather laborious, and we report here a simple and efficient synthesis of this useful derivative **10**, starting from versatile reagents.

In a first step, chloride ion of diethylchlorophosphate was easily displaced by ammoniac. The reaction was fast (1 h at –35 °C) and no by-product was formed. After centrifugation and removal of the solvent, diethylamidophosphate **9** was isolated without difficulty. The crude product was purified by distillation (Scheme 7).

The second step is the methoxycarbonylation of **9**. We have examined this reaction in some detail by ^{31}P-NMR spectroscopy. Deprotonation of **9**, in THF at 60 °C, with two equivalents of sodium hydride produced the anion **9′** which was stable at this temperature. The formation of **9′** was complete after 10 min. Addition of one equivalent of methylchloroformiate led to the formation of the stable intermediate **10′**. Two equivalents of NaH were essential for a quantitative formation of **10′**. Subsequent acidification of the reaction mixture with a saturated HCl ether solution at pH = 7, centrifugation and removal of the solvents led to pure compound **10** in good yield (79%) (Scheme 8).

This simple procedure constitutes a good phosphonocarbamate **10** synthesis, superior to known methods.

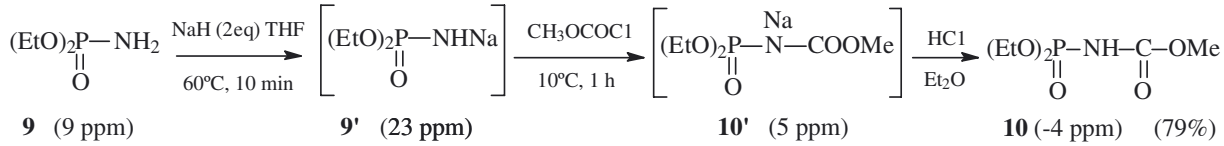
After demonstrating that this process gave an attractive access to **10** we investigated the chemioselective deprotection of the diethylphosphonyl group. The treatment of **10** with 8 equivalents of bromotrimethylsilane and a large excess of triethylamine afforded the methyl (ditrimethylsilylphosphono)carbamate. The complete silylation was slow (56 h). After basic methanolysis, compound **4b** was isolated. The crude product was purified by washing with CH_2Cl_2, AcOEt and Et_2O and then filtered. The solid residue was then triturated with the mixture MeOH/Me_2CO to eliminate NaBr. These operationally simple treatments led to the pure compound **4b** in 50% yield (Scheme 9).

3. Enzymology

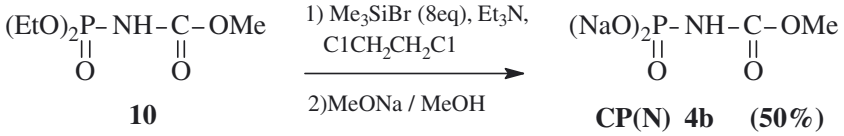
On the basis of the above-mentioned working hypotheses the influence of the different compounds obtained on the activity of *E. coli*, ATCase was examined under various conditions as indicated in Experimental, in the presence of 5 mM carbamylphosphate, 20 mM [^{14}C]-L-aspartate or otherwise indicated.

3.1. Influence of the bisubstrate analogues PALG, PALA(F) and PALA(FF) on ATCase activity

The response of the ATCase activity to increasing concentrations of PALG, PALA(F) and PALA(FF) is shown in



Scheme 8.



Scheme 9.

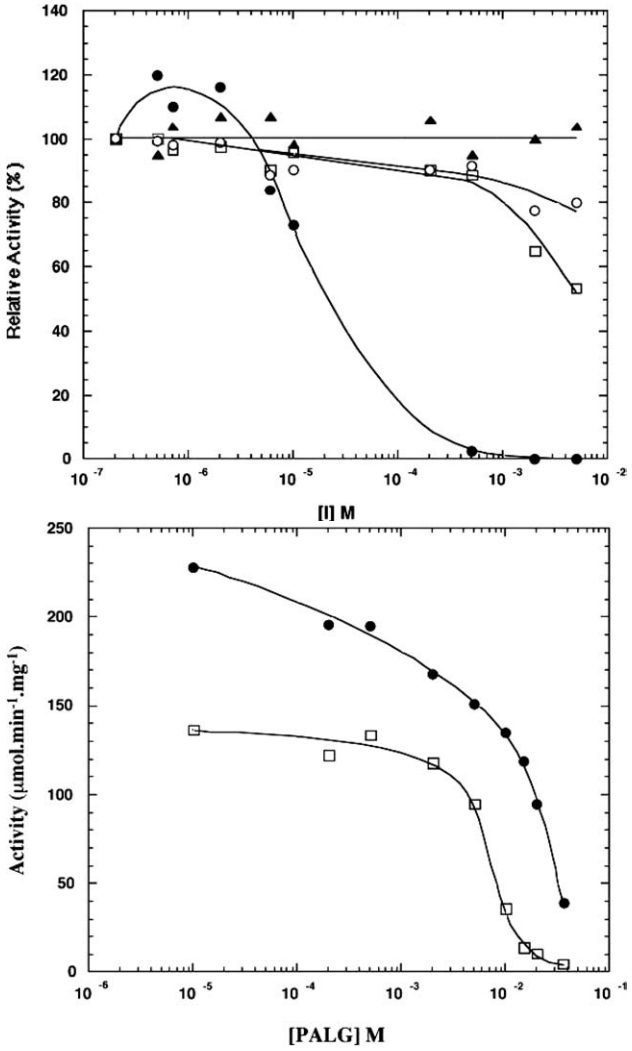


Fig. 5. Influence of the different substrate analogues on ATCase activity. The ATCase activity was measured as indicated in "Experimental" in the absence or presence of increasing concentrations of the different substrate analogues and PALA as a control. (A) Effect of the inhibitors in the presence of 5 mM aspartate. (○-○): PALG; (□-□): PALA(F); (Δ-Δ): PALA(FF); (●-●): PALA. (B) Effect of PALG on ATCase and its isolated catalytic subunits in the presence of 20 mM aspartate. (□-□): ATCase; (λ-λ): isolated catalytic subunits.

Fig. 5A in comparison with the influence of PALA under the same conditions. It appears that PALG did not provoke inhibition for concentrations lower than 1 mM. In order to test the significance of the 20% inhibition observed at 5 mM, the influence of this compound was measured in the presence of 20 mM aspartate, using both the native ATCase and the isolated catalytic subunits.

Fig. 5B shows that this increase in aspartate concentration does not alter the level of inhibition by PALG. In addition, the native enzyme and its isolated catalytic subunits exhibit the same sensitivity to this analog. Taken together, these observations indicate that the small inhibition observed is not due to the stabilization of the T conformation of the enzyme.

At a concentration of 5 mM, the inhibition by PALA(F) raises to about 45%, at the same concentration PALA(FF) does not provoke inhibition. This difference points to the importance of the stereo-electronic effects on the loose binding at the enzyme active site. PALA(FF) was previously reported to be an efficient inhibitor of mung bean ATCase [8e]. This difference is most probably due to the fact that plant ATCases have a much simpler organization than the homologous enzymes from *E. coli* and mammals, since they are simple catalytic trimers in which the catalytic site is more readily accessible.

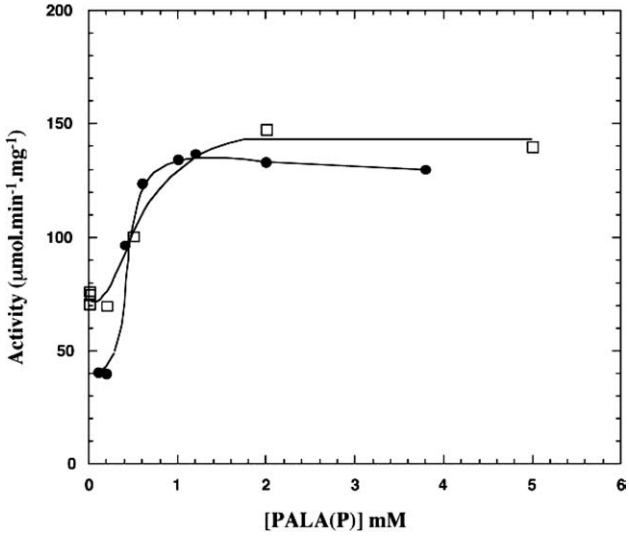


Fig. 6. Influence of the transition state analogues PALA(P) on ATCase activity. The ATCase activity was measured as indicated in "Experimental" in the presence of increasing concentrations of PALA(P). (●-●): in absence of ATP; (□-□): in the presence of 1 mM ATP.

The slight activation which is observed in the presence of low concentrations of PALA is known and expected. It results from the influence of this analogue on the T–R equilibrium which is not entirely shifted towards R in the presence of 5 mM aspartate.

3.2. Influence of the transition-state analogue PALA(P) on ATCase activity

The influence of the putative transition-state analogue PALA(P) on ATCase activity was measured, using the same methodology. It can be seen in Fig. 6 that, unexpectedly, this compound has no inhibitory effect on the native enzyme, but that at concentrations higher than 0.2 mM, it behaves as a activator whatever the aspartate concentration. Such an activation was not observed in the case of the isolated catalytic subunits, a result which suggested that this stimulation could result from a shift in the T–R equilibrium towards the R conformation. However, the fact that neither the aspartate concentration nor the presence of 1 mM ATP (Fig. 6) decreases the extent of this activation rules out this explanation.

*3.3. Influence of the competitive inhibitors CP(C) **4a** and CP(N) **4b** on the activity of ATCase*

In the presence of 20 mM aspartate and 10 mM carbamylphosphate, CP(C) and CP(N) appear to be very potent

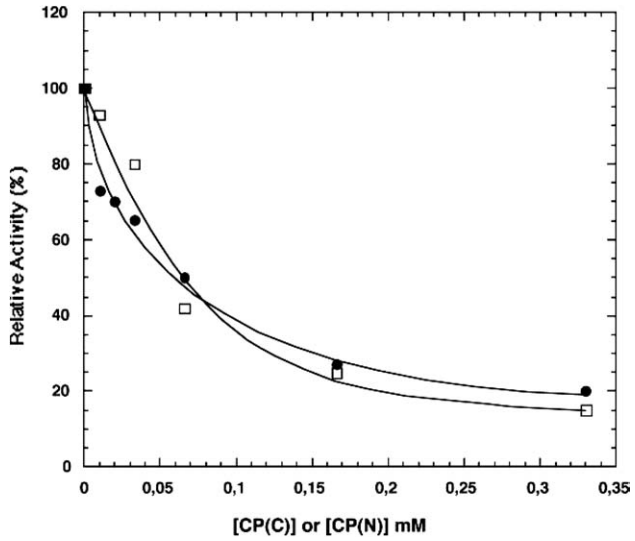


Fig. 7. Influence of the carbamylphosphate analogues CP(C) and CP(N) on the ATCase activity. The ATCase activity was measured as indicated in "Experimental" in the presence of either CP(C): (•-•) or CP(N): (□-□).

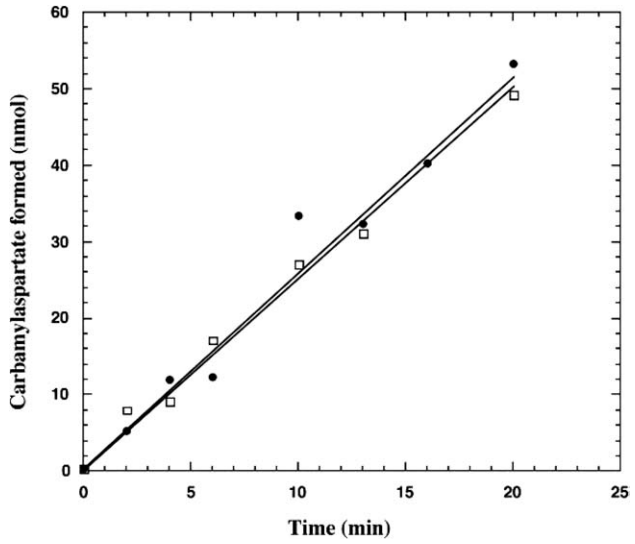


Fig. 8. Kinetic of the ATCase reaction in the presence of CP(C) or CP(N). The ATCase activity was measured as indicated in "Experimental" in the presence of either CP(C): (λ-λ) or CP(N): (□-□) at a concentration of 0.15 mM.

inhibitors of ATCase (Fig. 7). At a concentration of 0.05 mM, the inhibition is about 50% and raises to 80% at 0.33 mM.

In a preliminary attempt to detect a putative inactivation of the enzyme by these compounds the kinetics of the ATCase reaction were followed over 20 min in the presence of these two inhibitors at a concentration of 0.15 mM. The result of this experiment is shown in Fig. 8. No decrease of the rate of the reaction is observed over that period of time.

4. Conclusion

Among the different substrate analogues synthesized and tested in this study, only CP(C) and CP(N) appear to be strong inhibitors of the activity of ATCase, and these two compounds exhibit the same efficiency. The ATCase reaction proceeds through an ordered mechanism in which carbamylphosphate binds first and induces a conformational change which allows the subsequent binding of the second substrate aspartate. A consequence of this process is that the bi-substrate analogue PALA binds to the catalytic site of this enzyme in competition with carbamylphosphate. However the interaction between the two carboxyl groups of this compound with positively charged residues of the catalytic site contributes energetically to its affinity for this site. In contrast, CP(C) and CP(N) are analogues or carbamylphosphate alone.The fact that under similar conditions these two inhibitors and PALA provoke inhibitions which are of the same order of magnitude suggest that CP(C) and CP(N) would bind more tightly to the catalytic site of ATCase than carbamylphosphate. Further investigation is needed to determine whether these two new anti-metabolites are acting strictly as very potent inhibitors in pure competition with carbamylphosphate or as suicide substrates.

5. Experimental

5.1. Chemistry

5.1.1. General methods

Melting points were determined on an Electrothermal IA9100 digital apparatus. Infrared spectra were obtained using a Nicolet 205 spectrometer and are given in cm^{-1}. NMR spectra were recorded using a BRUKER AC250 spectrometer. For ^{1}H and ^{13}C-NMR data, chemical shifts are reported in parts per million (δ, ppm) downfield from $CHCl_3$ as an internal standard while ^{31}P-NMR are reported with 85% H_3PO_4 as an external standard. ^{19}F-NMR are reported from $CFCl_3$ as standard. NMR coupling constants (J values) are listed en hertz (Hz) and spin multiplicities are reported as singulet (s), doublet (d), triplet (t), multiplet (m) and broad (br). Mass spectra were obtained with a TRIO 5000 spectrometer. Organic solvents were purified according to the methods described by W.L.F. Armagero and D.D. Perrin [19]. All no aqueous reactions were performed in oven-dried glassware under nitrogen atmosphere. *n*-Butyllithium and *s*-butyllithium were purchased from Aldrich and were titrated in tetrahydrofuran for *n*-BuLi and benzene for *s*-BuLi according to the Watson and Eastham procedure [20]. Advancement of reactions was followed by ^{31}P-NMR spectroscopy.

5.1.2. Preparation of N-(phosphonoacetyl)-L-glutamic acid **1**

5.1.2.1. Dibenzyl methylphosphonate. To a suspension of freshly washed sodium hydride (NaH) (55% suspension in mineral oil, 1.90 g, 79.2 mmol) in toluene (10.0 ml) was added dropwise a solution of dibenzyl H-phosphonate (18.88 g, 72.0 mmol) in toluene (10.0 ml), under N_2 atmosphere, at room temperature and using an ice bath checked

temperature reaction. After stirring for 1 h, end of gas liberation was observed then iodomethane (10.22 g, 72.0 mmol) in toluene (10.0 ml) was added dropwise. Reaction mixture was stirring overnight at room temperature. After hydrolysis and extraction with dichloromethane (CH_2Cl_2), combined organic layers were dried over magnesium sulfate ($MgSO_4$), filtered and solvents were removed in *vacuum* to afford the expected crude compound in 70% yield: IR (film) ν 1050, 1250; ^{1}H-NMR ($CDCl_3$) δ 1.49 (d, 3H, $^2J_{HP}$ = 17.5 Hz, PCH_3), 4.90–5.20 (m, 4H, OCH_2), 7.35–7.45 (m, 10H, Ph); ^{31}P-NMR ($CDCl_3$) δ 29.3 (s, 1P); ^{13}C-NMR ($CDCl_3$) δ 11.0 (d, $^1J_{CP}$ = 144 Hz, PCH_3), 66.4 (d, $^2J_{CP}$ = 6 Hz, OCH_2), 127.3–128.0 (m, CH_{arom}), 135.7 (d, $^3J_{CP}$ = 2 Hz, C_{arom}).

5.1.2.2. (Dibenzylphosphono)acetic acid. *n*-Butyllithium (*n*-BuLi) (17.5 ml of a 1.6 M solution in hexane, 28.0 mmol) was added to tetrahydrofuran (THF) (28 ml) at –70 °C, under nitrogen. Then a solution of dibenzyl methylphosphonate (7.73 g, 28.0 mmol) in THF (6 ml) was added dropwise, at –70 °C. After mechanic stirring for 30 min at –70 °C, reaction solution was poured with stirring into a Dewar containing a saturated dry ice/diethyl ether (Et_2O, 200 ml) solution. A few minutes later, the mixture was poured into a beaker and allowed to warm to room temperature (2 h) with stirring. After hydrolysis with water (30 ml), organic layer was washed with a saturated aqueous sodium hydrogencarbonate solution ($NaHCO_3$). Combined aqueous layers were then washed twice with Et_2O, acidified to pH 1 with a 6 M HCl aqueous solution, saturated with NaCl and extracted with CH_2Cl_2. Organic layer was dried with $MgSO_4$ and solvents were removed under *vacuum* to give (dibenzylphosphono)acetic acid as an oil in 60% yield: IR (film) ν 1025, 1215, 1720, 2500–3200; ^{1}H-NMR ($CDCl_3$) δ 3.01 (d, 2H, $^2J_{HP}$ = 22 Hz, PCH_2), 5.08 (d, 4H, $^3J_{HP}$ = 8.5 Hz, $POCH_2Ph$), 7.20–7.39 (m, 10H, Ph), 10.00 (br s, 1H, COOH); ^{31}P-NMR ($CDCl_3$) δ 22.0 (s, 1P); ^{13}C-NMR ($CDCl_3$) δ 34.3 (d, $^1J_{CP}$ = 136 Hz, PCH_2), 68.5 (d, $^2J_{CP}$ = 6 Hz, OCH_2), 127.9 (s, CPh), 128.4 (s, CPh), 135.5 (s, CPh), 167.7 (d, $^2J_{CP}$ = 6 Hz, C_{arom}).

5.1.2.3. N-*[(Dibenzylphosphono)acetyl]*-L-*glutamic acid dibenzyl diester* ***5***. Triethylamine (Et_3N) (0.40 g, 4.0 mmol) was added to a solution of L-glutamic acid dibenzyl diester tosylate (H_3N-L-Glu(OBn)-OBn, TsO) (2.00 g, 4.0 mmol) in CH_2Cl_2 (40 ml). Then, (dibenzylphosphono)acetic acid (1.28 g, 4.0 mmol) in CH_2Cl_2 (20 ml), benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) (1.77 g, 4.0 mmol) in CH_2Cl_2 (20 ml) and Et_3N (0.40 g, 4.0 mmol) in CH_2Cl_2 (10 ml) were added successively. After stirring for 45 min at room temperature, CH_2Cl_2 (50 ml) was added. Organic layer was washed at first three times with a 10 N sulfuric acid solution, with a saturated aqueous NaCl solution, with a saturated aqueous $NaHCO_3$ solution then with a saturated aqueous NaCl solution again and dried with $MgSO_4$. After evaporation of solvent and flash chromatography purification (eluent: hexane/ethyl acetate), the expected phosphonopeptide **5** was obtained as an oil in 75% yield: R_f (hexane/acetone: 1/1) = 0.75; IR (film) ν 1010, 1250, 1680, 1743, 3200; ^{1}H-NMR ($CDCl_3$) δ 1.90–2.40 (m, 4H, CH_2CH, CH_2CO), 2.89 (d, 2H, $^2J_{HP}$ = 21 Hz, PCH_2), 4.60–4.70 (m, 1H, CH), 4.90–5.15 (m, 8H, OCH_2Ph), 7.20–7.40 (m, 20H, Ph); ^{31}P-NMR ($CDCl_3$) δ 20.6 (s, 1P); ^{13}C-NMR ($CDCl_3$) δ 28.5 (d, $^1J_{CP}$ = 175 Hz, PCH_2), 29.0 (s, $CHCH_2$), 31.5 (s, CH_2CO), 54.0 (s, *C*H), 69.2 (s, OCH_2), 127.7 (s, CPh), 127.8 (s, CPh), 128.2 (s, CPh), 136.0 (s, C_{arom}), 163.8 (s, CONH), 171.0 (s, COO), 172.1 (s, COO).

5.1.2.4. N-*(Phosphonoacetyl)*-L-*glutamic acid* ***1***. A solution of phosphonopeptide **5** (0.82 g, 1.3 mmol) in formic acid (50 ml) and 10% palladium on charcoal catalyst (0.10 g) was stirred at room temperature under hydrogen atmosphere for 36 h. Reaction advancement was followed by ^{31}P-NMR spectroscopy. Catalyst was removed by filtration using celite and evaporation of formic acid under *vacuum* provided the expected *N*-(phosphonoacetyl)-L-glutamic acid **1** as a solid in quantitative yield: IR (KBr) ν 1080, 1150, 1600, 1720, 2200–3700; ^{1}H-NMR (D_2O) δ 1.70–2.20 (m, 2H, CH_2CH), 2.38 (t, 2H, $^3J_{HH}$ = 7 Hz, CH_2COOH), 2.79 (d, 2H, $^2J_{HP}$ = 20 Hz, CH_2P), 4.10–4.30 (m, 1H, CH); ^{31}P-NMR (D_2O) δ 14.1 (s, 1P); ^{13}C-NMR (D_2O) δ 30.3 (s, $CHCH_2$), 35.2 (s, CH_2CO), 39.1 (d, $^1J_{CP}$ = 168 Hz, PCH_2), 57.8 (s, CH), 175.6 (s, COO), 182.3 (s, CONH). Anal. Calcd. for $C_7H_{12}NO_8P$: C, 31.24; H, 4.49; N, 5.20. Found: C, 30.85; H, 4.70; N, 5.50; P, 11. 90.

5.1.3. Preparation of N-*[(phosphono)fluoroacetyl)]*-L-*aspartic acid* (***2a***) *and* N-*[(phosphono) difluoroacetyl]*-L-*aspartic acid* ***2b***

5.1.3.1. Peptidic coupling reaction.

5.1.3.1.1. N-*[(Diethylphosphono)fluoroacetyl]*-L-*aspartic acid dimethyl diester* ***6a***. Phosphonopeptide **6a** was obtained according to the coupling procedure described for derivative **5** starting from L-aspartic acid dimethyl diester hydrochloride (H_3N-L-Asp(OMe)-OMe, Cl) (0.79 g, 4.0 mmol) and (diethylphosphono)fluoroacetic acid (0.86 g, 4.0 mmol). Reaction mixture was stirred for 45 min at room temperature. A flash chromatography purification (eluent: hexane/ethyl acetate) afforded the two diastereoisomers of phosphonopeptide **6a** as an oil in 82% yield: IR (Film) ν 1150, 1250, 1682, 1743, 3200; ^{1}H-NMR ($CDCl_3$) δ 1.36 (t, 6H, $^3J_{HH}$ = 7 Hz, OCH_2CH_3), 2.97 (dd, 1H, $^2J_{H-H}$ = 20 Hz, $^3J_{H-H}$ = 5 Hz, CH_2COO), 2.99 (dd, 1H, $^2J_{H-H}$ = 20 Hz, $^3J_{HH}$ = 5 Hz, CH_2COO), 3.71 (s, 3H, $COOCH_3$), 3.78 (s, 3H, $COOCH_3$), 4.20–4.32 (m, 4H, CH_2O), 4.93 (dt, 1H, $^3J_{HH}$ = 8 Hz, $^3J_{HH}$ = 5 Hz, CH), 5.23 (dd, 1H, $^2J_{HF}$ = 46 Hz, $^2J_{HP}$ = 11 Hz, PCHF), 7.43 (d, 1H, $^3J_{HH}$= 8 Hz, NH); ^{31}P-NMR ($CDCl_3$) δ 8.1 (d, 1P, $^2J_{PF}$ = 70 Hz), 8.4 (d, 1P, $^2J_{PF}$ = 70 Hz); ^{19}F-NMR ($CDCl_3$) δ 174.6 (dd, 1F, $^2J_{FP}$ = 70 Hz, $^2J_{FH}$ = 46 Hz), -174.3 (dd, 1F, $^2J_{FP}$ = 70 Hz, $^2J_{FH}$ = 46 Hz).

5.1.3.1.2. N-*[(Diethylphosphono)difluoroacetyl]*-L-*aspartic acid dimethyl diester* ***6b***. Phosphonopeptide **6a** was obtained according to the coupling procedure described for derivative **5** starting from L-aspartic acid dimethyl diester hydrochloride (H_3N-L-Asp(OMe)-OMe, Cl) (0.79 g, 4.0 mmol) and (diethylphosphono)difluoroacetic acid (0.93 g, 4.0 mmol). Reaction mixture was stirred for 2 h at room temperature. A flash chromatography purification (eluent: hexane/ethyl acetate) afforded the expected phosphonopeptide **6b** as an oil in 79% yield: IR (Film) *v* 1250, 1710, 1740, 3200; ^{1}H-NMR ($CDCl_3$) δ 1.39 (t, 6H, $^3J_{HH}$ = 7 Hz, OCH_2CH_3), 2.90 (dd, 1H, $^2J_{H–H}$ = 20 Hz, $^3J_{H–H}$ = 5 Hz, CH_2COO), 3.12 (dd, 1H, $^2J_{H–H}$ = 20 Hz, $^3J_{H–H}$ = 5 Hz, CH_2COO), 3.71 (s, 3H, $COOCH_3$), 3.78 (s, 3H, $COOCH_3$), 4.29–4.40 (m, 4H, CH_2O), 4.87 (dt, 1H, $^3J_{HH}$ = 8 Hz, $^3J_{HH}$ = 5 Hz, CH), 7.62 (d, 1H, $^3J_{HH}$= 8 Hz, NH); ^{31}P-NMR ($CDCl_3$) δ 0.6 (t, 1P, $^2J_{PF}$ = 96 Hz); ^{19}F-NMR ($CDCl_3$) δ 113.3 (d, 1F, $^2J_{FP}$ = 96 Hz).

5.1.3.1.3. N-*[(Diethylphosphono)fluoroacetyl]*-L-*aspartic acid di*-tert-*butyl diester* ***6′a***. Phosphonopeptide **6′a** was obtained according to the coupling procedure described for derivative **5** starting from L-aspartic acid di-*tert*-butyl diester hydrochloride (H_3N-L-Asp(O*t*Bu)-O*t*Bu, Cl) (1.13 g, 4.0 mmol) and (diethylphosphono)fluoroacetic acid (0.86 g, 4.0 mmol). Reaction mixture was stirred for 45 min at room temperature. A flash chromatography purification (eluent: hexane/ethyl acetate) afforded the two diastereoisomers of phosphonopeptide **6′a** as an oil in 82% yield: R_f (acetone/hexane: 1/1) = 0.70; IR (Film) *v* 1150, 1267, 1695, 1737, 3200; ^{1}H-NMR ($CDCl_3$) δ 1.19 (t, 6H, $^3J_{HH}$ = 7 Hz, OCH_2CH_3), 1.29 (t, 6H, $^3J_{HH}$ = 7 Hz, OCH_2CH_3), 1.38 (s, 18H, $(CH_3)3C$), 2.50–2.80 (m, 2H, CH_2COO), 4.10–4.20 (m, 4H, CH_2O), 4.60–4.70 (m, 1H, CH), 5.23 (dd, 1H, $^2J_{HF}$ = 46 Hz, $^2J_{HP}$ = 11 Hz, PCHF), 7.34 (br s, 1H, NH); ^{31}P-NMR ($CDCl_3$) δ 8.1 (d, 1P, $^2J_{PF}$ = 70 Hz), 8.8 (d, 1P, $^2J_{PF}$ = 70 Hz); ^{19}F-NMR ($CDCl_3$) δ 174.6 (dd, 1F, $^2J_{FP}$ = 70 Hz, $^2J_{FH}$ = 46 Hz), -174.3 (dd, 1F, $^2J_{FP}$ = 70 Hz, $^2J_{FH}$ = 46 Hz); ^{13}C-NMR ($CDCl_3$) δ 16.2 (d, $^3J_{CP}$ = 6 Hz, OCH_2CH_3), 27.7 (s, $(CH_3)3C$), 27.8 (s, $(CH_3)3C$), 37.1 (s, CH_2CO), 48.6 (s, CH), 48.8 (s, CH), 64.0 (d, $^2J_{CP}$ = 5 Hz, OCH_2CH_3), 81.7 (s, $(CH_3)3C$), 82.5 (s, $(CH_3)3C$), 86.2 (dd, $^1J_{CP}$ = 158 Hz, $^1J_{CF}$ = 206 Hz, PCHF), 163.6 (d, $^2J_{CP}$ = 17 Hz, CONH), 168.6 (s, COO), 168.7 (s, COO), 169.5 (s, COO), 169.6 (s, COO).

5.1.3.1.4. N-*[(Diethylphosphono)difluoroacetyl]*-L-*aspartic acid di*-tert-*butyl diester* ***6′b***. Phosphonopeptide **6′b** was obtained according to the coupling procedure described for derivative **5** starting from L-aspartic acid di-*tert*-butyl diester dibenzenesulfimide (H_3N-L-Asp(O*t*Bu)-O*t*Bu, $(PhSO_2)_2N$) (2.17 g, 4.0 mmol) and (diethylphosphono)difluoroacetic acid (0.93 g, 4.0 mmol). Reaction mixture was stirred for 2 h at room temperature. A flash chromatography purification (eluent: hexane/ethyl acetate) afforded the expected phosphonopeptide **6′b** as an oil in 90% yield: R_f (acetone/hexane: 1/1) = 0.68; IR (Film) *v* 1250, 1740, 3200; ^{1}H-NMR ($CDCl_3$) δ 1.25 (t, 6H, $^3J_{HH}$ = 7 Hz, OCH_2CH_3), 1.48 (s, 18H, $(CH_3)3C$), 2.90–3.00 (m, 2H, CH_2CO), 4.32–4.38 (m, 4H, CH_2O), 4.65–4.75 (m, 1H, CH), 7.60 (d, 1H, $^3J_{HH}$= 8 Hz, NH); ^{31}P-NMR ($CDCl_3$) δ 0.7 (t, 1P, $^2J_{PF}$ = 96 Hz); ^{19}F-NMR ($CDCl_3$) δ 117.7 (d, 1F, $^2J_{FP}$ = 96 Hz), -117.5 (d, 1F, $^2J_{FP}$ = 96 Hz); ^{13}C-NMR ($CDCl_3$) δ 14.0 (d, $^3J_{CP}$ = 6 Hz, OCH_2CH_3), 27.6 (s, $(CH_3)3C$), 27.8 (s, $(CH_3)3C$), 36.7 (s, CH_2CO), 49.3 (s, CH), 60.2 (d, $^2J_{CP}$ = 5 Hz, OCH_2CH_3), 81.8 (s, $(CH_3)3C$), 134.0–137.4 (m, CF_2), 168.4 (s, COO), 169.5 (s, COO), 170.6 (s, CONH).

5.1.3.2. Protective group removal.

5.1.3.2.1. N-*[(Phosphono)fluoroacetyl]*-L-*aspartic acid dimethyl diester* ***7a***. Bromotrimethylsilane (3.83 g, 25.0 mmol) was added to a solution of diethylphosphonopeptide dimethyl diester **6a** (3.60 g, 10.0 mmol) in dichloroethane (5 ml) under nitrogen atmosphere. Reaction mixture was stirred for 3 h at room temperature. After evaporation of solvent, excess of methanol was added. Then, removal under *vacuum* of solvent, the expected derivative **7a** was obtained in quantitative yield: ^{1}H-NMR ($CDCl_3$) δ 2.87–3.10 (m, 2H, CH_2CO), 3.71 (s, 3H, $COOCH_3$), 3.78 (s, 3H, $COOCH_3$), 4.84–5.10 (m, 1H, CH), 5.40 (dd, 1H, $^2J_{HF}$ = 46 Hz, $^2J_{HP}$ = 20 Hz, CHF), 7.43 (d, 1H, $^3J_{HH}$ = 8 Hz, NH), 11.20 (br s, 2H, OH); ^{31}P-NMR ($CDCl_3$) δ 6.0 (br s, 1P).

5.1.3.2.2. N-*[(phosphono)difluoroacetyl]*-L-*aspartic acid dimethyl diester* ***7b***. Compound **7b** was obtained according to the coupling procedure described for derivative **7a** starting from bromotrimethylsilane (3.83 g, 25.0 mmol) and diethylphosphonopeptide dimethyl diester **6b** (3.75 g, 10.0 mmol). Reaction mixture was stirred for 3 h at room temperature. The expected derivative **7b** was obtained in quantitative yield: ^{1}H-NMR ($CDCl_3$) δ 2.91–3.09 (m, 2H, CH_2CO), 3.71 (s, 3H, $COOCH_3$), 3.78 (s, 3H, $COOCH_3$), 4.84–5.10 (m, 1H, CH), 7.62 (d, 1H, $^3J_{HH}$ = 8 Hz, NH), 10.45 (br s, 2H, OH); ^{31}P-NMR ($CDCl_3$) δ 3.1 (br s, 1P, $^2J_{PF}$ = 95 Hz).

5.1.3.2.3. N-*[(Phosphono)fluoroacetyl]*-L-*aspartic acid* ***2a***. Bromotrimethylsilane (1.23 g, 8.0 mmol) was added to a solution of diethylphosphonopeptide di-*tert*-butyl diester **6′a** (0.88 g, 2.0 mmol) in dichloroethane (1 ml) under nitrogen atmosphere. After stirring for 6 h at room temperature, methanol (0.200 g, 8.0 mmol) was added, and then the reaction solution was allowed to stir for 2 h more. Evaporation under *vacuum* of solvents afforded the expected derivative **2a** as an oil in 85% yield: IR (Film) *v* 1090, 1235, 1615, 1750, 2200–3700; ^{1}H-NMR (CD_3CN) δ 1.64 (m, 2H, CH_2CO), 3.65–3.75 (m, 1H, CH), 4.16 (dd, 1H, $^2J_{H–P}$ = 12 Hz, $^2J_{H–F}$ = 46 Hz, PCHF), 6.23 (d, 1H, $^3J_{H–H}$ = 7 Hz, NH); ^{31}P-NMR (CD_3CN) δ 6.1 (br s, 1P); ^{19}F-NMR (CD_3CN) δ 179.6–180.5 (m, 1F).

Purification of **2a** was obtained by derivation to its tri(cyclohexyammonium) salt. To a solution of phosphonoacid **2a** (0.41 g, 1.5 mmol) in ethanol (2 ml) wad added dropwise successively cyclohexylamine (0.45 g, 4.5 mmol) and dry

acetone (50 ml). The solution was cooled at –18 °C and precipitation of a white solid was observed. After removing of liquid phase, solid residue was washed twice with diethyl ether and then dried by desiccation: IR (KBr) *ν* 1090, 1235, 1620, 1750, 2200–3700; ^{31}P-NMR (D_2O) δ 2.6 (t, 1P, $^2J_{PF}$ = 76 Hz); ^{19}F-NMR (D_2O) δ -114.0 (d, 1F, $^2J_{FP}$ = 76 Hz). Anal. Calcd. for $C_{24}H_{48}N_4O_8FP$: C, 50.52; H, 8.48; N, 9.82; F, 3.33; P, 5.43 Found: C, 50.92; H, 8.73; N, 9.50; P, 5.70.

5.1.3.2.4. N-*[(Phosphono)difluoroacetyl]*-L-*aspartic acid* ***2b***. Compound **2b** was obtained according to the procedure described for derivative **2a** starting from bromotrimethylsilane (1.23 g, 8.0 mmol) and diethylphosphonopeptide di-*tert*-butyl diester **6′b** (0.92 g, 2.0 mmol) and subsequent addition of methanol (0.200 g, 8.0 mmol). Compound **2b** was afforded as an oil in 85% yield: IR (Film) *ν* 1090, 1235, 1690, 1750, 2200–3700; ^{1}H-NMR (CD_3OD) δ 2.52 (dd, 1H, $^2J_{H–H}$ = 17 Hz, $^3J_{H–H}$ = 6 Hz, CH_2COO), 2.54 (dd, 1H, $^2J_{H–H}$ = 17 Hz, $^3J_{H–H}$ = 6 Hz, CH_2COO), 4.49 (t, 1H, $^3J_{HH}$ = 6 Hz, CH); ^{31}P-NMR (CD_3OD) δ 3.1 (t, 1P, $^2J_{PF}$ = 95 Hz); ^{19}F-NMR (CD_3OD) δ 116.9 (d, 2F, $^2J_{FP}$ = 95 Hz).

Tetra(cyclohexyammonium) salt of phosphonoacid **2b** was prepared according to the method carried out for **2a** starting from compound **2b** (0.44 g, 1.5 mmol) and cyclohexylamine (0.59 g, 6.0 mmol): IR (KBr) *ν* 1096, 1230, 1620, 1740, 2200–3700; ^{31}P-NMR (D_2O) δ 2.6 (t, 1P, $^2J_{PF}$ = 76 Hz); ^{19}F-NMR (D_2O) δ 114.1 (d, 1F, $^2J_{FP}$ = 76 Hz), -113.7 (d, 1F, $^2J_{FP}$ = 76 Hz). Anal. Calcd. for $C_{30}H_{60}N_5O_8F_2P$: C, 52.39; H, 8.79; N, 10.18; F, 5.52; P, 4.50 Found: C, 52.55; H, 9.05; N, 9.74. P, 4.85.

5.1.4. Preparation of N-*[(phosphonomethyl)phosphinato]*-L-*aspartic acid* ***3***

A solution of *N*-{[(diethylphosphono)methyl]ethoxyphosphinyl}-L-aspartic acid dimethyl diester **8** (1.25 g, 3.1 mmol) in CH_2Cl_2 (16 ml) and triethylamine (5.82 ml, 41.9 mmol) was cooled to 0–5 °C then bromotrimethylsilane (2.05 ml, 15.51 mmol) was added dropwise. Reaction mixture was stirred at room temperature for 3.5 days. A solution 1 M sodium hydroxide in methanol (31.0 ml) was then added and stirring was extended for 1 h at room temperature. After evaporation of solvents, residue was washed three times with a mixture CH_2Cl_2/EtOH (1/1). Three steps of work-up were realized successively as follow: residue in suspension in THF, or MeOH or EtOH was stirred, respectively, 1.5 h at room temperature, 4 h at 65 °C and 2 h at 65 °C. After last filtration, the expected compound **3** was obtained as a solid in 69% yield: IR (KBr) *ν* 1096, 1447, 1596, 1672; MS (positive electrospray) *m/z* 356 ($M+H^+–2Na^+$), 424.1 ($M+Na^+$); ^{1}H-NMR (D_2O) δ 2.7 (t, 2H, $^2J_{HP}$ = 18 Hz, PCH_2P), 2.38 (dd, 1H, $^2J_{H–H}$ = 14 Hz, $^3J_{H–H}$ = 5 Hz, CH_2CO) 2.45 (dd, 1H, $^2J_{H–H}$ = 14 Hz, $^3J_{H–H}$ = 9 Hz, CH_2CO), 3.65–3.70 (m, 1H, CHN); ^{31}P-NMR (D_2O) δ 22.6 (d, 1P, $^2J_{PP}$ = 9 Hz, PN), 14.1 (d, 1P, $^2J_{PP}$ = 9 Hz, PO); ^{13}C-NMR (D_2O) δ 32.0 (t, $^1J_{CP}$ = 114 Hz, PCH_2P), 44.4 (s, CH_2CO), 54.9 (s, CHN), 180.7 (s, COONa), 182.8 (s, COONa).

5.1.5. Preparation of methoxycarbonylmethylenephosphonic acid disodium salt ***4a***

5.1.5.1. Diethylphosphonoacetic acid methyl ester. Methyl bromoacetate (12.0 g, 78.4 mmol) was warmed to 80 °C and triethylphosphite (10.0 g, 60.2 mmol) was added dropwise swiftly. Increase of reaction temperature until 130 °C allowed to distil bromoethane. Stirring of reaction mixture was extended for 30 min. Distillation under reduced pressure afforded the expected diethylphosphonoacetic acid methyl ester as an oil in 88% yield: $Eb_{10\ mmHg}$ = 128 °C; IR (Film) *ν* 1052, 1120, 1742; ^{1}H-NMR ($CDCl_3$) δ 1.35 (t, 6H, $^3J_{HP}$ = 7 Hz, CH_3CH_2O), 2.98 (d, 2H, $^2J_{HP}$ = 22 Hz, PCH_2), 3.75 (s, 3H, OCH_3), 4.10–4.20 (m, 4H, CH_3CH_2O); ^{31}P-NMR ($CDCl_3$) δ 17.2 (s, 1P).

5.1.5.2. Methoxycarbonylmethylenephosphonic acid disodium salt ***4a***. Bromotrimethylsilane (9.11 g, 59.5 mmol) was added to a solution of diethylphosphonoacetic acid methyl ester (5.0 g, 23.8 mmol) in dichloroethane (12 ml). After stirring for 5 h, a 1 N MeONa in MeOH solution (60.0 ml, 59.5 mmol) was added. Reaction mixture was stirred for 15 min. After evaporation of solvents, residue was washed with MeOH. After evaporation, solid residue was dissolved in water and lyophilised to afforded the expected salt **4a** as a white solid in 99% yield: (KBr) *ν* 1702; ^{1}H-NMR (D_2O) δ 2.67 (d, 2H, $^2J_{HP}$ = 20 Hz, PCH_2), 3.69 (s, 3H, OCH_3); ^{31}P-NMR (D_2O) δ 10.5 (s, 1P); ^{13}C-NMR (D_2O) δ 40.6 (d, $^1J_{CP}$ = 109 Hz, PCH_2), 54.9 (s, OCH_3), 177.7 (s, COO).

5.1.6. Preparation of methoxycarbonylamidophosphonic acid disodium salt ***4b***

5.1.6.1. Diethyl amidophosphate ***9***. A solution of diethyl chlorophosphate (40.0 g, 231.6 mmol) and triethylamine (23.43 g, 231.6 mmol) in Et_2O (200.0 ml) was cooled to –35 °C. Gas ammoniac was added at –35 °C and with efficient stir for 1 h. Ammonium chloride was removed by centrifugation then evaporation of solvent followed by a distillation under reduced pressure gave the expected phosphoramide **9** as a white amorphous solid in 87% yield: $Eb_{0.02\ mbar}$ = 120 °C; IR (KBr) *ν* 1033, 1231; ^{1}H-NMR ($CDCl_3$) δ 1.34 (t, 6H, $^3J_{HP}$ = 7 Hz, CH_3), 2.80 (br s, 2H, NH2), 4.05–4.15 (m, 4H, CH_2); ^{31}P-NMR ($CDCl_3$) δ 6.8 (s, 1P); ^{13}C-NMR ($CDCl_3$) δ 16.0 (d, $^3J_{CP}$ = 7 Hz, CH_3), 62.1 (d, $^2J_{CP}$ = 5 Hz, CH_2).

5.1.6.2. Methyl N-*(diethylphosphono)carbamate* ***10***. A suspension of freshly washed sodium hydride (NaH) (55% suspension in mineral oil, 2.07 g, 43.12 mmol) in THF (15 ml) was stirred at 65 °C. Phosphoramide **9** (3.0 g, 19.6 mmol) in THF (10 ml) was then added dropwise. After stirring for 10 min, reaction temperature was allowed to cool to 0 °C. Methyl chloroformiate (1.85 g, 19.6 mmol) in THF (10 ml) was added dropwise at 0 °C. After stirring for 1 h, a saturated HCl diethyl ether solution was added until pH = 7 of reaction mixture. After centrifugation of NaCl and evaporation of

solvents, the expected derivative **10** was obtained as an oil in 79% yield: IR (KBr) *v* 1742; ^{1}H-NMR ($CDCl_3$) δ 1.34 (t, 6H, $^{3}J_{HP}$ = 7 Hz, CH_3), 3.71 (s, 3H, OCH_3), 4.15–4.25 (m, 4H, CH_2), 6.90 (br s, 1H, NH); ^{31}P-NMR ($CDCl_3$) δ 4.0 (s, 1P); ^{13}C-NMR ($CDCl_3$) δ 15.9 (d, $^{3}J_{CP}$ = 7 Hz, CH_3), 52.8 (s, OCH_3), 63.7 (d, $^{2}J_{CP}$ = 5 Hz, CH_2), 154.6 (d, $^{2}J_{CP}$ = 5 Hz, COO).

*5.1.6.3. Methoxycarbonylamidophosphonic acid disodium salt **4b**.* Compound **4b** was obtained according to the procedure described for derivative **4a** starting from bromotrimethylsilane (9.25 g, 60.4 mmol) and compound **10** (1.50 g, 7.1 mmol). Reaction mixture was stirred for 56 h at room temperature. Work-up was carried out a solution 1 M MeONa in MeOH (60.6 ml). The crude compound was washed successively with CH_2Cl_2, AcOEt and Et_2O, and filtered. Then trituration in a mixture MeOH/Me_2CO afforded the expected compound **4b** as a white solid in 50% yield: (KBr) *v* 1618, 1638; ^{1}H-NMR (D_2O) δ 4.98 (s, 3H, OCH_3); ^{31}P-NMR (D_2O) δ 9.3 (s, 1P); ^{13}C-NMR (D_2O) δ 51.8 (s, OCH_3), 184.0 (s, COO).

5.2. Enzymology

5.2.1. ATCase preparation and assay

Purified ATCase was prepared and assayed as previously described [21] and the isolated catalytic subunits were prepared by the method of Gerhart and Holoubek [22]. The enzymatic assay was performed at 37 °C, in the presence of 50 mM Tris–HCl pH = 8, 5 mM carbamylphosphate and 20 mM aspartate, or otherwise indicated.

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