Design, Synthesis, and Evaluation of 2β-Alkenyl Penam Sulfone Acids as Inhibitors of β-Lactamases†

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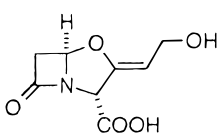
A general method for synthesis of 2β-alkenyl penam sulfones has been developed. The new compounds inhibited most of the common types of β-lactamase. The level of activity depended very strongly on the nature of the substituent in the 2β-alkenyl group. The inhibited species formed with the β-lactamase from *Citrobacter freundii* 1205 was sufficiently stable for X-ray crystallographic studies. These, together with UV absorption spectroscopy and studies of chemical degradation, suggested a novel reaction mechanism for the new inhibitors that might account for their broad spectrum of action. The (*Z*)-2β-acrylonitrile penam sulfone Ro 48-1220 was the most active inhibitor from this class of compound. The inhibitor enhanced the action of, for example, ceftriaxone against a broad selection of organisms producing β-lactamases. The organisms included strains of Enterobacteriaceae that produce cephalosporinases, which is an exceptional activity for penam sulfones.

Introduction

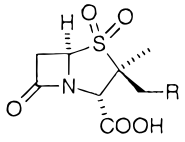
The most widespread mechanism of bacterial resistance to β-lactam antimicrobials is the expression of β-lactamases that degrade these antibiotics.[1] The β-lactamases exhibit considerable diversity in the range of antibiotics they attack and have been classified into four classes, from A to D, on the basis of their substrate specificity and genetics.[2] Class C β-lactamases and extended broad-spectrum class A β-lactamases are currently of particular clinical importance because strains of Enterobacteriaceae and *Pseudomonas aeruginosa* producing these enzymes have been selected by the extensive use of third-generation cephalosporins in antibacterial chemotherapy.[1−3] Thus, there is a growing interest in the development of effective inhibitors for these β-lactamases.[4] Commercially available inhibitors (clavulanic acid (**1**) or penam sulfones such as sulbactam (**2a)** and tazobactam (**2b**)) have potent activity against the class A β-lactamases, but they all lack activity against class C β-lactamases.[4−6]

The three-dimensional structures of several β-lactamases belonging to classes A and C have been solved by X-ray crystallography.[7−11] However, these are of limited use for the design, using computer-assisted molecular modeling, of new molecules based on the known types of inhibitor.[12,13] The principal reason for this is that the reactions of the inhibitors with β-lactamases involve branched pathways with many putative intermediates (Scheme 1), few of which have been positively identified.[6,14,15] Despite this uncertainty, we reasoned that the introduction of a certain degree of rigidity into the 2β-side chain of a penam sulfone (for example, by incorporating a double bond, such as in **3**) might improve the binding and slow down hydrolysis.

We report the synthesis and the properties of some 2β-alkenyl penam sulfone acid derivatives **3** that display improved activity against class C β-lactamases. Their combination with ceftriaxone exhibited useful activity against bacteria that were producing β-lactamases.

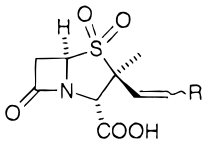


1 clavulanic acid



2a : R= H sulbactam

2b : R=  tazobactam



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Chemistry

Synthesis of the 2β-Formyl Penam Ester 10. 6β-Aminopenicillanic acid was transformed into the bromopenicillinate **4** by the method of Micetich[16] (Scheme 2). Tributylphosphine[17] in methanol was found to be superior to zinc in acetic acid for debromination to give the β-sulfoxide **5**. The disulfide **6** was obtained from **5** by reaction with 2-mercaptobenzothiazole according to Kamiya.[18] A 3:1 mixture of the penam **7** and the corresponding cepham **8** was produced by reaction of **6** with chloroacetic acid in the presence of silver acetate.[19] The unseparated mixture was treated with thiourea in the presence of pyridine to give a rather unstable penam alcohol (**9**) that was crystallized from the reaction mixture. Oxidation of **9** to the β-formyl penam ester **10** was performed using the conditions of Swern.[20]

Synthesis of 2β-Alkenyl Penam Sulfone Acids 3. The penam esters **11a**−**n** were obtained as *E*/*Z* mixtures by reaction of **10** with various ylides (Scheme 3). For **11g**,**h** (R = CN), the relative yields of *Z* isomer **11h** and *E* isomer **11g** depended on the reaction conditions: In standard Wittig conditions the ratio of **11h** to **11g** was

† Dedicated to Dr. Ivan Kompis on the occasion of his retirement as Head of Infectious Diseases Research at Hoffmann-La Roche.

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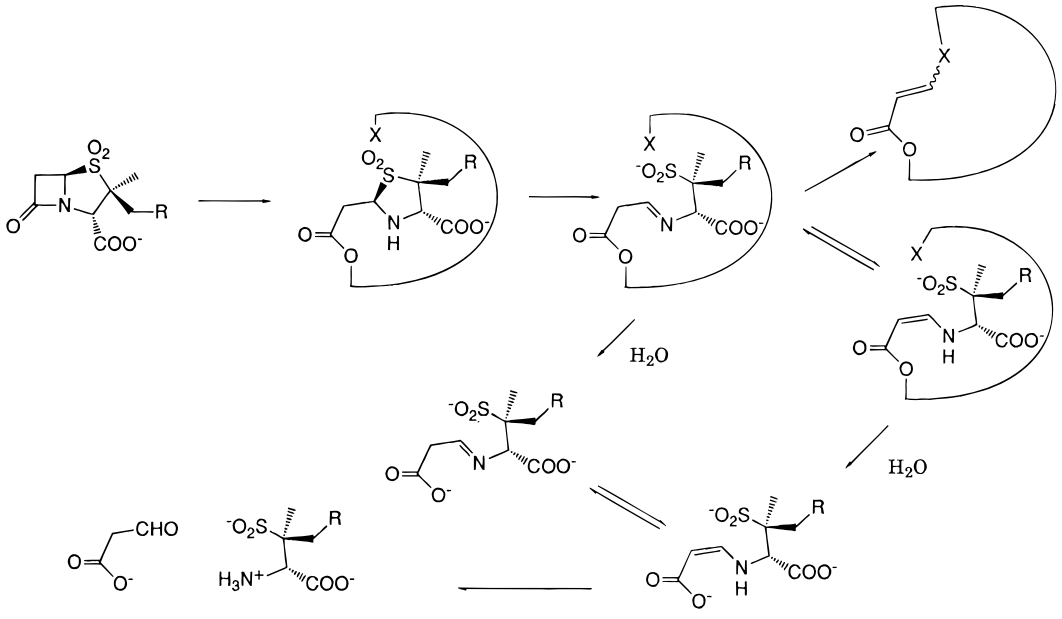
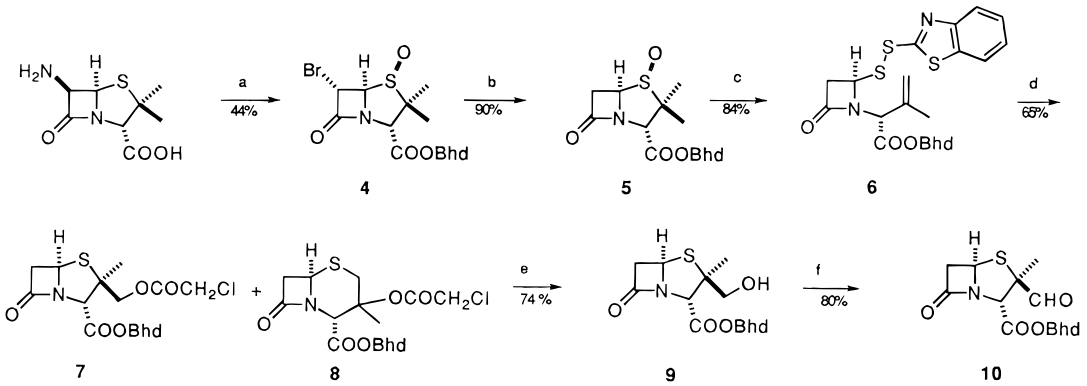
Scheme 1. Suggested Interactions of Penam Sulfones with β-Lactamases



Scheme 2[a]



[a] Reagents: (a) $NaNO_2$, KBr, H^+ then CH_3CO_3H, benzophenone hydrazone; (b) PBu_3, MeOH; (c) mercaptobenzothiazole, Δ; (d) $ClCH_2COOH$, AgOAc; (e) thiourea, pyridine; (f) DMSO, oxalyl chloride. Bhd: diphenylmethyl.

1:4, in MeCN at 0 °C in the presence of $LiClO_4$ it was 4:1, and in Wittig−Horner conditions it was 1:1. Peterson olefination gave only **11h** but in low yield. The allylic penam esters **11o**−**r** were produced by the reactions shown in Scheme 4. The unsaturated aldehyde **11f** was reduced to the allylic alcohol **11o** with DIBAH. This alcohol was reacted with dihydropyran to give the ether **11p**. The pyridinium derivative **11q** and the protected carbamate **11r** were obtained from **11o** by treatment with respectively triflate anhydride in pyridine and chloroacetyl isocyanate.

The corresponding penam sulfones **12** were synthesized by oxidation with *m*-chloroperbenzoic acid, $KMnO_4$, or RuO_2−$NaIO_4$ in a two-phase system and converted to the free acids **3** by removal of the benzhydryl blocking group in *m*-cresol at 50 °C (Table 1).

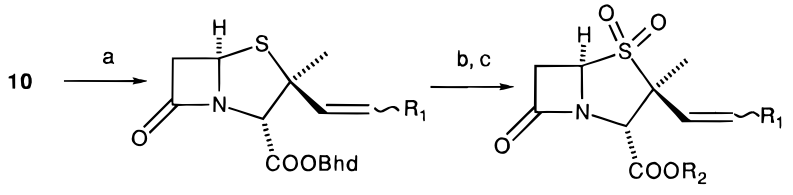
Biological Results and Discussion

Spectrum of Action of 2β-Alkenyl Penam Sulfone Acids. The 2β-alkenyl derivatives **3** had potent activity against most class A β-lactamases typical of penam sulfones.[5,6] Several compounds achieved a similar potency against the class C β-lactamase from *Citrobacter freundii* (Table 2) but not against more resistant class C enzymes such as the *Escherichia coli* AmpC β-lactamase. The exception was the nitrile analogue Ro 48-1220 (**3h**), which had a broad spectrum of activity encompassing most serine β-lactamases (Tables 2 and 3). Particularly striking is the relatively even coverage by **3h** of class C enzymes where other penam sulfone acids, such as tazobactam, are ineffective (Table 3).

Although the overall affinity for high molecular weight penicillin-binding proteins (transpeptidases) was rather low, there was some activity against the proteins from Gram-positive organisms (see Supporting Information). The pyridinium derivative **3q** showed relatively good activity against transpeptidases from Gram-positive bacteria that included proteins that had mutated to become resistant to cephalosporins (Table 4). The activity is not sufficient to result in a useful antibacterial against most pathogens. In a few organisms the reaction with penicillin-binding proteins is sufficient to result in intrinsic activity (data not shown), just as with other penam sulfone acids.[21]

The inhibitors were all able to afford protection to β-lactamase sensitive antibiotics when the combination was applied to organisms harboring class A β-lactamases susceptible to sulbactam and tazobactam (Table 5). The activity against organisms harboring more active class A β-lactamases like TEM-4 or relatively

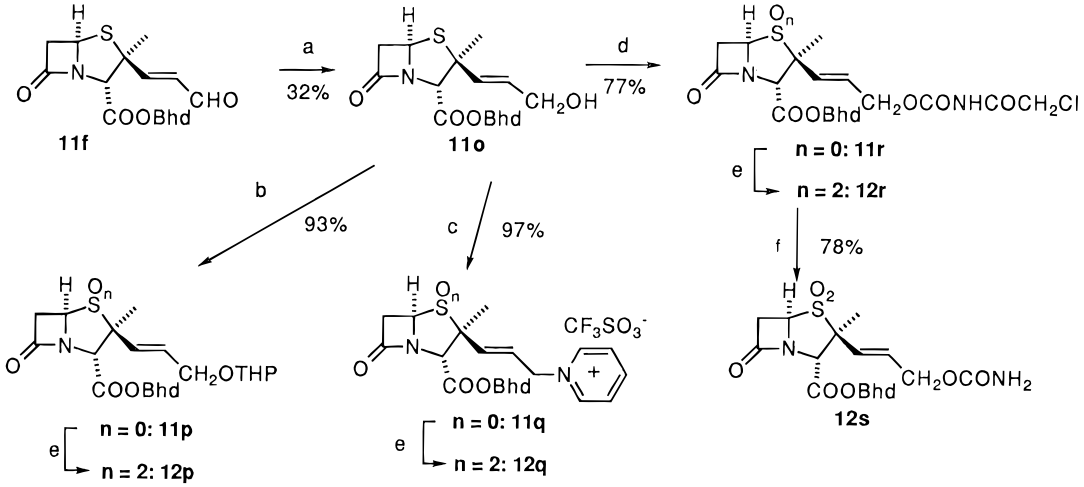
Scheme 3[a]



	R_1		Methods[a] (Yield, %) 11	12		R_1		Methods[a] (Yield, %) 11	12
a	COOEt	*(E)*	A (88)	D (60)	**h**	CN	*(Z)*	A (19)	D (7)
b	$CONH_2$	*(E)*	A (12)	E (30)	**i**	Cl	*(E)*	A (69)	C (30)
c	$CONH_2$	*(Z)*	A (8)	D (57)	**j**	Cl	*(Z)*		C (38)
d	CONMeOMe	*(E)*	A (80)	C (55)	**k**	2-Pyridinyl	*(E)*	A (28)	C (62)
e	COMe	*(E)*	A (52)	D (40)	**l**	2-Thiazolyl	*(E)*	B (62)	C (33)
f	CHO	*(E)*	A (13)		**m**	2-Thiazolyl	*(Z)*	B (17)	C (33)
g	CN	*(E)*	A (75)	D (23)	**n**	2-Oxadiazolyl	*(E)*	B (21)	C (40)

[a] Reagents employed according to general methods described in the text: (a) phosphorane (method A) or phosphonium bromide, butylene oxide (method B); (b) $NaIO_4$, RuO_2 (method C), or $KMnO_4$ (method D), or *m*-chloroperbenzoic acid (method E); (c) *m*-cresol, 50 °C (method F). Physical properties are given in the Supporting Information for intermediates **11** and **12** and in Table 1 for the penam sulfone acids **3**.

Scheme 4[a]



[a] Reagents and conditions: (a) DIBAH; (b) dihydropyran, *p*-TSS; (c) Tf_2O, pyridine; (d) chloroacetyl isocyanate; (e) $NaIO_4$, RuO_2 (method C); (f) $NaHCO_3$, MeOH.

susceptible class C β-lactamases (such as the *C. freundii* β-lactamase) showed more differentiation, but it was similar to that which can be obtained with sulbactam or tazobactam. A number of compounds, particularly **3h**,**c**,**j** (R = CN, $CONH_2$, and Cl, respectively), had significantly broader spectra of action. The biggest improvement in activity was obtained against organisms such as *Enterobacter* and *Pseudomonas* that have more resistant class C β-lactamases and less permeable outer membranes. The nitrile derivative Ro 48-1220 (**3h**) had the broadest spectrum of action in combination with other β-lactam antibiotics, and the synergy with ceftriaxone against strains of *Citrobacter* and *Enterobacter* lowers the MIC values to a useful level.

Mode of Inhibition of β-Lactamases by 2β-Alkenyl Penam Sulfones. The 2β-alkenyl penam sulfones reacted rapidly with β-lactamases to form inhibited enzyme species that had increased UV absorbance typical for penam sulfones[6,22] (Figure 1). The subsequent reaction, however, resulted in products with a different UV spectrum, which suggested that the stable acyl intermediate formed with 2β-alkenyl penam sulfones may have a different structure from that formed with tazobactam.

The kinetic parameters describing the formation of the inhibited enzyme complexes were not very different from those obtained with tazobactam (Table 6). The dissociation constants observed with class C β-lactamases were slightly higher than those determined for tazobactam with the same enzymes. Thus, recognition

Table 1. Physical Properties of the Penam Sulfone Acids **3**

compd	method[a]	yield (%)	elemental anal.[b]	IR (cm^{-1})[c]	^{1}H NMR (D_2O) δ (J, Hz) 2-H[d]	5-H[e]	6-H[e]		CH=CH[f]	
3a	F	50	NA[g]	1782, 1715	4.62	5.15 (2.0, 4)	3.46 (16, 2)	3.71 (16, 4)	6.35	7.10 (16)
3b	F	80	NA	1782, 1684	4.62	5.14 (1.4, 4)	3.46 (17, 1.4)	3.71 (17, 4)	6.46	6.88 (16)
3c	F	70	NA	3434, 1781, 1668	4.68	5.10 (1.2, 4)	3.44 (16, 1.2)	3.69 (16, 4)	6.35	7.10 (16)
3d	F	67	$C_{12}H_{15}N_2O_7SNa$	1785, 1626	4.65	5.16 (1.2, 4)	3.47 (17, 1.2)	3.78 (17, 4)	6.95[d]	6.95[d]
3e	F	27	NA	1782, 1681, 1627	4.66	5.16 (2, 4)	3.46 (17, 2)	3.72 (17, 4)	6.56	7.03 (16)
3g	G	82	NA	2240, 1782, 1629	4.62	5.15 (2.0, 4)	3.47 (16, 2.0)	3.72 (16, 4)	6.07	7.08 (16)
3h	G	80	$C_{10}H_9N_2O_5SNa$	2222, 1782, 1629	4.80	5.19 (1.6, 4.4)	3.47 (16, 1.6)	3.73 (16, 4.4)	6.15	6.68 (12)
3i	F	32	NA	1780, 1626	NA					
3j	F	65	$C_9H_9ClNO_5SNa$	1779, 1626	4.88	5.10 (1, 4)	3.44 (17, 4)	3.70 (17, 1)	5.96	6.71 (8.4)
3k	G	100	$C_{14}H_{13}N_2O_5SNa$	1784, 1629	4.66	5.15 (1, 4)	3.47 (16, 1)	3.72 (16, 4)	6.80	7.05 (16)
3l	G	98	$C_{12}H_{11}N_2O_5S_2Na$	1778, 1627	4.65	5.15 (1, 4)	3.47 (16, 1)	3.72 (16, 4)	6.80	7.16 (16)
3m	F	57	NA	1781, 1623	5.19	5.04 (1.2, 4.1)	3.42 (16, 1.2)	3.58 (16, 4.1)	6.06	7.14 (12.5)
3n	G	91	$C_{11}H_{10}N_3O_6SNa$	1787, 1627	4.70	5.17 (1.4)	3.47 (16, 1)	3.73 (14, 4)	7.03	7.10 (16)
3o	F	62	$C_{10}H_{12}NO_6SNa$	3428, 1780, 1622	4.48	5.08 (2, 4)	3.42 (16, 2)	3.68 (16, 4)	5.94	6.24 (16)[h]
3q	F	55	NA	1780, 1625	4.52	5.10 (1.5, 4)	3.42 (17, 1.5)	3.57 (17, 4)	6.14	6.37 (16)[i]
3s	F	62	NA	NA	4.48	5.08 (1.6, 4)	3.42 (17, 2)	3.69 (17, 4)	5.97	6.19 (16)[j]

[a] Methods F and G refer to general procedures described in the Experimental Section; all the derivatives were Na salt, except **3q**, obtained as inner salt. [b] C, H, N, S. [c] KBr. [d] Singlet. [e] Doublet of doublets. [f] Doublet. [g] NA = data not available. [h] 5.94 (doublet of triplets, J = 16, 0.8 Hz), 6.24 (doublet of triplets, J = 16, 4.5 Hz). [i] 6.37 (doublet of triplets, J = 16, 6 Hz). [j] 6.19 (doublet of triplets, J = 16, 4.5 Hz).

Table 2. Inhibition of β-Lactamases by Penam Sulfones

	IC_{50} (μM)					
	class A			class C		
compd	*B. licheniformis* 749/C	TEM-1	TEM-3	*E. coli* AmpC	*C. freundii* 1928	*P. aeruginosa* 18SH
3a	2.9	14	0.46	61	0.37	15
3b	6.0	13	0.054	5.3	2.3	4.9
3c	3.4	1.2	0.13	81	4.3	2.4
3d	4.4	12	0.18	72	2.5	5.8
3e	3.9	10	0.42	3.0	0.15	1.1
3g	0.32	1.3	0.69	21	0.70	7.5
3h	0.32	1.1	0.13	1.5	0.21	0.38
3j	6.6	5.6	0.06	1.7	0.6	1.1
3k	2.3	19	0.092	46	1.0	15
3l	0.54	13	0.054	2.7	0.41	8.9
3m	5.4	8.5	0.16	45	1.3	0.54
3n	1.7	12	0.22	5.2	0.49	11
3o	3.6	6.5	0.60	48	6.9	7.6
3q	1.8	3.6	0.14	330	16	75
3s	6.5	9.1	0.76	10	0.48	1.2

Table 3. Inhibition of β-Lactamases by **3h** (Ro 48-1220)

enzyme			IC_{50} (μM)	
Ambler	class (Bush[33])	source	tazobactam	Ro 48-1220
C	(1)	*Morganella morgani* U1627	0.19	0.21
		C. freundii 1982	0.93	0.21
		Enterobacter cloacae 908R	1.90	0.41
		Ent. cloacae 6300	85.0	3.40
		E. coli SNO3	18.0	1.51
		P. aeruginosa 18SH	0.82	0.38
		P. aeruginosa GN10362	2.11	0.86
		P. aeruginosa MK1184	36.0	1.97
		Bacteroides fragilis 98	98.0	3.70
A	(2a)	*Bacillus licheniformis* 749/C	0.51	0.32
		S. aureus PC1	0.50	0.25
A	(2b)	RTEM-1	0.42	1.07
		RTEM-2	0.56	0.98
A	(2b′)	RTEM-3	0.15	0.13
		SHV-2	0.93	0.54
A	(2c)	PSE-1	0.56	0.09
D	(2d)	OXA-1	0.24	0.10
		OXA-2	0.83	0.21
		B. fragilis 36	4.8	0.43
B	(3)	*Xanthomonas maltophila* 328	4000	24
		B. fragilis BF101	>10000	200

is not altered by the introduction of the double bond in the 2β-side chain. For both tazobactam and the 2β-alkenyl penam sulfones, the affinities with class C enzymes were often markedly less than the affinity either type of penam sulfone had with class A enzymes (Table 6). High affinity with class C enzymes is dependent on fulfilling interactions in the binding pocket provided for the acylamino side chain of penicillins and

Table 4. Inhibition of Penicillin-Binding Proteins by Selected Penam Sulfones

	IC_{50} (μM)									
	E. coli W3110[a]			*S. aureus* Schoch[a]				*St. pneumoniae*[b]		
compd	PBP 1b	PBP 2	PBP 3	PBP 1	PBP 2	PBP 3	PBP 4	PBP 2×	503[c]	604[c]
3h	340	34	>340	17	17	170	17	5	200	280
3k	>10000	145	>300	15	145	145	15	4.5	39	33
3l	>10000	143	286	14	143	286	14	4.8	22	14.1
3m	>10000	14	>300	14	28	28	143	50	100	80
3q	>10000	297	>300	15	15	30	15	3.9	8.2	8.1

[a] Penicillin binding assayed using membrane fragments and ^{14}C-labeled benzylpenicillin.[30] [b] Assayed using recombinant soluble protein.[31] [c] Cephalosporin resistant mutants of PBP 2×.[32]

Table 5. Antibacterial Activity of Ceftriaxone in Combination with Penam Sulfones

	MIC of ceftriaxone (mg/L) in a 1:4 combination with the compound				
				E. coli	
compd	*Ent. cloacae* 908R	*C. freundii* 1928	*P. aeruginosa* 18SH	pTEM-3	pTEM-4
none	128	64	>128	16	64
tazobactam	8	4	16	<0.25	2
3a	8	4	>16	<0.25	NA
3b	8	NA	16	<0.25	0.5
3c	4	2	4	0.25	2
3d	16	8	>16	0.5	2
3e	2	1	32	0.25	2
3g	4	2	16	<0.25	NA
3h	1	1	4	<0.25	1
3j	4	4	4	0.25	1
3k	32	8	>16	1	4
3l	16	16	>16	0.5	4
3m	>16	8	16	1	4
3n	8	4	16	0.5	2
3o	8	8	16	0.5	2
3q	8	4	>16	0.25	0.5
3s	8	4	8	1	4

Table 6. Kinetic Parameters Describing the Reaction of Ro 48-1220 (**3h**) with Representative β-Lactamases[a]

	Ro 48-1220			tazobactam		
enzyme	k_{on} (mM^{-1} s^{-1})	K_S (μM)	k_{off} (h^{-1})	k_{on} (mM^{-1} s^{-1})	K_S (μM)	k_{off} (h^{-1})
C. freundii 1928	64	125	<0.001	76	110	0.05
E. coli SNO3	56	377	<0.001	80	325	0.2
TEM-3	170	0.56	0.02	230	0.74	0.03
SHV-2	139	2.3	0.5	490	5	6.1

[a] The values for the rate of formation of the acyl−enzyme complex (k_{on}), the half-saturation constant derived from concentration dependence studies (K_S), and the rate of breakdown of the acyl−enzyme complex (k_{off}) were determined using published methods.[28,29]

cephalosporins. These comprise hydrogen bonds to main chain NH and the side chain of asparagine 152 as well as van der Waals interactions with tyrosine 221. Neither type of penam sulfone has a side chain that can utilize these interactions in the orientation the molecule must adopt for acylation to occur. Despite the disparate dissociation constants, class A and C enzymes come to similar apparent inhibition constants (Table 2) because of the different rates of deacylation, class A enzymes being significantly more rapid (Table 6).

The rate of recovery of the enzyme observed after all the sulfone had been consumed was slower with the 2β-alkenyl penam sulfones than with tazobactam. The activity eventually returned to the control activity (±10%), although this could take over 2 days. The acyl−enzyme complex formed with the 2β-alkenyl penam sulfones appears to be considerably more stable than those formed with sulbactam or tazobactam, particularly among the class C β-lactamases. Thus, it seems that the additional potency of these compounds stems from a property of the acyl−enzyme.

The reaction of sodium methoxide with some of the penam sulfones was studied as a model for the enzymic reaction. Treatment of a methanolic solution of the sodium salt of **3h** with a slight excess of sodium methoxide produced a rapid change in the UV absorption spectrum (Figure 2). After long incubation (>12 h), or in the presence of a larger excess of sodium methoxide (10 equiv), a new spectrum with increased absorption between 260 and 320 nm was established. Evaporation of the solvent after 15 h and gel chromatography allowed the isolation of the enamine **13** together with its corresponding amino acid **14** and the glyoxylate **15**. The UV absorption spectrum of **13** in methanol had a peak at 268 nm with a shoulder at 290 nm (Figure 2), and the absorption properties corresponded fairly well with the difference spectrum of the acyl−enzyme complex formed with *E. coli* β-lactamase. This suggests that the stable acyl intermediate formed with β-lactamases arises through elimination of SO_2 and electronic rearrangement to give the linear conjugated system found in **13** (Scheme 5).

The identity of the stable acyl−enzyme was confirmed by determining the X-ray crystal structure of complexes of **3h**,**c** with the class C β-lactamase from *C. freundii* (Figure 3). With **3c** the inhibitor molecule is well-defined by the electron density, which is consistent with the proposed *trans*-enamine structure **13**. The carbonyl

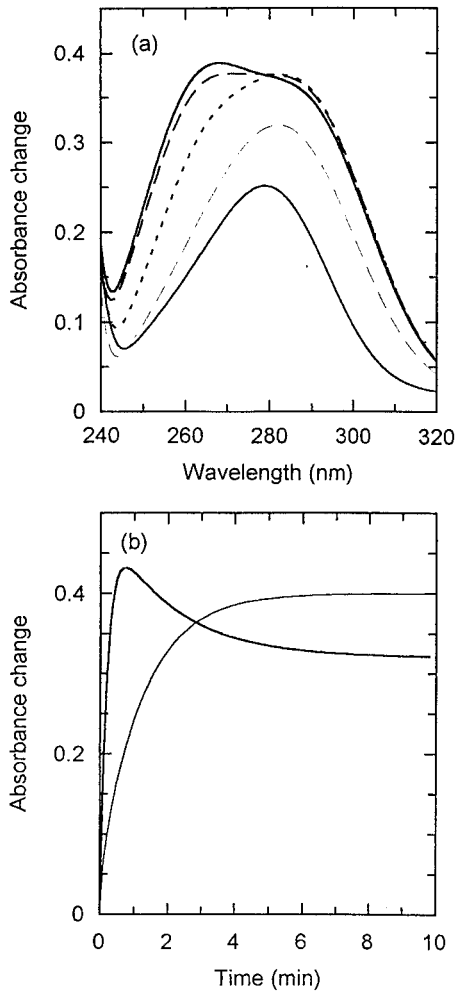


Figure 1. Changes in the UV absorption spectrum of Ro 48-1220 during the reaction with *E. coli* AmpC β-lactamase: (a) spectral changes after reaction for 0.1 (lowest curve), 0.5, 1, 1.5, and 2 (upper solid curve) min and (b) time courses of the changes at 265 nm (thin curve) and 285 nm (thick curve). Measurements were made using 1 μM protein and 100 μM Ro 48-1220 in 0.1 M sodium phosphate buffer, pH 7.0, according to general methods described in ref 28.

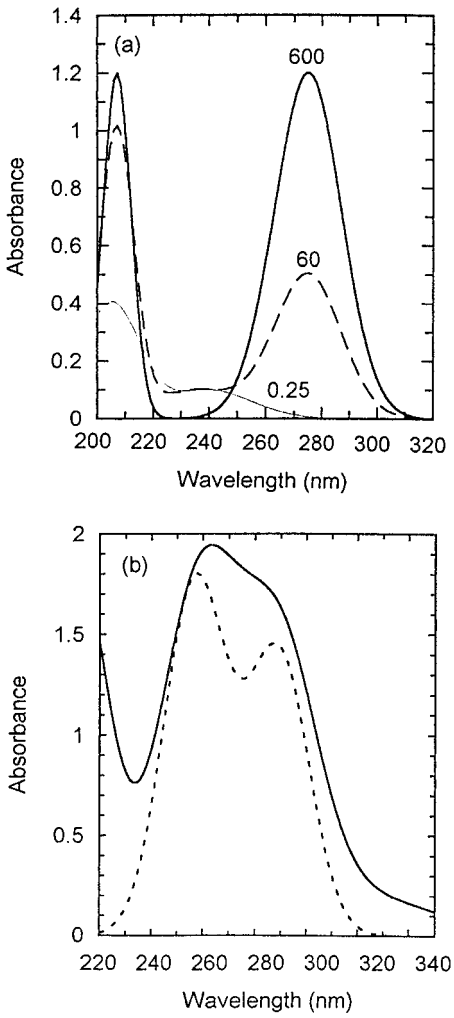


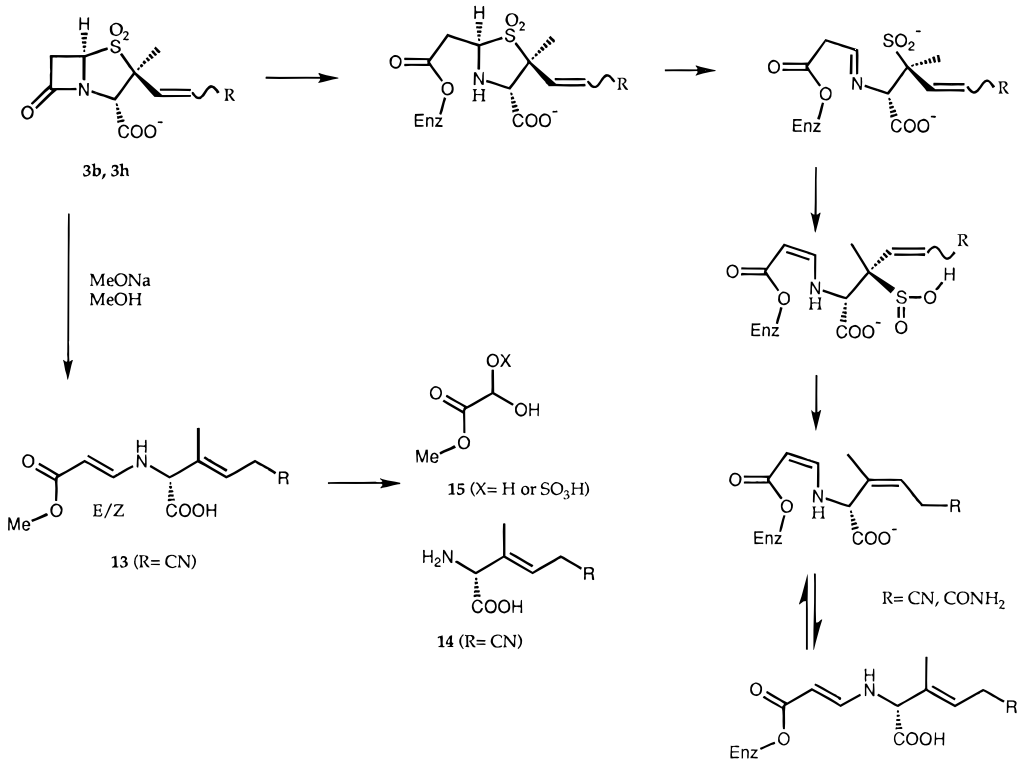
Figure 2. Changes in the UV absorption spectrum of Ro 48-1220 (**3h**) during methanolysis. (a) Treatment of 100 nmol of the sodium salt of Ro 48-1220 with 1.2 equiv of sodium methoxide in 1 mL of methanol. Temperature was maintained at 30 °C in a thermostated cuvette holder. Scans were taken at the times (in min) shown. (b) Comparison of the UV absorption spectrum of the isolated enamide **13** in methanol (solid line) with the difference spectrum of the reaction product of Ro 48-1220 with *E. coli* AmpC β-lactamase (dotted line). The difference spectrum was obtained using split cuvettes (0.8 cm total path length). Both sample and reference cuvettes contained 1 μM β-lactamase and 100 μM Ro 48-1200 (as for the experiment shown in Figure 1) in separate compartments. The reaction was initiated by mixing the contents of the sample cuvette and allowed to continue until no further change at 265 nm was observed.

oxygen of the acyl ester is located in the oxyanion hole[7−11] and forms two hydrogen bonds with the main chain NH of Ser64 and Ser318. The carboxamide group appears engaged in two hydrogen bonds with the protein, one with its oxygen to the main chain NH of Gly320 and the other with its NH_2 group to the Oγ of Ser212. Despite these apparently good interactions, the electron density indicated a second conformation of this terminal carboxamide, as illustrated in Figure 3a, top. The middle part of the inhibitor is in van der Waals contact with the protein, but neither the carboxylate substituent nor the NH of the enamine make hydrogen bonds to the protein. The side chain of Gln120, a candidate for such interactions at first sight, is not well-defined and may exist in more than one conformation. A number of favorable nonpolar contacts are made between the middle part of the elongated inhibitor and the protein, most noticeably between the methyl substituent of the inhibitor and the side chains of Thr319 and Tyr221.

For the complex with **3h**, the difference density at the oxyanion hole was as strong as that of **3c** but with the rest of the inhibitor poorly defined. Nevertheless, it was apparent that the conformation adopted by **3h** and its location in the active site were very similar to those observed with **3c** (Figure 3).

Chen and Herzberg[15] have described the X-ray crystal structure of an intermediate complex formed during the reaction of clavulanic acid with the class A β-lactamase from *Staphylococcus aureus*. Clavulanic acid undergoes a similar set of reactions as the penam sulfones, resulting in the formation of the enamine.[4] The acyl−enzyme complex, trapped at low temperature, contained electron density that could be interpreted as a mixture of the *cis*- and *trans*-enamines. The elongated *trans*-enamine was placed in the same orientation in the active site as the acyl moieties of the penam sulfones described here, while the *cis*-isomer was in the opposite, more expected orientation. The point in the reaction of the penam sulfones with class C β-lactamases at

Scheme 5



which the reorientation of the inhibitor moiety occurs is not clear. Productive binding must occur with the side chain in the opposite orientation (pointing toward the top in Figure 3). Relaxation of *cis*-enamine to the intrinsically more stable *trans*-isomer and the interactions in the side chain binding pocket would both provide a driving force for the reorientation.

Conclusion

A series of 2β-alkenyl penam sulfones have been synthesized and evaluated for their potency as inhibitors of β-lactamases and as partners for a β-lactamase-labile antibiotic. Several of these compounds, especially Ro 48-1220, had a broad spectrum of action against β-lactamases including both class A enzymes, normally sensitive to such compounds, and class C enzymes, normally resistant to such compounds. This activity against the isolated enzymes could be realized as potentiation of the antibacterial activity of a β-lactam antibiotic susceptible to attack by β-lactamases. The mechanism of inhibition underlying the extended spectrum of action of the 2β-alkenyl penam sulfones was investigated by spectroscopy and X-ray crystallography. It was demonstrated that the inhibited species of the enzyme is a linear vinologous enamine formed by elimination of SO_2 and electronic rearrangement. Such a species has not been hitherto suggested as an intermediate in the reaction of penam sulfones with β-lactamases.

Experimental Section

General Synthetic Methods. Solvents were dried by filtration through Al_2O_3 (neutral, Brockmann, no. 1) when necessary and stored over a bed of molecular sieves (3 Å). All the organic solutions obtained after extraction were washed with water and brine and dried over anhydrous $MgSO_4$. The solvent was evaporated under reduced pressure with a water bath temperature below 35 °C. Chromatography was performed using Merck silica gel 60 (particle size 40−63 μm), the fractions containing the substance of interest were pooled, and the solvent was evaporated. Water soluble substances were purified by gel filtration (Mitsubishi Kasei Corp. MCI gel CHP20P 75−150 μm) using a gradient of MeCN in water. TLC was performed on Merck TLC plates (silica gel 60 F_{254}), and spots were visualized with aqueous $KMnO_4$. ^{1}H NMR spectra were recorded on a Bruker AC250 spectrophotometer. Chemical shifts (δ) are reported in ppm relative to Me_4Si as internal standard, and J values are given in Hz. IR spectra were recorded from KBr pellets using a Nicolet FTIR spectrometer. Mass spectra were determined on a MS9 spectrometer with SS 300 Finnigan MAT data system. Elemental analyses are indicated by the symbols of the elements; analytical results were within 0.4% of the theoretical values. Melting points were determined with a Buchi 510 melting point apparatus and are uncorrected.

1. Preparation of the 2β-Formyl Penam Ester 10. (2*S*,4*S*,5*R*,6*S*)-6-Bromo-3,3-dimethyl-4,7-dioxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic Acid Benzhydryl Ester (4). **4** was prepared according to the method of Micetich.[16] After workup, the crystalline residue was washed with cold AcOEt and dried, giving a yield of 44%: mp 151−152 °C (lit.[16] mp 65−70 °C); IR 1796, 1743, 1490, 1209 cm^{-1}; MS 481.6 (M + NH_4^+). Anal. ($C_{21}H_{20}BrNO_4S$) C, H, N, S.

(2*S*,4*S*,5*R*)-3,3-Dimethyl-4,7-dioxo-4-thia-1-azabicyclo-[3.2.0]heptane-2-carboxylic Acid Benzhydryl Ester (5). To a suspension of the bromide **4** (131 g, 0.28 mol) in MeOH (1.4 L) was added dropwise PBu_3 (67.2 g, 85% grade, 0.28 mmol) while maintaining the temperature below 40 °C. The reaction mixture was stirred at room temperature for 45 min and then diluted with Et_2O (0.3 L) and cooled to 0 °C. After crystallization the total yield was 90%: mp 157−158 °C (lit.[16] mp 145−148 °C); IR 1793, 1756, 1200 cm^{-1}; MS 401.4 (M + NH_4^+), 406.4 (M + Na^+). Anal. ($C_{21}H_{21}NO_4S$) C, H, N, S.

(*R*)-2-[(*R*)-2-(Benzothiazol-2-yldithio)-4-oxoazetidin-1-yl]-3-methylbut-3-enoic Acid Benzhydryl Ester (6). **6** was prepared using a minor modification of the method of Micetich.[16] The oily residue obtained after workup was diluted with Et_2O and crystallized by addition of Et_2O/hexane (1:1). The total yield was 84.2%: mp 76−77 °C; IR 1772, 1740, 1652, 1486, 1240 cm^{-1}; MS 531 (M − H); NMR ($CDCl_3$) 1.91 (s, 3H), 3.21 (dd, 1H, J = 15, 2.3), 3.43 (dd, 1H, J = 15, 5), 4.90 and

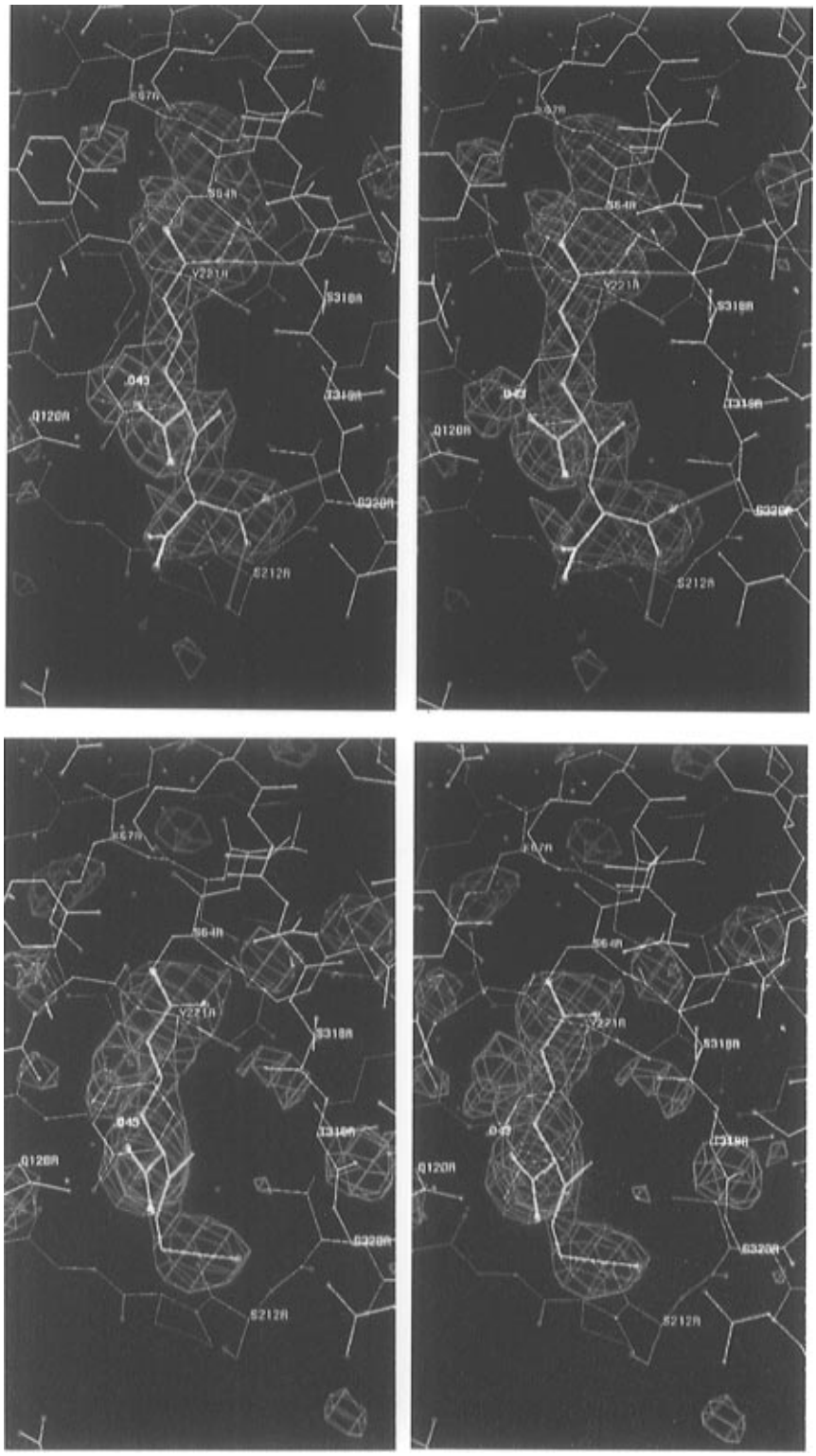


Figure 3. Structures of acyl−enzyme complexes of *C. freundii* class C β-lactamase with penam sulfones. (Top) Stereoview of an omit-difference electron density map for **3c** contoured at 0.15 e·Å^{-3}. The atoms of Ser64 and of the covalently attached inhibitor together with water molecules in the active site area were omitted for the calculation of structure factors. The active site of only one of the two crystallographically independent molecules is shown, but the density distribution in the other active site is comparable. The inhibitor is emphasized as a ball-and stick-model in green and the alternative conformation of its terminal carboxamide function in magenta. Hydrogen bonds between inhibitor and protein are indicated with red lines. Selected protein residues are labeled at their C-α-position. (Bottom) Stereoview of an F_{obs}(inhibitor) − F_{obs}(native) difference electron density map for **3h** contoured at 3 σ. The phases of the difference map were computed with the model of the uncomplexed enzyme structure omitting all water molecules. The manually placed model of the inhibitor is seen to account fairly well for the observed density features.

5.00 (2s, 2 × 1H), 5.15 (br, s, 1H), 5.39 (dd, 1H, J = 2.3, 5), 6.90 (s, 1H), 7.30−7.90 (m, 14H). Anal. ($C_{28}H_{24}N_2O_3S_3$) C, H, N, S.

(2*S*,3*R*,5*R*)-3-[(Chloroacetoxy)methyl]-3-methyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic Acid Benzhydryl Ester (7) and (2*R*,3*S*,6*R*)-3-(Chloroacetoxy)-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]octane-2-carboxylic Acid Benzhydryl Ester (8). $ClCH_2CO_2H$ (762 g,

8.07 mol) was added to a suspension of AcOAg (82.1 g, 0.49 mol) in CH_2Cl_2 (3 L). The suspension was treated with a solution of the disulfide **6** (172 g, 0.323 mol) in CH_2Cl_2 (0.3 L). After 6 h, the suspension was filtered through Dicalite and the solvent was evaporated. The residue was taken up in AcOEt and filtered through Dicalite. The filtrate was cooled to 0 °C, and the pH of the solution was brought to pH 6.5 with $NaHCO_3$ (a mixture of solid and saturated aqueous solution). The organic phase was separated, and the aqueous layer was extracted with AcOEt. The combined organic layers were worked up and chromatographed (AcOEt/hexane, 3:7), giving 131 g (88.2%) of a foam (as a 3:1 penam/cepham mixture). This mixture was used for the next step.

(2*S*,3*R*,5*R*)-3-(Hydroxymethyl)-3-methyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic Acid Benzydryl Ester (9). The mixture of **7** and **8** (total 131 g, 0.29 mol) was dissolved in DMF (0.27 L) and cooled to 0 °C, and pyridine (135 mL) was added. Thiourea (68.6 g, 0.9 mol) was added to the solution, which was stirred at 0 °C until the thiourea was completely dissolved. The reaction mixture was then allowed to reach room temperature, and the solvent was evaporated. The residue was dissolved in AcOEt and, after workup, concentrated to about 0.5 L and diluted with hexane (0.5 L). The resulting crystals were recrystallized from AcOEt/hexane. The mother liquor was evaporated and the residue quickly chromatographed (AcOEt/hexane, 3:7). The total yield was 74%: mp 129−130 °C; IR 3430, 1770, 1738, 1496, 1200 cm^{-1}; MS 401.3 $(M + NH_4)^+$, 406.2 $(M + Na)^+$; NMR ($CDCl_3$) 1.24 (s, 3H), 2.22 (pseudo-t, 1H, $J = 6$), 3.08 (dd, 1H, $J = 16$, 1.7), 3.53−3.61 (pseudo-m, 3H), 5.37 (dd, 1H, $J = 16$, 1.7), 6.94 (s, 1H), 7.30−7.38 (m, 10H). Anal. ($C_{21}H_{21}NO_4S$) C, H, N, S.

(2*S*,3*R*,5*R*)-3-Formyl-3-methyl-7-oxo-4-thia-1-azabicyclo-[3.2.0]heptane-2-carboxylic Acid Benzhydryl Ester (10). A solution of oxalyl chloride (31.8 g, 0.25 mol) in CH_2Cl_2 (1 L) was cooled under argon to −70 °C, and anhydrous DMSO (22.2 g, 284 mmol) was added dropwise. The solution was stirred at this temperature for 15 min; then a solution of the alcohol **9** (64 g, 167 mmol) in CH_2Cl_2 (0.2 L) was added dropwise while maintaining the temperature below −60 °C. The reaction mixture was stirred for 3 h at −70 °C, before addition of TEA (59.1 g, 584 mmol). The mixture was then allowed to reach −10 °C over 20 min. The reaction was quenched with 1 N HCl (0.8 L), and the organic layer was separated. The aqueous layer was washed with CH_2Cl_2, and the solvent from the combined organic layers was evaporated. The residue was crystallized from Et_2O/hexane affording 51.5 g of **10** (80.8%): mp 111.8−112.8 °C; IR 1786, 1740, 1707, 1204 cm^{-1}; MS 339.0 $(M - CH_2CO)$; NMR ($CDCl_3$) 1.26 (s, 3H), 3.06 (dd, 1H, $J =$ 16.2, 2), 3.54 (dd, 1H, $J = 16.2$, 4), 5.34 (s, 1H), 5.42 (dd, 1H, $J = 2$, 4), 6.95 (s, 1H), 7.30−7.38 (m, 10H), 9.20 (s, 1H). Anal. ($C_{21}H_{19}NO_4S$) C, H, N, S.

2. Preparation of the 2*β*-Alkenyl Penam Esters 11. (*E*/*Z*)-(2*S*,3*S*,5*R*)-3-(2-Cyanoethenyl)-3-methyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic Acid Benzhydryl Ester (11g,h) (Method A). A solution of the aldehyde **10** (33 g, 87 mmol) in MeCN (0.3 L) was added dropwise at −20 °C to a suspension of (cyanomethylene)triphenylphosphorane (28.8 g, 95.6 mmol) and 0.4 M $LiClO_4$ in MeCN (0.24 L). After 4 h, the solvent was evaporated. The residue was dissolved in AcOEt, worked up, and chromatographed (CH_2Cl_2) giving 33.4 g of an oil (yield 95%). The two isomers were separated by chromatography (AcOEt/hexane, 3:7) for analytical purposes. **11g:** TLC $R_f = 0.42$ (AcOEt/hexane, 3:7); IR (KBr) 2220, 1781, 1743 cm^{-1}; MS 422.2 $(M + NH_4^+)$. **11h:** TLC R_f = 0.40 (AcOEt/hexane, 3:7); IR (Film) 2219, 1779, 1745 cm^{-1}; MS 422.4 $(M + NH_4^+)$.

(*E*)-(2*S*,3*S*,5*R*)-3-Methyl-3-[2-(1,2,4-oxadiazol-3-yl)vinyl]-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic Acid Benzhydryl Ester (11n) (Method B). A suspension of [(1,2,4-oxadiazol-3-yl)methyl]triphenylphosphonium chloride[23] (1.38 g, 3.6 mmol) and the aldehyde **10** (1.14 g, 3.0 mmol) in 1,2-epoxybutane (15 mL) was refluxed for 10 h. After filtration, the solvent was evaporated and the residue was chromatographed (CH_2Cl_2) giving 0.28 g (21%) of **11n**: IR 1792, 1753, 1658, 1492, 1200 cm^{-1}; MS 465.3 $(M + NH_4)^+$.

(*E*)-(2*S*,3*S*,5*R*)-3-(3-Hydroxypropenyl)-3-methyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic Acid Benzhydryl Ester (11o). To a solution of the aldehyde **11f** (5.80 g, 14.2 mmol) in toluene (0.24 L) was added dropwise, under argon and at 0 °C, a solution of DIBAH in toluene (14.2 mL, 20%, 21.3 mmol). After stirring at room temperature for 6 h, the reaction was quenched with saturated aqueous NH_4-Cl (0.15 L) and the mixture extracted with CH_2Cl_2. The organic layer was worked up and chomatographed (AcOEt/hexane, 9:16) yielding 1.90 g (32%) of **11o**: IR (film) 3480, 1778, 1750, 1202 cm^{-1}; MS 427.6 $(M + NH_4)^+$.

(*E*)-(2*S*,3*S*,5*R*)-3-Methyl-7-oxo-3-[3-[(R)- and (S)-(tetrahydropyran-2-yl)oxy]propenyl]-4-thia-1-azabicyclo-[3.2.0]heptane-2-carboxylic Acid Benzhydryl Ester (11p). To a solution of the alcohol **11o** (620 mg, 1.50 mmol) in CH_2-Cl_2 (30 mL) was added *p*-toluenesulfonic acid (4.5 mg, 0.024 mmol) followed by 3,4-dihydro-2*H*-pyran (0.25 mL, 2.70 mmol). The solution was stirred for 1 h; then the solvent was evaporated. The residue was chromatographed (AcOEt/hexane, 9:16) yielding 700 mg (93%) of **11p**: IR (film) 3031, 1782, 1749, 1257 cm^{-1}; MS 511.6 $(M + NH_4)^+$.

(*E*)-(2*S*,3*S*,5*R*)-1-[3-[2-[(Benzhydryloxy)carbonyl]-3-methyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptan-3-yl]allyl]-pyridinium Trifluoromethansulfonate (11q). A solution of the alcohol **11o** (410 mg, 1.0 mmol) in CH_2Cl_2 (6 mL) was cooled to −40 °C, and $(CF_3SO_2)_2O$ (0.25 mL, 1.5 mmol) was added followed by pyridine (0.2 mL, 2.5 mmol). After 1 h at −40 °C, the solvent was evaporated and the residue taken up in CH_2Cl_2 for workup yielding 600 mg (97%) of **11q**: IR 1777, 1743, 1630, 1160 cm^{-1}; MS 471.6 (M^+).

(*E*)-(2*S*,3*S*,5*R*)-3-[3-[[[(Chloroacetyl)amino]carbonyl]-oxy]propenyl]-3-methyl-7-oxo-4-thia-1-azabicyclo[3.2.0]-heptane-2-carboxylic Acid Benzhydryl Ester (11r). Chloroacetyl isocyanate (0.09 mL, 1.05 mmol) was added under argon to a solution of the alcohol **11o** (300 mg, 0.73 mmol) in THF (10 mL). After 3 h, the solvent was evaporated and the residue was chromatographed (AcOEt/hexane, 9:16) affording 300 mg (77%) of **11r**: IR 3300, 1779, 1753, 1730, 1495, 1203 cm^{-1}; MS 546.4 $(M + NH_4)^+$.

3. Preparation of the 2*β*-Alkenyl Penam Sulfone Esters 12. (*Z*)-(2*S*,3*S*,5*R*)-3-(2-Cyanoethenyl)-3-methyl-4,4,7-trioxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic Acid Benzhydryl Ester (12h) (Method C). $NaHCO_3$ (47.3 g, 0.56 mol) was added to a solution of $NaIO_4$ (151.1 g, 0.71 mol) in water (1.8 L) at 0 °C. The solution was diluted with MeCN (1 L) and CH_2Cl_2 (1.6 L), and RuO_2 hydrate (364 mg, 0.75 mmol) was added. A solution of the penam esters **11g**,**h** (57.1 g, 0.141 mol) in CH_2Cl_2 (0.45 L) was added in one portion to the mixture which was then vigorously stirred at room temperature for 30 min. Charcoal was added, and the reaction mixture was diluted with brine and filtered. The filtrate was extracted with CH_2Cl_2, and the combined organic layers were worked up, chromatographed (AcOEt/hexane, 1:1), crystallized from *tert*-butyl methyl ether, and recrystallized from CH_2Cl_2/hexane giving 10.2 g of **12h** (*Z* isomer). The solvents from combined mother liquors were evaporated; the residue was chromatographed (AcOEt/hexane, 3:7) and crystallized from *tert*-butyl methyl ether affording 4.73 g of pure **12g** (*E* isomer). Complete separation of the two isomers was achieved by repeated chromatography and crystallization, giving total yields of 11.0 g (17.8%) of **12g** and 43.8 g (71.2%) of **12h**. **12g:** TLC $R_f = 0.48$ (AcOEt/hexane, 3:7); mp 145.5−146.1 °C; IR (KBr) 2228, 1802, 1758, 1334, 1192 cm^{-1}; MS 454.2 $(M + NH_4)^+$. Anal. ($C_{23}H_{20}N_2O_5S$) C, H, N, S. **12h:** TLC $R_f = 0.53$ (AcOEt/hexane, 3:7); mp 169−170 °C; $[\alpha]_D$ = +43.7° ($c = 1$, $CHCl_3$); IR 2220, 1797, 1753, 1334 cm^{-1}; MS 435.3 $(M - H)^-$. Anal. ($C_{23}H_{20}N_2O_5S$) C, H, N, S.

(*Z*)-(2*S*,3*S*,5*R*)-3-(2-Carbamoylvinyl)-3-methyl-4,4,7-trioxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic Acid Benzhydryl Ester (12c) (Method D). To a solution of the penam ester **11c** (0.37 g, 0.87 mmol) in CH_2Cl_2 (10 mL) were added AcOH (8 mL) and then, dropwise, a solution of $KMnO_4$ (414 mg, 2.63 mmol) in water (30 mL). The reaction mixture was stirred at room temperature for 30 min; then 30% H_2O_2 was added until the mixture was decolorized. The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were worked up yielding

225 mg (57%) of **12c**: IR 1799, 1750, 1678, 1326 cm^{-1}; MS 455.2 $(M + H)^+$.

(*E*)-(2*S*,3*S*,5*R*)-3-(2-Carbamoylvinyl)-3-methyl-4,4,7-trioxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic Acid Benzhydryl Ester (12b) (Method E). A solution of *m*-chloroperbenzoic acid (1.86 g, 7.55 mmol) in CH_2Cl_2 (30 mL) was added dropwise at 0 °C to a solution of the penam ester **11b** (0.64 g, 1.51 mmol) in CH_2Cl_2 (30 mL). After stirring at room temperature for 6 h, the solution was washed with a 3% Na_2SO_3 solution in saturated aqueous $NaHCO_3$, worked up, and crystallized from CH_2Cl_2/hexane affording 210 mg (30%) of **12b**: mp 186−188 °C; IR 1799, 1756, 1686, 1329 cm^{-1}; MS 455.3 $(M + H^+)$.

(*E*)-(2*S*,3*S*,5*R*)-3-[3-(Carbamoyloxy)propenyl]-3-methyl-4,4,7-trioxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic Acid Benzhydryl Ester (12s). A solution of $NaHCO_3$ (135 mg) in water (3 mL) was added under argon to a solution of the protected carbamate **12r** (450 mg, 0.80 mmol) in THF (5 mL) and MeOH (1.7 mL). After 24 h the solvents were evaporated, and the residue was taken up in AcOEt and brine. The aqueous solution was extracted twice with AcOEt, and the combined organic layers were worked up and chromatographed (AcOEt/hexane, 2:1) yielding 200 mg (78%) of **12s**: IR 3481, 1799, 1749, 1731, 1601, 1329 cm^{-1}; MS 484 $(M + NH_4)^+$.

4. Preparation of the 2β-Alkenyl Penam Sulfone Acids 3. (*E*)-(2*S*,3*S*,5*R*)-3-(3-Hydroxypropenyl)-3-methyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic Acid Sodium Salt (3o) (Method F). A solution of the penam sulfone ester **12r** (0.5 g, 0.95 mmol) in *m*-cresol (15 mL) was stirred for 6 h at 50 °C. After cooling to room temperature, isobutyl methyl ketone (50 mL) and 2 N sodium 2-ethylcaproate (0.55 mL, 1.1 equiv) were added, and the reaction mixture was extracted twice with water (10 mL). The combined aqueous solutions were washed with isobutyl methyl ketone and lyophilized. The residue was dissolved in water (3 mL) and purified by gel filtration giving 165 mg (62%) of **3o**: IR 3428, 1780, 1622, 1398, 1311 cm^{-1}; MS 274.3 $(M - Na)^-$. Anal. ($C_{10}H_{12}NO_6SNa$) C, H, N.

(*Z*)-(2*S*,3*S*,5*R*)-3-(2-Cyanoethenyl)-3-methyl-4,4,7-trioxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic Acid (3h) (Method G). A solution of the penam sulfone ester **12h** (20.2 g, 46.3 mmol) in *m*-cresol (60 mL) was stirred at 50 °C for 6 h. The reaction mixture was diluted with hexane and cooled to 0 °C. The crystals were collected by filtration and dissolved in AcOEt. The solution was treated with Fuller's earth, filtered, and concentrated to 0.13 L. It was then diluted with hexane (0.13 L) and crystallized overnight: total yield 10.8 g (86.4%); mp 166 °C; $[\alpha]_D = +23.4°$ ($c = 1$, H_2O); IR 2220, 1808, 1762, 1712, 1331 cm^{-1}; MS 161.2 $(M - COOH + SO_2)$; NMR (DMSO-d_6) 1.86 (s, 3H), 3.31 (dd, 1H, $J = 15, 2.5$), 3.72 (dd, 1H, $J = 15, 5$), 4.98 (s, 1H), 5.36 (m, 1H), 6.36 and 6.77 (2d, 2 × 1H, $J = 7.5$), 13.92 (1H, br). Anal. ($C_{10}H_{10}N_2O_5S$) C, H, N, S.

Formation of the Corresponding Sodium Salt. The free acid **3h** (0.5 g, 1.85 mmol) was added in one portion to a stirred solution of $NaHCO_3$ (155 mg, 1.85 mmol) in water (20 mL). After 10 min the solution was lyophilized giving 541 mg (100%) of the hygroscopic sodium salt: IR 2222, 1781, 1629, 1394, 1323 cm^{-1}; MS 269.2 $(M - Na)^-$. Anal. ($C_{10}H_9N_2O_5SNa$) C, H, N.

5. Methanolysis of the Penam Sulfone Acid. A solution of the acid **3h** (100 mg, 0.371 mmol) in MeOH (10 mL) was treated with 1 M NaOMe (1.1 mL). After 48 h, the solvent was evaporated and the residue was purified by gel filtration giving, after lyophilization, the enamides **13** (50 mg, 51%), the amino acid **14** (20 mg, 30%), and the glyoxylate **15** (12 mg, 17%).

(3*E*)-5-Cyano-2-[[2-(methoxycarbonyl)vinyl]amino]-3-methylpent-3-enoic acid (13): *E* isomer NMR (DMSO-d_6) 1.56 (s, 3H), 3.28 (d, 2H, $J = 7$), 3.46 (s, 3H), 3.80 (d, 1H, $J = 4$), 4.25 (b, 1H), 5.30 (t, 1H, $J = 7$), 7.06 (br, 1H), 7.40 (dd, 1H, $J = 7, 13$); *Z* isomer NMR (DMSO-d_6) 1.64 (s, 3H), 3.25 (d, 2H, $J = 7$), 3.58 (s, 3H), 3.95 (d, 1H, $J = 6$), 4.30 (d, 1H, $J = 10$), 5.38 (t, 1H, $J = 7$), 6.55 (dd, 1H, $J = 10, 17$), 7.05 (dd, 1H, $J = 6, 17$); IR 2250, 1671, 1605 cm^{-1}; MS(ISN) 237.2 (M − Na).

(*E*)-2-Amino-5-cyano-3-methylpent-3-enoic acid (14): NMR (DMSO-d_6) 1.65 (s, 3H), 3.3 (d, 2H, $J = 7$), 3.61 (s, 1H), 5.42 (t, 1H, $J = 7$).

3-Hydroxy-3-sulfoxypropionic acid methyl ester (15, tentatively X = OSO_3Na): NMR (DMSO-d_6) 2.35 (dd, 1H, $J = 9, 14$), 2.73 (dd, 1H, $J = 3, 14$), 3.54 (s, 3H), 4.22 (dd, 1H, $J = 3, 9$), 5.63 (br exchangeable, 1H).

6. X-ray Crystallography. Crystals of the class C β-lactamase from *C. freundii* were grown in 40% phosphate buffer at pH 8.0 as described previously.[8] For inhibitor binding studies, crystals were soaked in 0.3 mL of 50% phosphate buffer at pH 8.0 containing the respective inhibitor at a concentration of 3−5 mM for 3 h.

Data were collected at 15 °C with a Nicolet/Xentronics area detector mounted on an Elliott GX21 rotating copper anode X-ray generator operated at 30 kV, 90 mA with a 0.3 mm focal spot and a graphite monochromator. The crystal−detector distance was 120 mm; 0.15° frames were measured for 90 s and processed using the XDS primary data reduction program.[26] Further data reduction was performed using the CCP4 crystallographic package (Collaborative Computing Project No. 4, A Suite of Programs for Protein Crystallography, Daresbury Laboratory, Warrington, England, 1994). Analysis and refinement of the structure were performed with X-Plor version 3.1[24] and Moloc[27] on Indigo 2 workstations (Silicon Graphics Inc.). Data collection and refinement statistics are given in the Supporting Information.

As reported,[8] the uncomplexed enzyme structure has been refined at 2.0 Å resolution, and the final model comprises 2 molecules/asymmetric unit and 250 water molecules. Refinement of the acyl−enzyme complex with **3c** started from this model with the water molecules omitted. After rigid body refinement of the two enzyme molecules, the inhibitor molecules were readily fitted into a $F_{obs} - F_{calc}$ difference electron density map and added to the model. Several rounds of positional and B-factor refinement interrupted by manual corrections to the model and the addition of well-determined water molecules led to the final model, documented in the Supporting Information.

For the complex with **3h**, starting with the coordinates of the refined structure of the complex with **3c** produced a significantly better fit to the data than starting with those of the uncomplexed enzyme did. $F_{obs} - F_{calc}$ difference maps calculated at this stage showed only very weak density in the area expected, by analogy with the **3c** complex, to be occupied by **3h**. Refinement of this complex was not pursued further as first attempts showed that no improvement of the free *R*-factor could be obtained[25] and the *B*-factors of the inhibitor atoms were refined to very high values except those of the serine acyl ester moiety (see discussion section).

In subunit A of the noncrystallographic dimer, density differences consistent with **3h** occupying a position similar to that occupied by **3c** could be seen at rather low contour levels (1.5σ), while nothing interpretable was seen in the active site of subunit B. However, in both subunits the highest difference density is seen as a peak extending from Oγ of Ser 64 into the oxyanion hole. The peak height is in both cases consistent with full occupancy of this site. A considerably clearer difference density is obtained if the Fourier coefficients are taken as the differences between the observed amplitudes of inhibitor-soaked and native crystals. This is not surprising in view of the fact that the two data sets scale with a mean fractional difference of 0.107 as compared to 0.20 for calculated and observed amplitudes.

7. *In Vitro* Antibacterial Activity. The minimal inhibitory concentrations (MICs) of the test compound or combination were determined according to the standard method by a serial 2-fold dilution method using Müller−Hinton broth (Difco laboratories, Detroit, MI). The inoculum size was approximately 10^5 cfu/mL. The MIC of a compound or a combination was defined as the lowest concentration that prevented visible growth of bacteria after incubation at 37 °C for 18 h.

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Supporting Information Available: One table with the physical properties of compounds **11** and **12**, including ^{1}H NMR data, one table with the elemental analyses, one table with IC_{50} values for inhibition of penicillin-binding proteins from *E. coli*, *S. aureus*, and *St. pneumoniae*, and two tables with statistical data from X-ray crystallographic analysis of the acyl−enzyme complexes formed with compounds **3c**,**h** (5 pages). Ordering information is given on any current masthead page.

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