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Discovery of novel lipophilic inhibitors of OXA-10 enzyme (class D β-lactamase) by screening amino analogs and homologs of citrate and isocitrate

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ABSTRACT

Aminocitrate (and homolog) derivatives have been prepared by bis-alkylation of glycinate Schiff bases with bromoacetates (and ethyl acrylate), followed by N-acylation and esters (partial or complete) deprotection. Aminoisocitrate was similarly obtained by *mono*-alkylation with diethyl fumarate. Evaluation against representative β-lactamases revealed that the free acid derivatives are modest inhibitors of class A enzymes, whilst their benzyl esters showed a good inhibition of OXA-10 (class D enzyme). A docking experiment featured hydrophobic interactions in the active site.

The extensive and uncontrolled use of β-lactam antibiotics has resulted in the evolution of resistance in many strains of bacteria.[1] The bacterial resistance is now a major medical concern and there is a pressing need to discover new anti-infective agents, or to increase the therapeutic efficacy of the existing drugs.[2]

The transpeptidases involved in the biosynthesis of peptidoglycan, a major constituent of the bacterial cell wall, are serine enzymes which become inactive by binding penicillin and other β-lactam antibiotics.[3] The production of β-lactamases represents the most widespread and often the most efficient mechanism devised by bacteria to escape the lethal action of β-lactam antibiotics.[4] These defense enzymes have been grouped into four classes on the basis of their primary structures and catalytic mechanisms.[5] β-Lactamases of classes A, C and D are serine enzymes, whereas class B β-lactamases are metalloproteins which require zinc(II) ions for their activity. All β-lactamases catalyze very efficiently the hydrolysis of the β-lactam function of antibiotics, rendering them ineffective.[6]

Our research focuses on the synthesis and biochemical evaluation of potential inhibitors of β-lactamases. Several strategies have been considered to overcome the action of these β-lactam hydrolyzing enzymes.[7] Beside modified penicillin/cephalosporin-derived β-lactamase inhibitors,[8] non traditional derivatives are also developed, which do not feature a β-lactam ring.[9] The present work belongs to the latter approach and is based on a fortuitous observation made by Fonze et al. during the crystallographic study of *Bacillus licheniformis* BS3 β-lactamase, a class A enzyme.[10]

The crystallization of BS3 from a 100 mM citrate buffer revealed the unexpected role of this hydroxy acid which is perfectly located in the enzymic active site, as shown by the X-ray diffraction analysis. Similarly, isocitrate could be co-crystallized with BS3; in both cases, one β-carboxylate group of (iso)citrate interacts with the two catalytic serine residues, Ser70 and Ser130.[11] Citrate and isocitrate are modest inhibitors of BS3 β-lactamase with K_i values at pH 5 of 490 μM and 2200 μM respectively.[11] These experimental data prompted us to consider citrate and isocitrate as 'hits' for the conception of new affinity inhibitors of class A β-lactamases. We envisaged to increase the binding in the enzymic cavity by replacing the alcohol function of (iso)citrate with an amine function on which a variety of lateral chains could be fixed. Homologation, that is, the lengthening of the acidic chains, was also considered in view to possibly create novel interactions. The target molecules, depicted in Figure 1, are non natural amino acid derivatives related

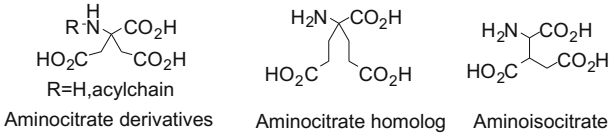

Figure 1. Target molecules (acids and corresponding esters).

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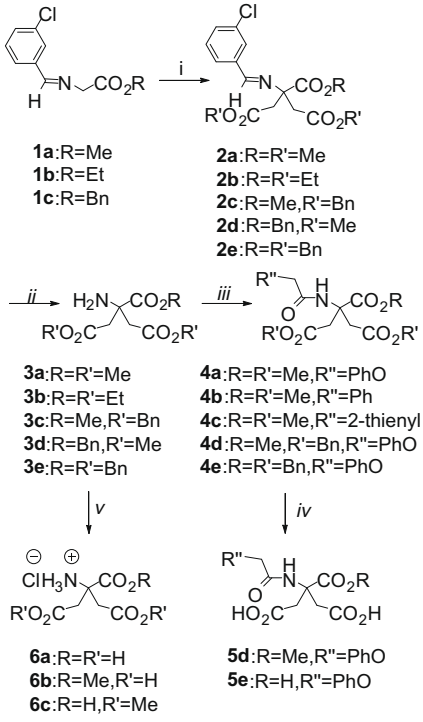
to glutamate and aspartate. Such compounds have traditionally been developed for drug discovery in the CNS (central nervous system) and anticancer research fields.[12] To our knowledge, analogs and homologs of (iso)citrate were not considered as potential inhibitors of β-lactamases before our work.[13]

Aminocitrate (and homolog) derivatives are α,α′-symmetrically bis-substituted α-amino acids, for which at least three synthetic strategies could be applied, namely the bis-alkylation of anions derived from oxazolin-5-ones,[14] nitroacetates[15] and glycinate Schiff bases.[16] We found that the last method was the most practical and versatile one, allowing a rapid access to the library of compounds of interest.

The Schiff bases **1**, prepared as usual by condensation of glycine esters with *m*-chlorobenzaldehyde, were treated with potassium carbonate, triethylammonium bromide (phase transfer catalyst)[16] and (m)ethyl bromoacetate (method A) or with LDA at low temperature and benzyl bromoacetate (method B),[11] to furnish the crude imines **2** (Scheme 1). Yields are quantitative for **2a–c**, but compounds **2d–e** (R = Bn) were contaminated with 10–20% of *mono*-alkylated products (from ^{1}H NMR analysis). The amines **3**, obtained after acidic hydrolysis,[17] were acylated with different acid chlorides in the presence of pyridine. Amides **4a–e** were recovered in about 60% overall yields after chromatographic purifications. Deprotection of the methyl esters **4a–c** posed unexpected problems: numerous acidic and basic conditions were tested, but competitive hydrolysis of the amide bond could not be avoided and the use of nucleophilic conditions was ineffective. Fortunately, hydrogenolysis of benzyl esters **4d–e** led quantitatively to the amides **5d–e** featuring two and three free carboxyl functions, respectively. Partially deprotected amine derivatives **6b–c** were also prepared by hydrogenolysis in aqueous HCl of the corresponding benzyl esters **3c–d** and isolated as hydrochloride salts. The fully deprotected aminocitrate **6a** could be obtained, either by hydrogenolysis of the tris-benzyl precursor **3e**, or by acidic hydrolysis of the tris-methyl or tris-ethyl precursors **3a–b**. A practical protocol using 6 N HCl at reflux was previously set up.[11]



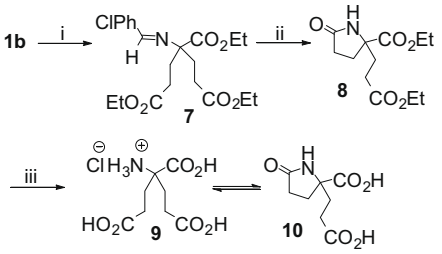
Scheme 1. Synthesis of aminocitrate derivatives. Reagents and conditions: (i) K_2CO_3, Bu_4NBr, excess $BrCH_2CO_2R'$, CH_3CN, 50 °C or LDA, excess $BrCH_2CO_2R'$, THF, −78 °C to 20 °C; (ii) 1 N HCl, CH_3CN, rt, 30 min, then basic work-up; (iv) pyridine, $R''CH_2COCl$, rt, 5 h; (iv) H_2, Pd/C, EtOAc, rt, 4 h; (v) H_2, Pd/C, HCl concd, EtOAc, rt, 5 h.

The synthesis of products possessing longer acidic chains was based on the bis-alkylation of Schiff base by Michael addition.[18] Thus, imine **1b** was treated with potassium carbonate and ethyl acrylate in excess to furnish compound **7** with 90% crude yield (Scheme 2). Imine hydrolysis led to the formation of pyrrolidinone **8**, isolated with 50% yield after purification by chromatography.[19] This was treated with 6 N HCl at reflux in view of producing the target-molecule **9**. In fact, we recovered a mixture of aminocitrate homolog **9** and unprotected pyrrolidinone **10**, in a 80:20 ratio from ^{1}H NMR analysis. Treatment with Dowex-H^+ resin did not improve the amount of **9**.

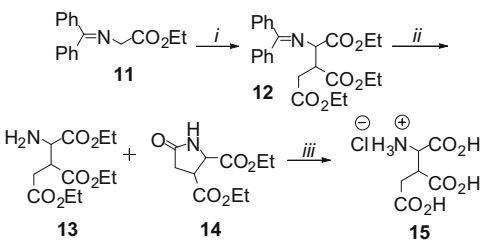
Finally, we prepared aminoisocitrate **15** (also named α-amino tricarballylic acid[20]) by the same strategy, as a racemic mixture of diastereoisomers (Scheme 3). The *mono*-alkylation of glycine Schiff bases requires the use of a sterically hindered derivative of benzophenone.[21] Thus, imine **11** was reacted with sodium ethoxide as catalyst and diethylfumarate in ethanol to obtain the *mono*-alkylated product **12** with 95% crude yield and a diastereoisomeric ratio of 27/73 determined by ^{1}H NMR analysis. The acidic hydrolysis of **12** led to a mixture of products, aminoisocitrate **13** and pyrrolidinone **14** (ratio 85/15), with 66% yield after chromatography. Tris-ethyl aminoisocitrate **13**[22] was isolated as a 46/54 mixture of diastereoisomers, whereas ethyl 3-ethoxycarbonylpyrroglutamate **14**[23], recovered in the last chromatographic fractions, was mainly one diastereoisomer. The previous mixture was hydrolyzed with 6 N HCl to give the racemic aminoisocitric acid **15** (50:50 ratio of diastereoisomers) with 80% yield.

Protocols and spectroscopic data for compounds **1**-**6** (Scheme 1), **7**–**10** (Scheme 2) and **12**-**15** (Scheme 3) are given as Supplementary data.

All the amino(iso)citrate derivatives (esters and acids) were evaluated for their activity against a series of β-lactamases of class A (TEM-1,[24] BS3,[10] NMCA[25]), class C (P99[26]), class D (OXA-10[27]), and class B (BcII[28], VIM-4[29]). Most of them are clinically reprenstative. Class A enzymes are primarily penicillinases, susceptible to inhibition by the presently marketed β-lactamase inhibitors, namely potassium clavulanate, sulbactam and tazobactam, which



Scheme 2. Synthesis of aminocitrate homolog. Reagents and conditions: (i) K_2CO_3, $CH_2{=}CHCO_2Et$, EtOH, rt, 16 h; (ii) 1 N HCl, CH_3CN, rt, 30 min, then basic work-up; (iii) 6 N HCl, 120 °C, 24 h.



Scheme 3. Synthesis of aminoisocitrate. Reagents and conditions: (i) NaOEt, diethyl fumarate, EtOH, rt, 1 h; (ii) HCl 1 N, CH_3CN, rt, 30 min, then basic work-up; (iii) 6 N HCl, 120 °C, 24 h.

are efficient only against a few class D β-lactamases. Some class D enzymes present rather a carbapenemase activity. Class C enzymes are primarily cephalosporinases, resistant to inhibition by the previous agents. There is no clinically useful inhibitor for class B enzymes that emerge now as worldwide source of carbapenem resistance.

The enzymes were incubated with the tested compounds during 30 min at 37 °C, at pH 7 or pH 5. Then nitrocefin (a chromogenic substrate) was added and the hydrolysis rate of this substrate was followed by spectrophotometry at 482 nm. In this way, the residual activity of the β-lactamases could be determined.[30,31] The results shown in Table 1 are expressed as percentages of β-lactamase initial activity. The compounds were tested at a concentration of 100 μM (unless otherwise mentioned); low% values indicate a very active compound.

None of the tested compounds inhibited BcII and VIM-4 (class B, zinc β-lactamases) nor P99 (Class C), except **6c** (pH 5). The activity of (iso)citrate-type derivatives against class A β-lactamases (TEM-1, BS3, NMCA) has been confirmed[11] and promising results were recorded for the inhibition of class D β-lactamase OXA-10.

Among the aminocitrate esters (**3**), **3b** (entry 1) inhibited TEM-1 and NMCA, **3c** (entry 2) inhibited NMCA and **3e** (entry 4) was a good inhibitor of OXA-10, whereas the less lipophilic ester **3d** (entry 3) was not active against OXA-10. Partially or totally deprotected aminocitrates **6a** (entry 10), **6b** (entry 11) and **6c** (entry 12) were poorly active or inactive. Among amide derivatives (**4**) fully protected as tris-esters, **4d** (entry 6) and **4e** (entry 7), but not **4b** (entry 5), inactivated OXA-10, confirming the importance of the lipophilic character of inhibitors to interact with this β-lactamase. As a matter of fact, partially or totally deprotected amides **5d** (entry 8) and **5e** (entry 9) were not active against OXA-10. However, bis-acid **5d** and tris-acid **5e** inhibited NMCA and BS3 respectively. Pyrrolidinones **8** (entry 13) and **14** (entry 16) were inactive. Aminocitrate homolog **9** (entry 14) was an inhibitor of TEM-1, whereas amino(iso)citrates **13** (entry 15) and **15** (entry 17) were devoid of significant activities. The selectivity of the tris-carboxyl derivatives **6a** and **9** for class A β-lactamases versus DD-peptidases was controlled by measuring their capacity to inhibit R39 enzyme,[32] a low molecular weight DD-transpeptidase/carboxypeptidase. No significant activity could be detected (data not shown).

Our initial hypothesis had been to improve the activity of (iso)-citrates versus class A β-lactamases by replacing the hydroxyl function with an amine/amide substituent, and/or by the homologation of two acid chains. Aminocitrate **6a**[33] was indeed slightly more active than citrate and its co-crystallization with BS3 β-lactamase showed, in a preliminary communication, that this inhibitor adopts a different position in the active site, the amine group interacting directly with the Ser70 and Ser130 catalytic residues.[11] The corresponding amide **5e**, featuring the side chain of penicillin V, was also an inhibitor of BS3. Lastly, the homolog **9**[33] of aminocitrate inhibited TEM-1, another class A β-lactamase. Compounds **6a**, **5e** and **9** are tricarboxylic acid derivatives, like citrate. This structural characteristic seems however not to be essential for inhibiting class A enzymes, since **3b**, **3c** (aminocitrate triesters) and **5d** (amidocitrate monoester) are also active compounds, against NMCA. Unfortunately, all attempts to co-crystallize the previous inhibitors with class A enzymes failed; thus structural data of the enzyme-inhibitor complexes are not available.

Interestingly, we have discovered novel inhibitors of OXA-10, a class D β-lactamase for which only few inhibitors were previously described in the literature.[34–37] Tribenzyl aminocitrate **3e**, and dibenzyl amidocitrates **4d** and **4e** were efficient against OXA-10 (the enzyme has a residual activity inferior to 50% in the presence of these compounds), whereas corresponding derivatives **3** and **4** bearing smaller ester groups were inactive (for instance **3b**, **3c**, **3d**, **5d**).

Thus, from our initial screening (Table 1), three compounds emerged, namely **3e**, **4e** and **4d**. They were further investigated for determining kinetic parameters. Compound **3e** is an inactivator of OXA-10, as shown by the inactivation graph (k_i vs [I]) of Figure 2, with a K_i value of 95 ± 25 μM (pH 7). On the other hand, compound **4e** behaves as a competitive inhibitor with a K_i value of 20 ± 4 μM (pH 7). The Hanes linearization has been used to assess the inhibition type (see Supplementary data).[38] The last compound, **4d**, is less efficient than **3e** and **4e** for inhibiting the β-lactamase.

There is a hydrophobic pocket in the active site of OXA-10 in which the molecules equipped with lipophilic benzyl groups may interact. The residues located in this hydrophobic core are essential for the catalytic efficiency of class D β-lactamases. In order to propose a mode of interaction of this series of compounds, manual docking of molecule **4e** in the active site has been carried out on

Table 1
Inhibition of β-lactamases by aminocitrate derivatives at pH 7 (pH 5)

Entry	Compound[a]	TEM-1	BS3	NMCA	P99	OXA-10	BcII	VIM-4[e]
1	**3b**[11]	**71**[b] (X)	82 (X)	**60** (X)	87 (X)	98 (X)	92 (93)	X
2	**3c**	81[b] (X)	100 (X)	**63** (X)	100 (X)	100 (X)	100 (93)	X
3	**3d**	X	X	X	X	100 (X)	X (100)	X
4	**3e**	100 (X)	100 (X)	X	X	**10** (X)	X (90)	X
5	**4b**	X	X	X	X	X	100 (X)	X
6	**4d**	X	X	X	X	**10** (X)	X	X
7	**4e**	X	X	X	X	**10** (X)	X	X
8	**5d**	98[d] (X)	96 (X)	**66** (X)	85 (X)	100 (X)	86 (100)	100
9	**5e**	96 (100)	80 (**70**)	96 (85)	97 (100)	84 (82)	X (100)	100
10	**6a**[11]	95[d] (X)	88 (X)	94 (X)	81 (X)	100 (X)	83 (X)	100
11	**6b**	99 (92)	96 (97)	94 (96)	100 (93)	96 (83)	100 (90)	100
12	**6c**	99 (87)	95 (100)	95 (99)	100 (73)	100 (79)	100 (100)	100
13	**8**	94 (80)	85 (96)	100 (100)	100 (100)	97 (98)	X (100)	96
14	**9 + 10**	88 (**69**)	100 (100)	100 (97)	100 (81)	100 (100)	100 (93)	96
15	**13 + 14**	94 (80)	100 (100)	100 (98)	100 (99)	82 (88)	X (79)	100
16	**14**	100 (86)	100 (100)	100 (93)	100 (100)	91 (87)	X (100)	94
17	**15**	100 (90)	85 (97)	100 (86)	100 (94)	92 (85)	X (100)[c]	94

Results expressed as percentages of initial activities.
[a] Compounds were tested at pH 7 in 50 mM phosphate buffer and at pH 5 in 50 mM acetate buffer at a concentration of 100 μM, otherwise mentioned.
[b] 0.2 mM.
[c] 1 mM.
[d] 5 mM
[e] It is not possible to test the activity of VIM-4 at pH 5; X = not tested. The standard deviation for the percentages of initial activities is below 5%.

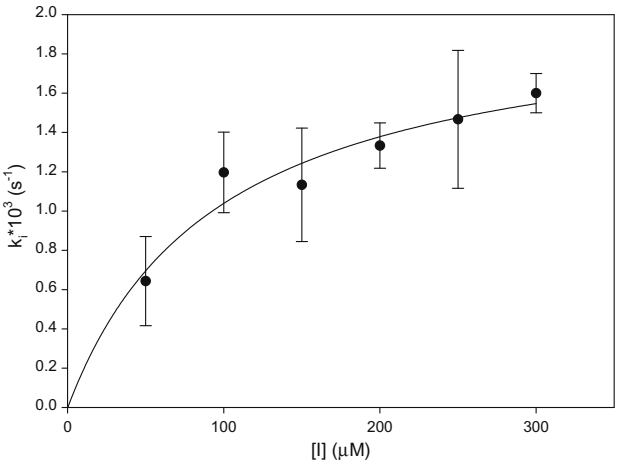


Figure 2. Interaction between **3e** and OXA-10. Variation of k_i at 50, 100, 150, 200, 250 and 300 μM in inhibitor.

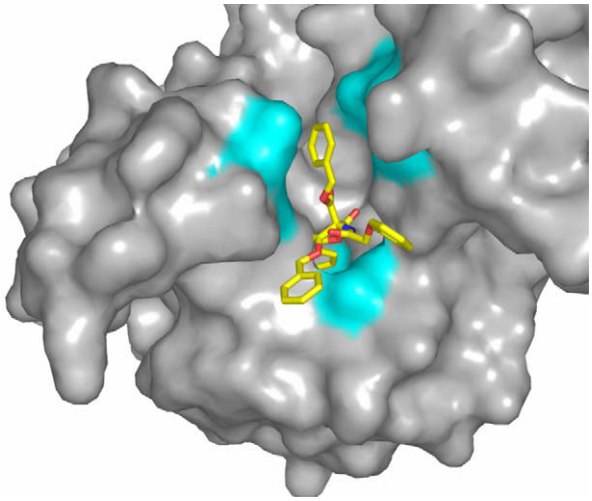


Figure 3. Docking of **4e** in OXA-10 enzyme. The hydrophobic residues in the active site are colored in cyan.

the basis of the known position of the amide chain in the complex of benzylpenicillin with OXA-10.[37] As shown in Figure 3, the aromatic rings are well located in the hydrophobic pocket.

This model remains to be confirmed by co-crystallization experiments. Nevertheless, the 2-aminopropane-1,3-di(benzyloxycarbonyl)-2-carboxylic acid motif represents a novel hydrophobic scaffold for the inhibition of class D β-lactamases. Usually, the non β-lactamic inhibitors of serine β-lactamases are designed to covalently interact with the active serine residue, as exemplified by Pratt and co-workers with α-ketoheterocycles,[39] phenacetu-rates[40] and diaroyl phosphates.[35] Recently, They have described amido ketophosph(on)ates acting as micromolar inhibitors of class D β-lactamases and suggested that these inhibitors interact at the active site in the carbonyl form rather than by formation of tetrahedral adducts.[36] Our work discloses original inhibitors of class D β-lactamases which mechanism is most probably based on non covalent interactions. Mechanistic studies are in progress.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.04.149.

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30. Protocol of biochemical evaluation: The enzymes were produced and purified as previously described.[10,24–29] The enzymes (1-100 nM) were incubated with the tested compounds (100 μM, otherwise mentioned) and the chromogenic substrate[31] (nitrocefin, 100 μM) in phosphate buffer (50 mM, pH 7) or acetate buffer (50 mM, pH 5). In the case of metallo-enzymes (class B), HEPES buffer (10 mM, pH 7) added with $ZnCl_2$ (50 μM) was used. Tested compounds were dissolved in DMSO at 10–100 mM and then diluted with the buffer (final concentration of DMSO in the testing = 2%); DMSO = 2% had no effect on the enzyme activity. The hydrolysis rate of nitrocefin was followed by spectrophotometry at 482 nm with UVIKON 860, 940 and XL apparatus connected to a computer via a RS232 line. The residual activity was obtained by comparison with the variation of the absorbance of the reference (sample without inhibitor) and indicated in Table 1. Results are expressed as % of initial activities. The standard deviation is below 5%. All experiments were performed three times.
31. The catalytic parameters (k_{cat}, K_m, k_{cat}/K_m) of tested β-lactamases versus nitrocefin are given as Supplementary data.
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33. Plot v_0/v_i (ratios of hydrolysis in the absence and in the presence of inhibitors) versus inhibitor concentration gave the inhibition constant K_i. For **6a**,[11] K_i = 250 μM (BS3, pH 5) and 150 μM (TEM-1, pH 5); for **9** + **10**, K_i = 105 μM (TEM-1, pH 5).
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