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Kinetic Study of Interaction between BRL 42715, β-Lactamases, and D-Alanyl-D-Alanine Peptidases

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A detailed kinetic study of the interactions between BRL 42715, a β-lactamase-inhibiting penem, and various β-lactamases (EC 3.5.2.6) and D-alanyl-D-alanine peptidases (DD-peptidases, EC 3.4.16.4) is presented. The compound was a very efficient inactivator of all active-site serine β-lactamases but was hydrolyzed by the class B, Zn^{2+}-containing enzymes, with very different k_{cat} values. Inactivation of the *Streptomyces* sp. strain R61 extracellular DD-peptidase was not observed, and the *Actinomadura* sp. strain R39 DD-peptidase exhibited a low level of sensitivity to the compound.

The production of β-lactamases by clinical isolates continues to be the main mechanism of bacterial resistance to β-lactam antibiotics. Whilst antibacterial therapy with β-lactamase-stable compounds has been partially successful in containing the problem, absolute stability has not been achieved with any one agent. Clinical experience has established that a second approach, using a specific β-lactamase inhibitor coadministered with a β-lactamase-labile antibiotic, offers a viable therapeutic option for combatting β-lactamase-mediated resistance. Although the three β-lactamase inhibitors in clinical use (clavulanic acid, sulbactam, and tazobactam) are effective inactivators of a wide range of β-lactamases, they do not inhibit all of the clinically important enzymes (4, 25).

Of recent interest is the penem β-lactamase inhibitor, BRL 42715 (Fig. 1A), which is synthesized following a stereocontrolled reaction sequence starting from the chiral synthon, 6-aminopenicillanic acid (23). BRL 42715 alone possesses no appreciable antibacterial activity (MIC, 16 to 64 μg/ml) except against the methicillin-sensitive staphylococci (MIC, 1 μg/ml). In cell-free β-lactamase assays, this inhibitor shows intrinsic activity which is superior to that of clavulanic acid, sulbactam, or tazobactam. Significantly, it is able to inactivate the plasmid-mediated OXA-1 β-lactamase and the chromosomally mediated enzymes of *Enterobacter*, *Citrobacter*, *Pseudomonas*, *Serratia*, and *Morganella* species, against which the other inhibitors show poor activity. Its potent inhibitory activity is reflected in its ability to potentiate the antibacterial properties of a wide range of β-lactamase-susceptible β-lactam antibiotics, including third generation cephalosporins, at very low concentrations. Higher concentrations (≈1 μg/ml) are required, however, against those strains producing elevated levels of class C β-lactamases (5, 7). The BRL 42715 diffusion rate through the outer membranes of gram-negative bacteria is similar to those of sulbactam and tazobactam but lower than that of clavulanic acid (11). However, thanks to its superior intrinsic activity, it remains the most active inhibitor against whole cells. In contrast to other penems, it is not readily hydrolyzed by the renal dehydropeptidase (6).

The kinetics of inhibition of β-lactamases of *Staphylococcus aureus* NCTC 11561 (class A), *Escherichia coli* JT4 (TEM-1, class A), and *Enterobacter cloacae* P99 (class C) by BRL 42715 has been investigated previously (12). The data show that inhibition occurs rapidly and is essentially irreversible and that the stoichiometry of inhibition is approximately 1:1, indicating almost zero turnover. With the staphylococcal enzyme, the enzyme-inhibitor complex decays very slowly, eventually regenerating the active enzyme. In the case of TEM-1 and class C β-lactamases, even after prolonged incubation not all the enzyme activity was restored, suggesting the formation of a permanently inactivated enzyme-inhibitor complex in addition to a transiently stable complex (12). A hypothetical pathway for the interaction mechanism, involving a complex active-site rearrangement, has been proposed on the basis of the results obtained from base- and β-lactamase-catalyzed hydrolysis of BRL 42715 (3). The major product in these hydrolysis experiments was the dihydrothiazepine rearrangement product (Fig. 1B). Thus, transient or irreversible inhibition of β-lactamases

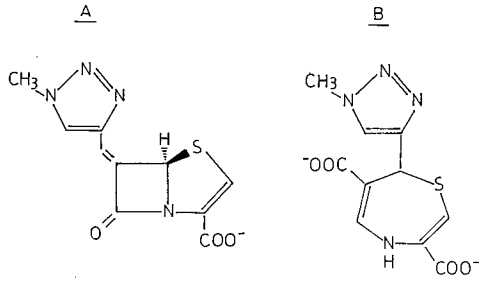

FIG. 1. Structure of BRL 42715 [(A) sodium (5*R*)-(Z)-6-(1-methyl-1,2,3-triazol-4-ylmethylene)penem-3-carboxylate] and of the chromogenic dihydrothiazepine obtained from base- or enzyme-catalyzed hydrolysis of this compound (B).

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TABLE 1. Enzymes utilized in this study

Enzyme	Class	Reference(s)
β-Lactamase from *S. albus* G	A	22
TEM-1 β-lactamase (plasmid pTAC11)	A	24, 2
β-Lactamase from *B. cereus* 5/B/6	B	8
β-Lactamase from *Pseudomonas maltophilia* 511	B	13
β-Lactamase from *A. hydrophilia* A2	B	13
β-Lactamase from *E. cloacae* 908R	C	22
OXA-2 β-lactamase (plasmid pDML303)	D	21
DD-peptidase from *Streptomyces* sp. strain R61	DD-peptidase	14
DD-peptidase from *Actinomadura* sp. strain R39	DD-peptidase	18

by BRL 42715 appears to involve the formation of a cyclic β-aminoacrylate–enzyme complex.

In this report, we present kinetic data concerning the interactions between BRL 42715 and representative β-lactamases of classes A, B, C, and D. The *Streptomyces* sp. strain R61 and *Actinomadura* sp. strain R39 D-alanyl-D-alanine peptidases (DD-peptidases) which have been extensively utilized as model penicillin-binding proteins were also included in this study to evaluate the specificity of the inactivating properties of BRL 42715 against active-site serine β-lactamases and DD-peptidases, which constitute two closely related families of penicillin-recognizing enzymes.

MATERIALS AND METHODS

Compounds. BRL 42715 was prepared by Beecham Pharmaceuticals. Nitrocefin was purchased from Unipath (Basingstoke, Hants, United Kingdom), and oxacillin was a kind gift of Bristol Myers-Squibb (Brussels, Belgium). The thiolester DD-peptidase substrate C_6H_5-CO-NH-CH_2-CO-S-CH_2-COOH was synthesized in Liège, Belgium (1).

Enzymes. The various enzymes studied in the present work are listed in Table 1, which also includes the references in which the purification methods are described. All enzyme preparations were at least 95% pure.

Kinetic studies. UV and visible spectroscopic recordings were performed with the help of a Uvikon 860 spectrophotometer linked to a Copam PC microcomputer. The hydrolysis of BRL 42715 was directly monitored by recording the A_{350} variation. Fitting of the data to linear equations was performed with the help of the Enzfitter program (Elsevier Biosoft, Cambridge, United Kingdom) and of complete hydrolysis time courses as described before (9). Inactivation experiments were performed by the reporter substrate method (9): pseudo-first-order inactivation rate constants were measured at increasing inactivator concentrations. The reporter substrate was nitrocefin for all β-lactamases but the OXA-2 enzyme with which oxacillin was used. The different buffers and incubation temperatures used were as follows: for β-lactamases of classes A, C, and D, 50 mM sodium phosphate, pH 7.0, at 30°C; for β-lactamases of class B, 10 mM sodium cacodylate, pH 7.0, containing 0.1 mM $ZnCl_2$ at 30°C for the *Aeromonas hydrophila* enzyme or 35°C for the other enzymes (a lower temperature was used for the *Aeromonas* enzyme because of its significantly poorer heat stability); and for DD-peptidases, 10 mM sodium phosphate, pH 7.0, at 37°C.

Mass spectrometry. BRL 42715 (200 nmol) was incubated for 2 h at 37°C with 250 μg of *Bacillus cereus* 5/B/6 β-lactamase II (class B; Table 2) in 2 ml of 10 mM sodium cacodylate, pH 6.0, containing 50 μM Zn^{2+}. The pH was adjusted to 3.0 with 1 M HCl, and the solution was extracted with ethyl acetate. The organic phase was dry evaporated, and the solid residue was redissolved in 50 μl of ethanol. In a similar experiment, 200 nmol of BRL 42715 was incubated for 4 h at 37°C with 1.35 mg of the *Streptomyces albus* G β-lactamase in 500 μl of 50 mM sodium phosphate, pH 7.0. The sample was then acidified and extracted. Mass spectra were obtained with a Quattro VG mass spectrometer equipped with an electrospray interface (VG Biotech, Manchester, Cheshire, United Kingdom). Samples (4 μl of ethanol solution) were injected into the electrospray, and the mass spectra were scanned over the 50- to 600-Da mass range.

TABLE 2. Kinetic parameters of class B β-lactamases for BRL 42715

Class B β-lactamase source	k_{cat} (s^{-1})	K_m (μM ± SD)	k_{cat}/K_m (M^{-1} s^{-1})
A. hydrophila	0.22	40 ± 5	$5.5 \cdot 10^3$
P. maltophilia 511	0.55	270 ± 10	$2.0 \cdot 10^3$
B. cereus II	121	6 ± 1	$2.0 \cdot 10^7$

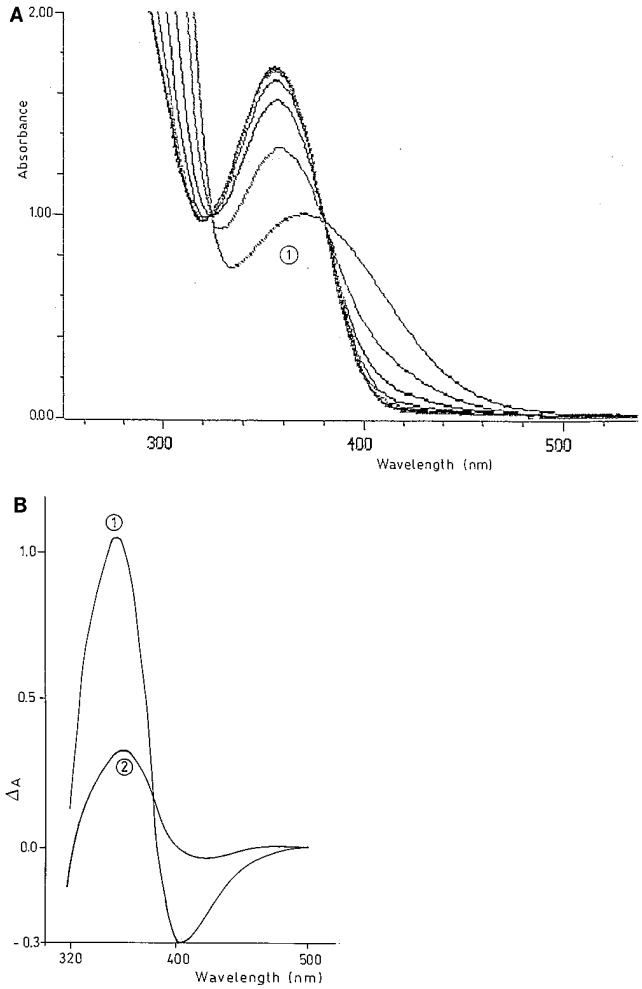

FIG. 2. Spectral changes recorded during hydrolysis of 500 μM BRL 42715. (A) hydrolysis by 50 mM sodium hydroxide. The nonhydrolyzed compound displayed the lowest absorbance values at around 350 nm. Spectra were recorded at 1-min intervals. The spectrum labelled 1 is that of the intact BRL 42715. (B) Difference spectra between the hydrolysis product and the intact BRL 42715 (curve 1) (superimposable difference spectra were obtained when the compound was hydrolyzed by 50 mM NaOH and the *S. albus* G and Zn^{2+} β-lactamases) and between the adduct obtained with 70 μM TEM β-lactamase and the starting compound (curve 2). ΔA, difference in absorbance spectra.

RESULTS

Class A β-lactamases: *S. albus* G and TEM-1. BRL 42715 was hydrolyzed by the *S. albus* G enzyme, and the time course of hydrolysis could be followed by UV-visible spectroscopy. Interestingly, the spectra of the product (P) were the same when obtained by using sodium hydroxide, the *S. albus* G, or one of the Zn^{2+} β-lactamases (see description below) as hydrolytic agents. The maximum absorbance variation occurred at 350 nm (Fig. 2). When monitored at this wavelength and when the initial BRL 42715 concentration ($[C]_0$) was at least 100 times that of the enzyme ($[E]_0$), a biphasic phenomenon, a rapid but partial hydrolysis of the β-lactam followed by a much

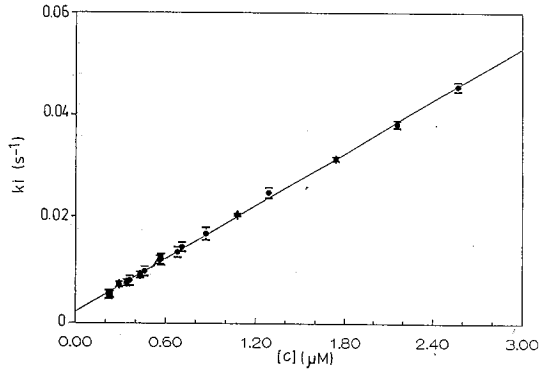

FIG. 3. Interaction between the *S. albus* G β-lactamase and BRL 42715. Variation of k_i with [C] from which an A/B value of 17,000 M^{-1} s^{-1} was obtained (see Results).

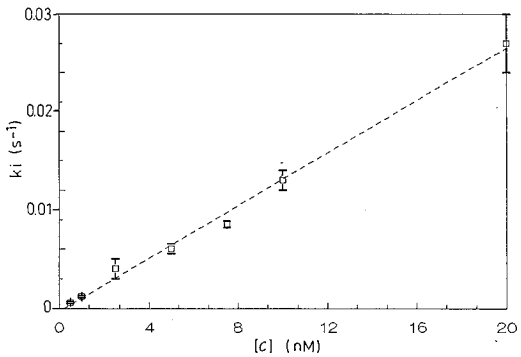

FIG. 4. Interaction between the TEM-1 β-lactamase and BRL 42715. The variation of k_i at low [C] values from which a k_2/K' value of 2.5 · 10^6 M^{-1} s^{-1} was deduced ± standard deviations is plotted.

slower turn-over, was observed. This pattern suggested a branched pathway, as in the model below:

$$\begin{array}{ccccccc} & & k_{+1} & k_2 & & k_3 & \\ \mathrm{E} + \mathrm{C} & \rightleftharpoons & \mathrm{EC} & \rightarrow & \mathrm{EC}^* & \rightarrow & \mathrm{E} + \mathrm{P} \\ & & k_{-1} & k_{+4} \;\; \downuparrows & k_{-4} & & \\ & & & & \mathrm{EC}_i & \rightarrow & \mathrm{E} + \mathrm{P}' \\ & & & & & k_5 & \end{array}$$

An approximate value of the k_3/k_{+4} ratio was obtained by measuring the residual activity after partial (and transient) inactivation at low $[C]_0/[E]_0$ ratios (16). The poor accuracy of the value obtained (40 ± 10) is due to the fact that the inactivated complex (EC_i) is not completely stable. This value was not significantly altered by the presence of high salt concentrations (0.5 M NaCl), in contrast with the results obtained with the same enzyme and β-halogenopenicillanates (10). The model admits two-limit cases, i.e., $k_{-4} = 0$ or $k_5 = 0$. The data presented here do not allow a choice between the various possibilities (general model or one of the limit cases). For simplicity's sake, the situation will be further discussed by assuming that $k_{-4} = 0$, but it should not be considered that this limit case is more likely than either the other one or the general model.

The reporter substrate method (with 100 μM nitrocefin) was used to follow the enzyme inactivation at high $[C]_0/[E]_0$ ratios. The dependency of the inactivation rate on the BRL 42715 concentration is shown by Fig. 3. On the basis of the above model (where $k_{-4} = 0$): $k_i = k_5 + A \cdot [C]/(B + [C])$, where $A = k_2 \cdot k_4/(k_2 + k_3 + k_4)$ and $B = (k_3 + k_4) \cdot K'/(k_2 + k_3 + k_4)$, with $K' = (k_{-1} + k_2)/k_{+1}$.

Our data thus allowed the computation of $k_5 = 2.2 \cdot 10^{-3}$ s^{-1} (extrapolation of the line to [C] = 0) and of $A/B = k_2 \cdot k_4/(k_3 + k_4) \cdot K' = 17{,}000$ M^{-1} s^{-1}. On the basis of the k_3/k_4 ratio determined above, it can be seen that $k_2/K' = 680{,}000 \pm 170{,}000$ M^{-1} s^{-1}. From the same data, the K_m value for the hydrolysis of BRL 42715 after establishment of the final steady state was determined. A value of 26 nM, corresponding to $K_m = k_5 \cdot (k_3 + k_4) \cdot K' / [k_2k_5 + k_2k_4 + k_5 \cdot (k_3 + k_4)]$ in model 1, was found.

With the TEM-1 enzyme, the situation was much more simple. Complete inactivation occurred at a 1/1 molar ratio. With 50 μM nitrocefin as a reporter substrate, the pseudo-first-order rate constant for inactivation remained proportional to BRL 42715 concentrations up to 20 nM (Fig. 4). From these data, and taking account of the protection by the reporter substrate, a k_2/K' value of (2.5 ± 0.3) · 10^6 M^{-1} s^{-1} was calculated. Figure 2B shows the difference in the spectra of the adduct and the starting compound, indicating clear similarities between the spectrum of the adduct and that of the hydrolyzed compound (Fig. 2A).

No lag was observed in the increase of A_{350}, which, on the basis of our model, reflected a very rapid rearrangement of the initially formed acyl enzyme (EC^*) to the stable species (EC_i). The model thus became rather simple, with k_{-4} and k_5 close to zero and k_{+4} much larger than both k_2 and k_3.

Class C β-lactamase: *E. cloacae* 908R. No hydrolysis was observed for the *E. cloacae* 908R enzyme, but the enzyme was rapidly inactivated, thus indicating a situation similar to that observed with the TEM-1 enzyme.

The rate of inactivation at various [C] values was measured with nitrocefin as a reporter substrate. A linear increase of k_i was found up to [C] = 100 nM ($k_i = 0.12$ s^{-1}), from which the following values were deduced: $k_2/K' = 1.2 \cdot 10^6$ M^{-1} s^{-1}; with $k_2 > 0.5$ s^{-1} and $k_3 < 10^{-3}$ s^{-1}.

Class D β-lactamase: OXA-2. Again, with OXA-2 no hydrolysis of the compound was observed, but rapid, total and irreversible inactivation was observed at $[C]_0/[E]_0$ values ranging from 15,000 to 1. With 1 mM oxacillin as a reporter substrate, the rate of inactivation was found to be independent of the inactivator concentration above 40 nM [$k_i = (2.1 \pm 0.2) \cdot 10^{-2}$ s^{-1}]. On the basis of our model, this would yield $k_2 = (2.1 \pm 0.2) \cdot 10^{-2}$ s^{-1}, $k_4 \gg k_2$, $k_2/K' > 0.5 \cdot 10^6$ $M^{-1}s^{-1}$, and both k_3 and $k_5 < 10^{-3}$ s^{-1}.

Class B: Zn^{2+} β-lactamases. In all cases, with Zn^{2+} β-lactamases BRL 42715 behaved as a substrate. The kinetic parameters are given in Table 2. They were derived from initial rate measurements and Hanes plots for the *A. hydrophila* enzyme and from complete time courses for the other two enzymes.

DD-peptidases. The *Actinomadura* sp. strain R39 enzyme was inactivated by BRL 42715. The inactivation was studied with the thiolester (100 μM) as a reporter substrate in the presence of 10 mM D-histidine to obtain a first-order hydrolysis of the thiolester (19). A k_2/K' value of 1,100 ± 30 M^{-1} s^{-1} was found (with $K' > 40$ μM). Conversely, the *Streptomyces* sp. strain R61 enzyme (1 μM) was not inactivated after over 5 min at a BRL 42715 concentration of 100 μM, indicating a k_2/K' value lower than 2 M^{-1} s^{-1}.

Identification of the reaction product. The mass spectra of the compound obtained after extraction of the acidified reaction mixtures exhibited clear peaks at an *m/e* of 279, for both

TABLE 3. Acylation rate constants of various active-site serine enzymes by BRL 42715

Source of enzyme	Enzyme type and class	k_2/K' (μM^{-1} s^{-1})	k_3/k_4	$(C_0/E_0)_i$[a]	$k_5(s^{-1})$
TEM-1	β-Lactamase, A	2.5, 6.4[b]	0	1	$1.6 \cdot 10^{-4}$[b]
S. aureus NCTC 11561	β-Lactamase, A	0.17[b]	0[b]	1	$3.6 \cdot 10^{-6}$[b]
S. albus G	β-Lactamase, A	0.68	40 ± 10	40 ± 10	$2.2 \cdot 10^{-3}$
K. pneumoniae K1	β-Lactamase, A	ND[c]	>1	>2	$6 \cdot 10^{-2}$[d]
E. cloacae P99	β-Lactamase, C	1.2, 2.5[b]	0	1	$1.2 \cdot 10^{-5}$[b]
OXA-2	β-Lactamase, D	>0.5	0	1	ND
Streptomyces sp. strain R61	DD-peptidase	—[e]	—	—	—
Actinomadura sp. strain R39	DD-peptidase	$1.1 \cdot 10^{-3}$	0	1	ND

[a] $(C_0/E_0)_i$, C_0/E_0 values leading to complete inactivation.
[b] Data from reference 12.
[c] ND, not determined.
[d] Data from reference 3.
[e] —, no interaction ($k_2/K < 2$ M^{-1} s^{-1}).

the *B. cereus* Zn^{2+} enzyme β-lactamase and the *S. albus* G β-lactamase. This corresponds to a molecular ion for dihydrothiazepine after the loss of one H. To show that the rearrangement was not an artifact due to the extraction procedure, an aliquot of the ethanol solution was dry evaporated and the residue was redissolved in 50 mM sodium phosphate, pH 7.0. The UV spectrum was superimposable on that of the product of the base- or enzyme-catalyzed hydrolysis (Fig. 2A), indicating that no further structure modification had occurred.

DISCUSSION

Like several other β-lactams which inactivate active-site serine β-lactamases, BRL 42715 was hydrolyzed by the Zn^{2+}-containing class B enzymes (13). Hydrolysis was particularly efficient with the *B. cereus* enzyme which, fortunately, does not represent a clinical problem. However, the fact that the three class B enzymes studied did hydrolyze the compound suggests that it might be a substrate for most metallo-β-lactamases and underlines the difficulties which might arise if these enzymes spread to a large number of pathogenic strains. In agreement with the results of Broom et al. (3), the spectral properties of the product were identical to those of the dihydrothiazepine (Fig. 1B) obtained after sodium hydroxide hydrolysis. The simplest explanation is that a rearrangement of the primary hydrolysis product, formed according to the usual (but still mysterious) catalytic pathway, takes place after its release from the enzyme. The mass spectrometry data also confirmed that the same dihydrothiazepine was obtained after hydrolysis by both the Zn^{2+} enzyme and the active-site serine *S. albus* G β-lactamases. As expected from its poor intrinsic antibacterial activity, BRL 42715 did not significantly react with the *Streptomyces* sp. strain R61 DD-peptidase and was a rather poor inactivator of the *Actinomadura* sp. strain R39 enzyme, which is generally very sensitive to most β-lactams. These properties were reminiscent of those of β-iodopenicillanate, which was also much more active against the latter DD-peptidase (17). The results obtained with the two model DD-peptidases were thus consistent with the rather poor intrinsic antibacterial activity exhibited by BRL 42715. By contrast, BRL 42715 was an extremely efficient inactivator of all tested active-site serine β-lactamases. Table 3 summarizes our results and those obtained earlier (3, 12), with which they were in excellent agreement. On the basis of the model (with $k_{-4} \approx 0$), these enzymes can be divided into two groups. Enzymes in the first group (*S. albus* G and *Klebsiella pneumoniae*) exhibited a branched pathway ($k_3 > k_{+4}$) and a nonnegligible hydrolysis of the chromophoric EC_i adduct ($k_5 \neq 0$). All the other enzymes belonged to the second group, in which the reaction pathway appears to be linear, with a value of $k_3 < k_{+4}$ and a very low k_5 value ($1.5 \cdot 10^{-4}$ s^{-1} or less). The spectral properties of the adduct formed with the TEM-1 enzyme also agreed with the formation of a seven-membered dihydrothiazepine chromophore.

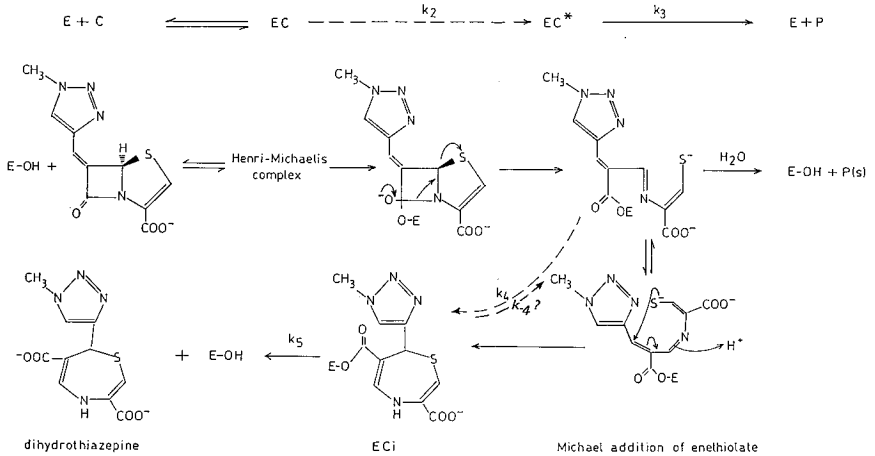

FIG. 5. General model for the interaction between BRL 42715 and the active-site serine β-lactamases (E-OH). The putative stable intermediates of the model presented in Results (EC* and EC_i) are tentatively identified. Broken lines represent the reaction steps actually detected by the kinetic and spectroscopic methods.

The data presently available indicate that branched pathways occur only with class A enzymes, but it should be stressed that only one representative each of class C and D has been studied. Figure 5 diagrams a proposed general interaction model based on the previously obtained chemical evidence and on our mass spectrometry results, where the initially formed acyl-enzyme (EC^*) can undergo both hydrolysis and rapid rearrangement to the more stable dihydrothiazepine-containing adduct. If this model is correct, the assumption that k_{-4} is ≈ 0 is probably justified. The hydrolysis product observed when k_3 is larger than k_4 also rapidly rearranges into the chromophoric dihydrothiazepine. All these results are very similar to those observed in the interactions between various β-lactamases and β-halogenopenicillanates (9, 15). In contrast to the situation prevailing with these compounds, the presence of high salt concentrations did not modify the k_3/k_4 ratio, indicating that this "salt effect" was a specific property of the rearrangement reaction of the acyl-enzyme formed with β-halogenopenicillanates (10). Finally, it was also noted earlier that the slow reactivation observed with the TEM and P99 (which is nearly identical to 908R) β-lactamases was not complete, suggesting the formation of an additional species even more stable than EC_i. The P99 adduct could arise from either EC^*, as hypothesized for the interaction between the TEM enzyme and clavulanic acid (20), or EC_i. Since the dihydrothiazepine moiety of the EC_i adduct appears to be rather stable, further modification of EC_i might involve an alteration of the enzyme tertiary structure.

The results presented here demonstrate that BRL 42715 is an extremely efficient inactivator of all active-site serine β-lactamases, a very unique, specific, and interesting property. Indeed, most other mechanism-based inactivators of active-site serine β-lactamases (e.g., clavulanic acid and sulbactam) generally exhibit rather poor activity against class C enzymes. BRL 42715 is however hydrolyzed by all the class B enzymes studied.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

While this paper was in press, Farmer et al. (Biochem. J. **303:**825–830, 1994) published results obtained with the same compound which were in excellent agreement with the present data.

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