SYNTHESIS AND β-LACTAMASE INHIBITORY ACTIVITIES OF SOME CLAVULANIC ACID ANALOGUES

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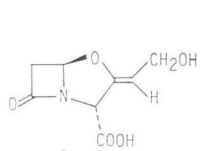
(Received for publication November 16, 1982)

Clavulanic acid analogs lacking the C-3 carboxyl group are potent inhibitors of both plasmid and chromosomally mediated β-lactamases. They exhibit only low intrinsic antibacterial activity, but potentiate the activity of ampicillin and cephaloridine against β-lactamase producing *Escherichia coli* and *Enterobacter cloacae in vitro*. No synergism was observed in β-lactamase negative strains. The *E. coli* TEM 1 and the *E. cloacae* P99 enzymes are inhibited in a progressive and irreversible manner by these compounds.

The isolation of clavulanic acid (**1**)[1], thienamycin (**2**)[2], and other "non-classical" bicyclic β-lactams from nature has created new interest in the search of novel structures and new synthetic routes to these compounds. In particular, the use of clavulanic acid as a β-lactamase inhibitor has found clinical applications[3] and prompted much investigation in this area. It has previously been reported that a number of analogs of **1** lacking the C-3 carboxyl group were found to be quite active as β-lactamase inhibitors[4]. Recently, OIDA and coworkers reported the synthesis of another type of analog (*e.g.* **3**), which they claimed to be devoid of antibacterial and β-lactamase inhibitory activity[5]. During the course of our synthetic program on chemotherapeutic agents, similar compounds were prepared and tested for β-lactamase inhibitory activity. We report our results with these derivatives in the present publication.



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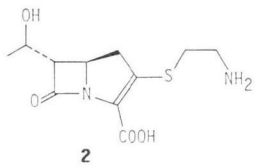

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Materials and Methods

Antibiotics

Sodium clavulanate was a generous gift of Beecham Pharmaceuticals, U.K. Nitrocefin was kindly supplied by Glaxo Group Research Ltd., U.K. Ampicillin and cephaloridine were obtained from Bayer AG, W. Germany, and Eli Lilly & Co. U.S.A., respectively.

Chemistry

Clavams **3a**～**3i** (see Table 1) were prepared by metal-catalyzed decomposition of the corresponding diazoketones **4a**～**4i**, which were synthesized according to previously employed conditions[6,7], from the respective azetidinon-1-yl acetic acids **5a**～**5i**, as outlined in Scheme 1.

The acids **5** were prepared by one of the following procedures:

1): Direct alkylation of the respective thio-substituted azetidinone **6a**～**6c** with methyl bromoacetate followed by alkaline hydrolysis (**5a**～**5c**).

2): Introduction of the respective mercapto function *via* chloroazetidinone **7**[8], followed by hydrolysis (**5d**～**5g**).

3): Reaction of azetidinone **6i**[9] with acetonylglyoxylate[5], followed by thionyl chloride treatment, zinc reduction, and alkaline hydrolysis (**5i**), as outlined in Scheme 2.

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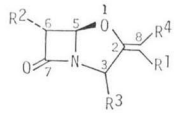
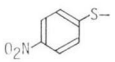
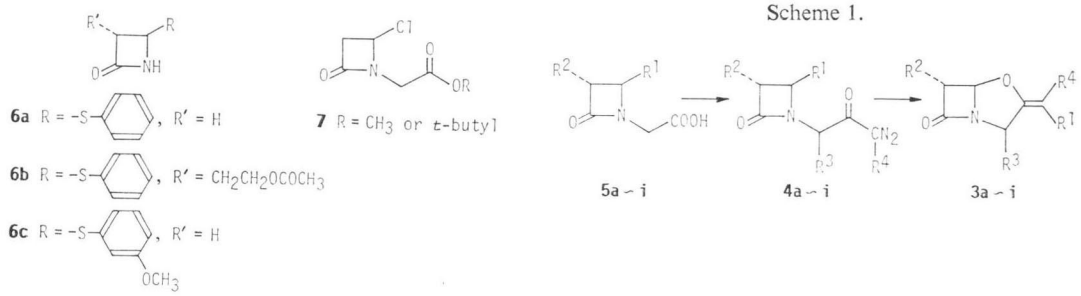
Table 1. ^{1}H NMR spectral data.[a)]



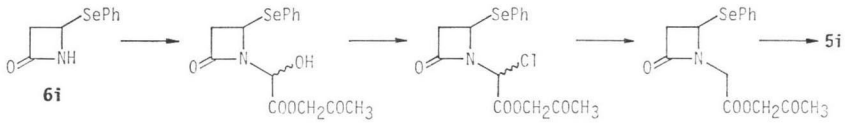
Compound					Chemical shifts (ppm)						Coupling constants (Hz)						
	R^1	R^2	R^3	R^4	H-8	H-3α	H-3β	H-5	H-6α	H-6β	8,3α	8,3β	3α,3β	3α,6α	5,6α	5,6β	6α,6β
3a		H	H	H	5.69	3.73	4.73	5.67	3.49	3.10	2	2	16.2	1	2.7	0.8	16.7
3b			H	H	5.68	3.70	4.70	5.46	—	3.36	2	2	16.2	—	—	0.5	—
3c		H	H	H	5.69	3.73	4.73	5.67	3.49	3.10	1.8	1.8	16.2	1	2.7	0.7	17
3d[b)]		H	CH_3	H	5.57 5.72	 4.30	4.95	5.66 5.38	3.40 3.29	3.02 3.09	— 2	1.5 —	— —	1 1	2.7 2.4	0.7 1.0	16.6 16.3
3e		H	H	H	5.64	3.82	4.60	5.72	3.52	3.14	2	2	16.2	1	2.7	0.7	16.2
3f		H	H	H	5.62	3.74	4.70	5.67	3.50	3.12	2	2	16	1	2.5	0.7	16
3f′		H	H	H	5.73	3.86	4.73	5.81	3.60	3.20	2	2	16	1	2.5	0.7	16
3g		H	H	H	5.70	3.72	4.67	5.63	3.49	3.08	2	2	16	1	2.5	0.7	17
3g′		H	H	H	5.78	3.32	4.68	5.73	3.57	3.13	1.8	1.8	16	1	2.5	0.5	17
3h′		H	H		—	4.20	5.15	5.81	3.60	3.08	—	—	16	1	2.5	1.0	17
3i		H	H	H	5.87	3.70	4.70	5.64	3.44	3.10	2	2	17	1	2.5	0.7	17
3j		H	H	H	5.91	3.97	5.14	5.71	3.49	3.10	2	2	16.2	1	2.7	0.8	16.7

a) NMR spectra of the sodium salts were determined in D_2O, otherwise in $CDCl_3$.
b) Mixture of isomers.

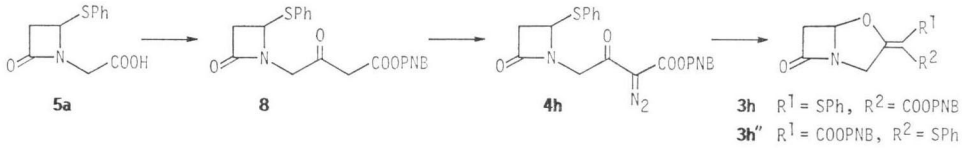
Scheme 1.



Scheme 2.



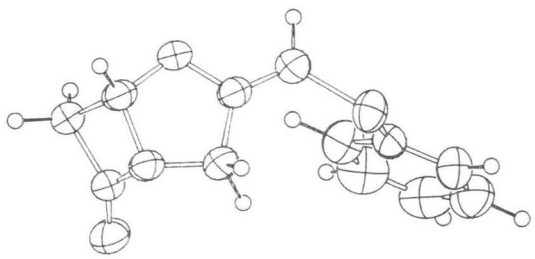
Scheme 3.



Diazoketone **4h** was prepared by diazo transfer using tosyl azide[9] from the β-keto ester **8**, which was obtained from the reaction of acid **5a** with malonic acid, *p*-nitrobenzylester, using the condition of MASAMUNE and coworkers[10].

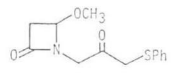
Decomposition of the diazoketone was routinely performed in hot benzene ($\sim$0.01 M), in the presence of a catalytic amount of copper acetylacetonate or rhodium acetate. The product was isolated by column chromatography of the crude mixture. With the exception of **4h**, which gave a 1 : 1 mixture of **3h** and **3h′′**, all other clavams were isolated as one single isomer,* whose structures were determined by spectroscopic analysis (see Table 1). However, we felt that the double bond geometry for the side chain of the clavams could not be conclusively assigned as the *E*-isomer, despite NMR correlations[5]. Therefore an X-ray analysis was performed on **3a**, and it was found then to be consistent with the assignments. A perspective view of the molecule is shown in Fig. 1. The sodium salts **3f′**, **3g′**, and **3h′** were prepared by catalytic hydrogenation of the corresponding *p*-nitrobenzyl ester, in the presence of one equivalent of sodium carbonate**. Sulfoxide **3j** was obtained by oxidation of **3a** with *m*-chloroperbenzoic acid.

Fig. 1. Perspective view of **3a** showing the 50% probability with thermal vibration ellipsoids of the heavy atoms (ORTEP drawing).



* Clavam **3d** contains an inseparable diastereomeric mixture (C-3 methyl). Otherwise all compounds are racemic, although one enantiomer was depicted for simplicity.

** Catalytic hydrogenation of clavam **3h′′** resulted in the exclusive formation of azetidinone **9**.



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β-Lactamase Preparation

The β-lactamases were from *Enterobacter cloacae* P99, *Escherichia coli* J5/R6K (TEM 1), *E. coli* p453 (SHV 1), *E. coli* J5/R46 (OXA 2), and *Staphylococcus aureus* NCIB 11195 (PC 1). The nomenclature of the Gram-negative β-lactamases follows that of SYKES and MATTHEW[11] and MATTHEW *et al.*[12]. The P99, TEM 1, and SHV 1 enzymes were isolated from exponentially growing cultures by osmotic shock treatment according to NOSSAL and HEPPEL[13], and not purified further. The OXA 2 enzyme was partially purified from the sonicate of an exponentially growing culture by chromatography on phosphocellulose P11 (Whatman), elution with 10 mM potassium phosphate buffer, pH 7.2, and subsequent ammonium sulfate precipitation. The staphylococcal β-lactamase PC 1 was partially purified by adsorption to phosphocellulose P11 according to RICHMOND[14]. One enzyme unit is defined as the amount of enzyme which hydrolyses 1 μmole of Nitrocefin per minute at 25°C, pH 7.0.

Assay of β-Lactamase Inhibition and Determination of I_{50} Values

The enzymes were incubated with various concentrations of the inhibitors in 2 ml of 0.1 M potassium phosphate buffer, pH 7.0, for 10 minutes at 25°C. The enzyme activity was measured by adding the chromogenic substrate Nitrocefin (25 μM)[15], and following the increase in absorbance at 486 nm on a Beckman DU 8 spectrophotometer. The residual enzyme activity was calculated by subtracting the enzyme activity of the inhibited reaction from the values of the uninhibited control reaction. Irrespective of the specific activities of the various enzyme preparations, the amount of enzyme added was adjusted to hydrolyze 5 nmole Nitrocefin per minute at 25°C. The concentration of inhibitor causing 50% inactivation (I_{50}) was derived from a graph in which the percentage of inhibition was plotted against the inhibitor concentration.

Kinetics of Irreversible Inhibition

Stock solutions (two units each) of TEM 1 and P99 β-lactamases were incubated with varying concentrations of the inhibitors at 25°C. At appropriate intervals 5 μl aliquots were removed and diluted into 2 ml of 0.1 M potassium phosphate buffer, pH 7.0. The residual enzyme activity was measured relative to the control, where the inhibitor was replaced by buffer, after adding 25 μM Nitrocefin, as described. In some experiments aliquots of the same incubation mixtures were removed after 10 and 60 minutes, cooled to 0°C, transferred to a Sephadex G-25 column (1 × 6.25 cm), and the enzyme eluted with 0.1 M potassium phosphate buffer, pH 7.0, at a flow rate of 40 ml/hour. The fractions containing enzyme activity were pooled, allowed to warm to 25°C, and the enzyme activity measured spectrophotometrically relative to controls, as described.

Determination of the Minimum Inhibitory Concentrations (MICs) and Synergistic Activity

The MICs were determined by the broth dilution technique on microtiter plates after incubation at 37°C for 18 hours. The inocula were 10^5 colony forming units (CFU). The test strains were *E. coli* J5 (sensitive) and the isogenic strain *E. coli* J5/R1 (producing TEM 1 β-lactamase), as well as the two isogenic strains of *E. cloacae,* one of them producing the P99 β-lactamase. The MICs were taken as the lowest concentrations which inhibit visible bacterial growth. The synergistic activity of the β-lactamase inhibitors with ampicillin or cephaloridine was determined by the conventional chequerboard technique. Synergy is defined as a greater than 4 fold reduction in the MICs of both agents.

Results

Inhibition of β-Lactamases

Most of the compounds showed significant inhibition of the isolated plasmid-mediated β-lactamases TEM 1, SHV 1, and OXA 2 after a 10-minute preincubation period (Table 2), the most active being **3f′**, **3g′** and **3h′**. The I_{50}-values of these derivatives compare well with those of clavulanic acid. In addition, they also inhibit the chromosomally mediated cephalosporinase P99 from *E. cloacae,* which is much less sensitive to clavulanic acid[1]. The staphylococcal β-lactamase PC 1, however, was not significantly inhibited by any of the tested derivatives, whereas clavulanic acid shows good inhibition

Table 2. Inhibition of isolated β-lactamases.

Inhibitor	I_{50} (μM)				
	E. cloacae P99	*E. coli* TEM 1	*E. coli* SHV 1	*E. coli* OXA 2	*S. aureus* PC 1
3a	27	0.2	0.3	7	25
3c	19	0.3	0.5	6	37
3e	20	0.4	1	10	19
3f′[a]	≤5	<0.05	<0.5	<5	50
3g′	2.5	0.03	0.06	3	22
3h′[a]	≤0.5	≤0.05	≤0.05	<0.5	n.t.[b]
3i	29	0.7	4	>50	>50
3j	50	0.2	0.5	5	40
Clavulanic acid	>50	0.05	0.04	2	0.1

[a] I_{50} values estimated from 10-fold dilutions of the compounds.

[b] n.t.=Not tested.

The I_{50}-values (concentration of inhibitor which inhibits 50% of the enzyme activity) derived from standard inhibition curves after 10-minute preincubation at 25°C, pH 7.0, without further dilution, as described in Materials and Methods.

Table 3. Minimum inhibitory concentrations (MICs) of ampicillin and cephaloridine alone and in combination against sensitive and β-lactamase producing bacteria.

	MIC (μg/ml)				
	A			B	
	Ampicillin	**3a**	**3g′**	Ampicillin+**3a**	Ampicillin+**3g′**
E. coli J5	6.25	>1,000	1,000	6.25+1,000	3.12+250
E. coli J5/R1 (TEM 1)	125	>1,000	1,000	3.12+6.25	6.25+3.9
	Cephaloridine	**3a**	**3g′**	Cephaloridine+**3a**	Cephaloridine+**3g′**
E. cloacae	6.25	>1,000	2,000	6.25+1,000	6.25+2,000
E. cloacae (P99)	500	>1,000	2,000	500 +1,000	50 +15.6

Panel A: The MIC values for ampicillin, cephaloridine, **3a** and **3g′** were determined by the broth dilution technique using an inoculum of 10^5 CFU as described in Materials and Methods. Panel B: The MIC values for the combinations of ampicillin and cephaloridine with the inhibitors **3a** and **3g′** were determined by the chequerboard technique and are recorded as the lowest combination concentrations at which no visible growth was observed.

of this enzyme as described by others[16]. Without preincubation of the β-lactamases these clavams showed significantly less inhibitory activities (data not shown), indicating a progressive type of inhibition. The sulfoxide derivative **3i** is about 4- to 10-fold less active against the plasmid-mediated β-lactamases when compared with **3a**. The activity towards the P99 enzyme did, however, not change and is low in both cases. Substitution with $-CH_3$ in the C-3 position (**3d**) does not seem to have any effect on the β-lactamase inhibitory activity, whereas the substituent in C-6 (**3b**) causes a dramatic loss of biological activity. At 50 μM, this derivative exhibited only 20% inhibition of the TEM enzyme (without preincubation), while at the same concentration all other compounds tested inactivated this enzyme >99% (data not shown).

Antibacterial Activity and Synergy

Two prototypes of this series of clavams, **3a** and **3g′**, were tested for antibacterial activity and synerg-

ism with ampicillin and cephaloridine. The intrinsic antibacterial activity of both derivatives is very low, the MICs being $\geq$1,000 μg/ml (Table 3). However, when combined with ampicillin, there was a marked decrease in the MIC of ampicillin against the TEM β-lactamase producing *E. coli*. **3g'** was approximately 10 times more active than **3a**, which is in agreement with the inhibitory activities of the two derivatives on the isolated enzyme. When the same combinations were tested on the β-lactamase negative strain no synergy was observed. It can therefore be concluded, that synergy is not due to the combined antibacterial action of two β-lactam antibiotics, but due to the inhibition of the β-lactamase, rendering the strain sensitive to ampicillin. **3g'** also showed strong synergy with cephaloridine against the β-lactamase producing *E. cloacae* strain, and not against the isogenic sensitive strain. The other derivative, **3a**, did not show the expected synergy with cephaloridine. This might be explained by poor penetration of the compound through the outer membrane of this strain.

Kinetics of Irreversible Inhibition

Clavulanic acid inhibits β-lactamases in a progressive manner[1], and, with the TEM 2 enzyme, produces two catalytically inactive forms: a transient complex and an irreversibly inhibited form in the presence of excess inhibitor[17]. Since preliminary experiments also indicated a progressive type of β-lactamase inhibition for our compounds, two derivatives, **3a** and **3g'**, were tested for their abilities to irreversibly inhibit the TEM enzyme. The two compounds were chosen because **3a** was the unsubstituted prototype of this series of compounds and **3g'** was one of the most active, water soluble derivatives. The β-lactamase was incubated with different concentrations of these derivatives, and at appropriate intervals aliquots were diluted 1/400 into the assay mixture. At this dilution the inhibitor concentrations were low enough not to further inactivate the β-lactamase. The residual enzyme activity was measured spectrophotometrically. The results are shown in Fig. 2. In the presence of low concentrations of both **3a** and **3g'**, a decrease in activity was observed initially, but after longer incubation times the enzyme activity recovered, but did not reach the values of the control reaction. At this stage we cannot say, whether the residual fraction of the inhibited enzyme consists of more than one species of inactivated enzyme. At 5×10^{-7} M of **3g'** 95% of the TEM β-lactamase remained inactivated even after 45 minutes (Fig. 2B). Irreversible inactivation of the TEM β-lactamase by **3g'** was also measured after filtration of the incubation mixture through a Sephadex G-25 column (data not shown). Incubation of the β-lactamase with 1.3×10^{-7} M and 5×10^{-7} M of **3g'** for 10 and 60 minutes, followed by gel-filtration, resulted in the

Fig. 2. Kinetics of reversible and irreversible inhibition of *E. coli* TEM 1 β-lactamase.

Two enzyme units of TEM 1 were incubated with varying concentrations of **3a** (A) and **3g'** (B) at 25°C. At the indicated time intervals aliquots were diluted 1/400 into the assay mixture (0.1 M potassium phosphate buffer, pH 7.0) and the residual enzyme activity measured from the hydrolysis of 25 μM Nitrocefin spectrophotometrically as described in Materials and Methods.

The amount of enzyme in the uninhibited reaction was equivalent to hydrolyze 5 nmole Nitrocefin per minute at 25°C. Inhibitor concentrations in the preincubation mixtures were:

□ 6×10^{-8} M, ● 1.3×10^{-7} M, ■ 2.6×10^{-7} M, ▲ 5×10^{-7} M, ○ 1×10^{-6} M.

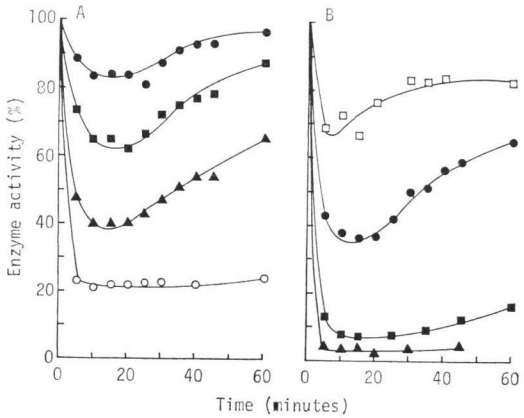
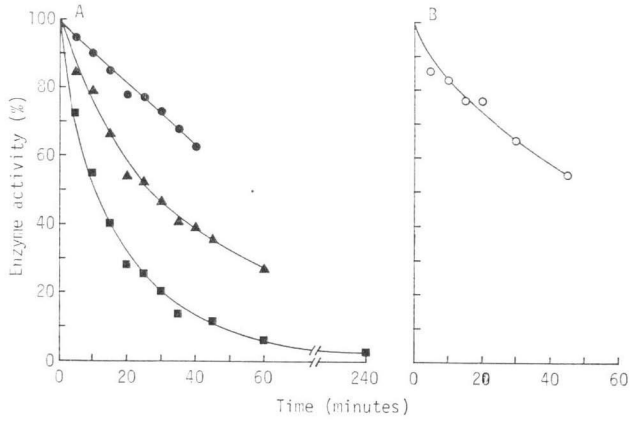


Fig. 3. Inactivation of *E. cloacae* P99 β-lactamase.

A: Two enzyme units of P99 β-lactamase were incubated with **3g′** at 25°C. At the indicated intervals aliquots were diluted 1/400 into the assay mixture (0.1 M potassium phosphate buffer, pH 7.0) and the residual catalytic activity measured from the hydrolysis of 25 μM Nitrocefin spectrophotometrically as described in Materials and Methods. The amount of enzyme in the assay mixture was equivalent to hydrolyze 5 nmole Nitrocefin per minute at 25°C. The concentrations of **3g′** in the preincubation mixtures were: ● 1.6×10^{-6} M, ▲ 3.1×10^{-6} M, ■ 6.3×10^{-6} M.

B: The P99 β-lactamase was incubated with clavulanic acid in 2 ml 0.1 M potassium phosphate buffer, pH 7.0, and the residual catalytic activity measured without further dilution by following the rate of hydrolysis of 25 μM Nitrocefin spectrophotometrically. The amount of enzyme activity was equivalent to hydrolyze 5 nmole Nitrocefin per minute at 25°C. The concentration of clavulanic acid was 1×10^{-4} M (○).



inactivation of the enzyme to a degree, which compared well with the results shown in Fig. 2B, where the residual enzyme activity was measured after dilution, as described above.

The derivative **3a** is about 4 times less active than **3g′**; at the highest concentration tested (1×10^{-3} M) 75% of the TEM β-lactamase is inactivated irreversibly (Fig. 2A).

When the same enzyme was incubated with either **3g′** or **3a** without dilution or gel-filtration, we did not observe recovery of the catalytic activity even after 60 minutes. Approximately one third of the inhibitor concentration is needed to inhibit the enzyme to the same degree as in Fig. 2. Under these conditions, however, the degree of inactivation could result from both transiently inhibited and irreversibly inactivated enzyme. This is also true for the inhibition with the other clavams, from which we calculated the I_{50}-values in Table 2.

In contrast to the TEM β-lactamase, prolonged incubation of the P99 enzyme with **3g′** did not result in a restoration of the catalytic activity, even after dilution (Fig. 3A) or gel-filtration. Clavulanic acid inhibits this enzyme also progressively, but only at very high concentrations (Fig. 3B).

Discussion

The continuing problem of resistance to penicillins and cephalosporins in bacteria caused by β-lactamases has also initiated our work on β-lactamase inhibitors as one way to overcome this type of resistance. The compounds described in this paper inhibit a broad spectrum of clinically important β-lactamases, both plasmid and chromosomally mediated. Unlike clavulanic acid[1], they show significant inhibition of the P99 cephalosporinase from *E. cloacae,* but they inhibit the staphylococcal β-lactamase only to a very limited degree. The activity against the plasmid mediated β-lactamases is for some derivatives comparable to that of clavulanic acid. Whether this shift in the spectrum of β-

lactamase inhibition is due to the lack of the C-3 carboxyl group remains an open question, because of missing comparative results with compounds which differ only in the presence or absence of the C-3 carboxyl group. Similar compounds have either been claimed to be devoid of β-lactamase inhibitory activity[5], or no details were published on their biological potency[4]. COLE[18] reported I_{50}-values of one analog of clavulanic acid, also lacking the C-3 carboxyl group, which indicate a 100-fold more potent inhibition of the P99 enzyme than with clavulanic acid, but without loss of inhibitory activity against the staphylococcal β-lactamase. From our results it can be concluded that clavams lacking the C-3 carboxyl group are potent inhibitors, but that a carboxyl group in the side chain in position 2 (**3f'**, **3g'** and **3h'**) increases the inhibitory activity 4- to 10-fold. The inhibition of the β-lactamases results in a synergistic activity with ampicillin or cephaloridine against β-lactamase producing *E. coli* and *E. cloacae*. No synergism was observed in the respective isogenic β-lactamase negative strains.

The derivatives **3a** and **3g'** inhibit the TEM 1 and P99 β-lactamases progressively and irreversibly, and resemble in this respect the mode of inactivation of clavulanic acid[1,17]. More detailed studies on purified enzymes are planned to further elucidate the type of inhibition by these clavulanic acid derivatives.

Acknowledgment

We thank Dr. H. P. WEBER for the X-ray analysis, Prof. A. VASELLA, Dr. W. J. LLOYD and Prof. G. HÖGENAUER for helpful discussions. The expert technical assistance of P. KNEUSSEL, K. ADLGASSER, K. WAGNER, W. NERUDA, K. BEDNARIK and E. JANZEK-HAWLAT is greatfully acknowledged.

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