Bioorganic & Medicinal Chemistry Letters 18 (2008) 3764–3768

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

2-Aminopropane-1,2,3-tricarboxylic acid: Synthesis and co-crystallization with the class A β-lactamase BS3 of *Bacillus licheniformis*

Joséphine Beck [a], Eric Sauvage [b], Paulette Charlier [b], Jacqueline Marchand-Brynaert [a,*]

[a] Unité de Chimie Organique et Médicinale, Université catholique de Louvain, Bâtiment Lavoisier, Place Louis Pasteur 1, B-1348 Louvain-la-Neuve, Belgium
[b] Centre d'Ingénierie des Protéines, Institut de physique B5a, Université de Liège, B-4000 Sart- Tilman, Belgium

ARTICLE INFO

Article history:
Received 31 March 2008
Revised 8 May 2008
Accepted 9 May 2008
Available online 16 May 2008

Keywords:
2-Aminopropane-1,2,3-tricarboxylic acid
Glycine bis-alkylation
β-Lactamase inhibitors
Aminocitrate-BS3 complex
X-ray diffraction analysis

ABSTRACT

The title compound **4** has been prepared in four steps from ethylglycinate in 63% overall yield. This amino analog of citric acid has been co-crystallized with the class A β-lactamase BS3 of *Bacillus licheniformis* and the structure of the complex fully analyzed by X-ray diffraction. Tris-ethyl aminocitrate **3** and the free tris-acid **4** have been tested against a member β-lactamase from all distinct subgroups. They are novel inhibitors of class A β-lactamases, still modest but more potent than citrate and isocitrate.

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.[1] Our research focuses on the synthesis and the biochemical evaluation of potential inhibitors of β-lactamases. Numerous β-lactamase inhibitors have been reported in the literature.[2] However, most of these drugs are β-lactam derivatives, and when exposed to such molecules, bacteria acquire resistance. To disrupt this vicious circle, non β-lactam inhibitors may be an alternative. The search of novel structures considered as 'hits' in medicinal chemistry proceeds from different strategies, such as the mechanism-based design and the screening of various chemical libraries, but also from serendipity. Our present work is precisely based on a fortuitous observation related to protein crystallography, when using 100 mM sodium citrate buffer in the crystallization protocols.

Citrate has been recently shown, by X-ray diffraction analysis, to perfectly fit into the active site of the *Bacillus licheniformis* BS3 β-lactamase, and to behave as a modest inhibitor of this serine enzyme with a micromolar K_i value.[3] We speculated that the replacement of the hydroxyl function of citrate with an amine function could enhance the affinity for the target enzyme. Moreover, the amine function could be used to introduce different side chains susceptible to modulate the interactions within the active site.

In this paper, we propose a simple method for preparing the amino analog of citric acid, namely 2-aminopropane-1,2,3-tricarboxylic acid, and we compare the structures of complexes formed between citrate or aminocitrate and the BS3 β-lactamase. The citrate regio-isomer, that is, isocitrate, has been also involved in the structural and biochemical studies. Our aim was to pave the route towards the discovery of novel anti-β-lactamase compounds (Fig. 1).

The previous syntheses of 2-aminopropane-1,2,3-tricarboxylic acid are based on two methods: (i) the hydrolysis of hydantoin prepared from diethyl or diisobutylacetone-dicarboxylate[4,5] and (ii) the double alkylation of nitro-acetate anion with haloesters followed by reduction and ester hydrolysis.[6,7] Here we disclose an alternative, more practical method.

Fully protected 2-amino-propane-1,2,3-tricarboxylic acid derivative **2** was prepared by bis-alkylation of the glycine imine precursor **1** (Scheme 1), a methodology originally developed by O'Donnell, and currently applied for the synthesis of particular amino acids.[8–10] The majority of glycine mono-alkylation reactions

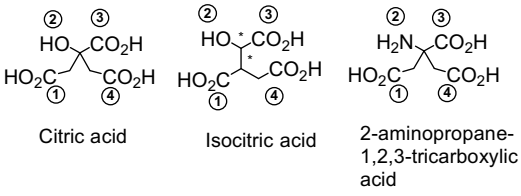
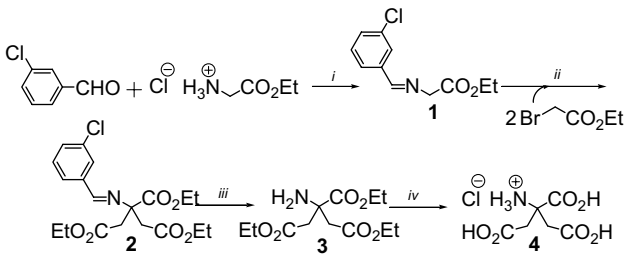


Figure 1. Structures of compounds of interest and numbering of their functional groups.

* Corresponding author. Tel.: +32 10 47 27 40; fax: +32 10 47 41 68.
E-mail address: jacqueline.marchand@uclouvain.be (J. Marchand-Brynaert).

0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.05.045



Scheme 1. Synthesis of aminocitrate. Reagents and conditions: (i) CH_2Cl_2, Et_3N, $MgSO_4$, 20 °C, 24 h; (ii) Method A: K_2CO_3, Bu_4NBr, CH_3CN, 50 °C, 1–2 days; Method B: NaH, DMF, 0–20 °C, two successive additions; Method C: LDA, THF, −78–20 °C; (iii) 1 N HCl, CH_3CN, 20 °C, then basic work-up; (iv) 6 N HCl.

makes use of benzophenone-derived imines, while the bis-alkylations are more readily performed on *m*-chlorobenzaldehyde-derived imines.[11,12] Thus, we prepared the Schiff base **1** from *m*-chlorobenzaldehyde and glycine ethyl ester hydrochloride. Crude imine **1** was then reacted with two equivalents of ethyl bromoacetate and an excess of potassium carbonate, under phase transfer conditions (Method A).[13] The completion of bis-alkylation required a very long reaction time, about 1–2 days of heating in acetonitrile at 50 °C. Crude compound **2** was quantitatively recovered.

We then considered alternative protocols versus the original O'Donnell's method. Using sodium hydride as base in dimethylformamide solution, we performed the deprotonation at 0 °C and the alkylation at 20 °C; the best results were collected by addition of the reagents in two successive fractions of one equivalent (Method B).[14] Lastly, we used lithium diisopropylamide (LDA, 2 equiv) for imine **1** deprotonation at low temperature (−78 °C) in tetrahydrofurane solution.[15] After 1 h, two equivalents of ethyl bromoacetate were added and the mixture was warmed to room temperature (Method C). This protocol furnished similar results to Method B. However, the Method C was preferred because it allowed working on larger quantities, always with good and reproducible yields, most probably due to the fact that the reactive medium remains homogeneous. Crude **2** was directly submitted to a smooth acidic hydrolysis followed by a basic work-up to furnish the free amine **3** which was purified by column chromatography on silica gel with 50% yield. This yield corresponds to pure product isolated after three steps of reaction from glycine ester.[16] The amine **3** is characterized in ^{1}H NMR by two doublets corresponding to the symmetrical methylenic protons (4H) around 2.66–2.88 ppm (J = 16 Hz, AB pattern).

Tris-ester **3** could be fully deprotected by treatment with 6 N HCl at reflux; the amino analog of citric acid was isolated as the hydrochloride salt **4** with 90% yield.[17] The sequence of reaction we propose now constitutes the best route towards this non natural amino acid (four steps from ethylglycinate with only one chromatographic purification; overall yield = 63%).

The biochemical activity of tris-ester **3** and tris-acid **4** has been evaluated at pH 7.2 against representative β-lactamases of class A (TEM-1,[18] BS3,[3] NMCA[19]), class C (P99[20]), class D (Oxa-10[21]), and class B (BcII[22a]); all are serine enzymes, except the last one which is a zinc enzyme working with two Zn(II) in the active site.[22b] The enzymes (1–100 nM) were incubated with the tested compounds during 30 min at 37 °C. Then a chromogenic substrate (nitrocefine) was added and the hydrolysis rate of this substrate was followed by spectrophotometry at 482 nm.[23] Results of Table 1 (residual activity) are expressed as percentages of β-lactamases initial activity. Aminocitrate derivatives **3** and **4** are modest inhibitors of class A and class C β-lactamases; they are inactive against class D β-lactamase and slightly active as inhibitors of zinc β-lactamase.

Table 1
Inhibition of β-lactamases by aminocitrate derivatives

Compound[a]	TEM-1	BS3	NMCA	P99	Oxa-10	BcII
3	71[b]	82	60	87	98	92
4	95[c]	88	94	81	100	83

Results expressed as percentages of initial activities.
[a] Compounds were tested at pH 7.2 in 50 mM phosphate buffer and at a concentration of 100 μM, otherwise mentioned.
[b] 0.2 mM.
[c] 2 mM.

Table 2
K_i values[a] (μM)

Compound	BS3	TEM-1
Citrate	490	730
Isocitrate	2200	1500
4	250	150

[a] Compounds were tested at pH 5 in 25 mM acetate buffer.

The K_i values have been measured in the cases of BS3 and TEM-1 enzymes for aminocitrate **4**, citrate and isocitrate. As shown in Table 2, the amino analog is the more active compound.

This result stimulates our interest in comparative crystallographic studies of BS3-**4** and BS3-isocitrate complexes with the known BS3-citrate complex.[3,24] The novel complexes were obtained by a similar method.[25]

Among the residues with a demonstrated role in the catalytic process of β-lactam hydrolysis by class A β-lactamases, Ser70 and Ser130 are directly involved in the catalytic mechanism. Thr235 and Arg244 interact with the substrate carboxylate and are thus involved in the positioning of the β-lactam antibiotic in the active site. Class A β-lactamases are also characterized by the presence of an oxyanion hole defined by the amide groups of Ser70 and Ala237, which draws the β-lactam carbonyl oxyanion in the course of hydrolysis of the β-lactam ring.[26] Citrate was firstly observed in the active site of BS3 enzyme as a co-crystallization product.[3]

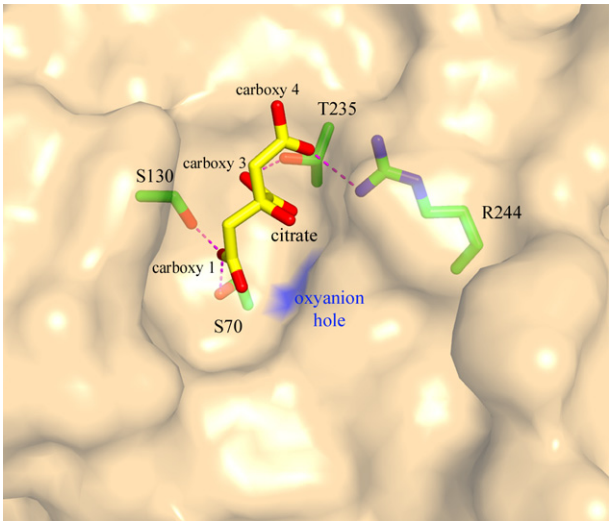


Figure 2. Inhibition of the class A β-lactamase of *B. licheniformis* BS3 by citrate. Orange molecular surface shows the active site cleft with a molecule of citrate (in yellow sticks). Residues of the active site interacting with the citrate molecule are shown in green stick. Oxygen atoms are red and nitrogen atoms are blue. Citrate carboxylates are numbered according to the Figure 1. Blue surface indicates the oxyanion hole. Colors are identical for Figures 3 and 4.

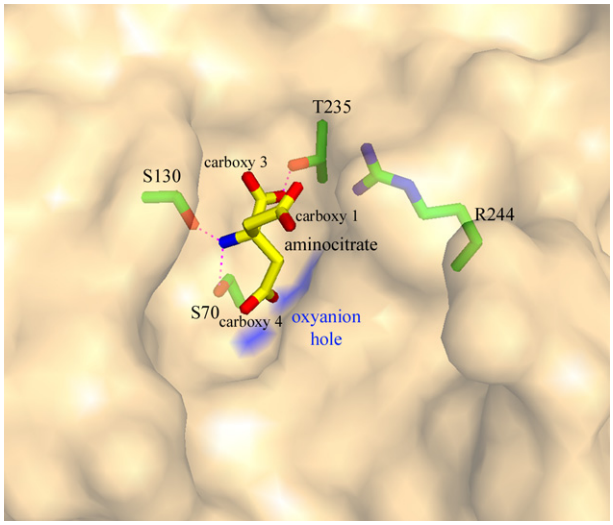


Figure 3. Inhibition of the class A β-lactamase of *B. licheniformis* BS3 by aminocitrate.

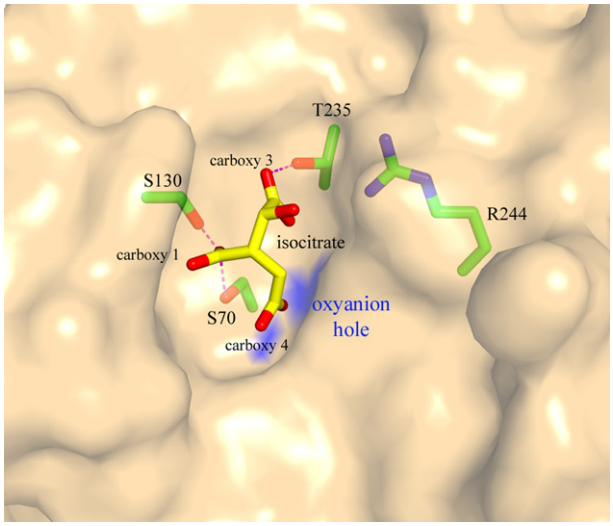


Figure 4. Inhibition of the class A β-lactamase of *B. licheniformis* BS3 by isocitrate.

As shown in Figure 2, an oxygen atom of the carboxylate 1 of the citrate molecule interacts with the two catalytic serines (2.5 Å to Ser70 Oγ and 2.4 Å to Ser130 Oγ). One oxygen atom of the carboxylate 3 is at 2.8 Å from Thr235 Oγ and at 3.2 Å from Ser130 Oγ, whereas the other oxygen atom interacts with Arg244 and Thr235. The hydroxyl group points out of the catalytic cleft and the carboxylate 4 salt bridges with Arg244. The carboxylate 3 occupies a position identical to the carboxylate group of a β-lactam antibiotic (for example in the acyl-enzyme complex between BS3 and cefoxitin).[3]

Although aminocitrate (Fig. 3) only differs from citrate by the substitution of the hydroxyl group with an amine group, its position and orientation in the active site and the interactions with the catalytic cleft residues are different from those of the citrate, except carboxylate 3 that makes similar interactions. The amine group of the aminocitrate directly interacts with Ser70 Oγ and Ser130 Oγ (3.2 Å and 2.3 Å, respectively) and an oxygen atom of the carboxylate 4 lies in the oxyanion hole.

In the case of isocitrate (Fig. 4), carboxylate 1 interacts with Ser70 Oγ and Ser130 Oγ, whereas an oxygen atom of the carboxylate 4 is in the oxyanion hole.[27]

Table 3 summarizes the direct interactions between amino acid residues and the inhibitors functional groups.

Clearly, citrate, isocitrate and aminocitrate adopt different conformations in the active site of BS3. However, the main difference concerns the functional group which interacts with the two catalytic serine residues (Ser70 and Ser130): a carboxylate for citrate and isocitrate and the amine group in the case of amino-citrate. This could probably explain the enhanced biochemical activity of **4** compared to (iso)citrate. Modification of the amine group of **4**, as initially proposed, seems now less appropriate than substitutions at the level of the carboxylic functions for further developments. As a matter of fact, the tris-ethyl ester **3** was found to be slightly more active than **4** in the competitive inhibition tests versus β-lactamases (Table 1).

The interest of using X-ray data for the design of structure-based small-molecule enzyme inhibitors remains an open question, the technique featuring as much advantages as drawbacks.[28] Considering citrate to identify new 'hits', the situation is quite ambiguous since the complexes formed with class A and class C β-lactamases showed different structures.[29]

In conclusion, we have set up a practical synthesis of 2-amino-1,2,3-tricarboxylic acid (amino analog of citric acid) and shown that this non natural aminoacid **4** behaves as a modest inhibitor of several β-lactamases. Beside its potential, but debatable, use as

Table 3
Summary of direct interactions between amino acid residues and functional groups **1**–4 (see Fig. 1)

Group-atoms	BS3-citrate[a]	BS3-aminocitrate[a]	BS3-isocitrate[a]
Carboxylate 1-O1	Ser130 Oγ (2.4) Ser70 Oγ (2.5)	—	Ser130 Oγ (2.1) Ser70 Oγ (2.6)
Carboxylate 1-O2	—	—	Thr235 Oγ (2.8)
Hydroxyl 2/amine 2	—	Ser130 Oγ (2.3) Ser70 Oγ (3.2)	—
Carboxylate 3-O1	Ser130 Oγ (3.2) Thr235 Oγ (2.8)	Thr235 Oγ (2.3) Arg244 NH1 (3.2)	Thr235 Oγ (3.1)
Carboxylate 3-O2	Arg244 NH1 (3.1) Thr235 Oγ (3.1)	—	Thr235 Oγ (2.8)
Carboxylate 4-O1	Arg244 NH1 (2.8)	Ala237 NH (2.9) Ser70 (2.9)	Ala237 NH (2.8) Ser70 (3.0)
Carboxylate 4-O2	—	—	—

[a] Distances between atoms in Å are indicated into parentheses.

novel 'hit' towards anti-β-lactamases, aminocitrate **4** has been already recognized as a valuable compound for cation complexation. Applications in the field of heavy metal sequestering agents have been disclosed,[30] that should benefit of our convenient synthesis.

Acknowledgments

The biochemical evaluations have been performed in the laboratories of professors J. M. Frère and M. Galleni (University of Liège). We thank R. Herman for protein crystallization work, and the staff of beamline BM30a at ESRF for assistance in X-ray data collection. This work was supported in part by the Belgian Program on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming (PAI 5/33 and PAI 6/19), by the Fonds National de la Recherche Scientifique (IISN 4.4505.00, FRFC 9.45/9.99, FRFC 2.4.508.01.F, FRFC 9.4.538.03.F, FRFC 2.4.524.03) and the University of Liège (Fonds spéciaux, Crédit classique). J.M.-B. is senior research associate of the FRS-FNRS (Belgium).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.05.045.

References and notes

1. Fisher, J. F.; Meroueh, S. O.; Mobashery, S. *Chem. Rev.* **2005**, *105*, 395.
2. Sandanayaka, V. P.; Prashad, A. S. *Curr. Med. Chem.* **2002**, *9*, 1145.
3. Fonze, E.; Vanhove, M.; Dive, G.; Sauvage, E.; Frere, J-M. J.-M.; Charlier, P. *Biochemistry* **2002**, *41*, 1877.
4. Durnow, A.; Rombusch, K. *Chem. Ber.* **1955**, *88*, 1334.
5. Connors, T. A.; Mauger, A. B.; Peutherer, M. A.; Ross, W. C. J. *J. Chem. Soc.* **1962**, 4601.
6. Kaji, E.; Zen, S. *Bull. Chem. Soc. Jpn* **1973**, *46*, 337.
7. Fu, Y.; Hammarstrom, L. G.; Miller, T. J.; Franczek, F. R.; Mc Laughlin, M. L.; Hammer, R. P. *J. Org. Chem.* **2001**, *66*, 7118.
8. O'Donnell, M. J. *Aldrichim. Acta* **2001**, *34*, 3.
9. O'Donnell, M. J.; Polt, R. L. *J. Org. Chem.* **1982**, *47*, 2663.
10. Allwein, S. P.; Secord, E. A.; Martins, A.; Mitten, J. V.; Nelson, T. D.; Kress, M. H.; Dolling, H. *Synlett* **2004**, 2489.
11. (a) O'Donnell, M. J.; Wu, S.; Huffman, J. C. *Tetrahedron* **1994**, *50*, 4507; (b) O'Donnell, M. J. *Acc. Chem. Res.* **2004**, *37*, 506.
12. Kitamura, M.; Shirakawa, S.; Maruoka, K. *Angew. Chem., Int. Ed.* **2005**, *44*, 1549.
13. O'Donnell, M. J.; Wojciechowski, K.; Ghosez, L.; Navarro, M.; Sainte, F.; Antoine, J. P. *Synthesis* **1984**, *4*, 313.
14. Park, K.-H.; Abbate, E.; Olmstead, M. M.; Kurth, M. J.; Najdi, S. *Chem. Commun.* **1998**, *16*, 1679.
15. Laue, K. W.; Haufe, G. *Synthesis* **1998**, *10*, 1453.
16. Preparation of compound **3**.
Step i: hydrochloride salt of ethyl glycinate (1 g, 7.16 mmol) and triethylamine (1 equiv, 1 mL, 7.16 mmol) were dissolved in dichloromethane (10 mL) and stirred for 6 h at 20 °C. Magnesium sulfate (1.5 g) and 3-chlorobenzaldehyde (0.9 equiv, 730 μL, 6.44 mmol) were then added and the mixture was further stirred overnight. After filtration, the organic solution was washed three times with brine, dried ($MgSO_4$) and concentrated under vacuum to give **1** as yellow oil (1.45 g, quantitative yield). HRMS (ESI): 226.0645 (calculated for $C_{11}H_{12}ClNO_2$+H: 226.0635); ^{1}H NMR ($CDCl_3$, 300 MHz): δ ppm, 1.32 (t, 3H, CH_3, J = 7.14 Hz), 4.26 (q, 2H, CH_2, J = 7.14 Hz), 4.41 (s, 2H, CH_2), 7.33–7.45 (m, 2H, Ar), 7.63 (d, 1H, Ar, J = 7.5 Hz), 7.82 (s, 1H, Ar), 8.25 (s, 1H, NCH). ^{13}C NMR ($CDCl_3$, 125 MHz): δ ppm, 14.36 (CH_3), 61.35 (CH_2), 62.07 (CH_2), 127.00 (CHAr), 128.23 (CHAr), 130.02 (CHAr), 131.31 (CHAr), 134.98 (CAr), 137.47 (CAr), 164.03 (NCH), 170.01 (CO_2Et).
Step ii (Method C): to the crude imine **1** (580 mg, 2.6 mmol) dissolved in dry THF (12 mL) and cooled at −78 °C, was added under argon atmosphere LDA (commercial solution in THF, 2.1 equiv, 2.7 mL, 5.42 mmol). After 1h at −78 °C, ethyl bromoacetate was added (2.1 equiv, 570 μL, 5.42 mmol) and the mixture was allowed to slowly warm up to 20 °C, overnight, under stirring. After addition of water, the solution was concentrated under vacuum. The oily residue was dissolved in ether and the organic layer was washed successively with aqueous $NaHCO_3$and brine. Drying over $MgSO_4$ and concentration under vacuum furnished **2** as a brown oil (1 g, quantitative yield). ^{1}H NMR ($CDCl_3$, 500 MHz): δ ppm, 1.24 (t, 6H, CH_3, J = 7.14 Hz), 1.29 (t, 3H, CH_3, J = 7.16 Hz), 3.13 (d, 2H, CH_2, J = 15.79 Hz), 3.29 (d, 2H, CH_2, J = 15.77 Hz), 4.14 (q, 4H, CH_2, J = 7.10 Hz); 4.26 (q, 2H, CH_2, J = 7.14 Hz), 7.34 (dd, 1H, Ar, J = 8 Hz and J = 7.6 Hz), 7.40 (ddd, 1H, Ar, J = 8 Hz, J = 2.17 Hz and J = 1.14 Hz), 7.57 (dt, 1H, Ar, J = 7.6 Hz and J = 1.3 Hz), 7.76 (s, 1H, Ar), 8.28 (s, 1H, NCH). ^{13}C NMR ($CDCl_3$, 125 MHz): δ ppm, 14.37 (CH_3), 40.83 (CH_2), 60.79 (CH_2), 61.94 (CH_2), 67.86 (C), 127.24 (CHAr), 128.19 (CHAr), 130.02 (CHAr), 131.39 (CHAr), 135.00 (CAr), 137.85 (CAr), 159.63 (N=CH), 170.65 (CO_2Bn), 171.00 (CO_2Me).
Step iii: the crude imine **2** (857 mg, 2.15 mmol) was dissolved in acetonitrile (3 mL) and 1 N HCl was added (1.5 equiv, 3.2 mL, 3.22 mmol). After 30 min at room temperature, the solution was concentrated under vacuum, and the aqueous phase was extracted three times with ether. The aqueous phase was then neutralized with $NaHCO_3$ and basified to pH 10 with 1 N NaOH. This was extracted five times with CH_2Cl_2. The organic layers were collected and washed with brine. After drying ($MgSO_4$) and concentration under vacuum, the residue was purified by column chromatography on silica gel to furnish **3** as yellow oil (296 mg, 50% yield). R_f = 0.5 (CH_2Cl_2/EtOAc, 1:1); HRMS (ESI): 276.1450 (calculated for $C_{12}H_{21}NO_6$+1: 276.1447); ^{1}H NMR ($CDCl_3$, 250 MHz): δ ppm, 1.30 (t, 9H, J = 7.13 Hz, CH_3), 2.56 (s, 2H, NH_2), 2.66–2.72 (d, 2H, J = 16.0 Hz, CH_2), 2.82–2.88 (d, 2H, J = 16.0 Hz, CH_2), 4.15 (q, 4H, J = 7.13 Hz, CH_2); ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ ppm, 14.3 (CH_3) 43.6 (CH_2), 55.8 (C), 61.0 (CH_2), 61.9 (CH_2), 170.5 (CO_2Et), 175.0 (CO_2Et).
17. Preparation of compound **4**. Amine **3** (100 mg) was treated with 6 N HCl (10 mL) at reflux for 1 night. After extraction with CH_2Cl_2, the aqueous phase was concentrated under vacuum and the residue was dried under high vacuum to furnish **4** as a white solid (75 mg, 90% yield). HRMS (ESI): 192.0503 (calculated for $C_6H_9NO_6$+1: 192.0508); ^{1}H NMR (D_2O, 250 MHz): δ ppm, 2.98 (s, 4H); ^{13}C NMR (D_2O, 62.5 MHz): δ ppm, 39.9 (CH_2), 59.3 (C), 173.7 (CO_2H).
18. Fonze, E.; Charlier, P.; To'th, Y.; Vermeire, M.; Raquet, X.; Dubus, A.; Frere, J. M. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1995**, *D51*, 682.
19. Swaren, P.; Maveyraud, L.; Raquet, X.; Cabantous, S.; Duez, C.; Pedelacq, J.-D.; Mariotte-Boyer, S.; Mourey, L.; Labia, R.; Nicolas-Chanoine, M.-H.; Nordmann, P.; Frere, J.-M.; Samama, J.-P. *J. Biol. Chem.* **1998**, *273*, 26714.
20. Dubus, A.; Ledent, P.; Lamotte-Brasseur, J.; Frere, J. M. *Proteins* **1996**, *25*, 473.
21. (a) Paetzel, M.; Danel, F.; De Castro, L.; Mosimann, S. C.; Page, M. G. P.; Strynadka, N. C. J. *Nat. Struct. Biol.* **2000**, *7*, 918; (b) Golemi, D.; Maveyraud, L.; Vakulenko, S.; Tranier, S.; Ishiwata, A.; Kotra, L. P.; Samama, J.-P.; Mobashery, S. *J. Am. Chem. Soc.* **2000**, *122*, 6132.
22. (a) Carfi, A.; Duee, E.; Galleni, M.; Frere, J.-M.; Dideberg, O. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1998**, *D54*, 313; (b) Llarrull, L. I.; Tioni, M. F.; Kowalski, J.; Bennett, B.; Vila, A. J. *J. Biol. Chem.* **2007**, *282*, 30586.
23. Determination of biochemical activity. The enzymes were produced and purified as previously described.[18–22] The enzymes (1–100 nM) were incubated with the tested compounds (100 μM, otherwise mentioned) and the chromogenic substrate nitrocefine (100 μM) in a phosphate buffer (50 mM, pH 7.2). The hydrolysis rate of this substrate was followed by spectrophotometry at 482 nm. The residual activity was obtained by comparison with the variation of the absorbance of a reference (sample without inhibitor) and indicated in Table 1. Results are expressed as % of initial activities; variations of results are within ±5%. Plot V/V_i versus inhibitor concentration (ratios of hydrolysis in the absence and in the presence of inhibitors) gave the inhibition constant indicated in Table 2. All experiments were performed three times.
24. Ledent, P.; Duez, C.; Vanhove, M.; Lejeune, A.; Fonze, E.; Charlier, P.; Rhazi-Filali, F.; Thamm, I.; Guillaume, G.; Samyn, B.; Devreese, B.; Van Beeumen, J.; Lamotte-Brasseur, J.; Frere, J.-M. *FEBS Lett.* **1997**, *413*, 194.
25. BS3 β-lactamase purification, crystallization, and data collection. The expression, purification and initial crystallization conditions of the BS3 enzyme (BS3-citrate) were described previously.[3,24] Typically, monoclinic crystals were obtained using the hanging drop vapor diffusion method with drops containing 5 μL of a protein solution (at a concentration of 40 mg/mL in 50 mM NaCl, 10 mM Tris buffer, pH 7.2) and 5 μL of 10% PEG 6000 in 100 mM sodium citrate buffer (pH 3.4), equilibrated against 1 mL of the latter solution at 20 °C. The BS3-isocitrate crystals were obtained in the same conditions, by replacing the citrate buffer by a 100 mM sodium isocitrate buffer at the same pH. The BS3-aminocitrate crystals were grown in drops containing 5 μL of a protein solution (at a concentration of 38 mg/mL in 50 mM NaCl, 10 mM Tris buffer, pH 7.2), 4 μL of 8% PEG 6000 in 100 mM sodium aminocitrate buffer (pH 3.4) plus 1 μL of 0.1 M urea additive, equilibrated against 1 mL of a 20% PEG 6000 solution, at 20 °C. X-ray diffraction experiments were carried out under cryogenic conditions (100 K) after transferring the crystals into a reservoir solution supplemented with 50% glycerol. The diffraction data for the BS3-isocitrate crystal were measured at ESRF (Grenoble, France) on the FIP-BM30a beamline (λ = 1.0 Å) using a MarResearch 165 mm CCD detector. Data for the BS3-aminocitrate crystal were collected with a Rigaku RU-200 rotating anode generator operating at 40 kV and 100 mA and a MarResearch Mar345 Imaging Plate (λ = 1.5418 Å). Intensities were indexed and integrated using MOSFLM version 6.01. The scaling of the intensity data was accomplished with SCALA of the CCP4 program suite and all corresponding statistics are available as Supplementary data. The atomic coordinates are available at the Protein Data Bank under the codes 1I2S (citrate), 1W7F (isocitrate) and 3B3X (aminocitrate).
26. Matagne, A.; Lamotte-Brasseur, J.; Frere, J.-M. *Biochem. J.* **1998**, *330*, 581.
27. One diastereoisomer, which configuration is (2*R*,3*S*), was found in the enzymic cavity: Heretsch, P.; Thomas, F.; Aurich, A.; Krautscheid, H.; Sicker, D.; Giannis, A. *Angew. Chem., Int. Ed.* **2008**, *47*, 1958.

28. Besong, G. E.; Bostock, J. M.; Stubbings, W.; Chopra, I.; Roper, D. I.; Lloyd, A. J.; Fishwick, C. W. G.; Johnson, A. P. *Angew. Chem., Int. Ed.* **2005**, *39*, 6403.
29. In the class C β-lactamase CMY2 of *Klebsiella pneumoniae* (β-lactamase structurally close to P99 enzyme), the citrate molecule seems to make a 180° rotation in the active site, in comparison with its position in the BS3 catalytic cleft (see Supplementary data). The atomic coordinates are available at the Protein Data Bank under the code 1ZC2.
30. (a) Brown, M. R.; Shankar, R.; Sallis, J. D. *J. Labelled. Compd. Radiopharm.* **1984**, *21*, 905; (b) Tsao, J. W.; Schoen, F. J.; Shankar, R.; Sallis, J. D.; Levy, R. J. *Biomaterials* **1988**, *9*, 393; (c) Shankar, R.; Crowden, S.; Sallis, J. D. *Atherosclerosis* **1984**, *52*, 191; (d) Shankar, R.; Brown, M. R.; Wong, L. K.; Sallis, J. D. *Experientia* **1984**, *40*, 265; (e) Yamamoto, H.; Koide, S.; Takayanagi, Y. PCT Int. Appl. WO 9635662, 1996.; (f) Takahashi, M. Jpn Kokai Tokkyo Koho JP 09059162, 1997.