Mechanism of Inactivation of β-Lactamases by Novel 6-Methylidene Penems Elucidated Using Electrospray Ionization Mass Spectrometry

Keiko Tabei,[†] Xidong Feng,[†] Aranapakam M. Venkatesan,[‡] Takao Abe,[§] Ushirogochi Hideki,[§] Tarek S. Mansour,[‡] and Marshall M. Siegel*,[†]

Wyeth Research, Chemical and Screening Sciences, Chemical Technologies and Medicinal Chemistry, Pearl River, New York 10965, and Wyeth KK, Medical Research Laboratories, Saitama, Japan

Received February 3, 2004

The reactions of 6-methylidene penems **4**−**7** with β-lactamases (TEM-1, SHV-1, Amp-C) were characterized by electrospray ionization mass spectrometry (ESI-MS). The kinetics of the reactions were monitored, demonstrating that only one penem molecule reacts to form an acyl−enzyme complex. For penem **5**, the ESI-MS/MS spectrum of the hydrolysis product produced in the reaction was identical to the spectrum generated from a synthesized dihydro[1,4]-thiazepine **10**, confirming the rearrangement of the penem ring system to a seven-membered dihydro[1,4]thiazepine structure. Gas-phase ESI-MS/MS fragmentation data were rationalized due to tautomerization between imine and enamine substructures. ESI-MS/MS analysis of the T-6 trypsin-digested fragments of TEM-1 and SHV-1 demonstrated that the penems were only attached to Ser-70 of these class A β-lactamases and that the penem ring structures were rearranged to seven-membered dihydro[1,4]thiazepines.

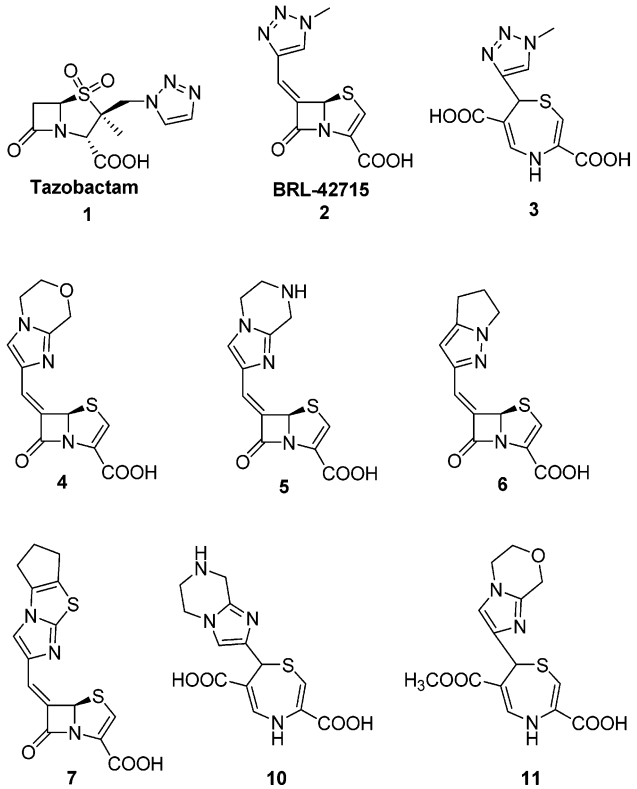
Introduction

An effective strategy to overcome the resistance of β-lactamase-containing pathogens to β-lactam antibiotics is to prescribe a combination of the β-lactam antibiotic together with a β-lactamase inhibitor.[1] The inhibitor selectively reacts quickly to inactivate the β-lactamase, leaving the β-lactam antibiotic free to perform its antibacterial function.

Several reviews of the mechanisms of action for the major β-lactamase inhibitor classes have been published recently.[2,3] The inhibition mechanism of class A β-lactamases by β-lactam inhibitors follows a general pattern. A transient acyl−enzyme complex is formed that subsequently undergoes rearrangement and degradation of the inhibitor portion of the complex followed by the release of low molecular weight degradation products. This occurs with inhibitors such as clavulanic acid and sulbactam. The use of mass spectrometric techniques to elucidate mechanistic aspects of biological events is receiving widespread attention.[4−6] These techniques are useful since they can simultaneously measure the masses of numerous products accurately, rapidly, and with great sensitivity, thus enabling the possibility for understanding the nature of intermediates and transformations in biological events. Our interest in new inhibitors[7] and the understanding of the mechanisms of inactivation of β-lactamases with β-lactam-based inhibitors prompted us to elucidate the mechanism of inactivation of PCI and TEM-1 (class A) β-lactamases by tazobactam (**1**) (Chart 1) using electrospray ionization mass spectrometry.[8] Similarly, Bonomo et al. applied HPLC-ESI-MS/MS analyses in their mechanistic studies of tazobactam with other β-lactamases.[9,10]

A new class of broad-spectrum penem inhibitors was recently disclosed.[11−15] The most widely mechanistically studied member of this class was BRL 42715 (**2**) which

Chart 1. Structures of Various Penem Based β-Lactamase Inhibitors and Rearranged Seven-Membered Dihydro[1,4]thiazepine Ring Products



* To whom correspondence should be addressed: Wyeth Research, 401 N. Middletown Rd., Building 222/Room 1043, Pearl River, NY 10965. Tel: 845-602-3417; fax: 845-602-2969; e-mail: siegelm@wyeth.com.

[†] Chemical Technologies, Chemical and Screening Sciences, Wyeth Research.

[‡] Medicinal Chemistry, Chemical and Screening Sciences, Wyeth Research.

[§] Medical Research Laboratories, Wyeth KK.

10.1021/jm049903j CCC: $27.50
Published on Web 05/28/2004

Table 1. In Vitro Activity [IC_{50} (nM)] of Tazobactam **1** and Penems **4a**−**7a** against Different Class A (TEM-1, Imi-1), Class B (CcrA), and Class C (Amp-C) β-Lactamases

compound	TEM-1 (IC_{50}, nM)	Imi-1 (IC_{50}, nM)	CcrA (IC_{50}, nM)	Amp-C (IC_{50}, nM)
Tazobactam, **1**	100 ± 8	30 ± 2	400000 ± 200	84000 ± 300
4a	0.4 ± 0.3	8 ± 2	66 ± 4	5.0 ± 1
5a	5.0 ± 1.0	28 ± 4	320 ± 6	6.0 ± 2
6a	1.0 ± 0.2	50 ± 3	14 ± 3	2.0 ± 1
7a	1.0 ± 0.3	72 ± 4	240 ± 10	2.0 ± 1

was found to form an acyl−enzyme complex with class A,[11−15] C,[13,15] and D[11] β-lactamases without the formation of decomposition products.[12−15] Broom et al.[15] isolated the BRL 42715 hydrolysis product when excess penem (**2**) was incubated over an extended period of time in the presence of K1 β-lactamase (class A). Using NMR data, they demonstrated that the penem five-membered thiazole ring system opened up upon hydrolysis at the C5−S bond and rearranged via a Michael addition to form a seven-membered dihydro[1,4]thiazepine ring system (**3**). Even though BRL 42715 is a potent broad-spectrum β-lactamase inhibitor, it was not developed commercially due to its chemical instability and short half-life in humans.

Recently, we have disclosed a number of 6-methylidene penem derivatives, including penems **4**−**7** bearing bicyclic and tricyclic heterocyclic moieties, that were shown to be broad-spectrum β-lactamase inhibitors.[16] Examination of the IC_{50} values, listed in Table 1, reveals that these classes of compounds are potent inhibitors of class A (TEM-1, Imi-1), class B (CcrA), and class C (Amp-C) β-lactamases and are significantly more potent than tazobactam **1**. High-resolution X-ray crystallographic structures of **4** with SHV-1 (class A) and GC1 (class C) β-lactamases revealed the formation of ring-expanded seven-membered dihydro[1,4]thiazepine in both enzymes with clear insight into the stereochemistry of the C7 carbon moiety.[17]

Electrospray ionization mass spectrometry is a useful technique for the analysis of penem inhibitors, including BRL 42715[12] (bearing a monocyclic 1,2,3-triazolyl moiety on the 6-methylidene linkage) but has never before been applied extensively. In the following studies, the mechanism of action of three penems **4**−**6** (bearing bicyclic heterocyclic moieties on the 6-methylidene linkages) and one penem **7** (bearing a tricyclic heterocyclic moiety on the 6-methylidene linkage) have been evaluated using electrospray mass spectrometry to demonstrate and confirm the formation of the seven-membered dihydro[1,4]thiazepine ring system and that Ser-70 is the site of attachment of the acyl−enzyme complex in class A enzymes. The choice of three bicyclic penems **4**−**6** with different heterocyclic moieties was made to eliminate possible complications due to fragmentation in this portion of the rearranged product whereas the choice of the tricyclic penem **7** provides the chemical diversity needed to establish the scope of the techniques employed in this work. Excess penems were reacted with different β-lactamases to produce the penem hydrolysis products. The ESI-MS/MS spectra for the hydrolyzed products and synthesized dihydro[1,4]thiazepines were compared to confirm the identity of the rearranged hydrolyzed products. Nanoelectrospray-MS and capillary-HPLC-ESI-MS spectra of the trypsin-digested acyl−enzyme complexes were used to identify the digested fragment containing the penem complexes. In addition, nanoelectrospray-MS/MS and capillary HPLC-ESI-MS/MS were used to determine the binding site of penem attachment and elucidate the structures of the inactivated penems. ESI-FT-MS and ESI-FT-MS/MS high-resolution exact-mass data were also used to determine the elemental formulas for the observed parent and fragment ions and for elucidating the fragmentation pathways.

Chemistry

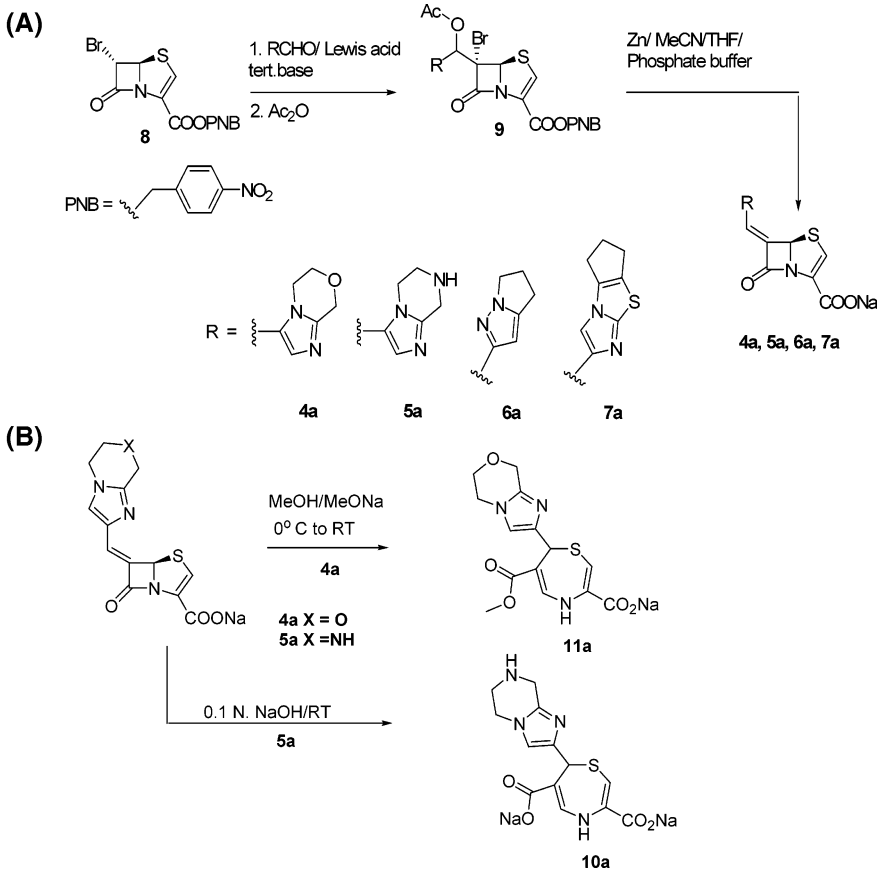
Penems **4a**−**7a** were synthesized by condensing a bromopenem **8** with the appropriate aldehydes, as depicted in Scheme 1A, in the presence of a tertiary amine and a Lewis acid. The resultant bromoacetates **9** were converted to desired penems **4a**−**7a** by a reductive elimination procedure.[18] The 6-methyl ester of the seven-membered dihydro[1,4]thiazepine ring system **11a** was prepared by reacting penem **4a** with sodium methoxide in the presence of anhydrous methanol, as illustrated in Scheme 1B. The seven-membered dihydro-[1,4]thiazepine ring compound **10a** was prepared by hydrolysis of **5a** in the presence of sodium hydroxide, at room temperature for 1 h (Scheme 1B).

Results

ESI-MS Time Course Studies of 5 with TEM-1 β-Lactamase. Penem **5** (MW 304) was incubated with TEM-1 class A β-lactamase (MW 28 907) at a molar ratio of 2:1 and analyzed during the time course of the reaction by ESI-MS. The transformed ESI mass spectra acquired for the high mass region are illustrated in Figures 1A-D for pure TEM-1 and after 5 min, 60 min, and 24 h, respectively. (The standard deviation in the measured TEM-1 masses was 3 Da.) The measured molecular weight for TEM-1 was 28 903 Da (Figure 1A). At 5 min into the reaction, a single product with molecular weight of 29 210 Da was observed (Figure 1B) corresponding to the addition of 307 Da to TEM-1. This mass difference is consistent with the covalent addition of one molecule of **5** to one TEM-1 enzyme since the ESI-MS experiments were conducted under protein denaturing conditions. After 60 min into the reaction (Figure 1C), besides the dominating covalent addition product with MW 29 210, a low abundance peak appears with MW 28 903, corresponding to regenerated TEM-1, which then dominates the mass spectrum after 24 h into the reaction (Figure 1D). All the lower abundance peaks in the transformed ESI mass spectra ∼41 Da greater than the above-indicated masses correspond to acetonitrile adducts of TEM-1 or TEM-1 conjugated with **5**.

The activity of TEM-1 can be estimated using the mass spectral data from the abundance of the TEM-1 peak relative to that of the sum of the abundances of

Scheme 1. (A) Synthesis of Penems **4a**−**7a**. (B) Synthesis of **10a** and **11**



the TEM-1 peak and its complex with **5**. The activity of TEM-1 calculated from the mass spectral data was initially 100% but rapidly declined to 0% at 5 min and gradually rose to 9% after 60 min and nearly recovered after 24 h to 85%. These mass spectral results were consistent with a biochemical assay for TEM-1 activity, viz., 2.5% after 5 min, 1% after 60 min, and 90% recovery after 24 h.

Penem **5** was also incubated with the class C β-lactamase Amp-C (molar ratio 2:1) and the time course of the reaction was monitored by ESI-MS. The ESI-MS measured molecular weight for Amp-C was 39 383 Da. The reaction products were sampled after 5 min, 1.25, 25, and 46 h, and the only detectable product in each experiment had a molecular weight of 39 688 Da, corresponding to the addition of one molecule of **5** to Amp-C. No low mass hydrolysis products of **5** from the covalent complex of **5** with the class C β-lactamase Amp-C appeared in the mass spectra.

A complimentary method for monitoring the reaction of **5** (MW 304) incubated with TEM-1 is to follow by ESI-MS the production of low mass product ions as a function of time. These experiments were conducted with an excess of inhibitor relative to β-lactamase, viz., **5**:TEM-1 molar ratio of 10:1. The ESI mass spectra (positive ion mode) acquired for the low mass region are illustrated in Figures 2 A-F for pure TEM-1 and after 1 min, 30 min, 1.5 h, 3.5 h, and 22 h, respectively. With increasing time, the relative abundance of the protonated molecular ion of **5**, *m*/*z* 305, decreases while the abundance of the ion at *m*/*z* 323 increases. The low mass ESI results correlate well with the high mass results in that the concentration of **5** initially decreases with time as it covalently binds to the TEM-1 β-lactamase, but with increasing time the molecule dissociates from TEM-1 with a mass 18 Da higher. The exact mass of the later ion was measured by high-resolution FT-MS and found to be *m*/*z* 323.08123 corresponding to the elemental formula $C_{13}H_{15}N_4O_4S$ (Δ = 0.38 mDa), consistent with the addition of water to **5**. These ESI mass spectral results demonstrate that **5** forms a transient

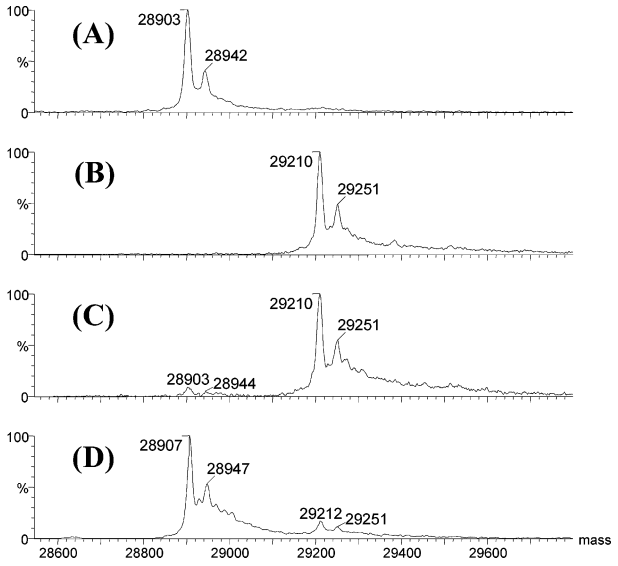


Figure 1. Transformed ESI mass spectra (high mass range, 28 540−29 800 Da) of **5** (MW 304) incubated with TEM-1 β-lactamase (MW 28 907) at a molar ratio of 2:1 and analyzed during the time course of the reaction (A) for pure TEM-1, (B) after 5 min, (C) after 60 min, and, (D) after 24 h.

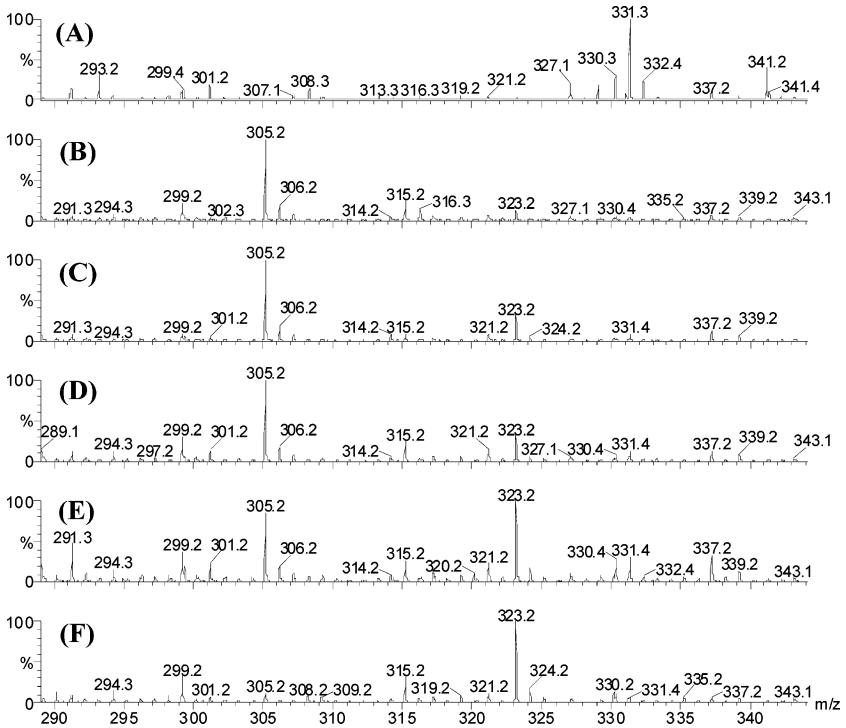


Figure 2. ESI mass spectra (low *m*/*z* range, *m*/*z* 290 to *m*/*z* 345) of **5** (MW 304) incubated with TEM-1 β-lactamase (MW 28 907) at a molar ratio of 10:1 and analyzed during the time course of the reaction (A) for pure TEM-1, (B) after 1 min, (C) after 30 min, (D) after 1.5 h, (E) after 3.5 h, and (F) after 22 h.

acyl−enzyme complex with TEM-1 β-lactamase which eventually is dissociated from the enzyme as a hydrolyzed product.

ESI-MS/MS Studies of Enyzmatically and Synthetically Produced Hydrolysis Products of 5 and 11. The hydrolysis product of **5**, produced after incubation of **5** with TEM-1 (10:1 molar ratio) for 22 h (*m*/*z* 323, Figure 2F), was analyzed by ESI-MS/MS under low-resolution conditions (Figure 3A) at neutral pH (conditions under which the residual penem does not hydrolyze). Likewise, a synthetic version of the seven-membered dihydro[1,4]thiazepine ring compound **10** was prepared by hydrolysis of **5** in the presence of sodium hydroxide, incubated at room temperature for 1 h, and analyzed under ESI-FT-MS/MS high-resolution exact-mass conditions (Figure 3B). The resulting spectra correlate extremely well, indicating that the enzymatically generated component is identical to that of **10**, the synthetic seven-membered dihydro[1,4]thiazepine ring system of hydrolyzed **5**. Table 2A lists the observed exact masses and elemental formulas for the fragment ions generated from **10**. The proposed fragmentation scheme for **10** is illustrated in Scheme 2A. A number of prominent features are observed in the fragmentation of the molecule besides the small molecule neutral losses of water and CO_2. The most notable is that all fragmentations are confined to the seven-membered ring system and that the side chain heterocyclic group does not undergo any fragmentation, serving principally as the charge source for all the observed fragment ions except for the ion at *m*/*z* 130. As a corollary, nearly all the fragment ions can be rationalized as originating from pairs of single bond cleavages of the seven-membered ring system. For this to occur, the double bonds of the seven-membered ring system have to tautomerize under the higher energy gas-phase ESI-MS/MS conditions to a variety of equilibrium substructures. A variety of enamine/imine tautomeric substructures, **12** and **13**, can be generated from **10**, centered on both sides of the seven-membered ring nitrogen atom. An enamine double bond could tautomerize between the R group and carboxylic acid stabilizing the system via conjugation. These rationalizations explain the fragmentations observed in the gas phase. However, condensed phase hydrogen/deuterium exchange experiments with the synthetic seven-membered dihydro[1,4]-thiazepine structure of **5**, when analyzed by ESI-MS and ESI-MS/MS, exhibited four exchangeable sites, consistent with that of a structure that does not undergo readily proton-shift tautomerism in the condensed phase and favors the enamine structure. Up to three additional exchangeable hydrogen atoms would have been observed in the condensed phase if the enamine/imine structures rapidly exchanged. These ESI-MS and ESI-MS/MS results are consistent with the seven-membered ring structures from previously reported NMR[15] and X-ray[17] data.

Figure 3C illustrates the low mass region of the ESI-MS/MS mass spectrum of the TEM-1 T-6 tryptic fragment covalently bound to penem **5**. (For discussion, vide infra.) All the *masses* of the starred (*) ions in Figure 3C are listed in Table 4 [column labeled **5**+H_2O **(10)**] and correlate with the masses generated by ESI-FT-MS/MS for **10** (Figure 3B, Table 2A) demonstrate that the penem ring system of **5** hydrolyzed and rearranged to a dihydro[1,4]thiazepine ring system as in **10**. To further model the fragmentation of the seven-membered dihydro[1,4]thiazepine ring system when covalently attached to Ser-70 of a β-lactamase, as reported in the X-ray data,[17] a 6-methyl ester thiazepine derivative of **4** was prepared, viz., structure **11**. The ESI-FT-MS/MS spectrum of **11** is illustrated in Figure 3D, and the

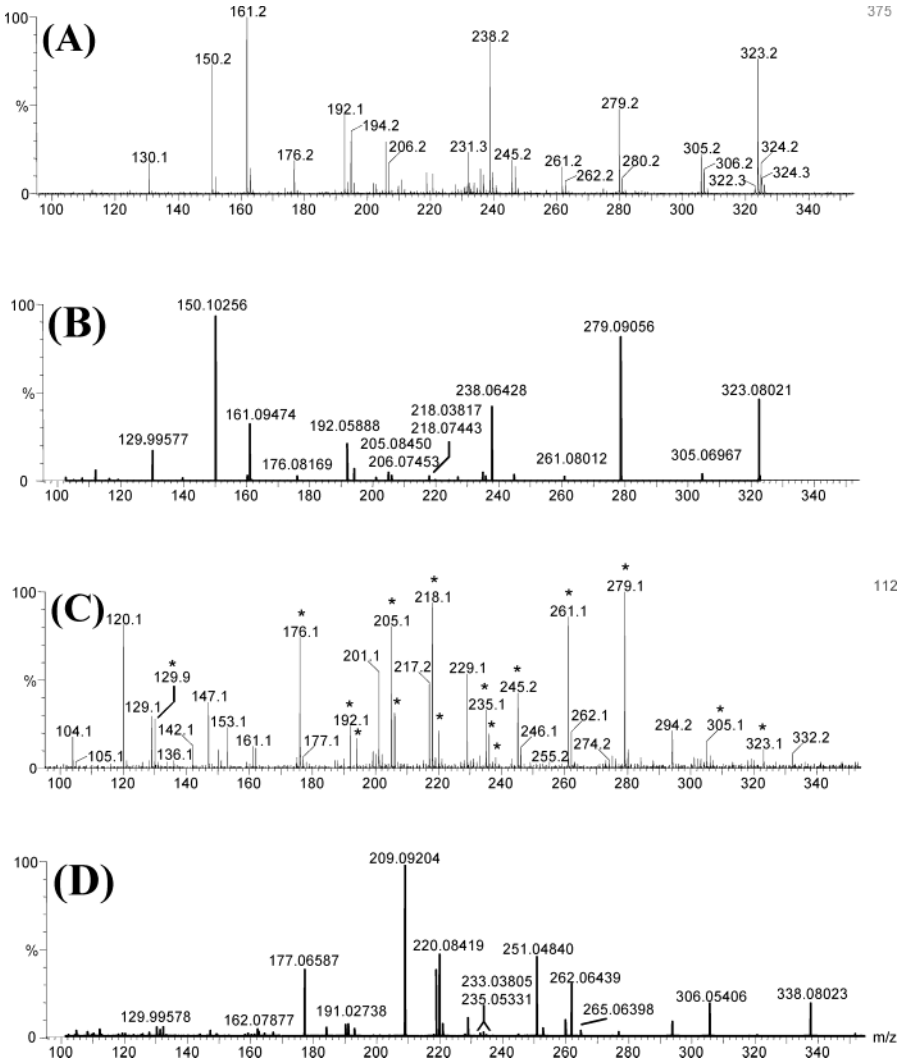


Figure 3. Mass spectra of the hydrolysis product of **5** (MW 322) produced under a variety of conditions. (A) ESI-MS/MS spectrum of *m*/*z* 323 generated during the incubation of **5** (MW 304) with TEM-1 β-lactamase (MW 28 907) at a molar ratio of 10:1 after 22 h. (Refer to Figure 2F for the spectrum containing the parent ion *m*/*z* 323.) (B) ESI-FT-MS/MS high-resolution exact-mass spectrum of *m*/*z* 323 generated from the synthetically prepared base hydrolysis product of **5** containing the seven-membered dihydro[1,4]-thiazepine ring system, **10** (MW 322). (C) ESI-MS/MS spectrum (low mass range *m*/*z* 100 to *m*/*z* 350) of the doubly charged ion, *m*/*z* 646.8, corresponding to the covalent addition of **5** to the T-6 trypsin-digested fragment of TEM-1 β-lactamase. Ions related only to the dissociated hydrolyzed **5** are labeled with stars (*). (D) ESI-FT-MS/MS high-resolution exact-mass spectrum of *m*/*z* 338 generated from the synthetically prepared methyl ester hydrolysis product of **4** containing the seven-membered dihydro[1,4]-thiazepine ring system, **11** (MW 337).

observed exact masses are listed in Table 2B. The fragmentation processes observed for **11** (Scheme 2B) are dominated by pairs of single bond cleavages of the dihydro[1,4]thiazepine ring system, losses of methanol from the methyl ester, and no fragmentation from the side chain heterocyclic group, observations consistent with the formation of multiple gas-phase enamine/imine tautomeric structures, **14** and **15**, as reported above for structure **10**.

ESI-FT-MS/MS Studies of 6-Methylidene Penems. The ESI-FT-MS/MS high-resolution exact-mass spectra of the β-lactamase inhibitors **4** (MW 305), **5** (MW 304), **6** (MW 289), and **7** (MW 345) are illustrated in Figure 4 and the exact-mass results listed in Table 3. The proposed fragmentation processes, illustrated in Scheme 3, are consistent with the elemental formulas calculated from the exact masses. All the 6-methylidene penem compounds underwent neutral losses of CO_2, β-lactam ring cleavages via a retro-Diels−Alder reaction with charge retention on the ketene side containing the basic R groups of high abundance and with lower abundance reaction products with charge retention on the thiazole ring. No fragmentations related to the bicyclic and tricyclic ring systems (R groups) were detected. The observed fragmentation processes for the 6-methylidene penems are consistent with those previously reported for β-lactam[19] and penem β-lactam[20,21] antibiotics. Note, however, the significant differences between these penem fragmentation processes and those reported above for the seven-membered dihydro-[1,4]thiazepine **10**.

Determination of the Binding Site of 6-Methylidene Penems to β-Lactamases. A variety of 6-methylidene penems were incubated individually with different β-lactamases. The products were digested in the presence of trypsin and analyzed directly by nanoelectrospray-MS or by capillary HPLC-ESI-MS. Likewise, the pure β-lactamases were digested with trypsin

Table 2. ESI-FT-MS/MS Observed Exact Masses, Calculated Elemental Formulas, and Proposed Structural Assignments for the Fragment Ions Generated from the Protonated Molecular Ions of (A) **10**, the Synthetic Seven-Membered Dihydro[1,4]thiazepine Ring System of Hydrolyzed **5**, and (B) **11**, the 6-Methyl Ester of the Synthetic Seven-Membered Dihydro[1,4]thiazepine Ring System of Hydrolyzed **4**

A

exp mass	pred mass	Δ (mmu)	Δ (ppm)	rel abund (%)	elemental formula[a]	structural assignments
323.08021	323.08085	−0.64	−1.98	48	$C_{13}H_{14}N_4O_4SB$	M + H (M = penem + H_2O)
305.06967	305.07029	−0.62	−2.03	5	$C_{13}H_{12}N_4O_3SB$	M + H − H_2O
279.09056	279.09102	−0.46	−1.65	84	$C_{12}H_{14}N_4O_2SB$	M + H − CO_2
261.08012	261.08046	−0.34	−1.30	3	$C_{12}H_{12}N_4OSB$	M + H − CO_2 − H_2O
245.10310	245.10330	−0.20	−0.82	4	$C_{12}H_{12}N_4O_2B$	M + H − CO_2 − H_2S
238.06428	238.06447	−0.19	−0.80	45	$C_{10}H_{11}N_3O_2SB$	M + H − CH=C(CO_2H)NH (85)
236.04855	236.04882	−0.27	−1.14	4	$C_{10}H_9N_3O_2SB$	M + H − CH_2=C(CO_2H)NH_2 (87)
235.10105	235.10119	−0.14	−0.60	6	$C_{11}H_{14}N_4SB$	M + H − 2CO_2
220.05370	220.05391	−0.21	−0.95	1	$C_{10}H_9N_3OSB$	M + H − CH=C(CO_2H)NH (85) − H_2O
218.07443	218.07464	−0.21	−0.96	4	$C_{11}H_{11}N_3SB$	M + H − 2CO_2 − NH_3
218.03817	218.03826	−0.09	−0.41	3	$C_{10}H7N_3OSB$	M + H − CH_2=C(CO_2H)NH_2 (87) − H_2O
206.07453	206.07464	−0.11	−0.53	4	$C_{10}H_{11}N_3SB$	M + H − HN=CHCO_2H (73) − CO_2
205.08450	205.08458	−0.08	−0.39	5	$C_{10}H_{10}N_3O_2B$	M + H − ·SCH=C(CO_2H)NH_2 (118)
194.09222	194.09240	−0.18	−0.93	6	$C_9H_{11}N_3O_2B$	M + H − SCH=C(CO_2H)N=CH (129)
194.07464	194.07464	0.00	0.00	8	$C_9H_{11}N_3SB$	M + H − CH=C(CO_2H)NH (85) − CO_2
192.05888	192.05899	−0.11	−0.57	22	$C_9H_9N_3SB$	M + H − CH_2=C(CO_2H)NH_2 (87) − CO_2
176.08169	176.08184	−0.15	−0.85	4	$C_9H_9N_3OB$	M + H − SCH=C(CO_2H)N=CH (129) − H_2O
161.09474	161.09475	−0.01	−0.06	35	$C_9H_{10}N_3B$	M + H − ·SCH=C(CO_2H)NH_2 (118) − CO_2
160.08697	160.08692	0.05	0.31	4	$C_9H_9N_3B$	M + H − HSCH=C(CO_2H)NH_2 (119) − CO_2
150.10256	150.10257	−0.01	−0.07	100	$C_8H_{11}N_3B$	M + H − SCH=C(CO_2H)N=CH (129) − CO_2
129.99577	129.99572	0.05	0.38	18	$C_4H_3NO_2SB$	(SCH=C(CO_2H)N=CH) (129) + H

B

exp. mass	pred. mass	Δ (mDa)	Δ (ppm)	rel abund (%)	elemental formula[a]	structural assignments
338.08023	338.08051	−0.28	−0.83	20	$C_{14}H_{15}N_3O_5SB$	M + H
321.05354	321.05397	−0.43	−1.34	1	$C_{14}H_{12}N_2O_5SB$	M + H − NH_3
306.05406	306.05430	−0.24	−0.78	19	$C_{13}H_{11}N_3O_4SB$	M + H − CH_3OH
294.09048	294.09069	−0.21	−0.71	8	$C_{13}H_{15}N_3O_3SB$	M + H − CO_2
277.06406	277.06414	−0.08	−0.29	3	$C_{13}H_{12}N_2O_3SB$	M + H − CO_2 − NH_3
265.06398	265.06414	−0.16	−0.60	3	$C_{12}H_{12}N_2O_3SB$	M + H − HN=CHCO_2H (78)
262.06439	262.06447	−0.08	−0.31	30	$C_{12}H_{11}N_3O_2SB$	M + H − CO_2 − CH_3OH
260.10287	260.10297	−0.10	−0.38	9	$C_{13}H_{13}N_3O_3B$	M + H − CO_2 − H_2S
253.06402	253.06414	−0.12	−0.47	5	$C_{11}H_{12}N_2O_3SB$	M + H − NHC(CO_2H)=CH (85)
251.04840	251.04849	−0.09	−0.36	45	$C_{11}H_{10}N_2O_3SB$	M + H − NH_2C(CO_2H)=CH_2 (87)
248.10230	248.10297	−0.67	−2.70	1	$C_{12}H_{13}N_3O_3B$	M + H − CO_2 − SCH_2
245.03790	245.03792	−0.02	−0.08	1	$C_{12}H_8N_2O_2SB$	M + H − CO_2 − CH_3OH − NH_3
235.05331	235.05357	−0.26	−1.11	1	$C_{11}H_{10}N_2O_2SB$	M + H − CH_3OH − NCCO_2H (71)
234.06947	234.06956	−0.09	−0.38	3	$C_{11}H_{11}N_3ODB$	M + H − CO_2 − HCO_2CH_3
233.03805	233.03792	0.13	0.56	2	$C_{11}H8N_2O_2SB$	M + H − CH_3OH − HN=CHCO_2H (73)
229.08452	229.08458	−0.06	−0.26	10	$C_{12}H_{10}N_3O_2B$	M + H − CO_2 − CH_3OH − HS.
228.07672	228.07675	−0.03	−0.13	1	$C_{12}H_9N_3O_2B$	M + H − CO_2 − CH_3OH − H_2S
221.09177	221.09207	−0.30	−1.36	3	$C_{11}H_{12}N_2O_3B$	M + H − SCH=C(CO_2H)NH (117)
221.03784	221.03792	−0.08	−0.36	7	$C_{10}H_8N_2O_2SB$	M + H − CH_3OH − CH_2C(CO_2H)=N (85)
220.08419	220.08424	−0.05	−0.23	49	$C_{11}H_{11}N_2O_3B$	M + H − SCH=C(CO_2H)NH_2 (118)
219.02224	219.02227	−0.03	−0.14	39	$C_{10}H_6N_2O_2SB$	M + H − CH_3OH − NH_2C(CO_2H)=CH_2 (87)
209.09204	209.09207	−0.03	−0.14	100	$C_{10}H_{12}N_2O_3B$	M + H − SCH=C(CO_2H)N=CH (129)
193.04286	193.04301	−0.15	−0.78	4	$C_9H_8N_2OSB$	M + H − CO_2 − CH_2(CO_2CH_3)CH=NH (101)
191.02738	191.02736	0.02	0.10	7	$C_9H_6N_2OSB$	M + H − CO_2 − CH_2(CO_2CH_3)CH_2NH_2 (103)
177.06587	177.06585	0.02	0.11	38	$C_9H_8N_2O_2B$	M + H − CH_3OH − SCH=C(CO_2H)N=CH (129)
149.07099	149.07094	0.05	0.34	1	$C_8H_8N_2OB$	M + H − CH_3O_2CH − SCH=C(CO_2H)N=CH (129)
147.05533	147.05529	0.04	0.27	4	$C_8H_6N_2OB$	M + H − CH_3O_2CH − HSCH=C(CO_2H)N=CH_2 (131)
129.99578	129.99572	0.06	0.46	5	$C_4H_3NO_2SB$	(SCH=C(CO_2H)N=CH) (129) + H
110.00594	110.00590	0.04	0.36	1	C_5H_3NSB	(SCH=CHN=CHCC) (110) + H

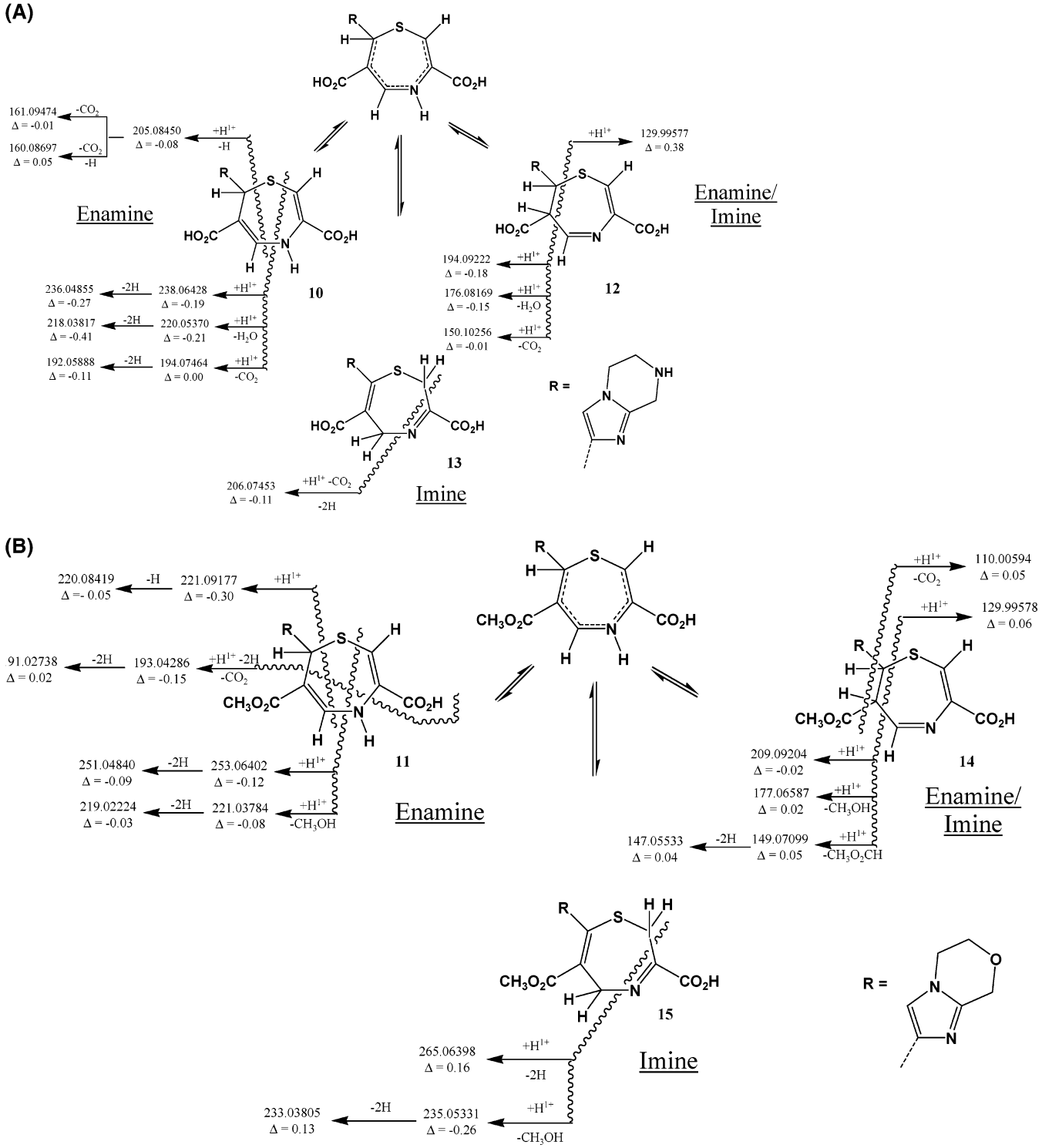
[a] B = H^{1+}.

and analyzed by ESI-MS. The resultant sets of mass spectra acquired with and without the penems were compared. In all cases studied, only one new set of peaks corresponding to the addition of a penem molecule to a trypsin-digested fragment was observed. These trypsin-digested fragments were then analyzed by ESI-MS/MS to determine the amino acid residue to which the penem was attached. In this fashion, **5** was studied in the presence of TEM-1 β-lactamase and **4**, **6**, and **7** were each studied in the presence of TEM-1 and SHV-1 β-lactamases, respectively.

Discussion

The nanoelectrospray mass spectra obtained for TEM-1 β-lactamase in the absence and presence of incubated **5** after trypsin digestion are nearly identical in that the predicted trypsin-digested fragments for TEM-1 can be clearly identified covering *all* of the amino acid residues in the molecule. The outstanding difference between the mass spectra is illustrated for the narrow mass region *m/z* 635−685 in Figures 5A and B for the trypsin digests of TEM-1 incubated without and with **5**, respectively. Note the appearance of the moderately abundant doubly charged ion with *m/z* 646.8 (Figure 5B). Also observed (not illustrated) was a triply charged ion of lower abundance at *m/z* 431.6 (partially overlapping with a secondary trypsin-digested fragment). These ions correspond to the doubly and triply charged T-6 trypsin-digested fragment [(*m/z* 494.7)$^{2+}$ and (*m/z* 330.2)$^{3+}$] covalently bound to **5** (MW 304). Similarly for the

Scheme 2. (A) The Proposed ESI-MS/MS Mass Spectral Fragmentation Pathways for **10** (MW 322), Prepared from the Base Hydrolysis of **5**. (B) The Proposed ESI-MS/MS Mass Spectral Fragmentation Pathways for **11** (MW 337)



TEM-1 and SHV-1 β-lactamases, doubly charged trypsin T-6 fragments covalently bound to **4** (MW 305), **6** (MW 289), and **7** (MW 345) were observed at *m/z* 647.3, *m/z* 639.3, and *m/z* 667.3, respectively. For the observed TEM-1 T-12 trypsin-digested fragments at *m/z* $(1303.2)^{2+}$ and $(869.1)^{3+}$, no corresponding T-12 fragments covalently bound to **5** were observed. The presence of only one trypsin-digested fragment containing the addition of a penem molecule suggests that there is only one reactive site to penems in the TEM-1 β-lactamase enzyme and that is the T-6 fragment which spans Ser-70. Note that the T-12 fragment, which spans Ser-130, is not reactive with the penems but was also reactive with tazobactam.[8]

The ESI-MS/MS spectrum for the doubly charged ion, *m/z* 646.8, corresponding to the covalent addition of **5** to the T-6 trypsin-digested fragment of TEM-1 β-lactamase, is illustrated in Figure 6. The tandem mass spectrum exhibits a large number of abundant fragment ions owing to the higher collision energy experienced by the doubly charged parent ion. These fragment ions can be classified into three types of ion series: (1) T-6

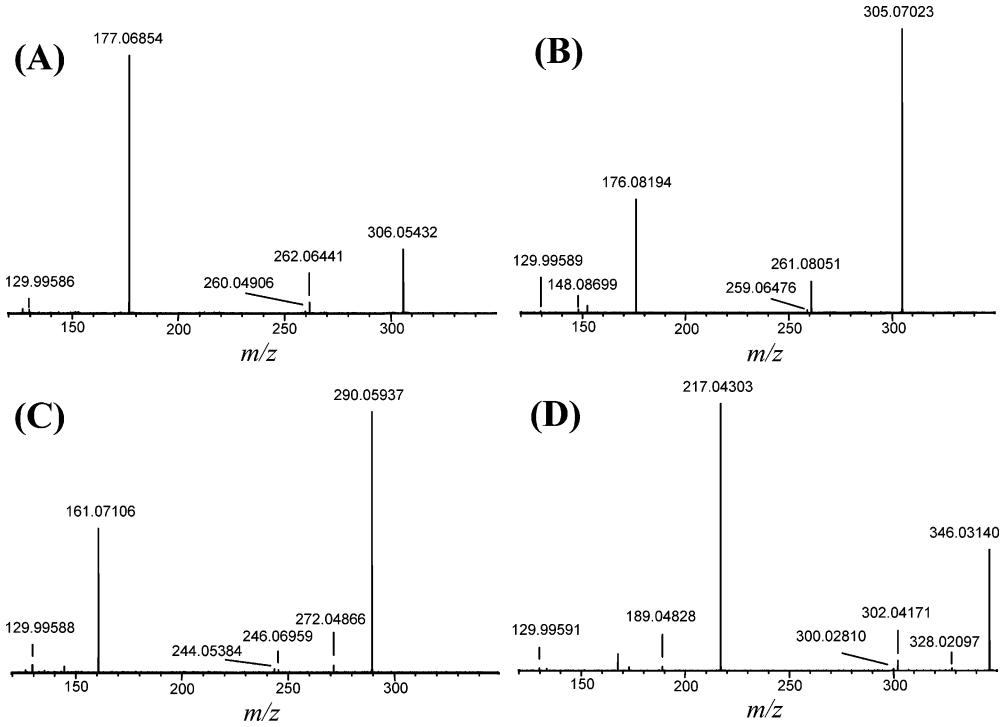


Figure 4. ESI-FT-MS/MS high-resolution exact-mass spectrum of proton adduct molecular ion of (A) **4** (MW 305), (B) **5** (MW 304), (C) **6** (MW 289), and (D) **7** (MW 345). (Unlabeled weak peaks are rf artifacts.)

Table 3. ESI-FT-MS/MS Observed Exact Masses, Calculated Elemental Formulas, and Proposed Structural Assignments for the Fragment Ions Generated from the Protonated Molecular Ions of **4** (MW 305), **5** (MW 304), **6** (MW 289), and **7** (MW 345). See Scheme 3

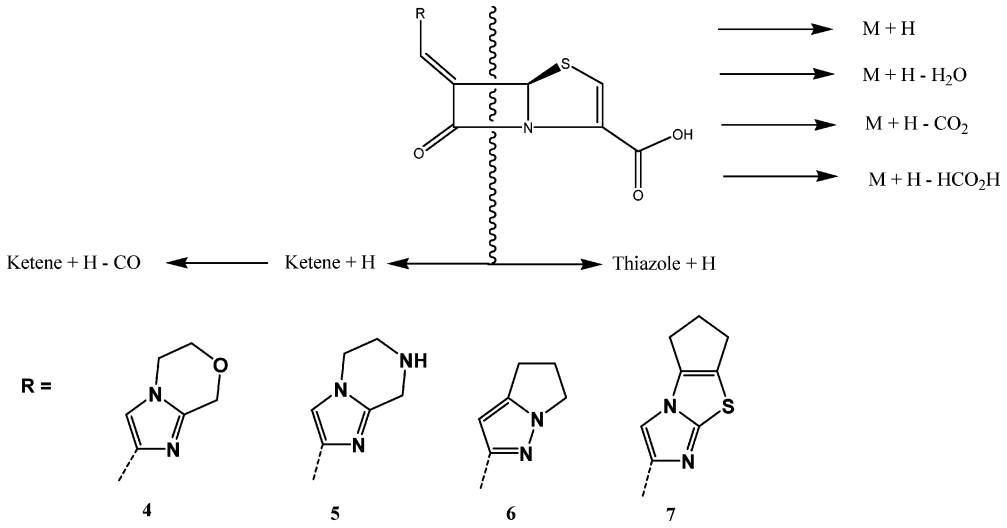
penem structure	exp mass (*m*/*z*)	pred mass (*m*/*z*)	Δ (mDa)	Δ (ppm)	rel inten (%)	elemental formula[a]	assignment
4	305.07023	305.07029	−0.06	−0.20	100	$C_{13}H_{12}N_4O_3SB$	M + H
	261.08051	261.08046	0.05	0.19	11	$C_{12}H_{12}N_4OSB$	M + H − CO_2
	259.06476	259.06481	−0.05	−0.19	1	$C_{12}H_{10}N_4OSB$	M + H − HCO_2H
	176.08194	176.08184	0.10	0.57	43	$C_9H_9N_3OB$	ketene + H
	148.08699	148.08692	0.07	0.47	1	$C_8H_9N_3B$	ketene + H − CO
	129.99589	129.99572	0.17	1.31	1	$C_4H_3NO_2SB$	thiazole + H
5	306.05432	306.05430	0.02	0.07	26	$C_{13}H_{11}N_3O4SB$	M + H
	262.06441	262.06447	−0.06	−0.23	5	$C_{12}H_{11}N_3O_2SB$	M + H − CO_2
	260.04906	260.04882	0.24	0.92	1	$C_{12}H_9N_3O_2SB$	M + H − HCO_2H
	177.06594	177.06585	0.09	0.51	100	$C_9H_8N_2O_2B$	ketene + H
	129.99586	129.99572	0.14	1.08	2	$C_4H_3NO_2SB$	thiazole + H
6	290.05937	290.05939	−0.02	−0.07	100	$C_{13}H_{11}N_3O_3SB$	M + H
	272.04886	272.04882	0.04	0.15	3	$C_{13}H_9N_3O_2SB$	M + H − H_2O
	246.06959	246.06956	0.03	0.12	1	$C_{12}H_{11}N_3OSB$	M + H − CO_2
	244.05384	244.05391	−0.07	−0.29	2	$C_{12}H_9N_3OSB$	M + H − HCO_2H
	161.07106	161.07094	0.12	0.75	58	$C_9H_8N_2OB$	ketene + H
	129.99588	129.99572	0.16	1.23	3	$C_4H_3NO_2SB$	thiazole + H
7	346.03140	346.03146	−0.06	−0.17	45	$C_{15}H_{11}N_3O_3S2B$	M + H
	328.02097	328.02090	0.07	0.21	1	$C_{15}H_9N_3O_2S2B$	M + H − H_2O
	302.04171	302.04163	0.08	0.26	4	$C_{14}H_{11}N_3OS2B$	M + H − CO_2
	300.02610	300.02598	0.12	0.40	1	$C_{14}H_9N_3OS2B$	M + H − HCO_2H
	217.04303	217.04301	0.02	0.09	100	$C_{11}H_8N_2OSB$	ketene + H
	189.04828	189.04810	0.18	0.95	2	$C_{10}H_8N_2SB$	ketene + H − CO
	129.99591	129.99572	0.19	1.46	1	$C_4H_3NO_2SB$	thiazole + H

[a] B = H^{1+}.

peptide sequence ions, (2) penem fragment ions, and (3) penem fragments bound to T-6 peptide sequence ions. The unique feature of these ion series is the absence of an ion series containing the intact 6-methylidene penem molecule.

The complete series of the predicted y_n" and b_n (n = 1, ..., 8) fragment ions for the TEM-1 T-6 trypsin-digested fragment are observed in the ESI-MS/MS spectrum for the isolated doubly charged T-6−**5** parent ion with *m*/*z* 646.8 and are labeled in Figure 6 and illustrated in Scheme 4. These ions correspond to the pure T-6 sequence fragment ions where the covalently bound **5** was released from the T-6 trypsin-digested fragment in a dehydrated state. These ions yield no information on the site to which the penem is bound. However, a corresponding limited set of dehydrated sequence ions were observed, corresponding to $y_n^* = y_n$" − H_2O (n = 4, ..., 8) and $b_n^* = b_n - H_2O$ (n = 5, ..., 8), and are also indicated in Scheme 4. Since the $y_n^* = y_n$" − H_2O series begins at the fourth amino acid residue from the C-terminus and the $b_n^* = b_n - H_2O$ series begins at the fifth amino acid residue from the N-

Scheme 3. The Proposed ESI−FT-MS/MS Mass Spectral Fragmentation Pathways for 6-Methylidene Penems **4**−**7**



Scheme 4. Peptide Backbone Sequence Ions (y_n", b_n, and a_n) and Dehydrated Backbone Sequence Ions (y_n* = y_n" − H_2O, b_n* = b_n − H_2O and a_n* = a_n − H_2O) and Internal Cleavage Ions b_{n-1} and b_{n-1}* = b_{n-1} − H_2O Observed in the ESI-MS/MS Parent Ion Spectrum of the T-6 Trypsin-Digested Fragment of TEM-1 β-Lactamase Covalently Bound to **5** (Identical fragment ions were also observed for TEM-1 β-lactamase covalently bound to **4**, **6**, and **7** except for y_5*.)

	F	**P**	**M**	**M**	**S**	**T**	**F**	**K**	
	8	7	6	5	4	3	2	1	n
	988	841	744	613	482	395	294	147	y''_n
	970	823	726	595	464				$y^*_n = y''_n - H_2O$
n	1	2	3	4	5	6	7	8	
b_n	148	245	376	507	594	695	842	988	
$b^* = b_n - H_2O$					576	677	824	970	
a_n	120	217	348	479	566	667	814		
$a^* = a_n - H_2O$					548	649	796		
b_{n-1}			229	360	447	548	695		
$b^*_{n-1} = b_{n-1} - H_2O$					429	530	677		

terminus, the T-6 serine residue (Ser-70) is the site of dehydration. This implies that the penem was originally covalently bound to Ser-70 but under the ESI-MS/MS conditions dissociated from the T-6 trypsin-digested fragment as a hydrated molecule of **5**. Also observed in Figure 6 are low abundance ion series corresponding to the a_n (n = 1, ..., 7) sequence ion series and the internal cleavage acylium ion series where the N-terminal phenylalanine residue is missing, indicated as the b_{n-1} (n = 3, ..., 7) fragment ion series. Further verification that Ser-70 is the site of penem attachment are the dehydrated fragment ion series a_n* = a_n − H_2O (n = 5, 6, 7) and b_{n-1}* = b_{n-1} − H_2O (n = 5, 6, 7). The masses for these observed sequence ions are also tabulated in Scheme 4. Identical y_n" and b_n sequence ions and y_n* and b_n* dehydrated sequence ions were observed in the ESI-MS/MS mass spectra of the doubly charged T-6 fragments of TEM-1 complexes with **4**, **6**, and **7** at m/z 647.3, m/z 639.3, and m/z 667.3, respectively, except for the y_5* = y_5" − H_2O fragment ion at m/z 595. These data also verify that the site of penem attachment is Ser-70. Similar ESI-MS/MS observations of dehydrated sequence ions were used to assign the binding site of tazobactam to β-lactamases at Ser-70.[8]

A unique feature of the low-mass region of the ESI-MS/MS mass spectra of the T-6-penem covalent complexes is the appearance of relatively abundant fragment ions related only to the dissociated penem molecule. These penem related ions are indicated with stars (*) in Figures 6 and 3C (exploded view) for the low-mass region of the ESI-MS/MS spectrum of the isolated doubly charged T-6−**5** parent ion with m/z 646.8. For comparison, note the series of identical ions observed in the ESI-FT-MS/MS spectra for **10** (Figure 3B). Clearly, the two spectra are nearly identical, within experimental error, demonstrating that the covalently linked penem to the T-6 trypsin-digested fragment is also the seven-membered dihydro[1,4]thiazepine ring analogue of **5**. The fragment ion assignments for the ESI-FT-MS/MS exact-mass data for **10** are illustrated in Scheme 2A and listed in Table 2A. Note that all the observed fragment ions originate from pairs of single bond cleavages of the seven-membered dihydro[1,4]-thiazepine ring while the R group stays intact during all these processes and serves principally as the charge site for the ions. Nearly identical series of ions are observed in the low mass regions of the ESI-MS/MS mass spectra of the isolated doubly charged T-6-penem complexes with **4**, **6**, and **7** with parent ions m/z 647.3, m/z 639.3, and m/z 667.3, respectively. The observed penem ions were shifted by the relative mass differences between the penem and **5**. The observed ions and structural assignments are listed in Table 4 and are consistent with ions observed in the ESI-MS/MS spectra of the synthetically prepared hydrated penem compound **10** containing the seven-membered dihydro[1,4]thiazepine ring. These ESI-MS/MS data confirm the NMR[15] and X-ray[17] data in that the penem underwent a rearrangement to form a seven-membered dihydro[1,4]thiazepine ring system.

Most of the remaining uninterpreted ions in the ESI-MS/MS mass spectrum of the TEM-1 T-6−**5** covalent complex correspond to singly and doubly charged ions of penem **5** fragments bound to trypsin peptide sequence ions. These ions are listed in Table 5 with their assignments, and many of the ions are labeled in Figure 6 with a caret (^). In all these cases, penem **5** undergoes some fragmentation, most often accompanied by peptide backbone cleavages producing sequence ions. No T-6−**5** sequence ions were observed containing the intact 6-methylidene penem **5** molecule. Also, the observed

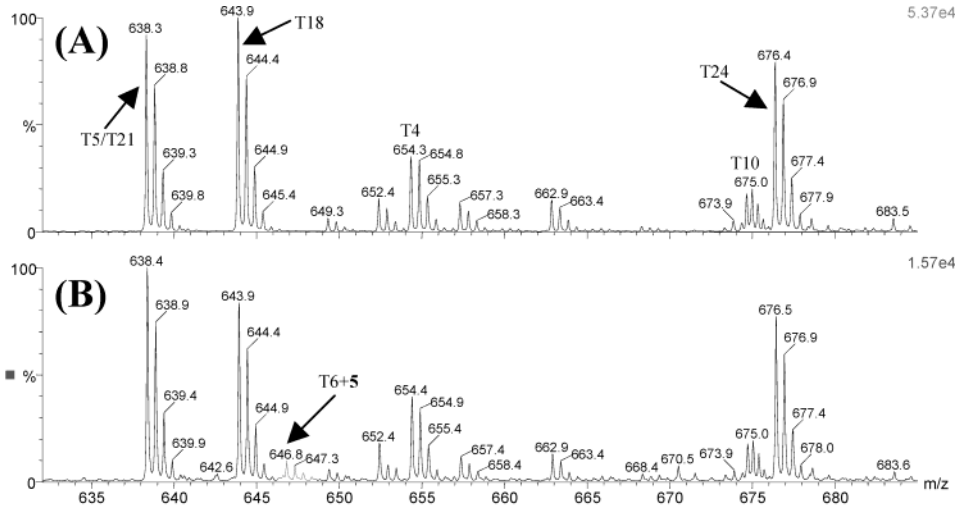


Figure 5 A,B

Figure 5. (A) ESI mass spectrum (narrow mass region) of TEM-1 *β*-lactamase after trypsin digestion. (B) ESI mass spectrum (narrow mass region) of TEM-1 *β*-lactamase complexed with **5** after trypsin digestion. The panels indicate the *m*/*z* region where the T-6 trypsin-digested fragment containing **5** was identified at *m*/*z* 646.8.

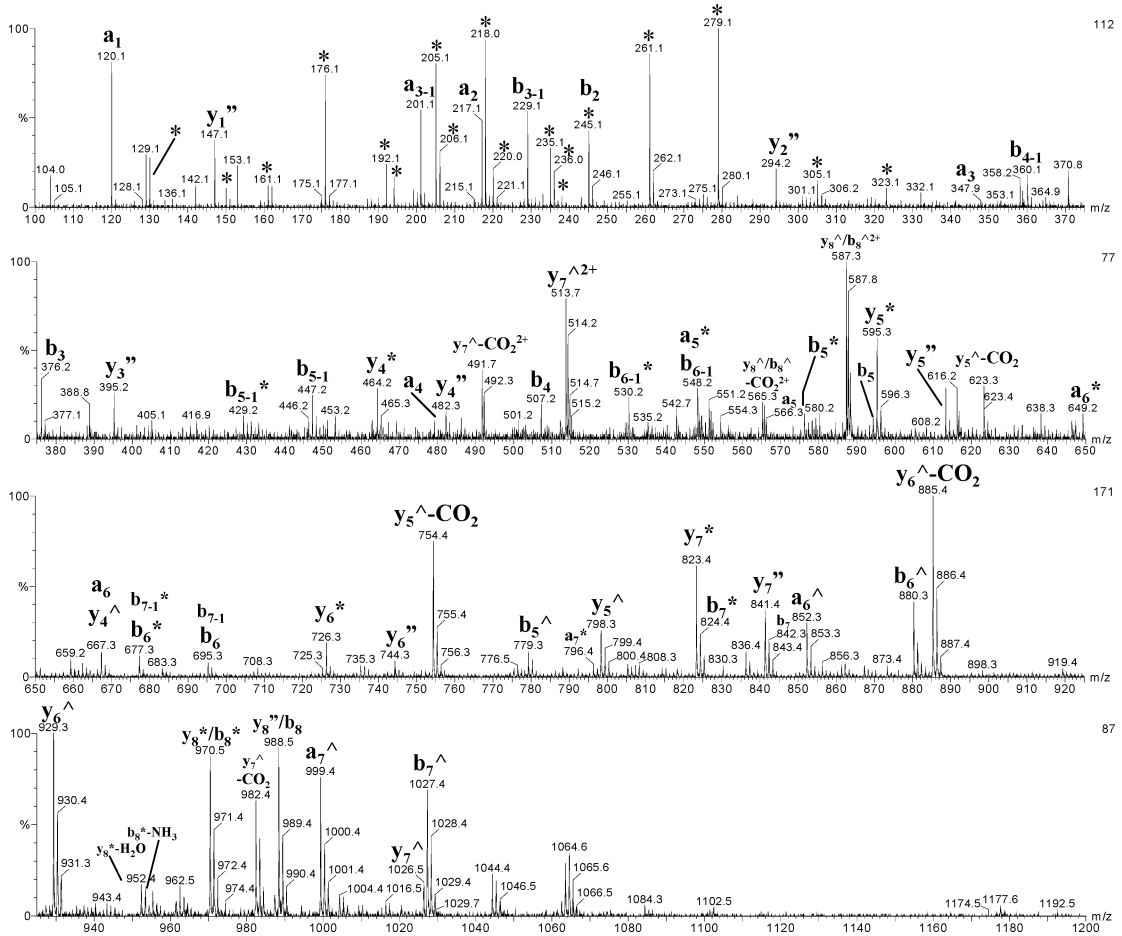


Figure 6. ESI-MS/MS spectrum for the doubly charged parent ion, *m*/*z* 646.8, of the covalent addition of **5** to the T-6 trypsin-digested fragment of TEM-1 *β*-lactamase. Note that the y_n", b_n, and a_n sequence ions and the related $y_n^* = y_n" - H_2O$, $b_n^* = b_n - H_2O$, and $a_n^* = a_n - H_2O$ sequence ions are tabulated in Scheme 4. Ions related only to the dissociated hydrolyzed **5** are labeled with stars (*) and are listed in Table 4 [column labeled **5** + H_2O (**10**)]. T-6 sequence ions incorporating fragments of hydrolyzed **5**, $y_n^\wedge = y_n" + \text{penem} - 119$, $b_n^\wedge = b_n + \text{penem} - 119$ and $a_n^\wedge = a_n + \text{penem} - 119$, are labeled and listed in Table 5. Internal cleavage acylium ions, corresponding to the loss of the phenyl alanine residue at the N-terminus, b_{n-1} and as dehydrated ions $b_{n-1}^* = b_{n-1} - H_2O$, are labeled and the masses listed in Scheme 4.

penem fragment ions for the T-6 trypsin-digested fragment of TEM-1 *β*-lactamase complexed at Ser-70 with penems **4**, **6**, and **7** are listed in Table 5.

An outstanding feature of all the ESI-MS/MS spectra of the TEM-1 T-6−penem complexes is that for any given fragmentation process, the ions of the respective

Table 4. Low Mass Fragment Ions Observed in the ESI-MS/MS Spectrum of the Protonated Parent Ion of TEM-1 T6 Complexed with Penems **4**−**7**, Respectively, Corresponding to Decomposition Products of Hydrolyzed **4** (MW 323), Hydrolyzed **5** (**10**) (MW 322), Hydrolyzed **6** (MW 307), and Hydrolyzed **7** (MW 363)

4 + H_2O	**5** + H_2O (**10**)	**6** + H_2O	**7** + H_2O	structural assignments
324	323	308	364	M′ + H (M′ = penem + H_2O)
306	305	290	346	M′ + H − H_2O
280	279	264	320	M′ + H − CO_2
262	261	246	302	M′ + H − CO_2 − H_2O
246	245	ND[a]	286	M′ + H − CO_2 − H_2S
239	238	ND	279	M′ + H − CH═C(CO_2H)NH (85)
237	236	221	277	M′ + H − CH_2═C(CO_2H)NH_2 (87)
236	235	220	276	M′ + H − 2CO_2
221	220	205	261	M′ + H − CH═C(CO_2H)NH (85) − H_2O
219	218	203	259	M′ + H − 2CO_2 − NH_3 and/or M′ + H − CH_2═C(CO_2H)NH_2 (87) − H_2O
207	206	191	247	M′ + H − HN═CH(CO_2H) (73) − CO_2
206	205	190	246	M′ + H − ·SCH═C(CO_2H)NH_2 (118)
195	194	179	235	M′ + H − SCH═C(CO_2H)N═CH (129) and/or M′ + H − CH═C(CO_2H)NH (85) − CO_2
193	192	177	233	M′ + H − CH_2═C(CO_2H)NH_2 (87) − CO_2
177	176	161	217	M′ + H − SCH═C(CO_2H)N═CH (129) − H_2O
162	161	ND	202	M′ + H − ·SCH═C(CO_2H)NH_2 (118) − CO_2
151	150	ND	191	M′ + H − SCH═C(CO_2H)N═CH (129) − CO_2
130	130	130	130	(SCH═C(CO_2H)N═CH) (129) + H

[a] ND, none detected.

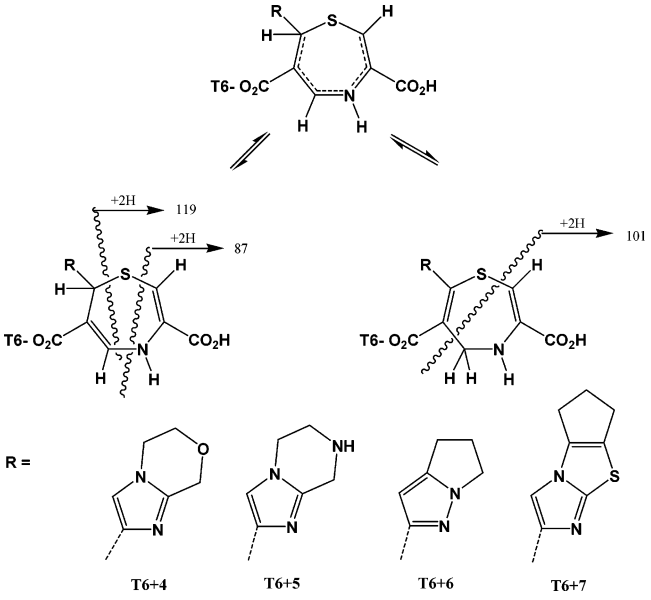
Table 5. Sequence Ions Observed in the ESI-MS/MS Spectrum of the Protonated Parent Ion of TEM-1 T6-Complexed with Penems **4**−**7**, Respectively, Containing Fragments of the Penem Portion of Each Molecule (all *m*/*z* values listed as singly charged ions)[a,b]

T6 + **4**	T6 + **5**	T6 + **6**	T6 + **7**	structural assignments[d]
1293.6	*1292.6*	*1277.6*	*1333.6*	M + H
ND[c]	ND	1233.8	ND	M + H − CO_2 w
1232.6	*1231.6*	*1216.6*	*1272.6*	M + H − 61 (CO_2+NH_3) w
ND	ND	*1190.6*	*1246.6*	M + H − 87 (CH_2═C(CO_2H)NH_2) w
ND	ND	*1189.8*	*1245.8*	M + H − 88 (2CO_2) w
1188.6	*1187.6*	*1172.6*	ND	M + H − 87/88 − H_2O/NH_3 w
1174.6	*1173.6*	*1158.6*	*1214.6*	M + H − 119 (HSCH═C(CO_2H)NH_2) [y_8/b_8 + penem − 119]
1130.6	1129.5	*1114.6*	*1170.6*	M + H − 119 − CO_2 [y_8/b_8 + penem − 119 − CO_2]
1102.6	1101.4	*1086.6*	*1142.6*	y_7 + penem − CO_2
1085.6	1084.4	*1069.6*	*1125.6*	y_7 + penem − CO_2 − NH_3
1059.6	1058.4	*1043.6*	*1099.6*	y_7 + penem − 87 (CH_2═C(CO_2H)NH_2)
1046.6	1045.5	1030.6	1086.6	b_7 + penem − 101 (CH_2═C(CO_2H)NHCH_3)
1045.6	1044.5	1029.6	1085.6	y_7 + penem − 101 (CH_2═C(CO_2H)NHCH_3)
1028.5	1027.4	1012.6	1068.5	b_7 + penem − 119
1027.6	1026.4	1011.6	1067.6	y_7 + penem − 119
1000.5	999.4	984.6	1040.6	a_7 + penem − 119
983.6	982.4	967.6	1023.6	y_7 + penem − 119 − CO_2
930.5	929.4	914.5	970.5	y_6 + penem − 119
886.4	885.3	870.4	926.5	y_6 + penem − 119 − CO_2
881.4	880.3	865.4	921.5	b_6 + penem − 119
868.4	867.4	852.4	908.4	y_6 + penem − 119 − CO_2 − H_2O
864.4	863.3	848.4	904.4	b_6 + penem − 119 − NH_3
853.4	852.3	837.4	893.4	a_6 + penem − 119
837.4	836.3	821.4	877.4	b_6 + penem − 119 − 44 ?
799.4	798.3	783.4	839.4	y_5 + penem − 119
780.4	779.3	764.4	820.4	b_5 + penem − 119
755.4	754.3	739.4	795.4	y_5 + penem − 119 − CO_2
752.4	751.3	736.4	792.4	a_5 + penem − 119 w
736.4	735.3	720.4	776.4	b_5 + penem − 119 − 44 ?
668.4	667.3	652.4	708.4	y_4 + penem − 119
624.4	623.3	608.4	664.4	y_4 + penem − 119 − CO_2

[a] Masses in italics were computed from the observed doubly charged species. [b] Masses underlined were observed as both singly and doubly charged ions. [c] ND, not detected. [d] w, ions of low abundance.

compounds are each shifted by the mass difference between the compounds (Table 5). This occurs because the neutral losses for each of the fragmentation processes are the same for each of the compounds and that no fragmentation occurs in the heterocyclic (R) portion of the hydrolyzed penem. All fragmentation is confined exclusively to the seven-membered dihydro[1,4]thiazepine ring system and to the peptide backbone. Assuming all the CO_2 and NH_3 losses are confined to the peptide backbone, nearly all the fragment ions can be rationalized. The loss of 87 Da corresponds to the loss of CH_2═C(CO_2H)NH_2, and the loss of 101 Da corresponds to the loss of CH_2═C(CO_2H)NHCH_3. A series of y_n", b_n, and a_n sequence ions accompanied by the neutral loss of 119 Da from the penem were observed, viz., $y_n\wedge = y_n$" + penem − 119 (n = 4, ..., 8), $b_n\wedge = b_n$ + penem − 119 (n = 5, ..., 8) and $a_n\wedge = a_n$ + penem − 119 (n = 5, 6, 7). These data verify that each of the penems are covalently bound to Ser-70 and that the fragmentation is confined to the seven-membered dihydro[1,4]thiazepine ring

Scheme 5. Fragmentation Pathways for the Neutral Structural Losses of 87, 101, and 119 Da from the T-6 Trypsin-Digested Fragment of TEM-1 Covalently Attached to the Seven-Membered Ring System of Hydrolyzed 6-Methylidene Penems **4**−**7**



system. Another prominent ion series observed corresponds to the $y_n\wedge - CO_2 = y_n" + \text{penem} - 119 - CO_2$ ($n = 4, ..., 8$) series where the CO_2 loss originates from the sequence terminating carboxylic acid group. The loss of 119 Da corresponds to the loss of a $HSCH{=}C(CO_2H)\text{-}NH_2$ moiety from the seven-membered dihydro[1,4]-thiazepine ring system. Scheme 5 illustrates the fragmentation pathways for the neutral structural losses of 87, 101, and 119 Da from the T-6 trypsin-digested fragment of TEM-1 covalently attached to the seven-membered ring system of hydrolyzed penems **4**−**7**. Under the ESI-MS/MS experimental conditions, the fragmentation of the penems when covalently attached to a peptide sequence was an interesting and unexpected result. Sugars,[22] phosphates,[23] sulfates, and β-lactams[8] covalently attached nonterminally to peptides often completely dissociate from peptides under CID and IRMPD MS/MS conditions, leaving no strong indication of their site of attachment, requiring electron capture dissociation[22−24] experiments to generate sequence ions containing undissociated moieties.

Conclusions

Electrospray mass spectrometry has been used to characterize the kinetics of reaction of the 6-methylidene penem class of β-lactamase inhibitors with class A and C β-lactamases, signifying that a single acyl−enzyme complex is formed rapidly which hydrolyzes over time for the class A β-lactamases (TEM-1, SHV-1) while for the class C β-lactamase studied (Amp-C) appears to form a relatively stable covalent complex. These results are consistent with recently reported X-ray crystallographic data[17] that exhibited the presence of a water molecule in the vicinity of the acyl−enzyme ester bond for class A enzymes and its absence in class C enzymes. This water molecule is believed to be responsible for the hydrolysis of the acyl−enzyme ester bonds in class A β-lactamases.

The hydrolyzed products of the reactions of 6-methylidene penems with class A β-lactamases were analyzed by ESI-MS/MS and the resulting spectra compared to the respective ESI-MS/MS spectra of synthetically prepared seven-membered dihydro[1,4]thiazepine base hydrolysis product **10**. The ESI-MS/MS mass spectra for the hydrolyzed products were consistent with the formation of a seven-membered dihydro[1,4]thiazepine ring structure. Fourier transform ESI-MS/MS experiments confirmed the elemental formulas of the proposed structures for the fragment ions observed under low-resolution ESI-MS/MS conditions further validating the formation of the seven-membered dihydro-[1,4]thiazepine structure. The ESI mass spectral data for hydrolyzed penems, subjected to hydrogen/deuterium exchange, demonstrated that tautomerization did not take place in the condensed phase. However, the fragment ions observed for the seven-membered dihydro-[1,4]thiazepine ring system, in the higher energy gas-phase ESI-MS/MS spectra, were consistent with enamine/imine tautomeric ring structures.

ESI-MS/MS was also a powerful method for demonstrating that the penems were attached to Ser-70 of the β-lactamases upon the analysis of the T-6-penem trypsin-digested fragment. Besides exhibiting sequence ions, unusual features of these ESI-MS/MS spectra were that the sites of attachment were graphically demonstrated from sequence ions exhibiting dehydration at Ser-70 and sequence ions exhibiting *fragmentation* of the dihydro-[1,4]thiazepine ring at Ser-70. Strikingly, no T-6-penem sequence ions were observed containing the *intact* penem molecule. The trypsin-digested fragment (T-12 for TEM-1 β-lactamase) that contains Ser-130, another possible site for the attachment of the penem to the β-lactamase, was not observed to contain the covalent attachment of the penem in a trypsin digest mixture. These ESI-MS and ESI-MS/MS results are consistent with the previously reported NMR[15] and X-ray[17] findings.

Mass spectrometry has been demonstrated to be a most powerful method for characterizing the transformations taking place when β-lactamases interact with 6-methylidene penem inhibitors. This work clearly illustrates the value of mass spectrometric techniques in understanding details of the interactions of inhibitors with enzymes. Comparison of our approach with results obtained by NMR or X-ray crystallography reaffirms the versatility, accuracy, and speed of mass spectrometric techniques, particularly in cases where NMR and X-ray crystallography techniques cannot be employed.

Experimental Section

Enzymes. Clinical isolates producing TEM-1 (class A, MW 28,910),[25,26] SHV-1 (class A, MW 28 874), Imi-1 (class A), CcrA (class B), and Amp-C (class C, MW ∼39 400) β-lactamases were identified by isoelectric focusing of cellular extracts and were purified to near homogeneity using Sephadex G-75 column chromatography. Sequencing grade trypsin (modified) was purchased from Promega (Madison, WI). Ambler's numbering system for the amino acid sequence of PC1[27] was used for TEM-1 and SHV-1 throughout the text.

Biological Testing. Penems **4**−**7** were tested in vitro against TEM-1, Imi-1, CcrA, and Amp-C β-lactamases for their inhibitory ability. The inhibitory activities of the β-lactamases were determined spectrophotometrically using nitrocefin as the substrate and tazobactam **1** as a standard.[28,29] IC_{50} values were

calculated using WinNonlin software (Opharsight Corp., Mountainview, CA).

Biochemical Reactions Prior to ESI-MS. For intact protein analysis, enzyme stock solutions of TEM-1 were prepared in HPLC grade water and solutions of SHV-1 and Amp-C were prepared in buffer (50 mM PBS, pH 7.4). Samples of the penems were reacted with the β-lactamases by incubating the enzyme with inhibitor at inhibitor/enzyme molar ratios of 2:1 and/or 10:1 in H_2O or buffer at 25 °C. The reactions were subsampled periodically for the time course measurements. Inhibition of the β-lactamases by respective penem inhibitors was confirmed by testing for enzyme activity using the chromogenic substrate nitrocefin and monitoring at 495 nm using a Beckman Model DU7400 spectrophotometer.[30]

Proteolysis. The endoproteinase trypsin was used to cleave the peptide bonds C-terminally at lysine and arginine residues. The selected β-lactamase and 6-methylidene penem were dissolved in 50 mM NH_4HCO_3, pH 7.8, at concentrations of 50 μM and 100 μM, respectively. The selected penem and β-lactamase were mixed (inhibitor:enzyme, molar ratio 2:1) and incubated for 15 min at 25,°C. The penem β-lactamase reaction mixture and β-lactamase control (without penem) were each individually digested with trypsin at a molar ratio of 30:1 (β-lactamase:trypsin) for 10 h at 25°C.

Electrospray Ionization Mass Spectrometry (ESI-MS). Electrospray ionization mass spectra of the β-lactamase samples were obtained in the positive ionization mode with a Micromass LCT time-of-flight mass spectrometer. The samples were prepared at ~10 pmol/μL in 0.1% formic acid in H_2O:ACN (1:1, v/v). The β-lactamases were flow injected (2−5 μL) into the source of the mass spectrometer at a rate of 20 μL/min utilizing a carrier solvent of H_2O:ACN (1:1, v/v) with 0.025% formic acid. Protein data were acquired over the mass range of m/z 100−2500. The cone voltage was set to 35 V. Nitrogen was used as the nebulizing and drying gas with flow rates of 2.5 and 8 L/min, respectively. Ten to twenty scans were averaged, smoothed, baseline-subtracted, and transformed using the Micromass transform or maximum entropy programs.

Capillary HPLC-Electrospray Ionization Mass Spectrometry (Capillary HPLC/ESI-MS and -MS/MS). A Waters capillary HPLC pump (CapLC), equipped with a Spark Holland b.v. (Emmen, Netherlands) microvolume autosampler, was employed together with a Micromass Q-Tof hybrid quadrupole-time-of-flight mass spectrometer for all capillary HPLC/ESI-MS and -MS/MS experiments. Five microliters of trypsin-digested pure β-lactamase and penem-β-lactamase complex were injected and concentrated on to a LC Packing (San Francisco, CA) microguard C18 column. Using column and flow switching, the digests were desorbed using a solvent gradient of ACN:H_2O from 5:95 to 95:5 (v/v) containing 0.05% TFA at a flow rate of 0.20 μL/min for 60 min through a LC Packing NAN100−15-C18 0.1 × 150 mm C18 capillary column. Nitrogen was used as the drying gas. ESI-MS spectra in the positive ionization mode were acquired over a range of m/z 100−2500 at 3 s/scan with a cone voltage of 25 V. ESI-MS/MS spectra were obtained using a collision energy of 30 eV with argon as the collision gas at a pressure of 1.5×10^{-3} mBar. The notation used for the sequence ions observed in the of ESI-MS/MS data followed the proposal of Roepstorff and Fohlman[31] where the ion series y''_n and b_n represent the cleavage of the amide bond from the nth amino acid residue from the C-terminus and N-terminus, respectively, the double prime ($''$) represents the addition of two hydrogen atoms, and the ion series a_n represents the loss of the carbonyl group from the b_n ion series.

Nanoelectrospray-MS and -MS/MS. Nanoelectrospray-MS and -MS/MS[32] experiments were performed with β-lactamase digests on a Micromass Q-Tof mass spectrometer equipped with a Micromass Nanospray Accessory using New Objective (Woburn, MA) gold-coated glass needle nanovials (Offline PicoTip BG10-78-4-CE-20). The needle voltage was set to ~1200 V, and the flow rate was calculated to be ~100 nL/min. The Micromass Q-Tof was operated using conditions similar to the ones described above.

High-Resolution Exact-Mass ESI Fourier Transform Mass Spectrometry (FT-MS and -MS/MS). The instrument used was a Bruker-Daltonics Apex II Fourier transform mass spectrometer equipped with a Bruker-Magnex 9.4 T superconducting magnet and an Apollo electrospray source.[33] The ESI source was operated in the nanoelectrospray mode. Typically, a 5 μL sample was loaded into a glass tip/needle with conductive coating (New Objective, Woburn, MA), and about −700 V was applied between the glass tip/needle and the glass capillary ESI interface. The ions produced were externally accumulated in a hexapole ion guide in the ESI source region for 2.0 s and then pulsed into the FT-MS cell through a set of ion lenses. Gas-assisted dynamic trapping (GADT) was employed to trap a detectable number of ions inside the analyzer cell. Argon gas was pulsed into the ultrahigh vacuum chamber at a level up to 10^{-6} mbar to facilitate the trapping and thermalization of the ions. The front trap plate voltage of the analyzer cell was lowered (0 V) to permit the entry of the ions into the cell and raised afterward (4 V) to trap the ions. After four such analyzer cell filling cycles the trap plate voltages were lowered to about 0.5 V. The trapped ions in the range m/z 100 to 2000 were then excited and detected.

FT-MS/MS experiments were performed using SORI-CID (***s***ustained ***o***ff-resonance ***i***rradiation with ***c***ollision-***i***nduced ***d***issociation).[34] The protonated precursor ion was isolated using a correlated sweep (with an isolation window of 1000 Hz) and then was selectively activated. For the SORI-CID experiments, argon gas was pulsed into the ultrahigh vacuum region containing the analyzer cell to a peak pressure of 1×10^{-6} mbar. An rf pulse (41 dB attenuation), off resonance from the precursor by 1000 Hz, was applied for 100 ms. The voltage of the SORI-CID rf pulse was adjusted to give nearly complete attenuation of the precursor ion signal. Several seconds were allowed for fragmentation and for pumping away the collision gas so that the fragments could be detected under high-resolution conditions at about 4×10^{-10} mbar. For the SORI-CID experiments, 128 scans were acquired in the time domain, consisting of 512K data points each, signal averaged, apodized, and Fourier transformed (magnitude mode).

Hydrogen/Deuterium Exchange ESI-MS. Labile hydrogen atoms were exchanged for deuterium atoms in **10**, the synthetically prepared seven-membered dihydro[1,4]thiazepine ring system, by incubating the sample for 10 min in 0.1% acetic acid-d_4 in D_2O at 25 °C. The sample was directly infused into the Micromass Q-Tof mass spectrometer using a Harvard Apparatus syringe pump (Model 22).

Mass Spectrometer Calibration. All the mass spectrometers used in these penem−β-lactamase studies were calibrated externally with the Agilent ESI-MS tuning mixture (Agilent PN G2421A). The mass errors (Δ) for the FT-MS and -MS/MS experiments are expressed in milli-Daltons [Δ(mDa) = (experimental mass − predicted mass) × 10^3] and parts-per-million [Δ(ppm) = (experimental mass − predicted mass) × 10^6/predicted mass].

General Synthetic Methods. Melting points were determined in open capillary tubes on a Meltemp melting point apparatus and are uncorrected. ^{1}H NMR spectra were determined with a Bruker DPX-300 spectrometer at 300 MHz. Chemical shifts δ are reported in parts per million (δ) relative to residual chloroform (7.26 ppm), TMS (0 ppm), or dimethyl sulfoxide (2.49 ppm) as an internal reference with coupling constants (J) reported in hertz (Hz). The peak shapes are denoted as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Electrospray ionization (ESI) mass spectra were recorded in the positive ionization mode on a Micromass LCT time-of-flight mass spectrometer. Combustion analyses were obtained using a Perkin-Elmer Series II 2400 CHNS/O analyzer. Chromatographic purifications were performed by flash chromatography using Baker 40-μm silica gel. Thin-layer chromatography (TLC) was performed on Analtech silica gel GHLF-250-M prescored plates. The terms "concentrated" and "evaporated" refer to removal of solvents using a rotary evaporator at water aspirator pressure with a bath

temperature equal to or less than 60 °C. Unless otherwise noted, reagents were obtained from commercial sources and were used without further purification.

Synthetic Details. The synthesis of the (5*R*,6*S*)-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester, **8**, has been prepared by a previously reported procedure.[16] This was condensed with different aldehydes and the resultant acetoxy bromohydrines **9** were subjected to a reductive elimination procedure to yield the final products **4a**−**7a**.[17,18] See Scheme 1A.

General Method To Prepare 6-Methylidene Penem Derivatives. Step 1: Aldol Condensation of the Respective Aldehydes and (5*R*,6*S*)-6-Bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic Acid 4-Nitrobenzyl Ester (8). The stirred solution of the appropriately substituted aldehyde derivative (1.00 mmol) and (5*R*,6*S*)-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitrobenzyl ester (1.2 mmol), **8**, in dried THF (20 mL)/acetonitrile (15 mL) was added anhydrous $MgBr_2$:$O(Et)_2$ (1.2 mmol) under an argon atmosphere at room temperature. After being stirred at room temperature for 30 min, the reaction mixture was cooled to −20 °C, and Et_3N (2.0 mL) was added in one portion. The reaction vessel was covered with aluminum foil to exclude light. The reaction mixture was stirred for 8 h at −20 °C and treated with acetic anhydride (1.04 mL) in one portion. The reaction mixture was warmed to 0 °C and stirred for 15 h at 0 °C. The mixture was diluted with ethyl acetate and washed with 5% citric acid aqueous solution, saturated sodium hydrogen carbonate, and brine. The organic layer was dried ($MgSO_4$) and filtered through a pad of Celite. The pad was washed with ethyl acetate. The filtrate was concentrated under reduced pressure. The residue was applied to silica gel column chromatography, and then the column was eluted with ethyl acetate:hexane (1:1). Collected fractions were concentrated under reduced pressure, and the mixture of diastereomers were taken to the next step.

Step 2: Reductive Elimination Reaction of the Above-Mentioned Aldol Products. The aldol products, **9**, obtained by the above-mentioned step (1.37 mmol), was dissolved in THF (20 mL) and acetonitrile (10 mL). Freshly activated Zn dust (5.2 g) was added rapidly with 0.5 M phosphate buffer (pH 6.5, 28 mL). The reaction vessel was covered with foil to exclude light. The reaction mixture was vigorously stirred for 2 h at 30 °C. The reaction mixture was filtered and cooled to 3 °C, and 0.1 N NaOH was added to adjust the pH to 8.5. The filtrate was washed with ethyl acetate, and the aqueous layer was separated. The aqueous layer was concentrated under high vacuum at 35 °C to give a yellow precipitate. The product was purified by HP21 resin reverse phase column chromatography. Initially the column was eluted with deionized water (2 L) and latter with 10% acetonitrile:water. The fractions containing the product were collected and concentrated under reduced pressure at room temperature. The yellow solid was washed with acetone, filtered, and dried.

Preparation of (5*R*,6*Z*)-6-(5,6-Dihydro-8*H*-imidazo[2,1-*c*][1,4]oxazin-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo-[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (4a). Starting from 5,6-dihydro-8*H*-imidazo[2,1-*c*][1,4]oxazine-2-carbaldehyde (1.2 g, 7.89 mmol) and (5*R*,6*S*)-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitrobenzyl ester **8** (3.4 g, 8.8 mmol) and following the above-mentioned procedure, the aldol product, (5*R*)-6-[acetoxy-(5,6-dihydro-8*H*-imidazo[2,1-*c*][1,4]oxazin-2-yl)methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid *p*-nitrobenzyl ester was isolated as spongy yellow solid. Subjecting this crude material to the reductive elimination procedure (step 2) gave 756 mg of compound **4a**. Yield 30%; yellow solid; mp 130 °C; (M + H) 328. ^{1}H NMR (DMSO-d_6) δ 3.98−4.01 (m, 2H), 4.04−4.07 (m, 2H), 4.74 (AB, 2H, J = 15.3, 22.9 Hz), 6.40 (d, 1H, J = 0.8 Hz), 6.55 (s, 1H), 6.95 (d, 1H, J = 0.6 Hz), 7.54 (s, 1H); IR (KBr) 3412, 1741, 1672, 1592, 1549 cm^{-1}; λ^{max} (H_2O) 304 nm.

Preparation of (5*R*,6*Z*)-7-Oxo-6-(5,6,7,8-tetrahydroimidazo[1,2-*a*]pyrazin-2-ylmethylene)-4-thia-1-azabicyclo-[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (5a). Starting from 7-*p*-nitrobenzyloxycarbonyl-5,6,7,8-tetrahydroimidazo[1,2-*a*]pyrazine-2-carbaldehyde (1.6 g, 4.8 mmol) and (5*R*,6*S*)-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitrobenzyl ester **8** (1.8 g, 4.0 mmol) and following the above-mentioned procedure, 2.1 g of the aldol product, (5*R*)-6-[acetoxy-(7-*p*-nitrobenzyloxycarbonyl-5,6,7,8-tetrahydroimidazo[1,2-a]pyrazin-2-yl)-methyl]-6-bromo-7-oxo-4-thia-1azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid *p*-nitrobenzyl ester was isolated as spongy yellow solid. Yield: 59%. Subjecting this material to the reductive elimination procedure (step 2) gave 172 mg of compound **5a**. Yield 20%; yellow solid; mp 150 °C; (M + H) 327.^{1}H NMR (d, D_2O) δ 3.02 (t, 2H, J = 5.6 Hz), 3.82 (s, 2H), 3.89 (d, 2H, J = 5.6 Hz), 6.38 (s, 1H), 6.84 (s, 1H), 6.87 (s, 1H), 7.24 (s, 1H).

Preparation of (5*R*,6*Z*)-6-(5,6-Dihydro-4*H*-pyrrolo[1,2-*b*]pyrazol-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo[3.2.0]-hept-2-ene-2-carboxylic Acid, Sodium Salt (6a). Starting from 5,6-dihydro-4*H*-pyrrolo[1,2-*b*]pyrazole-2-carbaldehyde (1.36 g, 10 mmol) and (5R, 6S)-6-bromo-7-oxo-4-thia-1-aza-bicyclo-[3.2.0]hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester **8** (3.97 g, 10.2 mmol) and following the above-mentioned procedure, the aldol product, (5*R*)-6-[acetoxy-(5,6-dihydro-4*H*-pyrrolo[1,2-*b*]pyrazole-2-yl)methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]-hept-2-ene-2-carboxylic acid *p*-nitrobenzyl ester was isolated as spongy yellow solid. Subjecting this crude material to the reductive elimination procedure (step 2) gave 849 mg of compound **6a**. Yield 29%; yellow solid; mp 190 °C (dec); (M + H) 312; ^{1}H NMR (D_2O) δ 2.49 (m, 2H), 2.78 (t, 2H, J = 7.4 Hz), 4.02 (t, 2H, J = 7.4 Hz), 6.01 (s, 1H), 6.29 (s, 1H), 6.90 (s, 2H).

Preparation of (5*R*,6*Z*)-6-(6,7-Dihydro-5*H*-cyclopenta-[*d*]imidazo[2,1-*b*][1,3]thiazol-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (7a). Starting from (600 mg, 3.1 mmol) of 2-formyl-6,7-dihydro-5*H*-cyclopenta[*d*]imidazo[2,1-*b*][1,3]thiazole and (1.2 g, 3.1 mmol) of (5*R*,6*S*)-6-bromo-7-oxo-4-thia-1-aza-bicyclo-[3.2.0]hept-2-ene-2-carboxylic acid 4-nitrobenzyl ester **8** and following the above-mentioned procedure 850 mg of the aldol product was isolated as spongy yellow solid. Yield: 850 mg, 45%; M + H 620. Subjecting this material to the reductive elimination procedure (step 2) gave 138 mg of compound **7a**. Yield 29%; Yellow solid; mp 192 °C; (M + H) 368. ^{1}H NMR (DMSO-d_6) δ 8.2 (s, 1H), 7.1 (s, 1H), 6.55 (s, 1H), 6.4 (s, 1H), 3.01 (m, 2H), 2.51 (m, 4H).

Preparation of 7-(5,6,7,8-Tetrahydro-8*H*-imidazo[1,2-*a*][1,4]pyrazin-2-yl)-4,7-dihydro-[1,4]thiazepine-3,6-dicarboxylic Acid, 3,6-Disodium Salt (10a). To a stirred solution of (5*R*,6*Z*)-7-oxo-6-(5,6,7,8-tetrahydroimidazo[1,2-*a*]-pyrazin-2-ylmethylene)-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid, sodium salt **5a** (326 mg, 1 mmol) was added 0.1 N NaOH (15 mL) at room temperature. The reaction mixture was stirred for 1 h and concentrated. The red solid obtained was dissolved in water and loaded on to a HP21 resin reverse phase column chromatography. Initially the column was eluted with deionized water (2 L) and later with 5% acetonitrile:water. The fractions containing the product were collected and concentrated under reduced pressure at room temperature. The red solid was washed with acetone, filtered, and dried. Red amorphous solid; yield 200 mg, 54%; Mp: 225 °C (decomp); (M − 2Na + 3H) 323; ^{1}H NMR: (DMSO-d_6) δ 9.31 (s, 1H), 7.82 (s, 1H), 6.66 (s, 1H), 6.10 (s, 1H), 3.95−3.78 (m,3H), 2.9−3.1 (m,4H).

Preparation of 7-(5,6-Dihydro-8*H*-imidazo[2,1-*c*][1,4]-oxazin-2-yl)-4,7-dihydro-[1,4]thiazepine-3,6-dicarboxylic Acid 6-Methyl Ester, 3-Sodium Salt (11a). To a stirred solution of (5*R*),(6*Z*)-6-(5,6-dihydro-8*H*-imidazo[2,1-*c*][1,4]-oxazin-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt **4a** (500 mg, 1.4 mmol) in anhydrous methanol (50 mL) at 0 °C was added NaOMe (80.0 mg 1.5 mmol). The reaction mixture was stirred at room temperature for 2 h and concentrated. The product was purified by HP21 resin reverse phase column chromatography. Initially the column was eluted with deionized water (2 L) and

later with 10% acetonitrile:water. The fractions containing the product were collected and concentrated under reduced pressure at room temperature. The yellow solid was washed with acetone, filtered, and dried**.** Yellow amorphous solid; Yield 387 mg, 70%; Mp: 225 °C (Dec); (M + H) 360; ^{1}H NMR: (DMSO-d_6) δ 9.16 (d, 1H), 7.64 (d, 1H), 6.24 (s, 1H), 6.08 (s, 1H), 5.41 (s, 1H), 4.64 (q, 2H), 3.98−3.80 (m, 4H), 3.54 (s, 3H).

Acknowledgment. The authors greatly appreciate the assistance and biolchemical assay data provided by Youjun Yang and the helpful discussions with Beth A. Rasmussen and David M. Shlaes.

Supporting Information Available: C, H, N analyses of **4a**−**7a**, **10a**, and **11a**. This material is available free of charge via the Internet at http://pubs.acs.org.

Note Added after ASAP Posting. The version of this paper posted May 28, 2004, was missing underlines for 1027.6 and 983.6 in column 1 of Table 5. The corrected version of the paper was posted June 8, 2004.

References

(1) Micetich, R. G.; Salama, S. M.; Maiti, S. N.; Reddy, A. V. N.; Singh, R. β-Lactamases and their inhibitors: an update. *Curr. Med. Chem. Anti-infective Agents* **2002**, *1*, 193-214.

(2) Page, M. G. P. β-lactamase inhibitors. *Drug Resist. Updates* **2000**, *3*, 109−125.

(3) Sandanayaka, V. P.; Prashad, A. S. Resistance to β-lactam antibiotics: Structure and mechanism based design of β-lactamase inhibitors. *Curr. Med. Chem*. **2002**, *9*, 1145−1165.

(4) Dizdaroglu, M.; Jaruga, P.; Rodriguez, H. Oxidative damage to DNA: mechanisms of product formation and measurement by mass spectrometric techniques. *Crit. Rev. Oxidative Stress Aging* **2003**, *1*, 165−189.

(5) Ramtohul, Y. K.; Martin, N. I.; Silkin, L.; James, M. N. G.; Vederas, J. C. Synthesis of pseudoxazolones and their inhibition of the 3C cysteine proteinases from hepatitis A virus and human rhinovirus-14. *J. Chem. Soc., Perkin Trans*. **2002**, *11*, 1351−1359.

(6) Wang, H.; Wu, Y.; Zhao, Z. Fragmentation study of simvastatin and lovastatin using electrospray ionization tandem mass spectrometry. *J. Mass Spectrom*. **2001**, *36*, 58−70.

(7) Sandanayaka, V. P.; Prashad, A. S.; Yang, Y.; Williamson, R. T.; Lin, Y. I.; Mansour, T. S. Spirocyclopropyl β-lactams as mechanism-based inhibitors of serine β-lactamases. synthesis by rhodium-catalyzed cyclopropanation of 6-diazopenicillanate sulfone. *J. Med. Chem*. **2003**, *46*, 2569−2571.

(8) Yang, Y.; Janota, K.; Tabei, K.; Huang, N.; Siegel, M. M.; Lin, Y. I.; Rasmussen, B. A.; Shlaes, D. M. Mechanism of inhibition of the class A β-lactamases PC1 and TEM-1 by tazobactam. Observation of reaction products by electrospray ionization mass spectrometry. *J. Biol. Chem*. **2000**, *275*, 26674−26682.

(9) Ng, L. M.; Pagan-Rodriguez, D.; Zhou, X.; Simmons, R.; Bonomo, R. A.; Hujer, A. M.; Anderson, V. B. An ESI-MS/LC-MS analysis of the inhibition of SHV-1 β-lactamase with tazobactam. *Abstracts of Papers*, 224th American Chemical Society National Meeting, Boston, MA, Aug 18−22, 2002; American Chemical Society: Washington, DC, 2002; ANYL-099.

(10) Bonomo, R. A.; Liu, J.; Chen, Y.; Ng, L.; Hujer, A. M.; Anderson, V. E. Inactivation of CMY-2 β-lactamase by tazobactam: initial mass spectroscopic characterization. *Biochem. Biophys. Acta* **2001**, *1547*, 196−205.

(11) Coleman, K.; Griffin, D. R. J.; Page, J. W. J.; Upshon, P. A. In vitro evaluation of BRL 42715**,** a novel β-lactamase inhibitor. *Antimicrob. Agents Chemother*. **1989**, *33*, 1580−1587.

(12) Bulychev, A.; Massova, I.; Lerner, S. A.; Mobashery, S. Penem BRL 42715: an effective inactivator for β-lactamases. *J. Am. Chem. Soc*. **1995**, *117*, 4797−4801.

(13) Farmer, T. H.; Page, J. W. J.; Payne, D. J.; Knowles, D. J. C. Kinetic and physical studies of β-lactamase inhibition by a novel penem, BRL 42715. *Biochem. J*. **1994**, *303*, 825−830.

(14) Okafo, G. N.; Cutler, P.; Knowles, D. J.; Camilleri, P. Capillary electrophoresis study of the hydrolysis of a β-lactamase inhibitor. *Anal. Chem*. **1995**, *67*, 3697−3701.

(15) Broom, N. J. P.; Farmer, T. H.; Osborne, N. F.; Tyler, J. W. Studies on the mechanism of action of (5R)-(Z)-6-(1-methyl-1,2,3-triazol-4-ylmethylene)penem-3-carboxylic acid (BRL 42715), a potent inhibitor of bacterial β-lactamase. *J. Chem. Soc., Chem. Commun*. **1992**, *22*, 1663−1664.

(16) Takao, A.; Hiroshi, M.; Ado, M.; Sato, C.; Ushirogochi H.; Sato, K.; Takasaki, T.; Venkatesan, A. M.; Mansour, T. S. Process for preparing 6-alkylidene penem derivatives. Patent WO2003093277, 2003.

(17) Nukaga, M.; Abe, T.; Venkatesan, A. M.; Mansour, T. S.; Bonomo, R. A.; Knox, J. R. Inhibition of class A and class C β-lactamases by penems: crystallographic structures of a novel dihydrothiazepine intermediate. *Biochemistry* **2003**, *42*, 13152−13159.

(18) Abe, T.; Ushirogochi, H.; Yamamura, I.; Kumagai, T.; Mansour, T. S.; Venkatesan, A. M.; Agarwal, A.; Petersen, P. J.; Weiss, W. J.; Lenoy, E.; Yang, Y.; Shales, D. Novel penems bearing a methylidene linkage as broad spectrum β-lactamase inhibitors. *Abstracts of Papers of the 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC)*, Chicago, IL, Sept 14−17, 2003, F-538.

(19) Gioacchini, A. M.; Traldi, P. Mass spectrometry of β-lactam antibiotics. *Targets Heterocycl. Syst*. **2001**, *5* 327−355.

(20) Das, P. R.; Pramanik, B. N.; Girijavallabhan, V. M.; Ganguly, A. K. Ionization of penem β-lactam antibiotics using thermospray (filament-on) mass spectrometry. *J. Antibiot*. **1988**, *41*, 1268−1271.

(21) Triolo, A.; Bonelli, F. Structural characterization of a new class of penem β-lactam antibiotics by triple quadrupole tandem mass spectrometry**.** *Rapid Commun. Mass Spectrom*. **1995**, *9*, 707−711.

(22) Mirgorodskaya, E.; Roepstorff, P.; Zubarev, R. A. Localization of O-glycosylation sites in peptides by electron capture dissociation in a Fourier transform mass spectrometer. *Anal. Chem*. **1999**, *71*, 4431−4436.

(23) Shi, S. D.-H.; Hemling, M. E.; Carr, S. A.; Horn, D. M.; Lindh, I.; McLafferty, F. W. Phosphopeptide/phosphoprotein mapping by electron capture dissociation mass spectrometry**.** *Anal. Chem*. **2001**, *73*, 19−22.

(24) Zubarev, R.A.; Kelleher, N. L.; McLafferty, F. W. Electron capture dissociation of multiply charged protein cations. A nonergodic process. *J. Am. Chem. Soc*. **1998**, *120*, 3265−3266.

(25) Sutcliffe, J. G. Nucleotide sequence of the ampicillin resistance gene of Escherichia coli plasmid pB*R*322. *Proc. Natl. Acad. Sci. U.S.A*. **1978**, *75*, 3731−3741.

(26) Rasmussen, B. A.; Gluzman, Y.; Tally, F. P. *Escherichia coli* chromosomal mutations that permit direct cloning of the Bacteroides fragilis metallo-β-lactamase gene, ccrA. *Mol. Microbiol*. **1991** *5*, 1211−1219.

(27) Ambler, R. P.; Coulson, A. F. W.; Frere, J.-M.; Ghuysen, J.-M.; Joris, B.; Forsman, M.; Levesque, R. C.; Tiraby, G.; Waley, S. G. A standard numbering scheme for the class A β-lactamases. *Biochem. J*. **1991**, *276*, 269−270.

(28) Yang, Y.; Testa, R. T.; Bhachech, N.; Rasmussen, B. A.; Bush, K. Biochemical characterization of novel tetrahydrofuranyl-1-β-methylcarbapenems: stability to hydrolysis by renal dehydropeptidases and bacterial β-lactamases, binding to penicillin binding proteins and permeability properties. *Antimicrob. Agents Chemother*. **1999**, *43*, 2904−2909.

(29) Bush, K.; Macalintal C.; Rasmussen B. A.; Lee V. J.; Yang Y. Kinetic interactions of tazobactam with beta-lactamases from all major structural classes. *Antimicrob. Agents Chemother*. **1993**, *37*, 851−858.

(30) O'Callanghan, C. H.; Morris, A.; Kirby, S. M.; Shingler, A. H. Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. *Antimicrob. Agents Chemother*. **1972** *1*, 283−288.

(31) Roepstorff, P.; Fohlman, J. Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed. Mass Spectrom*. **1984**, *11*, 601.

(32) Valaskovic, G. A.; Kelleher, N. L. Miniaturized formats for efficient mass spectrometry-based proteomics and therapeutic development. *Curr. Topics Med. Chem. (Hilversum, Netherlands)* **2002**, *2*, 1−12.

(33) Palmblad, M.; Hakansson, K.; Hakansson, P.; Feng, X.; Cooper, H. J.; Giannakopulos, A. E.; Green, P. S.; Derrick, P. J. A 9.4 T Fourier transform ion cyclotron resonance mass spectrometer: description and performance. *Eur. J. Mass Spectrom*. **2000**, *6*, 267−275.

(34) Gauthier, J. W.; Trautman, T. R.; Jacobson, D. B. Sustained off-resonance irradiation for collision-activated dissociation involving Fourier transform mass spectrometry. Collision-activated dissociation technique that emulates infrared multiphoton dissociation. *Anal. Chim. Acta* **1991**, *246*, 211−225.

JM049903J