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Efficient synthesis of fluorothiosparfosic acid analogues with potential antitumoral activity

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Abstract—In this paper, we describe a short synthesis of *N*-(phosphonoacetyl)-L-aspartate (PALA) analogues. The mono- and difluorinated thioacetamide precursors were prepared in one step from methyl (diethoxyphosphono)di- and monofluoromethyldithioacetates **8** and **11** as starting materials. Antiproliferating properties on a L1210 strain and ATCase inhibition of these new compounds are disclosed. ThioPALA(FF) **5c** showed a remarkable cytotoxic activity towards murine leukemia L1210, when used as tetraester.

1. Introduction

Pyrimidines and purines, essential components of DNA and RNA, are needed for cell proliferation. In mammalian species, the first three steps of de novo pyrimidine biosynthesis are catalyzed by a trifunctional polypeptide named CAD including the carbamoyl-phosphate synthetase II (CPSase II; EC 6.3.3.5), aspartate transcarbamylase (ATCase; EC 2.1.3.2), and dihydrooratase (DHOase; EC 3.5.2.3). Elevated activities of these enzymes have been reported in rapidly proliferating cells; thus, inhibition of one of them constitutes a target for anticancer drug discovery.[1]

The *N*-(phosphonoacetyl)-L-aspartate (PALA) **1** has been designed to mimic the transition state of ATCase and is known to exhibit antitumor activities against cancers such as Lewis lung carcinoma and melanoma which could not be targeted by other antimetabolites (Fig. 1).[2] Nevertheless, in comparison with in vitro results, in vivo inhibition of pyrimidine biosynthesis was less efficient due to the difficult penetration of PALA in the cells under its tetra-anion state.[3] To overcome this main limitation, prodrugs containing either octadecyl carboxylic ester functions or enzyme-labile phosphonic esters (SATP) as well as polyethyleneglycol ether moieties have been synthesized and reported as efficient lipophilic anticancer agents.[4] The modifications of the structure by replacement of the carboxylic amino acid moiety by a phosphonic unit or the peptidic bond by a methylene-sulfide, -sulfoxide, -sulfone, or sulfonamide groups were unsuccessful.[5]

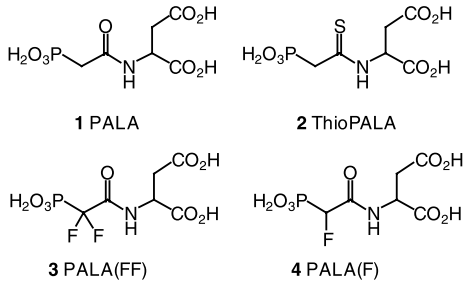

Figure 1. Representative sparfosic acid analogues.

However, other chemical modifications of PALA have been developed in order to increase its biological activity

Keywords: ATCase; L1210; Difluorophosphonates; PALA; Sparfosic acid.
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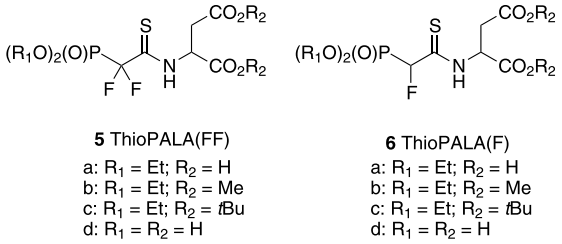


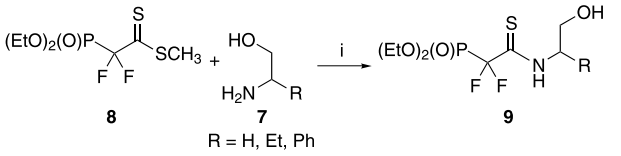
Figure 2. ThioPALA(FF) and thioPALA(F).

and bioavailability (Fig. 1). The replacement of the peptidic bond by a thiopeptidic bond or the introduction of fluorine atoms on the α-carbon have both furnished potent inhibitors of ATCase. For example, the thioPALA analogue **2** containing a thioamide function was found to be two times more active towards *Escherichia coli* ATCase than PALA.[6] This suggests that the presence of a sulfur atom can promote lipophilicity of the molecule and reaction on the enzyme site by nucleophilic addition. On the other hand, the difluoromethylphosphonate PALA(FF) **3**, which acts as a good phosphate mimic,[7] was also reported as an inhibitor of plant ATCase (mung bean), but was five times less powerful than the PALA.[8] However, it has been shown recently that **3** did not provoke inhibition of *E. coli* ATCase, while its monofluorophosphonate analogue PALA(F) **4** was a good inhibitor.[9] To the best of our knowledge, any PALA analogue containing both a fluorine atom and a thiopeptidic bond was already described. In view of the good biological activities of **2**, **3**, and **4**, these simultaneous structural modifications appear attractive for finding new potent inhibitors. Herein, we describe an efficient preparation of a new range of modified sparfosic acid derivatives **5** and **6** (Fig. 2) and report results of their biological studies towards solid murine tumor (L1210 leukemia) and *E. coli* ATCase.

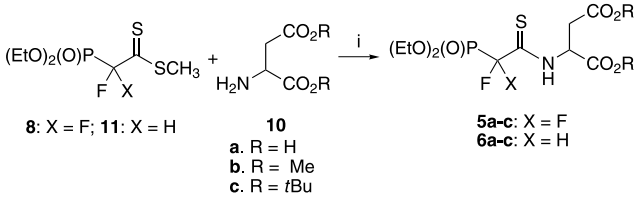
2. Results and discussion

2.1. Chemistry

The synthesis of difluoromethylphosphonates is well documented and the most widespread methods are based on the use of the *O*,*O*-diethyl phosphonodifluoromethyllithium.[10] On the other hand, the introduction of fluorine atoms by using electrophilic fluorinating reagents has emerged as an alternative strategy.[11] However, the latter was only efficient when activated methylenephosphonates were used.[12] The syntheses of modified sparfosic acid derivatives **5a–d** and **6a–d** were explored from phosphonofluorodithioesters **8** and **11**, respectively (Scheme 2). These starting materials were prepared from the *O*,*O*-diethyl phosphonodifluoromethyllithium according to reported methods.[13] Dithioesters are known as excellent N-thioacylating reagents for the synthesis of thioamides.[14] Previously, we have shown that phosphonodithioesters react readily with amines to produce phosphonamides.[15] Besides, phosphonodifluoromethylthioamides **9** were prepared in one step and in good yields from a variety



Scheme 1. Reagents and conditions: (i) CH_2Cl_2, 15–30 min, 20 °C (64–85%).



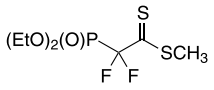
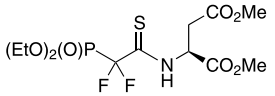
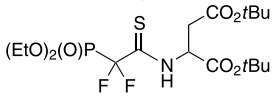
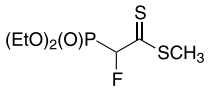
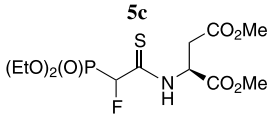
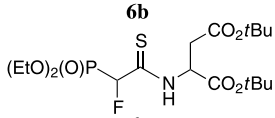
Scheme 2. Reagents and conditions: (i) CH_2Cl_2, 15 h, 20 °C (75–82%).

of β-amino alcohols **7** and the corresponding dithioester **8** (Scheme 1).[16]

This method was applied to the synthesis of **5a** (Scheme 2). The direct N-thioacylation of L-aspartic acid hydrochloride was carried out in the presence of triethylamine. The reaction was slower than in the case of β-amino alcohols, and completed after 48 h at room temperature. However, the thioamide **5a** was isolated in poor yields (<20%) due to its difficult purification and the formation of the by-product **11** (15–30%). Previously, we noticed that during the thioacylation of amines with difluorodithioester **8**, a competitive elimination of one fluorine atom occurred leading to the corresponding monofluorodithioester **11**.[17] Any modification of the experimental procedure (time, equivalent of amine, rate of addition, and temperature) was unsuccessful and **11** was still present in the crude mixture. Therefore, this study was extended to esters of amino acids. The dimethyl L-aspartate **10b**, prepared from L-aspartic acid and trimethylsilyl chloride in refluxing methanol,[18] was reacted with dithioester **8** in dichloromethane at room temperature (Scheme 2, Table 1). After 15 h of stirring, ^{19}F NMR analysis of the crude indicated a complete conversion of **8** and the formation of less than 5% of defluorinated product **11**. After purification by flash chromatography, the corresponding thioamide **5b** was isolated in 82% yield. As reported previously, no epimerization of the stereogenic center occurred during the thioacylation reaction.[16]

The study was extended to racemic aspartate ester to compare the biological activity of modified PALA under their pure optical form or their racemic form. The one-step synthesis of tetraester **5c** was investigated from the racemic commercially available di-*tert*-butyl aspartate **10c**. As noticed for the dimethyl aspartate, the reaction was completed after 15 h of stirring at room temperature, and only traces of by-product **11** were detected. The thioamide **5c** was isolated by flash column chromatography in 75% yield. The acidity of the phosphonic acid moiety was modulated by the preparations of monofluorophosphonate analogues following the

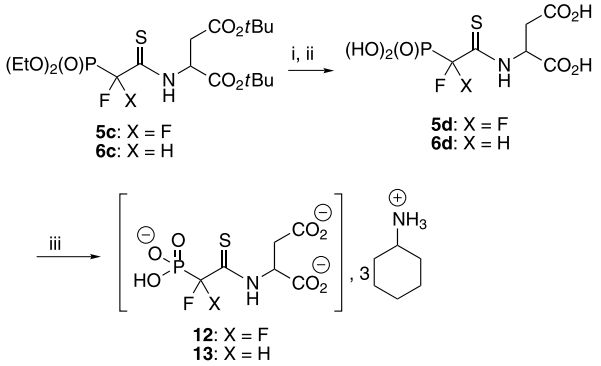
Table 1. One-step preparation of fluoro-thioPALA precursors

Dithioester	Amine	Product	Yield (%)
 **8**	**10b**	 **5b**	82
8	**10c**	 **5c**	75
 **11**	**10b**	 **6b**	83
11	**10c**	 **6c**	78

same route. From the monofluorodithioester **11**[17] and ester **10b**, a mixture of thioamide diastereomers **6b** was obtained and isolated in good yield (Table 1). As observed for **8**, the reaction, performed from the di-*tert*-butyl ester **10c** and dithioester **11**, afforded the corresponding thioamide **6c** also in good yield (Table 1).

The full deprotection of these tetraesters has been investigated to prepare acid derivatives **5d** and **6d**. Treatment of tetraesters **5b** and **6b** with trimethylsilyl bromide[19] led to a partial hydrolysis even after longer reaction times (15 h). Only the phosphonate ester function was hydrolyzed. Recently, we showed that both di-*tert*-butyl carboxylate and diethyl phosphonate functions of a sparfosic acid derivative could be converted in their corresponding acid form in a one-pot reaction.[20] This method has been applied to the di- and monofluorothioamides **5c** and **6c**.

The complete deprotection of the difluorinated tetraester **5c** was observed when treated with 5 equiv of bromotrimethylsilane in refluxing acetonitrile for 6 h. After hydrolysis of the crude mixture the corresponding acid **5d** was precipitated out by addition of cyclohexylamine. After filtration, the pure cyclohexylammonium salt **12** was isolated in 60% yield (Scheme 3).



Scheme 3. Reagents and conditions: (i) TMSBr, CH_3CN, reflux, 6 h; (ii) H_2O, 4 °C, overnight; (iii) cyclohexylamine, Et_2O, 30 min, (60%).

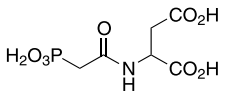
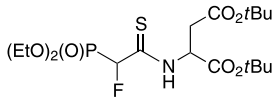
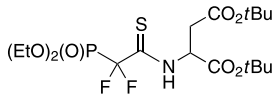
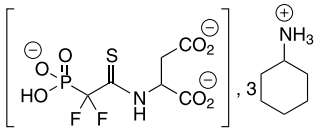
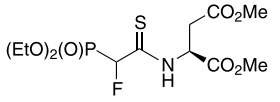
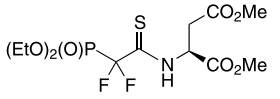
According to earlier observations mentioned in the literature, ^1H NMR analysis revealed the presence of three cyclohexylamine molecules in the salt **12**, indicating that the second acidity of the phosphonic function is not neutralized. After total hydrolysis of the monofluorinated analogue **6c**, under the same conditions, attempts to form the corresponding ammonium salt failed. No precipitate was observed and a gummy nonidentifiable product was formed. However, after a C-18 column chromatography of the crude acid **6d**, two nonseparable diastereomers were isolated in low yield (20%). When compared to our previous work on the synthesis of PALA, in the present case the ether cleavage reaction is slower (6 h vs 45 min). This could be related to a decrease of the nucleophilicity of the phosphonate function by the presence of fluorine atoms.

2.2. Biological studies

Antiproliferating activities of compounds **5b**,**c**, **6b**,**c**, and **12** were determined using the L1210 murine leukemia cell line, which is routinely used for in vitro screening of cytotoxic agents. PALA was used as the reference molecule. The IC_{50} values (μM) are reported in Table 2, for a given molecule, and ratios IC_{50}(PALA)/IC_{50} were calculated and are reported in Figure 3.

As observed for PALA, the cyclohexylammonium salt of the difluorinated thioPALA analogue **12** exhibited no activity toward proliferation of L1210 cells at 100 μM. This could be assigned to a weak penetration of this charged molecule in the cells. It has been shown that the tetraester of PALA could be used as lipophilic prodrug to enhance its cellular penetration.[4a] In agreement with these observations, high cytotoxic activities were observed from tetraalkyl esters **5b**,**c** and **6b**,**c**. The thioPALA(FF) analogues **5b** and **5c** showed inhibition of cell proliferation in the same range of concentration (31–32 μM) and are 13 times more active than PALA. The thioPALA(F) analogues **6b** and **6c** are also active, and are eight times more active than PALA.

Table 2. Cytotoxic activity of PALA and its analogues towards murine leukemia L1210 cell

Compound	Structure	IC_{50} (μM)
1		421
6c		51.8
5c		31.4
12		>100
6b		59.8
5b		32.1

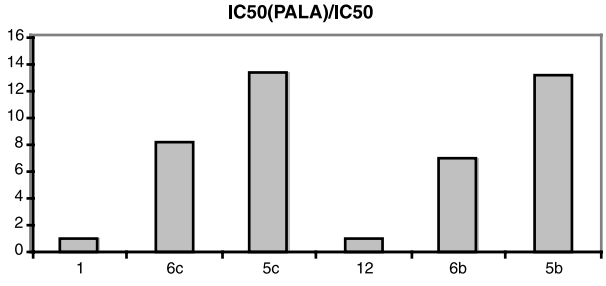


Figure 3. Comparison of cytotoxic activity of fluorophosphonates toward PALA. Calculated IC_{50} (PALA)/IC_{50} (fluorophosphonates) ratios.

The introduction of one supplementary fluorine atom enhances the lipophilicity of the tetraesters and the difluorophosphonate appears to be more efficient. This would be a consequence of better cell penetration by analogues **5b**,**c** than by **6b**,**c**. As shown for the optically pure form **5b**, and for the racemic form **5c**, even by changing the carboxylic ester group no influence of the configuration of the stereogenic centers was observed.

Due to the relatively high cytotoxicity of the fluorinated thiosparfosic ester analogues towards murine leukemia L1210 cells the inhibition of the *E. coli* aspartate transcarbamylase (ATCase) by difluorophosphonate tetraester **5c** and its corresponding acid tricyclohexylammonium salt **12** was investigated. The results were compared to those observed from PALA. The results of these tests are summarized in Figure 4. All experiments were conducted in the presence of 5 mM carbamoylphosphate and 5 mM L-[^{14}C]aspartate.

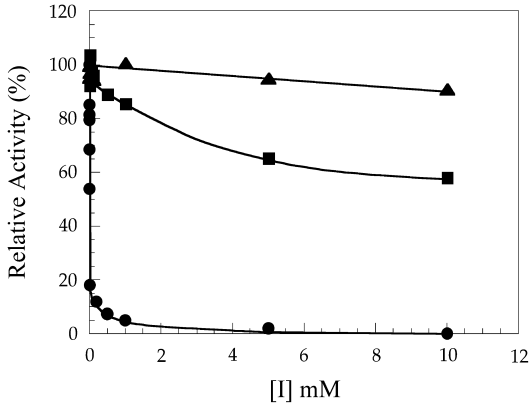


Figure 4. Influence of compounds **12** and **5c** on ATCase activity. The ATCase activity was measured as indicated in Section 4 in the absence or presence of increasing concentrations of the substrates analogues and PALA as a control. (●–●): PALA; (■–■): **12**; and (▲–▲): **5c**.

Figure 4 shows that the tetraester **5c** did not provoke inhibition of *E. coli* ATCase, and that the ammonium salt **12** has a significant effect, although it is considerably less active than PALA towards *E. coli* ATCase.

The antitumoral activity of PALA is attributed to the inhibition of ATCase. Therefore, the high cytotoxicity of tetraester **5c** compared to that of the sparfosic acid (PALA) is probably due to the better lipophilicity of **5c**, which makes this ester more prone to cross the cell membrane. Then, an intracellular hydrolysis of the ester functions could occur as observed previously.[4d] However, the relatively low ATCase inhibition observed with salt **12** does not allow the conclusion that the cytotoxicity of the fluorinated thiosparfosic acid derivatives results exclusively from the ATCase inhibition. The configuration of the catalytic site of ATCase and the amino acids which constitute this site are entirely conserved from bacteria and Archae to humans, an observation which justifies the use of the *E. coli* enzyme to test inhibition by the products synthesized in this study.[21] However, the molecular organization of these enzymes varies. In *E. coli*, the catalytic subunit of ATCase is associated with regulatory subunits while in mammals it is associated with carbamylphosphate synthetase and dihydro-orotase domains, which catalyze the first and the third reactions of the pyrimidine pathway. This difference raises the possibility that human ATCase might be more sensitive to the inhibitor than its bacterial homologue.

3. Conclusion

We have reported a facile synthesis of new sparfosic acid analogues. Mono- and difluorophosphonothioacetamides **6a–c** and **5a–c** were easily prepared from the methyl (diethoxyphosphono)di- or monofluorodithioacetate **8** and **11** and aspartic acid esters. In their tetraester forms, antiproliferating properties of these new PALA analogues have been demonstrated on a L1210 strain.

Compounds **5b**,**c** and **6b**,**c** exhibit an activity 8–13 times superior to the PALA itself. Although lower than that of PALA, the ATCase inhibition observed with the ammonium form of one of these thio- and difluoro- substituted compounds **12** suggests that the relatively high cytotoxicities of **5b**,**c** and **6b**,**c** on L1210 result from good cell penetration by these esters, which probably act as prodrugs of their corresponding acids.

4. Experimental

4.1. General methods

All reactions were carried out under a nitrogen atmosphere. Tetrahydrofuran (THF) was purified by distillation from sodium-benzophenone. Dichloromethane (CH_2Cl_2), diisopropylamine, cyclohexylamine, and triethylamine were distilled from calcium hydride. Reactions were monitored by thin-layer chromatography (TLC) using Merck silica gel 60 F_{254} plastic sheets. Flash column chromatography was performed over Merck silica gel Si 60 (40–63 μm). Reverse phase chromatography was realized with Polygoprep 60-50 C18 charge 8014 silica and TLC with RP18W/UV254 C18 silica gel. Carbon, proton, phosphorus, and fluorine NMR spectra were recorded on a Brücker DRX-250 spectrometer. The chemical shifts (δ) are expressed in ppm relative to internal tetramethylsilane for ^1H, ^{13}C nucleus, to H_3PO_4 for ^{31}P nucleus, and to $CFCl_3$ for ^{19}F nucleus. Splitter patterns are designated as: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; br, broad; dt, doublet of triplet; and dd, doublet of doublet. Low- and high-resolution mass spectra were recorded on a JEOL GC_{mate} spectrometer.

4.1.1. Methyl (diethoxyphosphoryl)difluoroethanedithioate (8). To a solution of LDA prepared from diisopropylamine (9 mL, 66.5 mmol) and BuLi 2.5 M (25.6 mL, 69.0 mmol) in 300 mL of anhydrous THF under N_2, was added slowly diethyl difluoromethylphosphonate (10 g, 53.0 mmol) at −78 °C. The mixture was stirred for 1 h at −78 °C, and carbon disulfide (16 mL, 265.0 mmol) was introduced dropwise. The solution was maintained at −78 °C for 30 min, and methyl iodine (16.5 mL, 265.0 mmol) was then added. The reaction mixture was stirred for 5 min, hydrolyzed with 1 M HCl, and extracted three times with Et_2O. The organic layers were dried with anhydrous $MgSO_4$, filtered, and concentrated. The resulting oil was purified by flash column chromatography (petroleum ether/ethyl acetate 6:4) affording the methyl (diethoxyphosphoryl)difluoroethanedithioate **8** (11.5 g, 78%) as a red oil. ^1H NMR ($CDCl_3$) δ: 1.38 (t, $^3J_{HH}$ = 7.0 Hz, 6H), 2.70 (s, 3H), 4.30 (m, 4H); ^{19}F NMR ($CDCl_3$) δ: −98.72 (d, $^2J_{FP}$ = 104.0 Hz); ^{31}P NMR ($CDCl_3$) δ: 3.63 (d, $^2J_{PF}$ = 104.0 Hz); ^{13}C NMR ($CDCl_3$) δ: 16.3 (d, $^3J_{CP}$ = 5.7 Hz), 19.4 (t, $^4J_{CP}$ = 2.5 Hz), 65.6 (d, $^2J_{CP}$ = 6.8 Hz), 117.2 (dt, $^1J_{CP}$ = 209.7 Hz, $^1J_{CF}$ = 271.0 Hz), 219.8 (dt, $^2J_{CP}$ = 17.2 Hz, $^2J_{CF}$ = 21.7 Hz); MS (EI, 70 eV) *m/z* (relative intensity) 278 ($M^{+\cdot}$ 17), 258 (20), 233 (16), 91 (100), 51 (30), 47 (66), 45 (86); HRMS (EI, 70 eV) *m/z* [M^+] Calcd for $C_7H_{13}F_2O_3PS_2$: 278.00115. Found: 278.00460.

4.1.2. Methyl (diethoxyphosphoryl)fluoroethanedithioate (11). In a 250 mL flask containing 150 mL of anhydrous THF and ethanethiol (2.7 mL, 35.9 mmol) stirred under N_2, BuLi 2.5 M (15 mL, 37.7 mmol) was added slowly at −78 °C. After 30 min, methyl (diethoxyphosphoryl)difluoroethanedithioate **8** (5 g, 17.9 mmol) was introduced, and the mixture was stirred for 15 h from −78 °C to rt. The solution was then hydrolyzed with 1 M HCl and extracted with CH_2Cl_2. The organic layer was dried with anhydrous $MgSO_4$, filtered, and concentrated. The methyl (diethoxyphosphoryl)fluoroethanedithioate **11** was isolated by flash chromatography (Et_2O/pentane 7:3) as a yellow oil (4.3 g, 92%). ^1H NMR ($CDCl_3$) δ: 1.38 (t, $^3J_{HH}$ = 7.0 Hz, 6H), 2.69 (s, 3H), 4.35 (m, 4H), 5.74 (dd, $^2J_{HP}$ = 12.6 Hz, $^2J_{HF}$ = 47.2 Hz, 1H); ^{19}F NMR ($CDCl_3$) δ: −183.28 (dd, $^2J_{FP}$ = 77.0 Hz, $^2J_{FH}$ = 47.2 Hz); ^{31}P NMR ($CDCl_3$) δ: 10.23 (d, $^2J_{PF}$ = 77.2 Hz); ^{13}C NMR ($CDCl_3$) δ: 16.7 (d, $^3J_{CP}$ = 6.1 Hz), 18.7 (d, $^4J_{CP}$ = 4.8 Hz), 64.7, 65.0 (d, $^2J_{CP}$ = 6.7 Hz), 98.0 (dd, $^1J_{CP}$ = 160.3 Hz, $^1J_{CF}$ = 200.9 Hz), 224.8 (d, $^2J_{CP}$ = 16.3 Hz); MS (EI, 70 eV) *m/z* (relative intensity) 260 ($M^{+\cdot}$ 97), 240 (28), 196 (62), 195 (43), 169 (46), 157 (27), 137 (25), 124 (33), 109 (100), 104 (32), 91 (68), 81 (49), 77 (26); HRMS (EI, 70 eV) *m/z* [M^+] Calcd for $C_7H_{14}FO_3PS_2$: 260.01058. Found: 260.01123.

4.1.3. *N*-[2-(Diethoxyphosphoryl)-2,2-difluoro-ethanethioyl] aspartic acid (5a). In a 50 mL flask, L-aspartic acid hydrochloride (295 mg, 2.2 mmol) and triethylamine (620 μL, 4.4 mmol) were dissolved in H_2O/THF 1:1 (5 mL). Difluorinated phosphonodithioester **8** (500 mg, 1.8 mmol) was added and the solution was stirred for 48 h at room temperature. The mixture was hydrolyzed with 1 M HCl and extracted with Et_2O. Organic layer was dried with anhydrous $MgSO_4$, filtered, and concentrated. The compound **5a** was isolated by flash chromatography (petroleum ether/ethyl acetate 6:4) as a yellow oil (130 mg, 20%). ^1H NMR ($CDCl_3$) δ: 1.38 (t, $^3J_{HH}$ = 7.0 Hz, 6H), 3.14 (d, $^3J_{HH}$ = 4.4 Hz, 2H), 4.35 (m, 4H), 5.39 (m, 1H), 9.38 (d, $^3J_{HH}$ = 7.4 Hz, 1H), 9.90 (s, 2H); ^{19}F NMR ($CDCl_3$) δ: −107.28 (d, $^2J_{FP}$ = 105.5 Hz); ^{31}P NMR ($CDCl_3$) δ: 3.60 (t, $^2J_{PF}$ = 105.7 Hz); ^{13}C NMR ($CDCl_3$) δ: 16.5 (d, $^3J_{CP}$ = 5.7 Hz), 34.3 (s), 54.1 (s), 67.2 (d, $^2J_{CP}$ = 6.9 Hz), 115.6 (dt, $^1J_{CP}$ = 204.1 Hz, $^1J_{CF}$ = 270.1 Hz), 172.4, 174.0 (s), 188.2 (dt, $^2J_{CP}$ = 17.2 Hz, $^2J_{CF}$ = 21.2 Hz).

4.1.4. Dimethyl *N*-[2-(diethoxyphosphoryl)-2,2-difluoro-ethanethioyl] aspartate (5b). In a 50 mL flask containing 20 mL of anhydrous CH_2Cl_2 and L-aspartate dimethyl ester (320 mg, 1.98 mmol) under N_2 the methyl (diethoxyphosphoryl)difluoroethanedithioate **8** (500 mg, 1.80 mmol) was introduced. The mixture was stirred for 15 h at room temperature and the solvent was evaporated. Flash chromatography (petroleum ether/ethyl acetate 1:1) furnished the difluorinated phosphonothioamide **5b** (577 mg, 82%) as a yellow oil. ^1H NMR ($CDCl_3$) δ: 1.39 (t, $^3J_{HH}$ = 7.0 Hz, 3H), 1.40 (t, $^3J_{HH}$ = 7.0 Hz, 3H), 3.13 (d, $^3J_{HH}$ = 4.3 Hz, 2H), 3.71, 3.81 (s, 3H), 4.32 (m, 4H), 5.38 (m, 1H), 9.13 (br s, 1H); ^{19}F NMR ($CDCl_3$) δ: −107.83 (dd, $^2J_{FP}$ = 103.5 Hz, $^2J_{FF}$ = 294.1 Hz, 1F), −106.48 (dd, $^2J_{FP}$ = 101.2 Hz, $^2J_{FF}$ = 294.2 Hz, 1F); ^{31}P NMR ($CDCl_3$) δ: 1.94 (t, $^2J_{PF}$ = 102.6 Hz); ^{13}C NMR

($CDCl_3$) δ: 14.7 (d, $^3J_{CP}$ = 5.7 Hz), 32.5 (s), 50.7, 51.6 (s), 52.1 (s), 64.2 (d, $^2J_{CP}$ = 6.2 Hz), 112.0 (dt, $^1J_{CP}$ = 206.0 Hz, $^1J_{CF}$ = 269.8 Hz), 167.7, 169.2 (s), 186.8 (dt, $^2J_{CP}$ = 16.9 Hz, $^2J_{CF}$ = 24.0 Hz); MS (EI, 70 eV) *m/z* (relative intensity) 391 ($M^{+\cdot}$ 34), 371 (92), 360 (48), 312 (96), 272 (34), 253 (48), 243 (75), 235 (30), 221 (34), 188 (34), 160 (61), 138 (26), 113 (98), 111 (40), 109 (61), 102 (51), 101 (35), 94 (39), 87 (34), 86 (27), 81 (56); HRMS (EI, 70 eV) *m/z* [M^+] Calcd for $C_{12}H_{20}F_2NO_7PS$: 391.0666. Found: 391.0656; $[\alpha]_D^{20} = -3.6$ (*c* 0.98, EtOH).

4.1.5. Di-*tert*-butyl *N*-[2-(diethoxyphosphoryl)-2,2-difluoroethanethioyl] aspartate (5c). In a 50 mL flask under N_2 containing 20 mL anhydrous CH_2Cl_2 were introduced D,L-aspartate di-*tert*-butyl ester hydrochloride (558 mg, 1.98 mmol) and triethylamine (275 μL, 1.98 mmol). The mixture was stirred at room temperature until a clear solution appeared and the methyl (diethoxyphosphoryl)difluoroethanedithioate **8** (500 mg, 1.80 mmol) was slowly added. After 15 h stirring, the solvent was concentrated and the obtained residue was purified by flash chromatography (pentane/Et_2O 55:45) leading the compound **5c** (641 mg, 75%) as a yellow oil. ^1H NMR ($CDCl_3$) δ: 1.39 (t, $^3J_{HH}$ = 7.1 Hz, 6H), 1.45, 1.48 (s, 9H), 2.94 (dd, $^3J_{HH}$ = 4.1 Hz, $^2J_{HH}$ = 15.1 Hz, 1H), 3.03 (dd, $^3J_{HH}$ = 4.3 Hz, $^2J_{HH}$ = 15.1 Hz, 1H), 4.33 (dt, $^3J_{HP} = {}^4J_{HF}$ = 7.0 Hz, 4H), 5.13 (m, 1H), 9.02 (br s, 1H); ^{19}F NMR ($CDCl_3$) δ: −107.47 (dd, $^2J_{FP}$ = 104.0 Hz, $^2J_{FF}$ = 295.0 Hz, 1F), −106.14 (dd, $^2J_{FP}$ = 103.0 Hz, $^2J_{FF}$ = 294.0 Hz, 1F); ^{31}P NMR ($CDCl_3$) δ: 2.04 (t, $^2J_{PF}$ = 103.7 Hz); ^{13}C NMR ($CDCl_3$) δ: 14.5 (d, $^3J_{CP}$ = 5.7 Hz), 26.1, 26.3 (s), 35.8 (s), 54.6 (s), 63.9 (d, $^2J_{CP}$ = 6.7 Hz), 63.9 (d, $^2J_{CP}$ = 6.6 Hz), 81.2, 81.5 (s), 111.8 (dt, $^1J_{CP}$ = 206.7 Hz, $^1J_{CF}$ = 270.1 Hz), 166.0, 167.6 (s), 185.9 (dt, $^2J_{CP}$ = 17.0 Hz, $^2J_{CF}$ = 24.0 Hz); MS (EI, 70 eV) *m/z* (relative intensity) 475 ($M^{+\cdot}$ 3), 363 (55), 346 (92), 343 (60), 329 (30), 301 (29), 300 (25), 299 (41), 244 (25), 188 (20), 129 (43), 113 (39), 101 (91), 84 (100); HRMS (EI, 70 eV) *m/z* [M^+] Calcd for $C_{18}H_{32}F_2NO_7PS$: 475.1605. Found: 475.16054.

4.1.6. Dimethyl *N*-[2-(diethoxyphosphoryl)-2-fluoroethanethioyl] aspartate (6b). This compound was prepared as described for compound **5b** from **11** (500 mg, 1.92 mmol) and L-aspartate dimethyl ester (380 mg, 2.11 mmol). Flash chromatography (pentane/Et_2O 55:45) furnished a mixture of two diastereomers as yellow oil (594 mg, 83%). *Diastereomer 1*: ^1H NMR ($CDCl_3$) δ: 1.28 (t, $^3J_{HH}$ = 7.0 Hz, 6H), 3.05 (d, $^3J_{HH}$ = 4.4 Hz, 2H), 3.63, 3.73 (s, 6H), 4.19 (m, 4H), 5.40 (m, 1H), 5.49 (dd, $^2J_{HP}$ = 11.0 Hz, $^2J_{HF}$ = 46.9 Hz, 1H), 8.87 (br s, 1H); ^{19}F NMR ($CDCl_3$) δ: −191.14 (ddd, $^4J_{FH}$ = 5.0 Hz, $^2J_{FH}$ = 47.0 Hz, $^2J_{FP}$ = 76.0 Hz); ^{31}P NMR ($CDCl_3$) δ: 11.53 (d, $^2J_{PF}$ = 76.0 Hz); ^{13}C NMR ($CDCl_3$) δ: 15.3 (d, $^3J_{CP}$ = 5.8 Hz), 33.6 (s), 51.2, 51.8 (s), 52.0 (s), 63.6 (d, $^2J_{CP}$ = 6.6 Hz), 91.0 (dd, $^1J_{CP}$ = 159.8 Hz, $^1J_{CF}$ = 205.0 Hz), 168.6, 169.9 (s), 190.2 (d, $^2J_{CF}$ = 14.0 Hz); MS (EI, 70 eV) *m/z* (relative intensity) 373 ($M^{+\cdot}$ 28), 363 (29), 353 (53), 346 (52), 343 (42), 342 (35), 299 (30), 294 (100), 268 (31), 266 (38), 226 (32), 217 (32), 195 (31), 176 (31), 160 (40), 157 (30), 143 (34), 138 (33), 114 (64), 113 (86), 109 (49), 102 (43), 101 (32), 81 (57), 77 (42). *Diastereomer 2*: ^1H NMR ($CDCl_3$) δ: 1.29 (t, $^3J_{HH}$ = 7.0 Hz, 6H), 3.05 (d, $^3J_{HH}$ = 4.4 Hz, 2H), 3.63, 3.73 (s, 6H), 4.19 (m, 4H), 5.40 (m, 1H), 5.49 (dd, $^2J_{HP}$ = 11.0 Hz, $^2J_{HF}$ = 46.9 Hz, 1H), 8.87 (br s, 1H); ^{19}F NMR ($CDCl_3$) δ: −190.87 (ddd, $^4J_{FH}$ = 4.0 Hz, $^2J_{FH}$ = 46.0 Hz, $^2J_{FP}$ = 74.0 Hz); ^{31}P NMR ($CDCl_3$) δ: 11.20 (d, $^2J_{PF}$ = 75.0 Hz); ^{13}C NMR ($CDCl_3$) δ: 15.3 (d, $^3J_{CP}$ = 5.8 Hz), 33.3 (s), 51.2, 51.9 (s), 52.0 (s), 63.3 (d, $^2J_{CP}$ = 6.7 Hz), 90.9 (dd, $^1J_{CP}$ = 159.1 Hz, $^1J_{CF}$ = 203.8 Hz), 168.4, 169.9 (s), 190.2 (d, $^2J_{CF}$ = 14.0 Hz); HRMS (EI, 70 eV) *m/z* [M^+] Calcd for $C_{12}H_{21}FNO_7PS$: 373.07602. Found: 373.07443.

4.1.7. Di-*tert*-butyl *N*-[2-(diethoxyphosphoryl)-2-fluoroethanethioyl] aspartate (6c). This compound was prepared as described for compound **5c** from **11** (500 mg, 1.92 mmol), triethylamine (295 μL, 2.11 mmol), and L-aspartate di-*tert*-butyl ester hydrochloride (596 mg, 2.11 mmol). Flash chromatography (Et_2O/pentane 6:4) furnished a mixture of two diastereomers as yellow oil (684 mg, 78%). *Diastereomer 1*: ^1H NMR ($CDCl_3$) δ: 1.36 (t, $^3J_{HH}$ = 7.0 Hz, 6H), 1.46 (s, 9H), 1.49 (s, 18H), 2.99 (m, 4H), 4.24 (m, 4H), 5.23 (dd, $^3J_{HH} = {}^3J_{HH}$ = 3.9 Hz, 1H), 5.55 (dd, $^2J_{HP}$ = 11.1 Hz, $^2J_{HF}$ = 46.9 Hz, 1H), 8.93 (br s, 1H); ^{19}F NMR ($CDCl_3$) δ: −190.90 (dd, $^2J_{FH}$ = 47.0 Hz, $^2J_{FP}$ = 75.0 Hz); ^{31}P NMR ($CDCl_3$) δ: 11.64 (d, $^2J_{PF}$ = 75.3 Hz); ^{13}C NMR ($CDCl_3$) δ: 16.6 (m), 28.1, 28.2, 28.3 (s), 36.0 (s), 53.7 (s), 64.6 (d, $^2J_{CP}$ = 6.6 Hz), 64.7 (d, $^2J_{CP}$ = 6.5 Hz), 82.2, 83.4 (s), 92.3 (dd, $^1J_{CP}$ = 158.9 Hz, $^1J_{CF}$ = 204.7 Hz), 168.4, 169.9 (s), 190.9 (d, $^2J_{CF}$ = 13.1 Hz); MS (EI, 70 eV) *m/z* (relative intensity) 457 ($M^{+\cdot}$ 7), 345 (47), 328 (100), 327 (31), 325 (38), 311 (26), 282 (44), 281 (73), 280 (78), 254 (28), 236 (52). *Diastereomer 2*: ^1H NMR ($CDCl_3$) δ: 1.35 (t, $^3J_{HH}$ = 6.9 Hz, 6H), 1.44 (s), 1.49 (s, 18H), 2.99 (m, 2H), 4.24 (m, 4H), 5.23 (dd, $^3J_{HH} = {}^3J_{HH}$ = 3.9 Hz, 1H), 5.55 (dd, $^2J_{HP}$ = 11.1 Hz, $^2J_{HF}$ = 46.9 Hz, 1H), 8.93 (br s, 1H); ^{19}F NMR ($CDCl_3$) δ: −190.61 (dd, $^2J_{FH}$ = 47.0 Hz, $^2J_{FP}$ = 74.0 Hz); ^{31}P NMR ($CDCl_3$) δ: 11.26 (d, $^2J_{PF}$ = 74.3 Hz); ^{13}C NMR ($CDCl_3$) δ: 16.6 (m), 28.1, 28.1, 28.2 (s), 36.3 (s), 53.9 (s), 64.7 (d, $^2J_{CP}$ = 6.7 Hz), 82.3, 83.9 (s), 92.5 (dd, $^1J_{CP}$ = 160.2 Hz, $^1J_{CF}$ = 205.7 Hz), 168.2, 169.6 (s), 190.7 (d, $^2J_{CF}$ = 13.2 Hz); HRMS (EI, 70 eV) *m/z* [M^+] Calcd for $C_{18}H_{33}FNO_7PS$: 457.1687. Found: 457.1710.

4.1.8. *N*-(2,2-Difluoro-2-phosphonoethanethioyl)-aspartatic acid (5d) and its cyclohexylammonium salt (12). In a 25 mL flask under N_2 containing **5c** (200 mg, 0.437 mmol) and 5 mL of anhydrous acetonitrile was introduced trimethylsilyl bromide (540 μL, 4.37 mmol). The solution was refluxed for 6 h and evaporated. The residue was dissolved in 3 mL of H_2O and kept at 4 °C overnight. The crude compound **5d**, obtained by lyophilization, was placed under N_2 and dissolved with 5 mL of anhydrous Et_2O. Cyclohexylamine (250 μL, 2.185 mmol) was slowly added. The mixture was stirred for 30 min, then filtered and washed with cold Et_2O to afford the cyclohexylammonium salt **12** as a yellowish solid (158 mg, 60%). *Compound 5d*: ^1H NMR (D_2O) δ: 2.98 (d, $^3J_{HH}$ = 5.9 Hz, 1H), 2.99 (d, $^3J_{HH}$ = 5.5 Hz, 1H), 5.26 (m, 1H); ^{19}F NMR (D_2O) δ: −108.83 (dd, $^2J_{FP}$ = 93.0 Hz, $^2J_{FF}$ = 287.0 Hz, 1F), −106.98 (dd, $^2J_{FP}$ = 93.0 Hz, $^2J_{FF}$ = 287.0 Hz, 1F);

^{31}P NMR (D_2O) δ: 0.00 (t, $^2J_{PF}$ = 92.6 Hz); ^{13}C NMR (D_2O) δ: 34.4 (s), 54.4 (s), 116.0 (dt, $^1J_{CP}$ = 186.8 Hz, $^1J_{CF}$ = 267.3 Hz), 172.5, 174.2 (s), 192.5 (dt, $^2J_{CP}$ = 15.1 Hz, $^2J_{CF}$ = 24.5 Hz). *Salt 12*: ^{1}H NMR (D_2O) δ: 1.23 (m, 3H), 1.36 (m, 15H), 1.82 (br s, 6H), 2.05 (br s, 6H), 2.81 (dd, $^3J_{HH}$ = 4.6 Hz, $^2J_{HH}$ = 16.4 Hz, 1H), 2.91 (dd, $^3J_{HH}$ = 5.3 Hz, $^2J_{HH}$ = 16.4 Hz, 1H), 3.05 (br s, 3H), 3.32 (m, 1H); ^{19}F NMR (D_2O) δ: −104.77 (dd, $^2J_{FP}$ = 83.0 Hz, $^2J_{FF}$ = 291.0 Hz, 1F), −103.10 (dd, $^2J_{FP}$ = 84.0 Hz, $^2J_{FF}$ = 292.0 Hz, 1F); ^{31}P NMR (D_2O) δ: 2.84 (t, $^2J_{PF}$ = 83.1 Hz); ^{13}C NMR (D_2O) δ: 25.0, 25.5, 31.5 (s), 39.7 (s), 51.5 (s), 60.3 (s), 177.6, 179.3 (s), 186.8 (dt, $^2J_{CP}$ = 16.9 Hz, $^2J_{CF}$ = 26.0 Hz).

4.1.9. *N*-(2-Fluoro-2-phosphonoethanethioyl) aspartic acid (6d). This compound was prepared as described for compound **5d** from **6 c** (375 mg, 0.820 mmol), acetonitrile (10 mL), and trimethylsilyl bromide (1.1 mL, 8.20 mmol). Reverse phase chromatography (H_2O) furnished a mixture of two diastereomers as yellow viscous residue (47 mg, 20%). ^{19}F NMR (D_2O) δ: −189.99 (dd, $^2J_{FH}$ = 47.0 Hz, $^2J_{FP}$ = 94.0 Hz, diastereomer 1), −189.97 (dd, $^2J_{FH}$ = 47.0 Hz, $^2J_{FP}$ = 94.0 Hz, diastereomer 2).

5. Cell culture and cytotoxicity

L1210 cells were provided by the NCI, Frederick, USA. They were cultivated in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mM HEPES buffer (pH 7.4). Cytotoxicity was measured by the microculture tetrazolium assay as described.[22] Cells were continuously exposed to graded concentrations of the compounds (nine serial dilution in triplicate) for 48 h. Results were expressed as IC_{50}, the concentration which reduced by 50% the optical density of treated cells with respect to untreated controls.

6. ATCase preparation and assay

Purified ATCase was prepared and assayed as previously described. The enzymatic assay was performed at 37 °C, in the presence of 50 mM Tris–HCl, pH 8, 5 mM carbamoyl-phosphate, and 5 mM aspartate as reported.[23]

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