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Bioorganic & Medicinal Chemistry Letters 17 (2007) 2086–2090
Bioorganic & Medicinal Chemistry Letters

Design, synthesis, and bioactivity of novel inhibitors of *E. coli* aspartate transcarbamoylase

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Received 30 October 2006; revised 12 December 2006; accepted 13 December 2006
Available online 21 December 2006

Abstract—A series of inhibitors of the aspartate transcarbamoylase, an enzyme involved in pyrimidine nucleotide biosynthesis, has been synthesized. These inhibitors are analogues of a highly potent inhibitor of this enzyme, *N*-phosphonacetyl-L-aspartate (PALA). Analogues have been synthesized with modifications at the α- and β-carboxylates as well as at the aspartate moiety. The ability of these compounds to inhibit the enzyme was evaluated. These studies, with functional group modified PALA derivatives, showed that amide groups can be a useful substitute of the carboxylate in order to reduce the charge on the molecule, and indicate that the relative position of the functional group in the β-position is more critical than the nature of the functional group. Some of the molecules synthesized here are potent inhibitors of the enzyme.

In mammals aspartate transcarbamoylase (ATCase) is a portion of a multifunctional enzyme (CAD)[1] which is required for de novo pyrimidine nucleotide biosynthesis. The ATCase portion of CAD catalyzes the second step in pyrimidine nucleotide biosynthesis, the reaction between carbamoyl phosphate and L-aspartate to give *N*-carbamoyl-L-aspartate and inorganic phosphate.[2] ATCase has become a target for the development of anti-proliferative drugs and inhibitors of ATCase are considered as potential anti-tumor agents, since the levels of ATCase have been shown to be elevated in cancer cells.[3]

N-(Phosphonacetyl)-L-aspartate (PALA), a bi-substrate analogue of ATCase,[4] is a potent inhibitor which shows strong anti-proliferative[5,6] and anti-tumor[7,8] activities in cell culture. It has been examined in clinical trials as a possible anti-proliferation agent, however, a considerable drop in its effectiveness was observed.[9] This is probably due to the difficult translocation of PALA into cells,[10] since the highly ionic nature of PALA makes it difficult for it to diffuse through the lipid bilayer of the cell membrane.[11]

No structural data are available for CAD or the ATCase portion of CAD, however the amino acid sequences of the mammalian and *Escherichia coli* enzymes are 43% identical, and therefore the structure of the *E. coli* enzyme has often been used as a model for the structure of the ATCase portion of CAD.[12] All of the side chains important for catalytic activity in the *E. coli* enzyme are also present in the mammalian enzyme. Finally, PALA has been shown to be an effective inhibitor of both the mammalian and the *E. coli* enzymes.[4,6]

Numerous analogues of PALA have been reported, unfortunately none of them are as potent as PALA.[13–21] Functional group modifications of PALA, without significant perturbations to the core structure, may be a promising method to design new inhibitors for ATCase. Although several research groups have been interested in the chemical and biological consequences of the modification of the carboxyl groups in the aspartic acid portion of PALA by other functional groups like phosphonic[22,23] or polyethyleneglycol monomethylether groups for a better prodrug,[24] few studies have been performed to evaluate the effect of replacing the carboxylate moieties of PALA. This study describes the synthesis of a series of PALA analogues with modifications on the aspartate unit and determines how effective these compounds are in binding to and inhibiting the enzyme. Functional group modifications reported here include the introduction of alcohol and amide groups

Keywords: Allosteric enzyme; Bi-substrate analogue; Aspartate carbamoyltransferase.
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doi:10.1016/j.bmcl.2006.12.050

instead of the carboxylates at the α- and β-positions of the aspartate moiety, along with the replacement of the entire aspartate moiety with other amino acids such as aminomalonate, threonine, tyrosine and serine. In this communication, we report the design, synthesis, and inhibitory ability of this unique class of structurally modified PALA analogues (Fig. 1), and describe aspects of their structure–activity relationship.

A series of functional group modified PALA molecules (**1**–**4**) were synthesized according to Schemes 1–3. Initially, a hydroxy group was introduced in the α- and/or β-positions of the aspartate moiety and then an amide was introduced in the α- or β-positions of the aspartate moiety.

The β-hydroxy or homoserine derivative (**2**) was synthesized from commercially available β-benzyl-L-aspartate (**10**) in six steps (Scheme 1). After protecting the α-carboxylic acid as the *tert*-butylester, chloroacetylation was performed on **11** to give the amide **12** in 68% yield. The reaction of chloroacetyl L-aspartate with triethyl phosphite under reflux conditions afforded the corresponding phosphonate ester (**13**) in good yield. Then

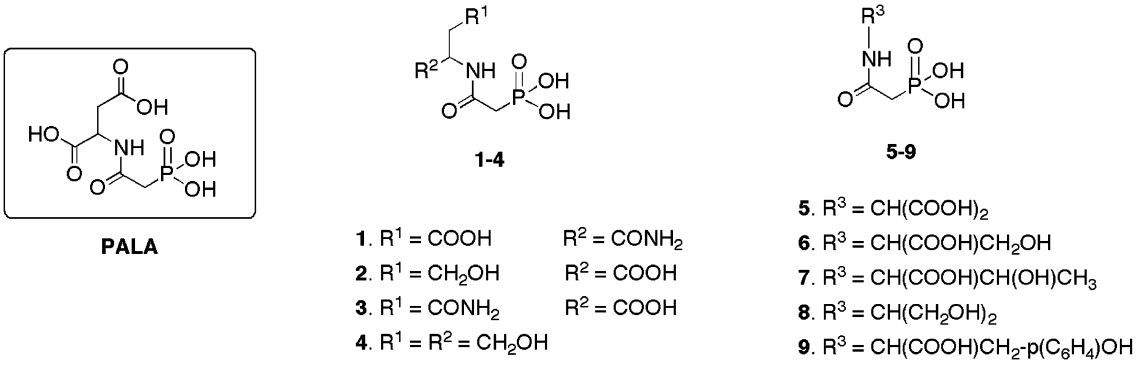
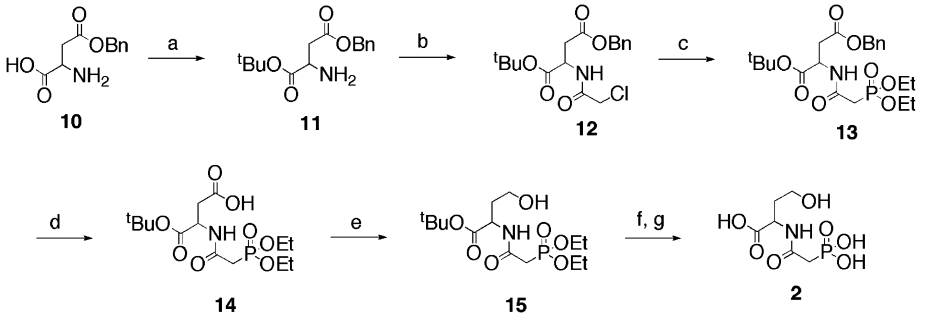
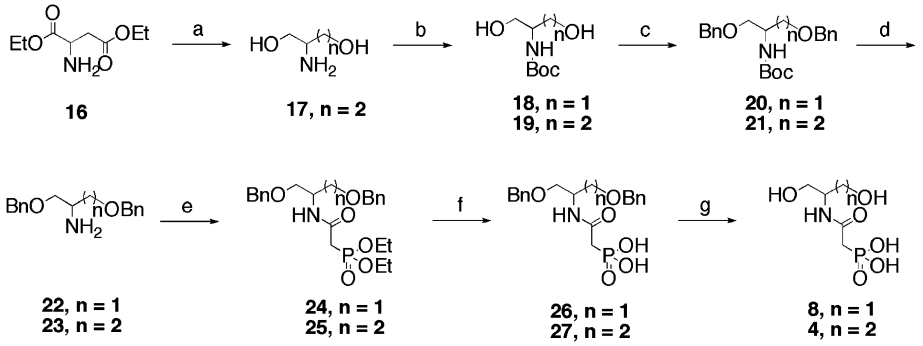


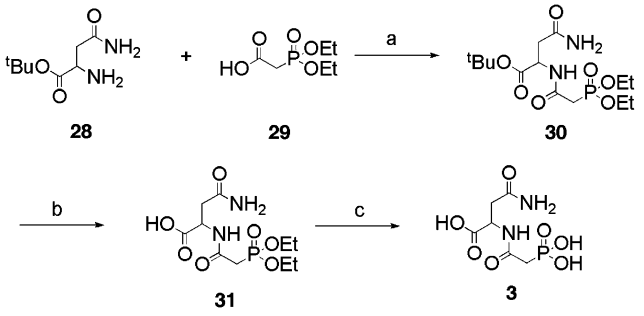
Figure 1. Structure of PALA, functional group modified analogues of PALA (**1**–**9**).



Scheme 1. Reagents and conditions: (a) H_2SO_4, isobutylene, dioxane, 20 h, 74%; (b) chloroacetic anhydride (2 equiv), pyridine (5 equiv), CH_2Cl_2, 4 h, 68%; (c) $P(OEt)_3$, 150 °C, 8 h, 97%; (d) 10% Pd/C, H_2, EtOH, overnight, 92%; (e) i—ethyl chloroformate (1.1 equiv), Et_3N (1.1 equiv), −17 °C, 40 min; ii—$NaBH_4$ (3.5 equiv), THF/H_2O (4:1 v/v), 5 h, 59%; (f) TFA, CH_2Cl_2, 3 h; (g) i—TMSBr (6 equiv), CH_3CN, 0 °C to rt, overnight; ii—H_2O, 1 h, 85%.



Scheme 2. Reagents and conditions: (a) $LiAlH_4$ (3 equiv), THF, 0 °C, then reflux for 30 min, 80%; (b) di-*tert*-butyl dicarbonate (1.3 equiv), MeOH/*t*-BuOH (1:1, v/v), 24 h, **18** (92%); **19** (85%); (c) BnBr (3.7 equiv), KOH (3.7 equiv), DMF, 4 h, **20** (59%); **21** (52%); (d) TFA, CH_2Cl_2, 3 h, **22** (83%); **23** (89%); (e) phosphonoacetic acid, Et_3N (1 equiv), DCC (1.1 equiv), HOBt (1 equiv), CH_2Cl_2, THF, overnight, **24** (74%); **25** (76%); (f) i—TMSBr (5.6 equiv), CH_3CN, 0 °C to rt, overnight, ii—H_2O, 1 h, 98%; (g) 10% Pd/C, H_2, EtOH, overnight, **8** (90%); **4** (77%).



Scheme 3. Reagents and conditions: (a) Et_3N (1 equiv), DCC (1.1 equiv), HOBt (1 equiv), CH_2Cl_2, THF, overnight, 85%; (b) 4 N HCl, dioxane, 0 °C to rt, 6 h, 100%; (c) i—TMSBr (5.6 equiv), CH_3CN, 0 °C to rt, overnight; ii—H_2O, 1 h, 81%.

the β-benzyl-protecting group was removed using hydrogenolysis and the acid obtained (**14**) was further reduced to the alcohol (**15**) using $NaBH_4$. The deprotection of *tert*-butyl and phosphonate esters of the alcohol provided the homoserine derivative of PALA (**2**).

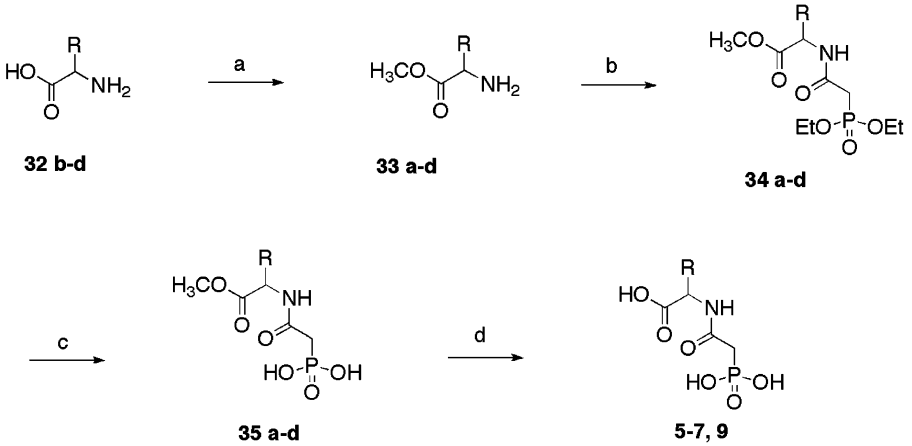
The bis hydroxy analogue of PALA (**4**) was synthesized by a different synthetic route starting from diethyl L-aspartate (Scheme 2). Reduction of ethyl ester of **16** using $LiAlH_4$ gave the corresponding bis alcohol (**17**).[25] After the sequential protection of the amino group as Boc and the hydroxy group as the benzyl ether, the Boc was cleaved with TFA and the resulting amine (**23**) was coupled with phosphonoacetic acid to afford the phosphonate ester (**25**) in 76% yield. Final deprotection of the phosphonate ester and benzyl ether under TMSBr conditions and hydrogenolysis, respectively, afforded the bis hydroxy analogue **4**.

The introduction of an amide group in the α- or β-positions resulted in significant changes in the ability of these compounds to inhibit ATCase. The synthesis and detailed studies of the α-amide derivative (**1**) have been reported[26] and **1** showed nanomolar level inhibition of ATCase. The β-amide derivative (**3**) was synthesized by a shorter route from commercially available starting material, *tert*-butyl L-asparaginate (**28**) (Scheme 3). Coupling of *tert*-butyl L-asparaginate with phosphonoacetic acid under standard amino acid coupling conditions afforded the phosphonate (**30**), which upon sequential deprotection of the *tert*-butyl and the phosphonate esters resulted in the β-amide analogue (**3**) of PALA.

All other structurally modified analogues were synthesized in three step reactions, except for (**8**), using similar synthetic routes (Scheme 4).[14] The malonate alcohol derivative (**8**) was synthesized according to a similar method adopted for the synthesis of inhibitor **4** (Scheme 2).

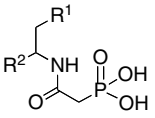
Different protected amino acid or alcohol starting materials, either purchased (**33a**) or prepared (**33b–d**), were reacted with phosphonoacetic acid to give the corresponding amino acid phosphonate derivatives (**34a–d**). The phosphonate esters were first deprotected under TMSBr conditions, and then the alkyl esters were deprotected with LiOH to provide the derivatives (**5–7,9**).

The ability of these compounds to inhibit ATCase (IC_{50}) (**1–9**) was evaluated against the catalytic subunit of *E. coli* aspartate transcarbamoylase (ATCase) by a colorimetric determination of the amount of *N*-carbamoyl-L-aspartate formed.[27] The results obtained are summarized in Table 1. These functional group modifications have a very large impact on the ability of these compounds to inhibit the enzyme. Compounds **1** and **3**

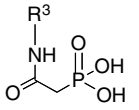


Scheme 4. Reagents and conditions: (a) $SOCl_2$, CH_3OH, 0 °C to rt, overnight, **33b** (96%); **33c** (92%); **33d** (86%); (b) phosphonoacetic acid, Et_3N (1 equiv), DIC (1.1 equiv), HOBt (1 equiv), CH_2Cl_2, THF, overnight, **34a** (73%); **34b** (38%); **34c** (83%); **34d** (73%); (c) i—TMSBr (5.6 equiv), CH_3CN, 0 °C to rt, overnight, ii—H_2O, rt, 1 h, **35a** (70%); **35b** (93%); **35c** (95%); **35d** (90%); (d) LiOH, CH_3OH, rt, 6 h, **5** (98%); **6** (98%); **7** (95%); **9** (96%).

Table 1. IC_{50} values of inhibitors **1**–**9** and PALA against the catalytic subunit of ATCase



Compounds 1-4



Compounds 5-9

Compound	R^1	R^2	R^3	IC_{50} (μM)
PALA	COOH	COOH		0.055
1	COOH	$CONH_2$		0.087
2	CH_2OH	COOH		3.900
3	$CONH_2$	COOH		0.225
4	CH_2OH	CH_2OH		2900
5			$CH(COOH)_2$	43.50
6			$CH(COOH)CH_2OH$	30.00
7			$CH(COOH)CH(OH)CH_3$	42.00
8			$CH(CH_2OH)_2$	12500
9			$CH(COOH)CH_2$–*p*-$(C_6H_4)OH$	63.00

showed inhibition at the nanomolar level, which is very close to the inhibition observed for PALA. Comparison of inhibitor **1** with PALA indicates that the amide modification in the α-position does not affect the inhibition significantly. Among the amide analogues, the α-amide showed more than twofold better inhibition than the β-amide, indicating that the β-carboxylate has more influence than the α-carboxylic group in the binding of the inhibitor. Though the analogues with an amide group did not make any dramatic changes in the observed inhibition as compared to PALA, introduction of the alcohol functionality resulted in analogues that were had substantially reduced ability to inhibit the enzyme. The mono alcohol **2** showed a 100-fold weaker inhibition compared to PALA, whereas the di-alcohol **4** only inhibited in the millimolar range. All the structurally modified compounds, except for **8**, exhibited inhibition at the micromolar level. Inhibitor **5**, which has one methylene unit less than PALA, showed an approximate 10^3-fold decrease in ability to inhibit the enzyme. A comparison of the IC_{50} values of inhibitors **5**, **6**, and **7** (see Table 1) indicates that the conversion of the carboxylate group into a primary or secondary alcohol does not have a significant influence on the inhibition, and also indicates that the relative position of the β-carboxylate moiety plays a crucial role in binding.

In summary, we described the synthesis of a series of novel inhibitors for ATCase with a variety of structural modifications. Also studied was the effect of these modifications on the ability of these analogues to inhibit the activity in ATCase. These studies with functional group modified PALA derivatives showed that amide groups can be a useful substitute of the carboxylate group thereby reducing the charge on the molecule. IC_{50} values of these analogues indicated that the methylene unit in the β-position is more critical than the functional group itself. Some of the newly synthesized molecules are potent inhibitors of ATCase and detailed functional and structural studies of these inhibitors of the enzyme are currently in process.

Acknowledgment

This work was supported by Grant GM26237 from the National Institutes of Health.

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