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Interaction between monobactams and model D-alanyl-D-alanine-cleaving peptidases

(Mode of action of β-lactam antibiotics; Zn^{2+} DD-carboxypeptidase; serine DD-carboxypeptidase/transpeptidase)

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1. SUMMARY

Several monobactams reacted with the serine DD-peptidases of *Streptomyces* R61 and *Actinomadura* R39 in a manner similar to that of bicyclic penicillins and cephalosporins. The dissociation constants of the Michaelis complexes formed between the R61 enzyme and sulfazecin (32 μM) and between the R39 peptidase and SQ 26324 (0.35 μM) had the lowest values ever observed with any β-lactam compound, suggesting an excellent fit of these two monobactams with the active sites of the respective enzymes. Azthreonam had a very poor inactivating potency, confirming its high selective reactivity towards the penicillin binding protein No. 3 of *Escherichia coli*. The Zn^{2+} DD-peptidase (from *Streptomyces albus* G) had a high intrinsic resistance to β-lactam compounds whether they possessed a mono- or a bicyclic structure.

2. INTRODUCTION

Bicyclic β-lactam compounds (penicillins and cephalosporins) interact with the D-alanyl-D-alanine-cleaving peptidases (in short DD-peptidases) according to:

$$E + I \overset{K}{\rightleftharpoons} E \cdot I \overset{k_{+2}}{\rightarrow} E\text{–}I^* \overset{k_{+3}}{\rightarrow} E + \text{degradation product(s)} \qquad \text{(model 1)}$$

where E = enzyme, I = β-lactam compound, K = dissociation constant of EI, k_{+2} and k_{+3} = first-order rate constants; E–I^* = inactive complex [1]. In the case of the serine DD-peptidases, E–I^* is a stable, ester-linked acyl enzyme intermediate. The higher the k_{+2}/K ratio value and the smaller the k_{+3} value, the better the β-lactam compound acts as an enzyme inactivator.

Following the discovery of nocardicin and sulfazecin [2–4], a wide variety of other monocyclic β-lactam compounds (characterized by a 2-oxo-azetidine-1-sulfonic acid moiety and called monobactams) were isolated and/or synthesized [5]. Monobactams were shown to bind covalently to the *Streptomyces* serine DD-peptidase in a manner similar to that of bicyclic β-lactam compounds, implying that the same serine residue was involved in the reaction [6]. As an extension of these studies, experiments were undertaken to determine the kinetic parameters of the interaction between several monobactams and both the

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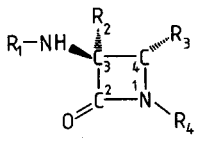
Streptomyces R61 and *Actinomadura* R39 serine DD-peptidases. The *Streptomyces albus* G Zn^{2+}-containing DD-peptidase, which is highly resistant to bicyclic β-lactam compounds, was also investigated.

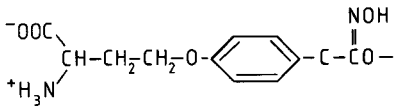
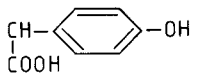
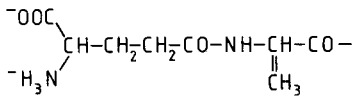
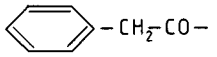
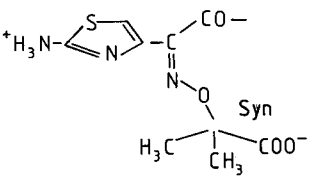
3. MATERIALS AND METHODS

Nocardicin A was from the Fujisawa Pharmaceutical Co. (Osaka, Japan), sulfazecin from Takeda Chemical Industries (Osaka), and azthreonam SQ26180 and SQ26324 were from the Squibb Institute for Medical Research (Princeton, NJ, U.S.A.) (Fig. 1). The enzymes were purified as described before [7–9] and the DD-carboxypeptidase activity was estimated at 37°C by measuring the amount of C-terminal D-Ala released from 2 mM N^{α}, N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala [10,11].

The k_{+3} values of model 1 were determined by incubating the enzyme for 30 min with the monobactam at a concentration sufficient to inactivate (completely or at least partially) the DD-carboxypeptidase activity. The excess of free monobactam was eliminated by dialysis (in the case of nocardicin) or by addition of 5 units of *Enterobacter cloacae* P99 β-lactamase [12] (one β-lactamase unit hydrolyzes 1 μmol of cephaloridin per min at

Structure of the monobactams



Name	R_1	R_2	R_3	R_4
Nocardicin A		H	H	
Sulfazecine		OCH_3	H	SO_3^-
SQ 26180	CH_3–CO–	OCH_3	H	SO_3^-
SQ 26324*		H	H	SO_3^-
Azthreonam		H	CH_3	SO_3^-

* SQ 26324 has the same R_1 side chain as the bicyclic penam benzylpenicillin

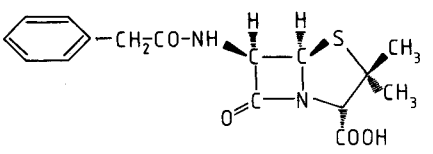


Table 1

Constants-values for the interaction between monobactams and the R61 and R39 serine DD-peptidases, at 37°C (unless otherwise stated) and in buffers as indicated in MATERIALS AND METHODS

Model reaction: $E + I \overset{K}{\rightleftharpoons} E \cdot I \overset{k_{+2}}{\rightarrow} E\text{-}I^* \overset{k_{+3}}{\rightleftharpoons} E$ + degradation product

Monobactam	R61 DD-peptidase				R39 DD-peptidase			
	K (μM)	k_{+2} (s^{-1})	k_{+2}/K ($M^{-1} \cdot s^{-1}$)	k_{+3}2h8 (s^{-1})	K (μM)	k_{+2} (s^{-1})	k_{+2}/K ($M^{-1} \cdot s^{-1}$)	k_{+3} (s^{-1})
Sulfazecin	32	1.3×10^{-3}	40	2×10^{-5}	> 0.1	$> 1 \times 10^{-3}$	6500	$< 1 \times 10^{-5}$
SQ26180	> 12	$> 1 \times 10^{-3}$	100	$< 1 \times 10^{-5}$	> 0.3	$> 1 \times 10^{-3}$	2500	$< 1 \times 10^{-5}$
SQ26324	> 10	$> 1 \times 10^{-3}$	70	2.5×10^{-5}	0.35	2.9×10^{-3}	8300	$< 1 \times 10^{-5}$
Azthreonam	12000	$< 2 \times 10^{-5}$	< 0.002	not determined	> 100	$> 1 \times 10^{-3}$	15	$< 0.5 \times 10^{-5}$

For comparison, the interaction between the R61 D-peptidase and benzylpenicillin (which has the same side chain as SQ26324) proceeds with $K = 12$ mM (at 20°C), $k_{+2} = 170$ s^{-1} (at 20°C), $k_{+2}/K = 14000$ $M^{-1} \cdot s^{-1}$ (at 20°C) and $k_{+3} = 1.4 \times 10^{-4}$ s^{-1} (at 37°C). Similarly, the k_{+2}/K value for the interaction between the R39 DD-peptidase and benzylpenicillin is 300000 $M^{-1} \cdot s^{-1}$ (at 37°C).

30°C and pH 7.0) and the recovery of the enzyme activity was measured after increasing periods of time. In turn, the apparent rates of enzyme inactivation (k_a) were determined by incubating the enzyme with varying concentrations of the selected monobactam. Samples were withdrawn after increasing periods of time, and, after elimination of the excess of free monobactam, they were assayed for residual DD-carboxypeptidase activity. With sulfazecin and the R61 enzyme and with SQ26324 and the R39 enzyme, the plots k_a vs. [I] were hyperbolic and the reciprocal plots $[I]/k_a$ vs. [I] permitted estimation of the individual K and k_{+2} values. In the other cases, k_a remained proportional to [I] and only the k_{+2}/K ratio values (and minimal values of K and k_{+2}) could be estimated.

The stability of sulfazecin, SQ26180 and SQ26324, at 37°C and in 1 M K_2HPO_4 pH 12, was measured as described by Frère et al. [13]. Benzylpenicillin binding was carried out by incubating the enzyme with 0.1 mM [^{14}C]benzylpenicillin and submitting the reaction mixture to thin-layer chromatography on Polygram Sil-G plates (Macherey Nagel and Co., Düren, F.R.G.) using the solvent n-butanol : 10; H_2O : 4; acetic acid : 3 ethanol : 3 (by vol.).

4. RESULTS

At a 5 mM concentration, none of the monobactams tested had any effect on the Zn^{2+}-containing DD-peptidase.

Sulfazecin, SQ26180 and SQ26324, inactivated both R61 and R39 serine DD-peptidases according to model 1. Azthreonam also inactivated the R39 enzyme but with the R61 enzyme, the k_{+2} value was extremely low an the K value was measured by steady-state kinetics. The values of the kinetic parameters are given in Table 1.

At 2 mM nocardicin A had no effect on the R61 enzyme. At 0.1 and 0.5 mM, and after 20 min of incubation, nocardicin A inactivated the R39 enzyme by 10 and 30%, respectively, and in parallel to this, the amount of [^{14}C]benzylpenicillin susceptible to be bound to the enzyme was decreased to a similar extent. The k_a values for enzyme inactivation were 0.7 and 2.5×10^{-3} s^{-1} and the k_{+3} value for enzyme recovery was 2.7×10^{-4} s^{-1}.

The interaction between nocardicin A and the R39 enzyme, however, was unusual in that the residual enzyme activity—as measured after the first 20 min of treatment—remained unchanged upon prolonged incubation times. This partial enzyme inactivation occurred in spite of a low k_{+3} value and thus could not be attributed to turnover of nocardicin A. Moreover, addition of fresh R39 enzyme to this partially inactivated enzyme sample resulted in a similar extent of enzyme inactivation, showing that nocardicin A was still present in a free and active form.

5. DISCUSSION

The partial inactivation of the R39 enzyme by nocardicin A cannot be attributed to degradation of the monobactam. The interaction thus seems not to obey the simple three-step reaction described by model 1 and cannot be explained by assuming that enzyme inactivation and inactivator turnover occur concomitantly (as observed during interaction between several β-lactamases and clavulanate or β-iodopenicillanate) [14–16]. The problem has not been further investigated because of the very low inactivating potency of nocardicin A.

Basically, the monobactams (except nocardicin A) react with the model Zn^{2+} and serine DD-peptidases in a manner similar to that of classical bicyclic β-lactam compounds (penicillins and cephalosporins). This conclusion rests upon the following observations: (i) The Zn^{2+} DD-peptidase shows high intrinsic resistance to both types of compounds; (ii) The monobactams sulfazecin, SQ 26180 and SQ26324 inactivate the R61 and R39 serine DD-peptidases according to model 1; (iii) The second-order rate constant (k_{+2}/K) values range between 40 and 100 $M^{-1} \cdot s^{-1}$ with the R61 enzyme and between 2500 and 8300 $M^{-1} \cdot s^{+1}$ with the R39 enzyme, thus confirming the high sensitivity of this latter enzyme when compared to the former, to β-lactam compounds (whether they possess a mono- or a bicyclic structure); (iv) in all cases, the E–I^* (acyl-enzyme) intermediates show high stability (low k_{+3} values).

In these two cases where the determinations could be made, the dissociation constants K (32 μM for the interaction between the R61 enzyme and sulfazecin; and 0.35 μM for the interaction between the R39 enzyme and SQ26324) have the lowest values ever observed (with bicyclic β-lactam compounds, the K values usually range between 1 and 10 mM). However, the values of the first-order rate constant k_{+2} of enzyme acylation (from 1 to 3×10^{-3} s^{-1}) are also very low resulting in low bimolecular rate constant (k_{+2}/K) values for enzyme inactivation. These low k_{+2} values cannot be attributed to a low intrinsic reactivity of the β-lactam ring in the monobactams since these compounds have, in 1 M K_2HPO_4, pH 12 (and at 37°C), half-lives which are shorter than those of classical penams or cephems (such as benzylpenicillin or cephalosporin C). These observations suggest: (i) an excellent fit of sulfazecin with the active site of the R61 serine DD-peptidase and, to a still much greater extent, of SQ26324 with the active site of the R39 serine DD-peptidase; but (ii) a low efficacy of the nucleophilic attack of the active serine hydroxyl group on C2 of the β-lactam ring due, perhaps, to unfavorable side chains resulting in a non-optimal positioning of the reagents.

The same interpretation might also apply to the interactions between sulfazecin and the R39 enzyme, SQ26180 and both R61 and R39 enzymes, and SQ26324 and the R61 enzyme, thus providing a possible explanation for the observed rather low k_{+2}/K values when compared to those obtained with benzylpenicillin (which has the same side chain as SQ26324) (see Fig. 1 and Table 1). Aztreonam, however, is peculiar in that it has virtually no activity on any of the model DD-peptidases tested, thus confirming the high and very selective reactivity that this monobactam exerts towards the membrane-bound penicillin-binding protein 3 of *Escherichia coli* and other aerobic, rod-shaped Gram-negative bacteria [17].

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