Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 14 (2004) 3979–3983
Bioorganic & Medicinal Chemistry Letters

Aza-boronic acids as non-β-lactam inhibitors of AmpC-β-lactamase ☆

Valentina Buzzoni,[a] Jesus Blazquez,[b] Stefania Ferrari,[a] Samuele Calò,[a] Alberto Venturelli[a] and M. Paola Costi[a,*]

[a]*Dipartimento di Scienze Farmaceutiche, Università degli Studi di Modena e Reggio Emilia, Via Campi 183, 41100 Modena, Italy*
[b]*Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnologia (CSIC), Campus UAM. Cantoblanco, 28049 Madrid, Spain*

Received 26 December 2003; revised 15 May 2004; accepted 21 May 2004
Available online 19 June 2004

Abstract—With the aim of improving the ability of non-β-lactam inhibitors to inhibit AmpC-β-lactamase, a series of 3-aza-phenyl-boronic acid derivatives was obtained using in parallel synthesis. The molecules were tested against *Escherichia coli* AmpC-β-lactamase. The best inhibitors, 3-(2-hydroxy-naphthalen-1-ylazo)-phenyl-boronic acid (**12**) and 3-(2,4-dihydroxy-naphthalen-1-ylazo)-phenyl-boronic acid (**14**), showed apparent inhibition constant values (K_i) of 0.3 and 0.45 μM and increased the potency of the semi-synthetic cephalosporin antibiotic, ceftazidime, lowering its minimum inhibitory concentration (MIC) value of 50%, against Gram-negative bacteria strains, producing high levels of AmpC-β-lactamase.

1. Introduction

β-Lactam antibiotics inhibit bacterial cell wall synthesis and therefore the proliferation of microorganisms. The over-expression of β-lactamases is one of the most common and well-studied mechanisms of β-lactam antibiotic resistance.[1–3] β-Lactamases compete with penicillin binding proteins (PBP) in binding β-lactam antibiotics. β-Lactamases deactivate the β-lactam molecules by hydrolyzing the β-lactam ring, thus preventing the interaction of the drug with the PBPs. Different classes of β-lactamases are known. The most clinically important are class A β-lactamases, which include the plasmid-based TEM penicillinase, and class C β-lactamases, represented by cephalosporinases, such as AmpC-β-lactamase.[4–6] To overcome the action of these enzymes, medicinal chemists have introduced 'β-lactamase resistant' β-lactams (e.g. aztreonam) or β-lactam-based β-lactamase inhibitors (e.g. clavulanic acid and sulbactam) (Fig. 1). Among the β-lactamase inhibitors, clavulanic acid and sulbactam (Fig. 1) can inhibit class A β-lactamases, but are mostly ineffective against class C β-lactamases. Class C β-lactamases are present in Gram-negative microorganisms, such as *Enterobacter cloacae* and *Pseudomonas aeruginosa*, which cause serious health problems. The urgency to develop new

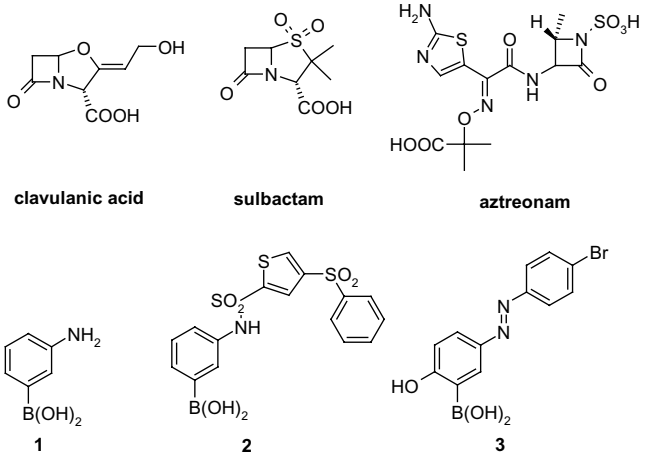


Figure 1. β-Lactamases inhibitors.

Keywords: AmpC-β-lactamase; Enzyme inhibitors; Phenyl-boronic acids; In parallel chemistry.
☆ Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2004.05.054
* Corresponding author. Tel.: +39-059-205-5134; fax: +39-059-205-5131; e-mail: costimp@unimore.it

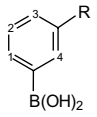
0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.05.054

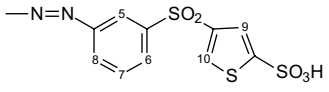
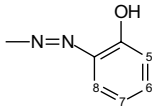
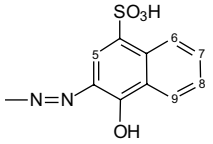
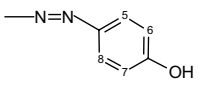
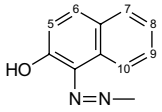
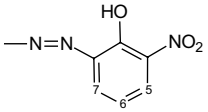
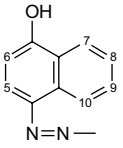
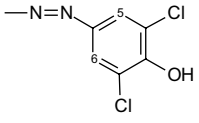
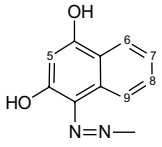
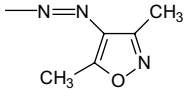
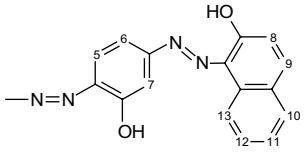
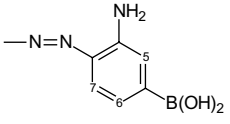
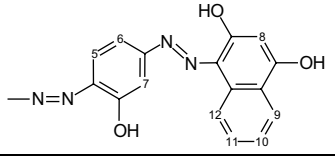
compounds that are able to inhibit class C β-lactamases[7,8] is clear.

In previous papers, the discovery of phenyl-boronic acid derivatives as new hits in the development of class C β-lactamases inhibitors, using a cycle of molecular modelling, synthetic chemistry and enzymatic assays[9,10] was described. These molecules act as transition state analogues competing with the β-lactam antibiotic and deactivating the enzyme.[11–15] Virtual screening of the commercially available boronic acid derivatives, using the DOCK 3.5 program, led to the identification of 3-amino-phenyl-boronic acid (**1**, Fig. 1), which shows a K_i of 7.3 μM against *Escherichia coli* AmpC-β-lactamase (βL).[10] This molecule was recognized to be a potential scaffold for new inhibitors: starting from this scaffold and using structure-based drug design approaches based on the *E. coli* AmpC-βL crystal structure, a library of 42 compounds was designed and synthesized.[16] The best compound, 3-(4-benzensulfonyl-thiophene-e-sulfonyl-amino)-phenylboronic acid (**2**, Fig. 1), showed a K_i of 0.08 μM, which is about one hundred-fold less than the K_i of the starting hit, **1**.[16] Further studies showed that some phenyl-aza derivatives, such as 5-(4-bromo-phenylazo)-2-hydroxy-phenyl-boronic acid (**3**, Fig. 1), inhibit *E. coli* AmpC-βL, with a K_i value of 3 μM (Shoichet B. K. personal communication).

With the aim of combining the 3-amino-phenyl-boronic acid feature with the phenyl-aza moiety, compound **1** was selected as the starting scaffold to design and synthesize a small library of 3-aza-phenyl-boronic acid derivatives. Visual inspection of the crystal structure of the complex *E. coli* AmpC-βL-**1** clearly suggested that a

Table 1. Apparent inhibition constants (K_i μM) of compounds **1**, **4**–**14**, **15a**, **16a** against *E. coli* AmpC-β-lactamase

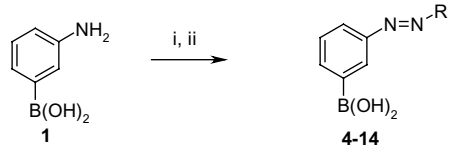


Compound	R	K_i (μM)	Compound	R	K_i (μM)
1	NH_2	7.3	**10**		2.0
4		3.5	**11**		5.0
5		0.7	**12**		0.3
6		1.5	**13**		0.9
7		3.0	**14**		0.45
8		1.0	**15a**		1.8
9		2.7	**16a**		0.7

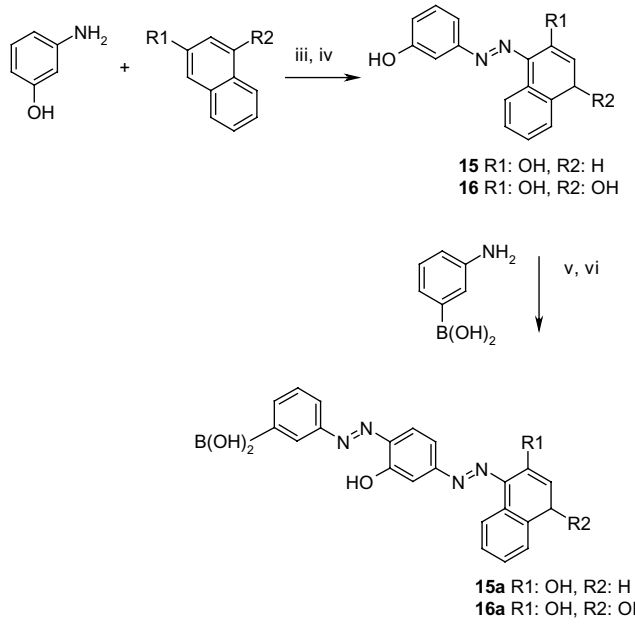
substituent in position 3 can fill the 'R1' pocket of the enzyme, which is an essentially unexplored binding site in the substrate binding domain, typically occupied by groups in position 6 of β-lactams.[11,14,16] Thirteen new compounds were obtained and tested against *E. coli* AmpC-βL. Some selected compounds were also tested against Gram-negative resistant bacteria: *E. coli* K12 AmpC-Ent, *E. coli* derepressed, *Enterobacter cloacae* derepressed 12991 ED, *Citrobacter freundii* derepressed 91/98-2, and *Pseudomonas aeruginosa* derepressed 88/98.

2. Chemistry

A small library of 13 aza-derivatives was synthesized using in parallel liquid phase chemistry (**4**–**14**, **15a**, **16a**)[17,18] (Table 1). The first library (**4**–**14**) was prepared starting from compound **1**, via diazotation of the amino group and further reaction with substituted aromatic rings (Scheme 1). Filtration under vacuum of the reaction mixtures provided compounds **12**–**14**, as highly pure material. Although the reaction was simple and quick, a column chromatography purification step was required to provide compounds **4**–**8**; for all the other compounds, liquid/liquid extraction was performed to provide highly pure compounds. The aza compounds **15** and **16** (Scheme 2) were prepared separately, starting from 3-amino-phenol via diazotation of the amino group in position 3, and further reaction with substituted aromatic rings. Subsequently, they were reacted with compound **1** to give compounds **15a**, **16a** (Scheme 2). The purity of all the synthesized compounds was determined by both TLC and elementary analyses. For all the compounds, structural characterization was achieved by nuclear magnetic resonance (^{1}H NMR). For a few compounds, UV spectra were also recorded (all the details may be found in supplementary materials).



Scheme 1. Reagents and conditions: (i) $NaNO_2$, HCl 5 N, 0 °C; (ii) R, NaOH 10%.



Scheme 2. Reagents and conditions: (iii) $NaNO_2$, HCl 5 N, 0 °C; (iv) R, NaOH 10%; (v) NaOH 10%; (vi) $NaNO_2$, HCl 5 N, 0 °C

3. Biological results and discussion

All the compounds were tested against *E. coli* AmpC-βL and apparent inhibition constant (K_i) values, in competition with the substrate cephalothin (Table 1), were obtained using spectrophotometric assay.[10] All the experiments were repeated at least three times and the standard error was under 20%. The K_i range of the newly synthesized compound was 0.3–5 μM and 6 out of 13 compounds were active in the sub-micromolar range (0.3–1 μM). The span for the K_i values is at least ten times. The naphthol derivatives, compounds **12** and **14** show the best inhibitory activity, with K_i values of 0.3 and 0.45 μM, respectively. Compound **13** shows an affinity that is three times lower, in comparison with the *ortho*-substituted compound **12** (K_i = 0.9 and 0.3 μM, respectively). Among the phenol derivatives, compound **5** is five times more potent, in comparison with the *ortho*-substituted compound **4** (0.7 μM, compared to 3.5 μM). A nitro group added in *ortho* to the hydroxyl of compound **4** (compound **6**), does not greatly modify the affinity of the molecule; on the contrary, two chlorine substituents on compound **5** (compound **7**), decrease the affinity fourfold. Among the naphthalene derivatives, the introduction of an acidic group, such as a sulfonylic group, was attempted in order to improve solubility in water, but only compound **11** was obtained. Unfortunately, the activity of compound **11** was about ten times lower than that of the other naphthalene derivatives (**12**–**14**). By combining the phenol and the naphthol moieties that proved to be the best (compounds **12** and **14**), compounds **15a** and **16a** were obtained. They showed K_i values lower than 2 μM, thus not seriously improving the affinity in comparison with the starting molecule **4**, (K_i = 3.5 μM). Compound **10** shows the same affinity for the enzyme as the mono-phenol derivatives. These results demonstrate that the active site is accessible to bulky groups, so a three-ring system can be allocated to the enzyme pocket, but the compounds are not as active as expected, based on simple synergistic considerations. It seems that the rigidity of the aza-bridge does not allow good adaptation to the enzyme binding site, as observed in a previous paper, where a new class of 3-amino-phenyl-boronic acid, with a sulfonamide and carbamide bridge, was synthesized.[16] Also in that case, the more rigid carbamide bridge inferred lower affinity in comparison with the highly flexible sulfonamide bridge. It seems that the flexible fragments

linked to the core skeleton are necessary for better molecular recognition, which requires the adaptation of the ligand to the enzyme.

For further studies, we considered compounds **12** and **14**, which showed the lowest K_i values. The minimum inhibitory concentration (MIC) of ceftazidime (CeftAZ), in the presence and absence of compounds **12** or **14**, was determined,[19] against several Gram-negative resistant clinical isolates that show an AmpC-derepressed phenotype, *E. coli* derepressed, *E. cloacae* derepressed 12991 ED, *C. freundii* derepressed 91/98-2, and *P. aeruginosa* derepressed 88/98, and the laboratory engineered *E. coli* K12 AmpC-Ent, which expresses AmpC from *E. cloacae* cloned in a multi-copy plasmid (Table 2, Fig. 2). The compounds were only weakly active against the Gram-negative bacteria. Compounds **12** and **14** lowered the CeftAZ MIC values by 50% against *E. coli* der. and *P. aureuginosa* der. 88/98. Although a reduction of this level is generally not considered significant, this result was obtained consistently in different experiments. Only compound **12** decreases the CeftAZ MIC value fourfold against *C. freundii* der. 91/98-2, thus the association of the antibiotic CeftAZ with these boronic compounds in vivo seems to strengthen the activity of the antibiotic alone.

Visual inspection[20] of the three-dimensional active site of *E. coli* AmpC-βL allowed us to understand how compounds **4**–**14**, **15a** and **16a** can interact with the protein and form some structure–activity relationships. The crystallographic structure of *E. coli* AmpC-βL in complex with compound **2**[16] was considered (Fig. 3). The ligand adopts a jack-knife structure and forms most of the interactions through the 3-sulfonyl-amino-phenyl-boronic acid fragment of the molecule. In particular, the borine forms a dative covalent bond with Ser64, the two hydroxyls of the boronic acid group are involved in hydrogen bonds with the main chain of Ser64, Ala318 and the side chain of Tyr150. The phenyl ring forms a dipole–quadrupole interaction with Asn152, while the sulfonamide nitrogen forms a dipole–quadrupole interaction with Tyr221 and the sulfonamide oxygen interacts with Tyr221 and Ser212. Moreover, four water molecules, one of which is highly conserved, contributes to the stabilization of this part of the complex. The distal part of the molecule forms a hydrogen bond with a water molecule through one sulfone oxygen molecule, whereas the phenyl ring interacts with Leu119 and Leu293.[16] This molecule shows a K_i value of 0.08 μM,[16] about four times lower than the best compound studied in this work, which is compound **12**.

Table 2. Minimum inhibitory concentration (MIC) (μg/mL) of ceftazidime (CeftAZ) for compounds **12** and **14**

	CeftAZ	CeftAZ/**12** (1/1)	CeftAZ/**14** (1/1)
E. coli K12 AmpC-Ent	64	64	64
E. coli derepressed	128	64	64
E. cloacae derepressed 12991 ED	256	256	256
C. freundii derepressed 91/98-2	64	16	64
P. aeruginosa derepressed 88/98	128	64	64

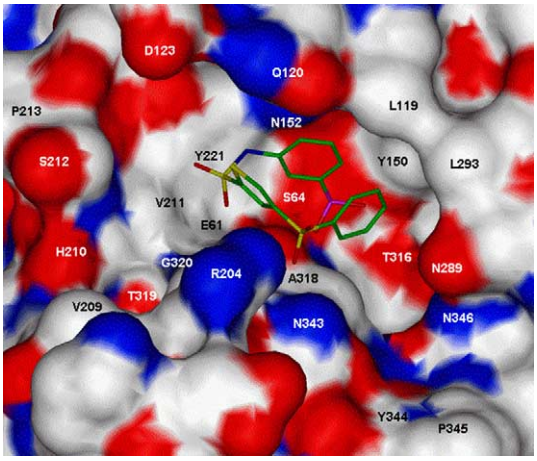


Figure 3. Surface of the active site of *E. coli* AmpC-βL with compound **2** bound.

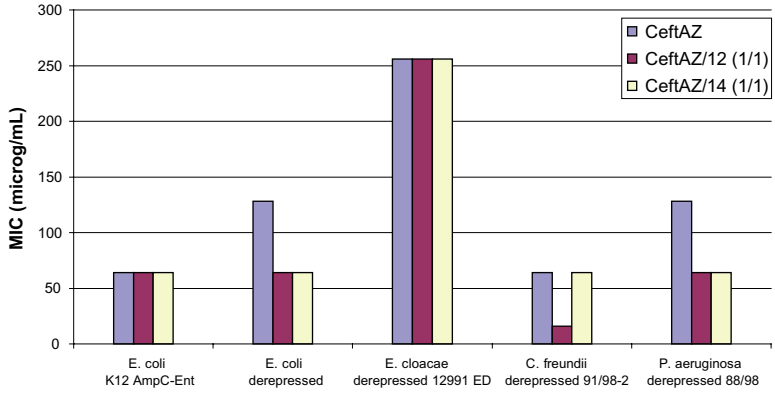


Figure 2. Minimum inhibitory concentration (MIC) (μg/mL) of ceftazidime (CeftAZ) for compounds **12** and **14**. The compounds were dissolved in 50% DMSO and dilutions were performed using growth medium. An adequate final concentration in which to determine the minimum inhibitory concentration (MIC) was obtained where the concentration of DMSO was maintained below 5%.

Compounds **4**–**14**, **15a** and **16a** were drawn three-dimensionally using InsightII:[20] their phenyl boronic acid moieties were rigidly superimposed on the same moiety as compound **2**, while the aza-substituents of the molecules were manually fitted inside the binding site of *E. coli* AmpC-βL. The aza bridge of these molecules imposes a rigid planar conformation, which is more difficult to allocate to the active site of the enzyme. Moreover, these compounds, lacking the sulfonamide bridge, do not interact with Tyr221 and Ser212. However, it is reasonable to consider that these molecules conserve the interaction using the boronic acid group, but in order to accommodate the aza moiety, the C–B bond of compounds **4**–**9**, **11**–**14** has to rotate by about 31° so that the second ring interacts with His210, Val211, Glu61 and Gly320. In this binding mode, the distance between Asn152 and the first phenyl ring increases. As a consequence, the dipole–quadrupole interaction is weaker. In this binding mode, the enzyme has to slightly change its conformation in the region of Val211 and Glu61.

In order to better accommodate these compounds, and also compounds **10**, **15a** and **16a**, which have a bulkier side chain, a second binding mode should be considered. In this case, the C–B bond should be rotated by about 106°, in order to locate the side chain in the region of Leu119, Leu293 and Asn289. In this case, only the boronic acid group conserves the interactions with Ser64, Ala318 and Tyr150, whereas the first phenyl ring interacts with Asn152.

On the basis of the two binding modes proposed, we can derive some general structure–activity relationships (SAR). A hydroxyl group in *para* on the second aromatic ring (compounds **13**, **5** and **7**) suggests the first binding mode in which the hydroxyl group could form a hydrogen bond with the backbone of His210 and Val211. In the case of compound **7**, showing a threefold lower K_i value in comparison with compound **5**, the hindrance of the two chlorine atoms in *meta*-positions cause the ligand to move slightly, resulting in new interactions of the hydroxyl group with His210 and Val211. A hydroxyl group in *ortho* (compounds **12**, **14**, **4**, **6**) suggests the second binding mode in which the hydroxyl group could form a hydrogen bond with Asn289. The one order difference in the K_i between compounds **4** and **12** could be due to nonspecific interactions with the enzyme, which are difficult to describe using the current model. In the case of compound **6**, the presence of the nitro group in *meta*-position causes the ligand to move slightly, and so affecting the interaction of the hydroxyl group with Asn289. A substituent in *meta*, such as the sulfonyl acid group of compound **11**, could also interact in the second binding mode with Asn289, but owing to its larger dimensions, it is reasonable that the ligand moves a little in order to avoid bad contacts, making the interactions with L119 and L293 weaker.

Acknowledgements

We would like to thank Dr. Tondi and Dr. Brian K. Shoichet (UCSF, San Francisco, USA) for reading the manuscript and Ely Lilly, Indianapolis for their financial support and NIH GM63815 B.K.S. grant. We also thank Dr. Stefano Ghelli for his help with the NMR experiments, R. Gallesi of the Microanalyses Laboratory, Centro Interdipartimentale Grandi Strumenti (CIGS) of Modena University and Centro Interdipartimentale di Calcolo Automatico ed Informatica Applicata (CICAIA) of Modena University.

References and notes

1. Hide, B. *ASM News* **1994**, *60*, 5.
2. Neu, H. C. *Science* **1992**, *257*, 1064.
3. Gerard, D. *Chem. Biol.* **2000**, *7*, 127.
4. Bush, K.; Jacoby, G. A.; Medeiros, A. A. *Antimicrob. Agents Chemother.* **1995**, *39*, 1211.
5. Pares, S.; Mouz, N.; Petillot, Y.; Hakenbeck, R.; Dideberg, O. *Nat. Struct. Biol.* **1996**, *3*, 284.
6. Anderson, J. W.; Pratt, R. F. *Biochemistry* **2000**, *39*, 12200.
7. Amicosante, G.; Felici, A.; Segatore, B.; Di Marzio, L.; Franceschini, N.; Di Girolomo, M. *J. Chemother.* **1989**, *1*, 394.
8. Dryjanski, M.; Pratt, R. F. *Biochemistry* **1995**, *34*, 3561.
9. Beesley, T.; Gascoyne, N.; Knott-Hunziker, V.; Petursson, S.; Waley, S. G.; Jaurin, B.; Grundstrom, T. *Biochem. J.* **1983**, *209*, 229.
10. Weston, G. S.; Blazquez, J.; Baquero, F.; Shoichet, B. K. *J. Med. Chem.* **1998**, *4*, 4577.
11. Lindquist, R. N.; Terry, C. *Arch. Biochem. Biophys.* **1974**, *160*, 135.
12. Bone, R.; Shenvi, A. B.; Kettner, C. A.; Agard, D. A. *Biochemistry* **1987**, *26*, 7609.
13. Sutton, L. D.; Stout, J. S.; Hosie, L.; Spencer, P. S.; Quinn, D. *Biochem. Biophys. Res. Commun.* **1986**, *134*, 386.
14. Usher, K. C.; Blaszczak, L. C.; Weston, G. S.; Shoichet, B. K.; Remington, S. J. *Biochemistry* **1998**, *37*, 16082.
15. Powers, R. A.; Blazquez, J.; Weston, G. S.; Morosini, M. I.; Baquero, F.; Shoichet, B. K. *Protein Sci.* **1999**, *8*, 2330.
16. Tondi, D.; Powers, R. A.; Caselli, E.; Negri, M. C.; Blazquez, J.; Costi, M. P.; Shoichet, B. K. *Chem. Biol.* **2001**, *8*, 593.
17. Sugimoto, K.; Ohki, S. *Chem. Pharm. Bull.* **1964**, *12*, 1375.
18. Hackmann, E.; Carvalho Ferreira, P. *Boll. Chim. Farm.* **1975**, *114*, 501.
19. *National Committee for Clinical Laboratory Standards*, Villanova, PA, 1997.
20. InsighII. http://www.accelrys.com., 2000.