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Short communication

Synthesis and biological evaluation of *S*-acyl-3-thiopropyl prodrugs of *N*-phosphonoacetyl-L-aspartate (PALA)

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Abstract

The synthesis of new prodrugs of PALA characterised by the presence of *S*-acyl-3-thiopropyl, as enzyme-labile groups on the phosphonate moiety of PALA, is reported. The cytotoxic activities of PALA prodrugs were determined against human cell line (SW1573 lung carcinoma cells). A number of prodrugs bearing *S*-pivaloyl as acyl groups displayed cytotoxic activity in the same order of magnitude of PALA.

Keywords: PALA; prodrugs; *S*-acyl-3-thiopropyl; lung carcinoma; calculated log *P*

1. Introduction

For many years, PALA (*N*-phosphonoacetyl-L-aspartate) has been considered to be of important therapeutic interest. PALA [1] is a rationally designed transition state analogue of the aspartate transcarbamylase (ATCase) subunit of CAD (Carbamylphosphate, Aspartate transcarbamylase, Dihydro-orotase), a multifunctional enzyme required for de novo pyrimidine biosynthesis and cell growth [2].

The spectrum of activity of PALA is unique in that it exhibits tumour-inhibitory activity against a number of transplantable solid murine tumours such as Lewis lung carcinoma (which is often insensitive to other treatments) and B16 melanoma [1], whereas L1210 and P388 leukaemias are relatively insensitive to the drug [3].

Over the past 25 years, PALA underwent a lot of structural modifications, motivated by the results obtained during in vivo studies. In order to improve the affinity of PALA towards ATCase, the different functions of the molecule were modified alternatively or simultaneously, leading to an important variety of compounds [4,5]. However, the affinity of PALA for ATCase does not seem to be increased by modifying its structure. Thus, other strategies appear to be necessary in order to improve the activity of PALA.

The de novo pyrimidine biosynthesis takes place in the cytosol of the cell and PALA has to penetrate inside the cell to act as an inhibitor of the aspartate transcarbamylase [6].

The ionic nature of PALA (Fig. 1) is a limiting factor for its diffusion through the lipidic bilayer of the cellular membrane [3b]. The use of vectors such as liposomes was postulated to allow the penetration of PALA into the cell [7]. The use of liposomes as vectors leads to a weak encapsulation of drugs (generally around 5% [7a]) and therefore alternative prodrug approaches can be used to overcome many problems associated with bioavailability and permeability of ionic molecules such as PALA.

The prodrug strategy will temporarily mask the anionic charges by a lipophilic ester protective group to enhance the hydrophobicity of the molecule and therefore improve the cellular membrane permeation. This protective group will then be selectively cleaved inside the cell in the presence of esterases.

We have demonstrated [8] that the *S*-acyl-3-thiopropyl groups (SATP) are new enzyme-labile protective groups of phosphonate esters. Indeed, SATP can be

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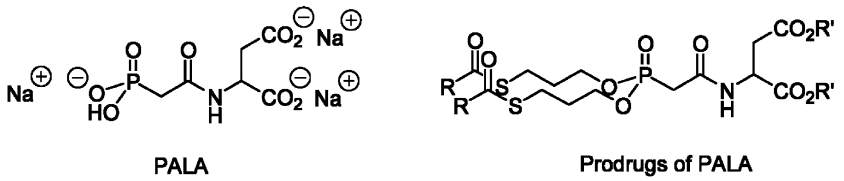

Fig. 1. PALA and its targeted prodrugs.

hydrolysed enzymatically by carboxyesterases which are more prevalent intracellularly.

In this study, we have linked the SATP group to the phosphonate moiety of PALA. One difficulty in prodrug approach is to find the fair balance between the lipophilicity necessary, for the transport through the cellular membrane and the hydrophilicity allowing the solubilisation in physiological media. The choice of the protective groups on the PALA carboxylate moieties was guided by the evaluation of the log *P* values of targeted molecules [9] (by the software ACD/LogP, ChemCAD). We chose monomethylether of polyethyleneglycol ($n = 1$–3) because the hydrophilicity of prodrugs is improved when they are linked to PEG [10]. We are presenting here the chemical synthesis of seven prodrugs of PALA as well as their cytotoxic activities against SW1573 lung carcinoma cells.

2. Chemistry

Among the several syntheses of PALA reported [1,2a,11], we designed a general synthetic procedure [12] in which the key step is an Arbuzov's reaction using the tris(trimethylsilyl)phosphite. At first, the introduction of enzyme-labile SATP groups was achieved using PALA dimethylester derivative **2** to obtain **4** bearing two *S*-pivaloyl-3-thiopropyl (^{t}BuSATP) groups on the phosphonate moiety. In this compound, the phosphonic acids were masked by an enzyme-labile ester and the carboxylic moieties of aspartic acid by methyl esters (Fig. 2).

The first step of the synthesis was the chloroacetylation of aspartate dimethylester by addition of chloroacetic anhydride [13] (Fig. 2). The phosphonylation of the resulting chloroacetyl–aspartate dimethylester **1** was then achieved according to the Arbuzov's reaction using tris(trimethylsilyl)phosphite [14] at 160 °C. The hydrolysis in a water–methanol mixture (1:1) afforded the expected phosphonic acid **2**.

The phosphonic acid **2** undergoes activation by oxalyl chloride in the presence of DMF, followed by the addition of the *S*-pivaloyl-3-thiopropanol **6**. The acylthiopropanols were obtained by radical addition of the corresponding thioacid to the allylic alcohol (Fig. 3).

The calculated log *P* value of compound **4** was 3.6 (Table 1) and the values of log *P* generally admitted to give good bioavailability are usually within the 2–3.5 range. Nevertheless, the solubilisation of compound **4** in aqueous media requires an additional 5% of DMSO. To improve the water solubility of these PALA prodrugs, we decided to introduce polyethyleneglycol monomethy-

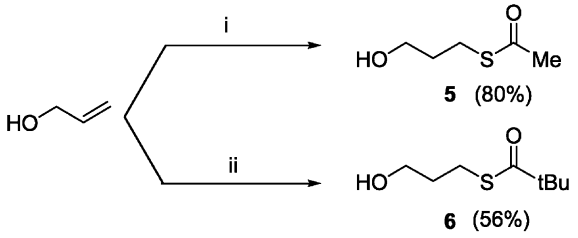

Fig. 3. Synthesis of *S*-acylthiopropanols. Reagents and conditions: (i) thioacetic acid, AIBN, benzene, reflux; (ii) thiopivaloic acid, AIBN, THF, UV: $\lambda = 254$ nm.

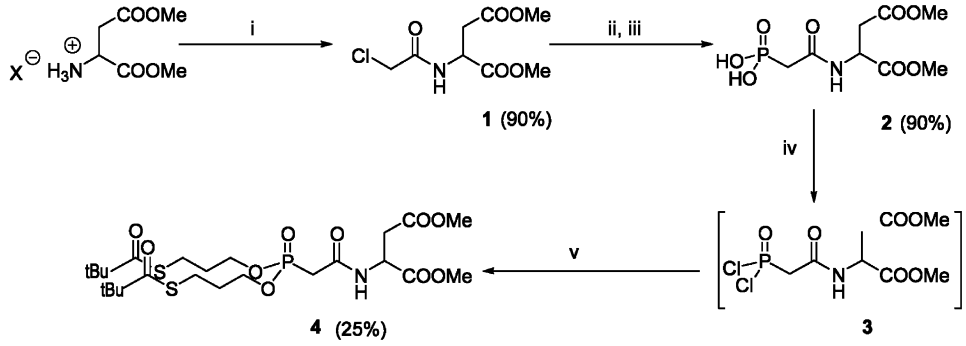

Fig. 2. Synthesis of bis-^{t}BuSATP–PALA dimethylester. Reagents and conditions: (i) $(ClCH_2CO)_2O$, CH_2Cl_2, pyridine; (ii) $P(OSiMe_3)_3$, 160 °C; (iii) H_2O–MeOH (1:1); (iv) $(ClCO)_2$, CH_2Cl_2, DMF; (v) **6**, Et_3N, CH_2Cl_2.

Table 1
Estimated log *P* values, yield and ^{31}P-NMR analysis of bis (*R*-SATP) polyethyleneglycol monomethylether *N*-phosphonoacetyl-L-aspartates **23**–**28**

Compounds	R (acyl)	*n*	Yield%	log $P_{calc} \pm 0.6$	δ ^{31}P (ppm)
23	Me	1	16	1.2	23.4
24	Me	2	53	0.4	23.5
25	Me	3	32	−0.3	23.6
26	^{t}Bu	1	25	3.6	23.4
27	^{t}Bu	2	53	2.9	23.4
28	^{t}Bu	3	27	2.2	23.6

lether groups ($n = 1$–3) on the carboxylate moiety of aspartic acid.

Attempts of esterification were conducted on *N*-chloroacetylaspartic acid (Fig. 4, path A), *N*-benzyloxycarbonyl aspartic acid (path B) or even on the aspartic acid bearing no protective groups on its nitrogen. Surprisingly (path A), the *N*-chloroacetylaspartic acid did not lead to the expected diesters **14**, **15** and **16**, while the path B via the benzyloxycarbonyl derivative **7** [15] provided the best results.

The polyethyleneglycol monomethylether groups were introduced by an esterification reaction involving DCC/DMAP [16] affording the diesters **8**, **9** and **10** in 75–80% yield.

The Cbz group was then removed by catalytic transfer hydrogenation leading in quantitative yields to **11**, **12** and **13**, respectively [17]. Chloroacetylation was then performed using chloroacetic anhydride in pyridine and the chloroacetylated compounds **14**, **15** and **16** were isolated in the range of 60–80% yields (Fig. 4).

The phosphonylation of chloroacetylated compounds **14**–**16** was performed as previously to give the bis-silylated phosphonates **17**–**19** which were not isolated (Fig. 4).

After distillation of the tris(trimethylsilyl)phosphite excess under reduced pressure, reaction of the crude mixture with oxalyl chloride in dichloromethane in the presence of catalytic amounts of DMF afforded the bis-chloro intermediates **20**–**22** [18]. The addition of acylthiopropanols **5** or **6** on the appropriate derivative **20**–**22** afforded the enzyme-labile prodrugs of PALA **23**–**28** (Fig. 5), the relative calculated lipophilicity of which is presented in Table 1.

The cytotoxic activities of the prodrugs **4**, **23**–**28** were determined using SW1573 lung carcinoma cell line. This human cell line of alveolar lung carcinoma was chosen because PALA had demonstrated a moderate inhibitory activity against human LX-1 lung carcinoma cell line [2c]. The tris-sodium salt of PALA (*N*-phosphonoacetyl-L-aspartate) was evaluated in parallel for comparison. Only the prodrugs **4**, **26**, **27** and **28** bearing *S*-pivaloyl-3-thiopropyl enzyme-labile groups on the phosphonate moiety displayed cytotoxic activity against SW1573 lung carcinoma cell line (Table 2).

The prodrugs **4**, **26**, **27**, **28**, induced the death of the cell to the same extent when compared to PALA. The introduction of *S*-pivaloyl-3-thiopropyl on the phosphonate moiety did not modify the cytotoxic activity by comparison with PALA. Nevertheless, the cytotoxic

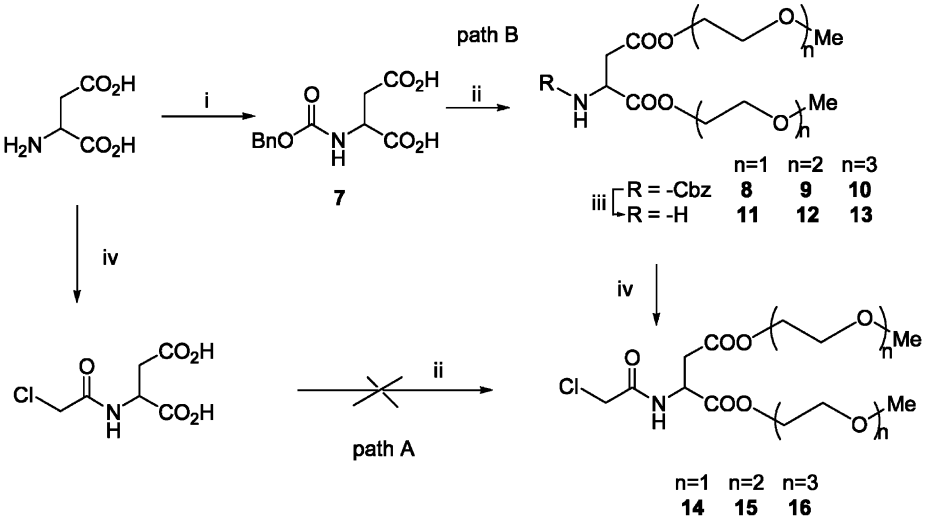

Fig. 4. Synthesis of *N*-chloroacetylaspartate (polyethyleneglycol monomethylether) diester. Reagents and conditions: (i) $ClCO_2Bn$, $NaHCO_3$; (ii) $HO(CH_2CH_2–O)_nMe$, DCC, DMAP, CH_2Cl_2; (iii) Pd/C, 1,4-cyclohexadiene, EtOH; (iv) $(ClH_2CCO)_2O$, CH_2Cl_2, pyridine.

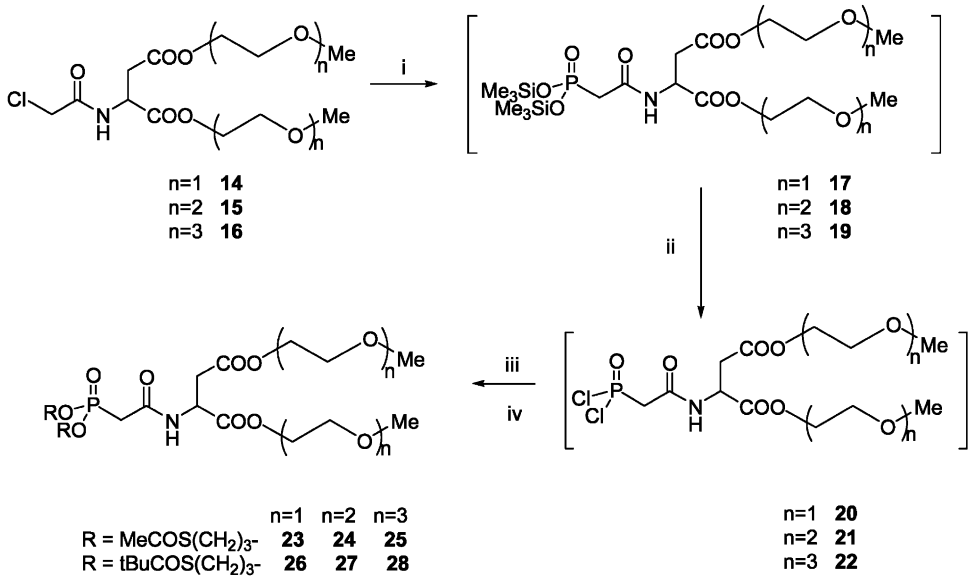

Fig. 5. Synthesis of bis (SATP) derivatives of PALA. Reagents and conditions: (i) $P(OSiMe_3)_3$, 160 °C; (ii) $(ClCO)_2$, CH_2Cl_2, DMF; (iii) **5**, Et_3N, CH_2Cl_2; (iv) **6**, Et_3N, CH_2Cl_2.

Table 2
Cytotoxic activities of PALA prodrugs against SW1573 lung carcinoma cells

Compounds	IC_{50} [b] M	log P_{calc}
4	$3.63 \times 10^{-4} \pm 5.5 \times 10^{-5}$ [a]	3.6
23	–/– [c]	1.2
24	–/–	0.4
25	–/–	−0.3
26	$3.50 \times 10^{-4} \pm 7.9 \times 10^{-5}$	3.6
27	$3.25 \times 10^{-4} \pm 2.5 \times 10^{-5}$	2.9
28	$3.50 \times 10^{-4} \pm 7.1 \times 10^{-5}$	2.2
PALA (Reference)	$2.88 \times 10^{-4} \pm 30.0 \times 10^{-5}$	$\ll 0$ [d]

[a] All data represent the average value of four separate experiments each one performed in triplicate.
[b] 50% inhibitory concentration.
[c] No inhibition observed.
[d] The software do not give fair estimated log *P* value for ionic molecule such PALA.

activity of the prodrug was lost when the *S*-pivaloyl was substituted by an *S*-acetyl in the SATP enzyme-labile group.

Several experiments confirmed the penetration of PALA into the cell through endocytosis [19]. On the other hand, prodrugs of PALA do not follow the same transport mechanism and they will use a passive transport mechanism to enter the cell [20]. The inhibitory activity of prodrugs bearing the *S*-pivaloyl group **4**, **26**–**28** could be explained by the passive transport through the cell membrane followed by an esterasic activity to release the active form of these prodrugs. The lack of activity observed for the prodrugs bearing the *S*-acetyl group **23**–**25** could be explained by a less effective passive transport due to low log *P* values, or by a quick release of the *S*-acetyl-3-thiopropyl groups into the culture medium leading to a diester by-product of PALA. Indeed, kinetic studies on nucleotidic prodrugs determined half-live in cellular extracts media to be shorter for *S*-acetyl than for *S*-pivaloyl derivatives [21].

3. Conclusion

A number of new prodrugs of PALA were synthesized and their cytotoxic activities determined against SW1573 lung carcinoma cells.

The take home message emerging from this study is the cytotoxic activity of the SATP prodrugs bearing *S*-pivaloyl as acyl groups. This cytotoxic activity against the SW1573 lung carcinoma cells is close to the PALA activity and can be explained by a passive transport of the prodrugs which release PALA inside the cells. It is noteworthy that only the prodrugs of estimated log *P* values higher than 2.5 showed a cytotoxic activity.

4. Experimental protocols

4.1. Chemistry

^1H- and ^{13}C-NMR were recorded on a Brucker apparatus DPX200, AC250 and DRX400. ^{31}P-NMR spectra were recorded on a Brucker DPX200. Mass spectra were recorded on a JEOL JMS-DX300. Log *P* was estimated by means of the software ACD/LogP calculator developed by ACD (Advanced Chemistry Development Inc.) and distributed by the company ChemCAD. Optical rotations were determined on a

Perkin–Elmer polarimeter PE 241. Microanalyses were performed in the analytical department of the CNRS (Vernaison, Rhône, France). Analyses indicated by the symbols were within $\pm 0.4\%$ of the theoretical values.

4.2. Dimethyl-*O*,*O*-bis(*S*-pivaloyl-3-thiopropyl)-*N*-phosphonoacetyl-L-aspartate (**4**)

The dimethyl *N*-phosphonoacetyl-L-aspartate (**2**) [12] (0.38 g, 1.34 mmol, 1 equiv.), was dissolved in 5 mL of dichloromethane and 10 drops of DMF were added as a catalyst. To the solution, cooled 10 min at 0 °C, oxalyl chloride (0.40 mL, 4.69 mmol, 3.5 equiv.) was added dropwise and the mixture was stirred for 3 h 30 min at room temperature. The volatile components were removed under reduced pressure and the *P*,*P*-dichloro-*N*-phosphonoacetyl-L-aspartate (**3**), obtained as a brown oil, was rapidly used without further purification. Complex **3** was dissolved in 10 mL of dichloromethane and cooled to 0 °C. A mixture of *S*-pivaloyl-3-thiopropyl alcohol (**6**) (0.48 g, 2.7 mmol, 2 equiv.) and triethylamine (1.12 mL, 8.04 mmol, 6 equiv.) in 5 mL of dichloromethane was added dropwise and the reaction was stirred for 2 h at room temperature. The mixture was diluted with 150 mL of dichloromethane and washed three times with a saturated solution of $NaHCO_3$ and twice with brine. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (ethyl acetate–methanol 90/10, 80/20) to give 0.198 g of compound **4** as yellow oil. Yield: 25%. $R_f = 0.28$ (ethyl acetate). ^{1}H-NMR $CDCl_3$/200 MHz: δ (ppm): 1.23 (s, 18H, $CH_{3\ (t\mathrm{Bu})}$); 1.95 (q^t, $^3J_{HH} = 6.0$ Hz, 4H, CH_2); 2.8–3.08 (m, 8H, CH_2S, PCH_2CO, CH_2CO_2); 3.70 and 3.76 (2s, $2 \times 3H$, $2CH_3$); 4.18 (m, 4H, $POCH_2$); 4.88 (td, $^3J_{HH} = 4.7$ Hz, $^3J_{HH} = 8.0$ Hz, 1H, $CHCO_2$); 7.44 (d, $^3J_{HH} = 8.0$ Hz, 1H, NH). ^{13}C-NMR $CDCl_3$/100 MHz: δ (ppm): 24.9 (s, CH_2S); 27.8 (s, $CH_{3(t\mathrm{Bu})}$); 30.9 (d, $^3J_{PC} = 6.3$ Hz, CH_2); 35.5 (d, $^1J_{PC} = 131.0$ Hz, PCH_2CO); 36.4 (s, CH_2CO_2); 46.9 (s, $C_{(t\mathrm{Bu})}$); 49.3 (s, $CHCO_2$); 52.5 and 53.2 (2s, $2CH_3$); 65.6 and 65.7 (2d, $^2J_{PC} = 5.6$ Hz, $POCH_2$); 164.2 (d, $^2J_{PC} = 4.5$ Hz, CONH); 171.5 and 171.6 (2s, CO_2); 206.9 (2s, COS). ^{31}P-NMR $CDCl_3$/200 MHz: δ (ppm): 24.3.

MS (Fab+/NBA): $m/z = 600$ $[M+H]^1$; 622 $[M+Na]^+$; 159 $[^t\mathrm{BuCOSCH_2CH_2CH_2}]^+$; 57 $[^t\mathrm{Bu}]^+$. Anal. $C_{24}H_{42}NO_{10}PS_2$ (C, H, N).

4.3. S-Acetyl-3-thiopropyl alcohol **5**

A solution of allyl alcohol (1.0 mL, 14.7 mmol, 1 equiv.), thioacetic acid (2.1 mL, 26.9 mmol, 2 equiv.) and AIBN (2.41 g, 14.7 mmol, 1 equiv.) in 20 mL of benzene was degazed 15 min by argon bubbling and then heated to reflux for 4 h. The mixture was cooled to room temperature, washed twice with a saturated solution of $NaHCO_3$, then twice with brine. The organic layer was dried with sodium sulphate and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (petroleum ether–ethyl acetate 70/30). 1.1 g of compound **6** was obtained as colourless oil in 56% yield. $R_f = 0.28$ (ethyl acetate–petroleum ether 50/50). ^{1}H-NMR $CDCl_3$/250 MHz: δ (ppm): 1.75 (q^t, $^3J_{HH} = 6.4$ Hz, 2H, CH_2); 2.28 (s, 3H, CH_3); 2.38 (s, 1H, OH); 2.93 (t, $^3J_{HH} = 6.8$ Hz, 2H, CH_2S); 3.57 (t, $^3J_{HH} = 5.7$ Hz, 2H, CH_2OH). ^{13}C-NMR $CDCl_3$/100 MHz: δ (ppm): 25.9 (s, CH_2S); 30.9 (s, CH_3); 32.8 (s, CH_2); 60.8 (s, CH_2OH); 197.8 (s, CO).

MS (Fab+/NBA): $m/z = 117$ $[CH_3COSCH_2CH_2CH_2]^+$; 43 $[CH_3CO]^+$.

4.4. S-Pivaloyl-3-thiopropyl alcohol **6**

In a quartz reactor, a solution of allyl alcohol (1.4 mL, 37.8 mmol, 1.8 equiv.), and thiopivaloic acid (4.2 mL, 37.8 mmol, 1.8 equiv.) in the presence of a catalytic amount of AIBN (50 mg) in 150 mL of THF was degazed for 15 min by argon bubbling. The solution was submitted to UV irradiation at $\lambda = 254$ nm during 3 h under inert atmosphere at room temperature. Then the mixture was diluted with 200 mL of ethyl acetate, washed twice with a saturated solution of $NaHCO_3$, then twice with brine. The organic layer was dried with sodium sulphate and concentrated under reduced pressure. The residual oil was purified by silica gel column chromatography (petroleum ether–ethyl acetate 70/30) to give 2.9 g of compound **6** as colourless oil in 80% yield. $R_f = 0.57$. ^{1}H-NMR $CDCl_3$/200 MHz: δ (ppm): 1.22 (s, 9H, CH_3); 1.79 (tt, $^3J_{HH} = 6.8$ Hz, $^3J_{HH} = 5.9$ Hz, 2H, CH_2); 2.68 (s, 1H, OH); 2.96 (t, $^3J_{HH} = 6.8$ Hz, 2H, CH_2S); 3.61 (t, $^3J_{HH} = 5.9$ Hz, 2H, CH_2OH). ^{13}C-NMR $CDCl_3$/100 MHz: δ (ppm): 25.2 (s, CH_2S); 27.8 (s, CH_3); 32.9 (s, CH_2); 46.9 (s, $C_{(t\mathrm{Bu})}$); 60.8 (s, CH_2OH); 208.9 (s, CO). MS (Fab+/NBA): $m/z = 199$ $[M+Na]^+$; 177 $[M+H]^+$; 85 $[^t\mathrm{BuCO}]^+$; 57 $[^t\mathrm{Bu}]^+$.

4.5. Bis (polyethyleneglycol monomethylether)-*N*-benzyloxycarbonyl-L-aspartate **8**–**10**

A solution of *N*-benzyloxycarbonyl-L-aspartic acid **7** (5 mmol, 1 equiv.) and polyethyleneglycol monomethylether (10 mmol, 2 equiv.) in 8 mL of dichloromethane was cooled for 10 min at 0 °C. Then DCC (16 mmol, 3.2 equiv.) dissolved in 25 mL of dichloromethane was added dropwise, followed by catalytic amount of DMAP. The mixture was stirred overnight at room temperature and the DCU precipitate was removed by filtration. The filtrate was washed twice with a 5% solution of $NaHCO_3$ then twice with 1 M solution of $KHSO_4$. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The crude product

was purified by silica gel column chromatography to give compounds **8**–**10** as colourless oil.

4.5.1. Bis-(2-methoxy-ethyl)-N-benzyloxycarbonyl-L-aspartate (**8**)

Yield: 75%. $R_f = 0.40$ (ethyl acetate). ^{1}H-NMR $CDCl_3$/200 MHz: δ (ppm): 3.01 (AB part of an ABX system, $\delta_A = 3.10$, $\delta_B = 2.91$, $^1J_{AB} = 17.3$ Hz, $^3J_{AX} = {}^3J_{BX} = 4.4$ Hz, 2H, CH_2CO_2); 3.34 and 3.35 (2s, 2×3H, CH_3O); 3.56 (m, 4H, $CH_2O_{(2,2')}$); 4.22 and 4.30 (2m, 2×2H, $CO_2CH_{2(1,1')}$); 4.67 (td, $^3J_{HH} = 4.4$ Hz, $^3J_{HH} = 8.8$ Hz, 1H, $CHCO_2$); 5.11 (s, 2H, CH_2Ph); 5.89 (d, $^3J_{HH} = 8.6$ Hz, 1H, NH); 7.34 (m, 5H, Ph). ^{13}C-NMR $CDCl_3$/100 MHz: δ (ppm): 36.8 (s, CH_2CO_2); 49.6 (s, $CHCO_2$); 59.3 (s, CH_3O); 64.3 and 65.1 (2s, $CO_2CH_{2(1,1')}$); 67.5 (s, CH_2Ph); 70.6 (2s, $CH_2O_{(2,2')}$); 128.5–128.9 (4s, C_2^{φ}, C_3^{φ}, C_4^{φ}); 136.6 (s, C_1^{φ}); 156.3 (s, CONH); 170.5 and 170.9 (2s, CO_2).

MS (Fab+/NBA): $m/z = 384$ $[M+H]^+$.

4.5.2. Bis-12-(2-methoxy-ethoxy)-ethyl]-N-benzyloxycarbonyl-L-aspartate (**9**)

Yield: 75%. $R_f = 0.40$ (ethyl acetate). ^{1}H-NMR $CDCl_3$/400 MHz: δ (ppm): 3.00 (AB part of an ABX system, $\delta_A = 3.08$, $\delta_B = 2.92$, $^1J_{AB} = 17.1$ Hz, $^3J_{AX} = 4.7$ Hz, $^3J_{BX} = 4.6$ Hz, 2H, CH_2CO_2); 3.36 and 3.38 (2s, 2×3H, CH_3O); 3.53 (m, 4H, $CH_2O_{(5,5')}$); 3.62 (m, 4H, $CH_2O_{(4,4')}$); 3.69 (m, 4H, $CH_2O_{(2,2')}$); 4.25 and 4.33 (2m, 2×2H, $CO_2CH_{2(1,1')}$); 4.68 (td, $^3J_{HH} = 4.6$ Hz, $^3J_{HH} = 8.5$ Hz, 1H, $CHCO_2$); 5.12 (s, 2H, CH_2Ph); 5.94 (d, $^3J_{HH} = 8.5$ Hz, 1H, NH); 7.34 (m, 5H, Ph).

^{13}C-NMR $CDCl_3$/100 MHz: δ (ppm): 37.1 (s, CH_2CO_2); 50.9 (s, $CHCO_2$); 59.4 (s, CH_3O); 64.4 and 65.2 (2s, $CO_2CH_{2(1,1')}$); 67.5 (s, CH_2Ph); 69.2 (2s, $CH_2O_{(2,2')}$); 70.8 and 70.9 (2s, $CH_2O_{(4,4')}$); 72.2 (2s, $CH_2O_{(5,5')}$); 128.5–128.9 (4s, C_2^{φ}, C_3^{φ}, C_4^{φ}); 136.6 (s, C_1^{φ}); 156.4 (s, CONH); 170.9 and 171.0 (2s, CO_2).

MS (Fab+/NBA): $m/z = 472$ $[M+H]^+$.

4.5.3. Bis 2-[2-(2-methoxy-ethoxy)-ethoxy]-ethyl-N-benzyloxycarbonyl-L-aspartate (**10**)

Yield: 80%. $R_f = 0.34$ (ethyl acetate). ^{1}H-NMR $CDCl_3$/400 MHz: δ (ppm): 2.97 (AB part of an ABX system, $\delta_A = 3.14$, $\delta_B = 2.80$, $^1J_{AB} = 17.1$ Hz, $^3J_{AX} = {}^3J_{BX} = 4.9$ Hz, 2H, CH_2CO_2); 3.35 (s, 6H, CH_3O); 3.52 (m, 4H, $CH_2O_{(8,8')}$); 3.63 (m, 16H, $CH_2O_{(2-7,2'-7')}$); 4.21 and 4.29 (2m, 2×2H, CO_2CH_2); 4.65 (td, $^3J_{HH} = 4.6$ Hz, $^3J_{HH} = 8.6$ Hz, 1H, $CHCO_2$); 5.10 (s, 2H, CH_2Ph); 5.91 (d, $^3J_{HH} = 8.8$ Hz, 1H, NH); 7.33 (m, 5H, Ph).

^{13}C-NMR $CDCl_3$/100 MHz: δ (ppm): 36.5 (s, CH_2CO_2); 49.3 (s, $CHCO_2$); 59.4 (s, CH_3O); 64.5 and 65.1 (2 s, $CO_2CH_{2(1,1')}$); 67.5 (s, CH_2Ph); 69.1 (2s, $CH_2O_{(2,2')}$); 71.0 (4s, $CH_2O_{(4-7,4'7')}$); 72.3 (s, $CH_2O_{(8,8')}$); 128.5–128.9 (4s, C_2^{φ}, C_3^{φ}, C_4^{φ}); 136.6 (s, C_1^{φ}); 156.4 (s, CONH); 170.2 and 171.3 (2s, CO_2). MS (Fab+/NBA): $m/z = 560$ $[M+H]^+$.

4.6. Bis(polyethyleneglycol monomethylether)-N-chloroacetyl-L-aspartate **14**–**16**

To a solution of compounds **8**–**10** (3 mmol, 1 equiv.) in 7 mL of ethyl alcohol, were successively added Pd/C 10% (w:w; 1:1) and cyclohexa-1,4-diene (30 mmol, 10 equiv.), stirring was continued for 1 h at room temperature. After filtration, the filtrate was concentrated under reduced pressure. Compounds **11**–**13**, obtained as pale yellow oil in quantitative yield, were used without further purification. Pyridine (18 mmol, 6 equiv.) was added to a solution of **11**–**13** (3 mmol, 1 equiv.) in 20 mL of dichloromethane. Then chloroacetic anhydride (6 mmol, 2 equiv.) was added to the mixture cooled at 0 °C. The mixture was stirred for 1 h at room temperature and then washed three times with cold water. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The crude oil was purified by silica gel column chromatography to give compounds **14**–**16** as pale yellow oil.

4.6.1. Bis-(2-methoxy-ethyl)-N-chloroacetyl-L-aspartate (**14**)

Yield: 64%. $R_f = 0.55$ (ethyl acetate). ^{1}H-NMR $CDCl_3$/200 MHz: δ (ppm): 3.00 (AB part of an ABX system, $\delta_A = 3.09$, $\delta_B = 2.91$, $^1J_{AB} = 17.2$ Hz, $^3J_{AX} = {}^3J_{BX} = 4.6$ Hz, 2H, CH_2CO_2); 3.29 and 3.31 (2s, 6H, CH_3O); 3.52 (m, 4H, $CH_2O_{(2,2')}$); 4.06 (s, 2H, CH_2Cl); 4.18 and 4.23 (2m, 2H, $CO_2CH_{2(1,1')}$); 4.84 (td, $^3J_{HH} = 4.6$ Hz, $^3J_{HH} = 7.8$ Hz, 1H, $CHCO_2$); 7.52 (d, $^3J_{HH} = 7.9$ Hz, NH). ^{13}C-NMR $CDCl_3$/100 MHz: δ (ppm): 36.4 (s, CH_2CO_2); 42.6 (s, CH_2Cl); 49.4 (s, $CHCO_2$); 59.3 (s, CH_3O); 64.4 and 65.1 (2s, $CO_2CH_{2(1,1')}$); 70.4 (2s, $CH_2O_{(2,2')}$); 164.7 (s, CONH); 170.5 and 170.9 (2s, CO_2). MS (Fab+/NBA): $m/z = 326$ $[M+H]^+$.

4.6.2. Bis-[2-(2-methoxy-ethoxy)-ethyl]-N-chloroacetyl-L-aspartate (**15**)

Yield: 70%. $R_f = 0.27$ (ethyl acetate). ^{1}H-NMR $CDCl_3$/200 MHz: δ (ppm): 3.00 (AB part of an ABX system, $\delta_A = 3.09$, $\delta_B = 2.91$, $^1J_{AB} = 17.3$ Hz, $^3J_{AX} = {}^3J_{BX} = 4.6$ Hz, 2H, CH_2CO_2); 3.37 (s, 6H, CH_3O); 3.53 (m, 4H, $CH_2O_{(5,5')}$); 3.63 (m, 4H, $CH_2O_{(4,4')}$); 3.68 (m, 4H, $CH_2O_{(2,2')}$); 4.06 (s, 2H, $ClCH_2$); 4.25 and 4.37 (2m, 2×2H, $CO_2CH_{2(1,1')}$); 4.85 (td, $^3J_{HH} = 4.6$ Hz, $^3J_{HH} = 8.1$ Hz, 1H, $CHCO_2$); 7.55 (d, $^3J_{HH} = 8.0$ Hz, NH). ^{13}C-NMR $CDCl_3$/100 MHz: δ (ppm): 36.6 (s, CH_2CO_2); 42.7 (s, $ClCH_2$); 49.4 (s, $CHCO_2$); 59.4 (s, CH_3O); 64.4 and 65.1 (2s, $CO_2CH_{2(1,1')}$); 69.2 (2s, $CH_2O_{(2,2')}$); 70.8 (2s, $CH_2O_{(4,4')}$); 72.2 (2s, $CH_2O_{(5,5')}$); 164.2 (s, CONH);

170.3 and 171.5 (2s, CO_2). MS (Fab+/NBA): $m/z = 414$ $[M+H]^+$.

4.6.3. Bis 2-[2-(2-methoxy-ethoxy)-ethoxy]-ethyl-N-chloroacetyl-L-aspartate (16)

Yield: 80%. $R_f = 0.70$ (ethyl acetate–methanol 90/10). ^{1}H-NMR $CDCl_3$/400 MHz: δ (ppm): 3.01 (AB part of an ABX system, $\delta_A = 3.09$, $\delta_B = 2.93$, $^1J_{AB} = 17.2$ Hz, $^3J_{AX} = 4.7$, $^3J_{BX} = 4.6$ Hz, 2H, CH_2CO_2); 3.37 (s, 6H, CH_3O); 3.55 (m, 4H, $CH_2O_{(8,8')}$); 3.65 (m, 12H, $CH_2O_{(4-7,4'-7')}$); 3.70 (m, 4H, $CH_2O_{(2,2')}$); 4.08 (s, 2H, $ClCH_2$); 4.22–4.38 (m, 4H, $CO_2CH_{2(1,1')}$); 4.88 (td, $^3J_{HH} = 4.6$ Hz, $^3J_{HH} = 8.1$ Hz, 1H, $CHCO_2$); 7.62 (d, $^3J_{HH} = 8.0$ Hz, NH). ^{13}C-NMR $CDCl_3$/100 MHz: δ (ppm): 36.4 (s, CH_2CO_2); 42.7 (s, $ClCH_2$); 49.3 (s, $CHCO_2$); 59.4 (s, CH_3O); 64.5 and 65.2 (2s, $CO_2CH_{2(1,1')}$); 69.1 and 69.2 (2s, $CH_2O_{(2,2')}$); 70.9 (4s, $CH_2O_{(4-7,4'-7')}$); 72.3 (s, $CH_2O_{(8,8')}$); 166.4 (s, CONH); 170.3 and 171.5 (2s, CO_2). MS (Fab+/NBA): $m/z = 502$ $[M+H]^+$.

4.7. Bis(polyethyleneglycol monomethylether)-O,O-bis-(S-acyl-3-thiopropyl)-N-phosphonoacetyl-L-aspartate 23–28

In a round bottom flask fitted with a distillation system, a mixture of chloroacetyl-L-aspartate **14**–**16** (2.0 mmol, 1 equiv.) and tris(trimethylsilyl)phosphite (3.6 mmol, 1.8 equiv.) was heated at 160 °C. 30 min later the formed trimethylsilylchloride began to distil. After 2 h, the mixture was allowed to cool at 60 °C and the excess of tris(trimethylsilyl)phosphite was removed under reduced pressure. Compounds **17**–**19** were obtained as pale yellow oil and were used without further purification. Derivatives **17**–**19** ($\sim$2 mmol, 1 equiv.) were dissolved in 7 mL of dichloromethane and 10 drops of DMF were added as a catalyst. To the solution, cooled for 10 min at 0 °C, 0.590 mL of oxalyl chloride (7.0 mmol, 3.5 equiv.) was added dropwise and the mixture was stirred for 3 h 30 min at room temperature. The volatile components were removed under reduced pressure and the *P*,*P*-dichloro-*N*-phosphonoacetyl-L-aspartate **20**–**22**, obtained as a brown oil, was rapidly used without further purification. Compounds **20**–**22** ($\sim$2 mmol, 1 equiv.) was dissolved in 10 mL of dichloromethane and cooled in an ice bath. A mixture of *S*-acyl-3-thiopropyl alcohol **5** or **6** (6.0 mmol, 3 equiv.) and triethylamine (12.0 mmol, 6 equiv.) in 10 mL of dichloromethane was added dropwise and the reaction was stirred for 2 h at room temperature. The mixture was diluted in 150 mL of dichloromethane and washed three times with brine. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography to give compounds **23**–**28** as brown oil.

4.7.1. Bis-(2-methoxy-ethyl)-O,O-bis-(S-acetyl-3-thiopropyl)-N-phosphonoacetyl-L-aspartate (23)

Yield: 16%. $R_f = 0.50$ (ethyl acetate–methanol 90/10). ^{1}H-NMR $CDCl_3$/200 MHz: δ (ppm): 1.90 (q^{t}d, $^3J_{HH} = 6.9$ Hz, $^4J_{HP} = 2.7$ Hz, 4H, CH_2); 2.27 (s, 6H, CH_3CO); 2.82–3.03 (m, 8H, CH_2CO_2, PCH_2CO, CH_2S); 3.29 and 3.31 (2s, 6H, CH_3O); 3.52 (m, 4H, $CH_2O_{(2,2')}$); 4.09 (m, 4H, $POCH_2$); 4.17 and 4.23 (2m, 2×2H, $CO_2CH_{2(1,1')}$); 4.82 (td, $^3J_{HH} = 4.6$ Hz, $^3J_{HH} = 8.0$ Hz, 1H, $CHCO_2$); 7.37 (d, $^3J_{HH} = 7.9$ Hz, NH). ^{13}C-NMR $CDCl_3$/100 MHz: δ (ppm): 25.6 (s, CH_2S); 30.8 (d, $^3J_{PC} = 6.3$ Hz, CH_2); 31.0 (s, CH_3CO); 35.4 (d, $^1J_{PC} = 131.5$ Hz, PCH_2CO); 36.6 (s, CH_2CO_2); 49.4 (s, $CHCO_2$); 59.3 (s, CH_3O); 64.3 and 65.1 (2s, $CO_2CH_{2(1,1')}$); 65.4 and 65.5 (2d, $^2J_{PC} = 6.1$ Hz, $POCH_2$); 70.5 (2s, $CH_2O_{(2,2')}$); 164.2 (d, $^2J_{PC} = 4.6$ Hz, CONH); 170.5 and 170.9 (2s, CO_2); 196.0 (2s, COS). ^{31}P-NMR $CDCl_3$/250 MHz: δ (ppm): 23.4. MS (Fab+/NBA): $m/z = 604$ $[M+H]^+$, 117 $[MeCOSCH_2CH_2CH_2]^+$.

$[\alpha]_D = +20.4°$ ($c = 0.2$; $CHCl_3$). Anal. $C_{22}H_{38}NO_{12}PS_2$ (C, H, N).

4.7.2. Bis-[2-(2-methoxy-ethoxy)-ethyl]-O,O-bis(S-acetyl-3-thiopropyl)-N-phosphonoacetyl-L-aspartate (24)

Yield: 53%. $R_f = 0.34$ (ethyl acetate–methanol 95/5). ^{1}H-NMR $CDCl_3$/200 MHz: δ (ppm): 1.94 (q^{t}d, $^3J_{HH} = 6.6$ Hz, $^4J_{HP} = 2.6$ Hz, 4H, CH_2); 2.31 (s, 6H, CH_3CO); 2.86–3.05 (m, 8H, CH_2CO_2, PCH_2CO, CH_2S); 3.36 (2s, 6H, CH_3O); 3.56 (m, 4H, $CH_2O_{(5,5')}$); 3.61 (m, 4H, $CH_2O_{(4,4')}$); 3.67 (m, 4H, $CH_2O_{(2,2')}$); 4.13 (m, 4H, $POCH_2$); 4.23 and 4.30 (2m, 22H, $CO_2CH_{2(1,1')}$); 4.85 (td, $^3J_{HH} = 4.8$ Hz, $^3J_{HH} = 7.9$ Hz, 1H, $CHCO_2$); 7.43 (d, $^3J_{HH} = 7.4$ Hz, NH). ^{13}C-NMR $CDCl_3$/100 MHz: δ (ppm): 25.6 (s, CH_2S); 30.8 (d, $^3J_{PC} = 6.4$ Hz, CH_2); 31.0 (s, CH_3CO); 35.4 (d, $^1J_{PC} = 131.7$ Hz, PCH_2CO); 36.6 (s, CH_2CO_2); 49.4 (s, $CHCO_2$); 59.4 (s, CH_3O); 64.4 and 65.1 (2s, $CO_2CH_{2(1,1')}$); 65.4 and 65.5 (2d, $^2J_{PC} = 5.9$ Hz, $POCH_2$); 69.2 (2s, $CH_2O_{(2,2')}$); 70.8 (2s, $CH_2O_{(4,4')}$); 72.2 (2s, $CH_2O_{(5,5')}$); 164.3 (d, $^2J_{PC} = 4.3$ Hz, CONH); 170.5 and 170.9 (2s, CO_2); 196.0 (2s, COS). ^{31}P-NMR $CDCl_3$/250 MHz: δ (ppm): 23.5. MS (Fab+/NBA): $m/z = 692$ $[M+H]^+$. $[\alpha]_D = +15.2°$ ($c = 0.2$; $CHCl_3$). Anal. $C_{26}H_{46}NO_{14}PS_2$ (C, H, N).

4.7.3. Bis 2-[2-(2-methoxy-ethoxy)-ethoxy]-ethyl-O,O-bis(S-acetyl-3-thiopropyl)-N-phosphonoacetyl-L-aspartate (25)

Yield: 32%. $R_f = 0.17$ (ethyl acetate–methanol 90/10). ^{1}H-NMR $CDCl_3$/400 MHz: δ (ppm): 1.90 (q^t, $^3J_{HH} = 6.6$ Hz, 4H, CH_2); 2.27 (s, 6H, CH_3CO); 2.80–3.00 (m, 8H, CH_2CO_2, PCH_2CO, CH_2S); 3.31 (s, 6H, CH_3O); 3.48 (m, 4H, $CH_2O_{(8,8')}$); 3.57 (m, 12H, $CH_2O_{(4-7,4'-7')}$); 3.62 (m, 4H, $CH_2O_{(2,2')}$); 4.09 (m, 4H, $POCH_2$); 4.17 and 4.24 (2m, 2×2H, $CO_2CH_{2(1,1')}$); 4.81 (td, $^3J_{HH} = 4.8$ Hz, $^3J_{HH} = 7.9$ Hz, 1H, $CHCO_2$); 7.47 (d, $^3J_{HH} = 7.9$

Hz, NH). ^{13}C-NMR $CDCl_3$/100 MHz: δ (ppm): 25.6 (s, CH_2S); 30.8 (d, $^3J_{PC} = 6.4$ Hz, CH_2); 31.0 (s, CH_3CO); 35.7 (d, $^1J_{PC} = 132.7$ Hz, PCH_2CO); 36.7 (s, CH_2CO_2); 49.4 (s, $CHCO_2$); 59.4 (s, CH_3O); 64.5 and 65.2 (2s, $CO_2CH_{2(1,1')}$); 65.4 (d, $^2J_{PC} = 4.1$ Hz, $POCH_2$); 65.5 (d, $^2J_{PC} = 4.4$ Hz, $POCH_2$); 69.2 (2s, $CH_2O_{(2,2')}$); 70.9 (3s, $CH_2O_{(4-7,4'-7')}$); 72.3 (s, $CH_2O_{(8,8')}$); 164.4 (d, $^2J_{PC} = 4.8$ Hz, CONH); 170.5 and 170.9 (2s, CO_2); 196.0 (2s, COS). ^{31}P-NMR $CDCl_3$/250 MHz: δ (ppm): 23.6. MS (Fab+/NBA): $m/z = 780$ $[M+H]^+$, 117 $[MeCOSCH_2CH_2CH_2]^+$, 43 $[MeCO]^+$. $[\alpha]_D = +17.5^\circ$ ($c = 0.2$; $CHCl_3$). Anal. $C_{30}H_{54}NO_{16}PS_2$ (C, H, N).

4.7.4. Bis-(2-methoxy-ethyl)-O,O-bis(S-pivaloyl-3-thiopropyl)-N-phosphonoacetyl-L-aspartate (**26**)

Yield: 25%. $R_f = 0.33$ (ethyl acetate–methanol 95/5). ^{1}H-NMR $CDCl_3$/200 MHz: δ (ppm): 1.16 (s, 18H, $CH_{3(t\,Bu)}$); 1.87 (q^td, $^3J_{HH} = 6.9$ Hz, $^4J_{HP} = 2.5$ Hz, 4H, CH_2); 2.80–3.02 (m, 8H, CH_2CO_2, PCH_2CO, CH_2S); 3.29 and 3.31 (2s, 6H, CH_3O); 3.52 (m, 4H, $CH_2O_{(2,2')}$); 4.08 (m, 4H, $POCH_2$); 4.17 and 4.23 (2m, 22H, $CO_2CH_{2(1,1')}$); 4.82 (td, $^3J_{HH} = 4.6$ Hz, $^3J_{HH} = 8.0$ Hz, 1H, $CHCO_2$); 7.38 (d, $^3J_{HH} = 8.0$ Hz, NH). ^{13}C-NMR $CDCl_3$/100 MHz: δ (ppm): 25.0 (s, CH_2S); 27.8 (s, $CH_{3(t\,Bu)}$); 30.9 (d, $^3J_{PC} = 6.2$ Hz, CH_2); 35.5 (d, $^1J_{PC} =$ 131.5 Hz, PCH_2CO); 36.6 (s, CH_2CO_2); 46.8 (s, $C_{(t\,Bu)}$); 49.4 (s, $CHCO_2$); 59.3 (s, CH_3O); 64.3 (s, $CO_2CH_{2(1')}$); 65.1 (s, $CO_2CH_{2(1)}$); 65.6 and 65.7 (2d, $^2J_{PC} = 6.3$ Hz, $POCH_2$); 70.4 (2s, $CH_2O_{(2,2')}$); 164.2 (d, $^2J_{PC} = 4.2$ Hz, CONH); 170.5 and 170.9 (2s, CO_2); 206.9 (2s, COS). ^{31}P-NMR $CDCl_3$/250 MHz: δ (ppm): 23.4. MS (Fab+/NBA): $m/z = 688$ $[M+H]^+$, 159 $[^t BuCOSCH_2CH_2CH_2]^+$, 85 $[^t BuCO]^+$. $[\alpha]_D = +19.5^\circ$ ($c = 0.2$; $CHCl_3$). Anal. $C_{28}H_{50}NO_{12}PS_2$ (C, H, N).

4.7.5. Bis-[2-(2-methoxy-ethoxy)-ethyl]-O,O-bis(S-pivaloyl-3-thiopropyl)-N-phosphonoacetyl-L-aspartate (**27**)

Yield: 53%. $R_f = 0.30$ (ethyl acetate–methanol 95/5). ^{1}H-NMR $CDCl_3$/200 MHz: δ (ppm): 1.16 (s, 18H, $CH_{3(t\,Bu)}$); 1.87 (q^td, $^3J_{HH} = 6.6$ Hz, $^4J_{HP} = 2.7$ Hz, 4H, CH_2); 2.80–3.00 (m, 8H, CH_2CO_2, PCH_2CO, CH_2S); 3.31 and 3.32 (2s, 6H, CH_3O); 3.47 (m, 4H, $CH_2O_{(5,5')}$); 3.56 (m, 4H, $CH_2O_{(4,4')}$); 3.62 (m, 4H, $CH_2O_{(2,2')}$); 4.08 (m, 4H, $POCH_2$); 4.18 and 4.27 (2m, 2×2H, $CO_2CH_{2(1,1')}$); 4.80 (td, $^3J_{HH} = 4.7$ Hz, $^3J_{HH} = 7.9$ Hz, 1H, $CHCO_2$); 7.38 (d, $^3J_{HH} = 7.9$ Hz, NH). ^{13}C-NMR $CDCl_3$/100 MHz: δ (ppm): 25.0 (s, CH_2S); 28.8 (s, $CH_{3(t\,Bu)}$); 30.9 (d, $^3J_{PC} = 6.3$ Hz, CH_2); 35.4 (d, $^1J_{PC} =$ 132.3 Hz, PCH_2CO); 36.6 (s, CH_2CO_2); 46.8 (s, $C_{(t\,Bu)}$); 49.4 (s, $CHCO_2$); 59.4 (2s, CH_3O); 64.4 and 65.1 (2s, $CO_2CH_{2(1,1')}$); 65.6 (2d, $^2J_{PC} = 6.1$ Hz, $POCH_2$); 69.2 and 69.3 (2s, $CH_2O_{(2,2')}$); 70.8 and 70.9 (2s, $CH_2O_{(4,4')}$); 72.2 (2s, $CH_2O_{(5,5')}$); 164.3 (d, $^2J_{PC} = 4.6$ Hz, CONH); 170.5 and 170.9 (2s, CO_2); 206.9 (s, COS). ^{31}P-NMR $CDCl_3$/250 MHz: δ (ppm): 23.4. MS (Fab+/NBA): $m/z = 776$ $[M+H]^+$. $[\alpha]_D = +17.6^\circ$ ($c = 0.2$; $CHCl_3$). Anal. $C_{32}H_{58}NO_{14}PS_2$ (C, H, N).

4.7.6. Bis-[2-(2-methoxy-ethoxy)-ethyl]-O,O-bis(S-pivaloyl-3-thiopropyl)-N-phosphonoacetyl-L-aspartate (**28**)

Yield: 27%. $R_f = 0.45$ (ethyl acetate–methanol 90/10). ^{1}H-NMR $CDCl_3$/400 MHz: δ (ppm): 1.16 (s, 18H, $CH_{3(t\,Bu)}$); 1.86 (q^td, $^3J_{HH} = 6.6$ Hz, 4H, CH_2); 2.82–2.95 (m, 8H, CH_2CO_2, PCH_2CO, CH_2S); 3.31 (s, 6H, CH_3O); 3.49 (m, 4H, $CH_2O_{(8,8')}$); 3.57 (m, 12H, $CH_2O_{(4-7,4'-7')}$); 3.62 (m, 4H, $CH_2O_{(2,2')}$); 4.09 (q, $^3J_{HH} = {}^3J_{HP} = 7.1$ Hz, $POCH_2$); 4.17 and 4.24 (2m, 2×2H, $CO_2CH_{2(1,1')}$); 4.80 (td, $^3J_{HH} = 4.9$ Hz, $^3J_{HH} =$ 8.2 Hz, 1H, $CHCO_2$); 7.45 (d, $^3J_{HH} = 7.9$ Hz, NH). ^{13}C-NMR $CDCl_3$/100 MHz: δ (ppm): 25.0 (s, CH_2S); 27.8 (s, $CH_{3(t\,Bu)}$); 30.9 (d, $^3J_{PC} = 6.1$ Hz, CH_2); 35.5 (d, $^1J_{PC} = 132.7$ Hz, PCH_2CO); 36.6 (s, CH_2CO_2); 46.8 (s, $C_{(t\,Bu)}$); 49.4 (s, $CHCO_2$); 59.4 (2s, CH_3O); 64.5 and 65.2 (2s, $CO_2CH_{2(1,1')}$); 65.7 (2d, $^2J_{PC} = 4.1$ Hz, $POCH_2$); 69.2 (2s, $CH_2O_{(2,2')}$); 70.9 and 70.9 (2s, $CH_2O_{(4-7,4'-7')}$); 72.3 (2s, $CH_2O_{(8,8')}$); 164.4 (d, $^2J_{PC} = 4.7$ Hz, CONH); 170.5 and 170.9 (2s, CO_2); 208.0 (s, COS). ^{31}P-NMR $CDCl_3$/250 MHz: δ (ppm): 23.6. MS (Fab+/NBA): $m/z = 864$ $[M+H]^+$, 159 $[tBuCOSCH_2CH_2CH_2]^+$. $[\alpha]_D = +18.1^\circ$ ($c = 0.2$; $CHCl_3$). Anal. $C_{36}H_{66}NO_{16}PS_2$ (C, H, N).

5. Biological studies

5.1. Cell culture

SW1573 lung carcinoma cell line [22] (obtained from ATCC) was maintained in Dubelcco's modified Eagle medium supplemented with 2 mM glutamine, 10% fetal calf serum and gentamycin at 37 °C in 5% CO_2 humidified atmosphere for one week. After trypsination cells were seeded at 2×10^4 cells/well in 96 wells plate and maintained in medium for24 h.

5.2. Cytotoxicity assay

Cells were treated with PALA or derivatives at various concentrations for 48 h. Medium was replaced by the one containing neutral red for 4 h. After two washes in phosphate buffered saline (PBS), cells were lysed in 50% ethanol, 1% acetic acid solution and incubated for 15 min at room temperature. Absorbance at 540 nm was determined and used for the measurement of the proportion of surviving cells. The data represent the average value of four separate experiments each one performed in triplicate.

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