IZUMENOLIDE—A NOVEL β-LACTAMASE INHIBITOR PRODUCED BY *MICROMONOSPORA*

II. BIOLOGICAL PROPERTIES

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Izumenolide is a potent inhibitor of β-lactamases, especially from Gram-negative bacteria. The I_{50} value of 0.01 μg/ml for TEM-2 β-lactamase, after 10 min preincubation, corresponds to a ratio of 7.6 moles inhibitor per mole of enzyme. The initial inhibitory reaction with TEM-2 β-lactamase exhibits mixed reaction kinetics, suggesting a possible overlapping binding site with the active center. TEM-2 β-lactamase is irreversibly inactivated by izumenolide in a biphasic reaction. Carbenicillin offers partial protection against inactivation. Izumenolide exhibits limited antibiotic activity against some Gram-negative bacteria. Against β-lactamase producing bacteria izumenolide provides protection to ampicillin and cephaloridine but the protection is limited due to permeability problems associated with izumenolide entry into the cells.

β-Lactamases are often responsible for causing increased resistance to β-lactam antibiotics in many bacteria. The search for naturally occurring β-lactamase inhibitors has been intensive in the past few years, resulting in the discovery of clavulanic acid[1], the olivanic acids[2~5], the epithienamycins[6,7] and PS-5[8]. All of these inhibitors, which possess antibiotic activity of their own, have been identified as bicyclic compounds containing a β-lactam ring. At least in the case of clavulanic acid[9~11], and probably PS-5[12], inactivation of specific β-lactamases may occur when the β-lactam reacts as a suicide substrate at the active site of the enzyme.

Izumenolide (**1**) is a highly specific β-lactamase inhibitor, especially for β-lactamases from Gram-negative bacteria. This compound, which was isolated from *Micromonospora chalcea* subsp. *izumensis* as described in the previous paper[13], is unique in that it is a sulfate ester-containing macrolide which bears little or no resemblance to a β-lactam[14]. In this paper the biological properties of izumenolide are discussed.

Materials and Methods

Enzymes

TEM-1 and TEM-2 β-lactamases were purified according to a modification of the procedure of MELLING and SCOTT[15]. TEM-2 and *Staphylococcus aureus* (PEN+) β-lactamases were >95% homogeneous. *Enterobacter cloacae* (P-99+) β-lactamase was purified to homogeneity as described by ROSS and BOULTON[16]. *Klebsiella aerogenes* (K-1+) β-lactamase was prepared by passing a clarified sonicate through Sephadex G-75. *Bacillus cereus* β-lactamases were obtained from Sigma and used without further purification.

Enzyme Assays

β-Lactamase activity was determined using spectrophotometric assays[17]. The following substrates were utilized with hydrolysis monitored at the indicated wavelength: benzylpenicillin, 240 nm; ampicillin, 232 nm; cephaloridine, 295 nm ($e=890\ M^{-1}\ cm^{-1}$); nitrocefin, 495 nm. All assays were performed in 0.1 M phosphate buffer, pH 7.0, at 25°C using a Gilford 250 recording spectrophotometer.

Substrates

The following compounds were obtained as indicated: ampicillin and benzylpenicillin from E. R. Squibb & Sons; cephaloridine from Eli Lilly; carbenicillin from Beecham; nitrocefin (87/312) from Glaxo.

Kinetic Analysis

Initial reaction rates were determined in triplicate using six substrate concentrations with three or four inhibitor levels. Kinetic parameters were obtained from LINEWEAVER-BURK plots and DIXON plots, using linear regression analysis (Hewlett-Packard HP-97 calculator).

Electrophoresis

Isoelectric focusing was performed using an LKB Multiphor with prepared polyacrylamide PAG plates in pH ranges of 4.0~6.5 or 3.5~9.5. Electrophoresis in SDS-polyacrylamide slab gels was carried out according to LAEMMLI[18].

Susceptibility-Synergy Testing

Agar dilution susceptibility tests were performed at an inoculum level of 10^4 colony-forming units (CFU) using a Denley Multipoint Inoculator (Denley Instruments, Sussex, U.K.). Media employed were Yeast Extract Agar (YEA; 0.5% yeast extract, 1.5% agar) and MUELLER-HINTON Agar (MH; Difco Laboratories). Tests were read after 18 hours at 37°C.

The enhancement of the antibacterial activity of β-lactam antibiotics by izumenolide was also demonstrated using an agar dilution procedure. Serial dilutions of ampicillin or cephaloridine were prepared with or without 10 μg/ml of izumenolide and added to YEA. An inoculum level of 10^6 CFU was employed and tests were read after 18 hours at 37°C.

Results and Discussion

I_{50} Determination with β-Lactamases

Izumenolide inhibits a variety of β-lactamases as shown in Table 1. β-Lactamases from Gram-negative organisms were affected to a much greater extent than β-lactamases from Gram-positive bacteria. High specificity for the TEM-2 enzyme was especially evident, as the I_{50} value obtained after preincubation of inhibitor and enzyme corresponded to an inhibitor to enzyme ratio of 7.6. In all systems

Fig. 1. Effect of izumenolide on the activity of β-lactamases from Gram-positive bacteria.

Izumenolide was incubated 10 minutes at 25°C with β-lactamase from either *S. aureus* (20 μM, 90 activity units with benzylpenicillin) or *B. cereus* (3.75 μg protein, 150 activity units with benzylpenicillin) in a volume of 1.01 ml. Assays were initiated by the addition of 50 μl of benzylpenicillin (final concentration, 0.5 mM).

Rates of hydrolysis were compared to incubated controls containing no izumenolide.

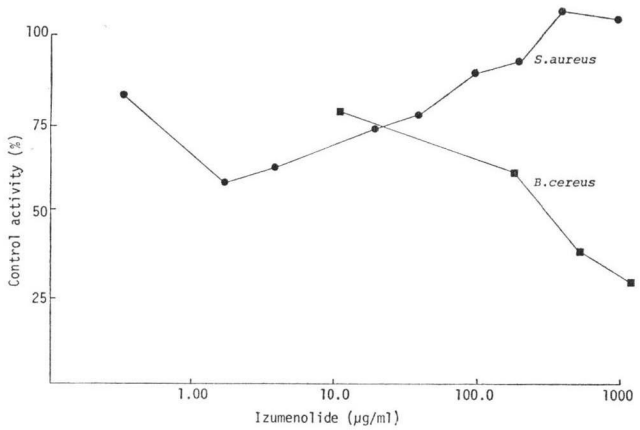


Table 1. I_{50} Values for izumenolide with β-lactamases from various sources.

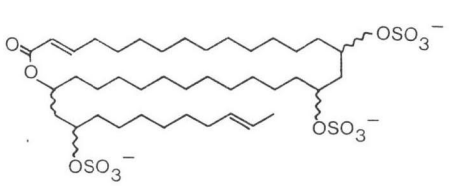
β-Lactamase	Substrate*	I_{50} (μg/ml)	
		0 min. incubation	10 min. incubation
Bacillus cereus I	Pen G	>1,000	280
Bacillus cereus II	CER	>1,000	660
Staphylococcus aureus (Pen+)	Pen G	>1,000**	>1,000**
Enterobacter cloacae (P-99+)	CER	0.6	0.1
Escherichia coli (TEM-1)	Pen G	10	0.4
Escherichia coli (TEM-2)	Pen G	10	0.01
Klebsiella aerogenes (K1+)	Pen G	230	0.2

* Pen G=Benzylpenicillin at 0.5 mM; CER=Cephaloridine at 1.0 mM.
** See Discussion.

inhibition was markedly improved after preincubation, thereby showing that izumenolide behaves as a progressive inhibitor.

Activity curves which contrast inhibition characteristics for the *Staphylococcus aureus* and *Bacillus cereus* I β-lactamases in the presence of izumenolide are shown in Fig. 1. Both *B. cereus* β-lactamases I and II exhibited inhibition curves quite similar qualitatively to those observed for the β-lactamases from Gram-negative bacteria. However, the β-lactamase from *S. aureus* behaved anomalously in the presence of izumenolide. Maximum inhibition was observed at about 2 μg/ml if enzyme and inhibitor were preincubated and at about 200 μg/ml in the absence of preincubation. However, this inhibition was reversed in the presence of high concentrations of izumenolide. These results suggest the presence of a secondary binding site on the *S. aureus* β-lactamase which may serve as a stabilizing (or possibly activating) site on the enzyme.

Fig. 2. Derivatives of izumenolide.



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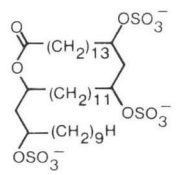

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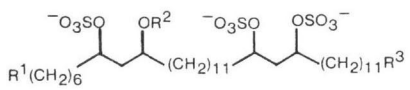


Compound	R^1	R^2	R^3
3	H_3C–HC=CH–	–H	–CH=$CHCO_2^-$
4	$H(CH_2)_3$–	–H	–$(CH_2)_3OH$
5	$H(CH_2)_3$–	$-CH_3$	–$(CH_2)_3OCH_3$

Several compounds derived from izumenolide[13] (Fig. 2) were also tested for inhibition using TEM-2 β-lactamase. I_{50} values are given in Table 2. From these results it is apparent that maximal inhibition is observed when the cyclic structure remains intact. Opening of the ring diminishes the activity somewhat, although derivatives **3**, **4**, and **5** are still good β-lactamase inhibitors. Therefore,

Table 2. I_{50} Values for izumenolide derivatives with TEM-2 β-lactamase.

Compound and TEM-2 β-lactamase (1.4 nM final concentration) were incubated at 25°C for 10 minutes and then added to ampicillin (0.2 mM) for assay.

Compound	I_{50} (μg/ml)	$\frac{I_{50}}{E}$
1	0.01	7.6
2	0.01	7.6
3	0.04	29
4	0.03	23
5	0.03	22

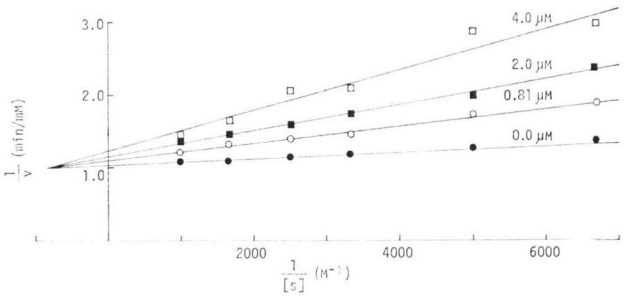
the specificity associated with izumenolide may be related to the spatial orientation of the sulfate ester moieties.

Inhibition of TEM-2 β-Lactamase

Inhibition of initial reaction rates by izumenolide using TEM-2 β-lactamase with benzylpenicillin as substrate resulted in a mixed kinetic pattern as shown in Fig. 3. A similar study with nitrocefin, analyzed using both a LINEWEAVER-BURK plot and a DIXON plot, suggested that hyperbolic competitive reaction kinetics was occurring with this cephalosporin substrate. Inhibition studies with TEM-2 β-lactamase were also performed in the presence of carbenicillin, a poor substrate (Fig. 4). Carbenicillin was shown to offer partial protection against progressive inhibition by izumenolide: the initial rate of inactivation was decreased at least ten-fold in the presence of carbenicillin. These kinetic studies together, therefore, suggest that the izumenolide binding site may overlap the enzymatic active site, but the two sites are non-identical.

Fig. 3. LINEWEAVER-BURK plot of initial reaction rates obtained with TEM-2 β-lactamase in the presence of izumenolide.

Enzyme (6.3 mM) was added to benzylpenicillin (0.15~1.0 mM) containing izumenolide. Initial reaction rates were followed for less than 1 minute.



Progressive inhibition of TEM-2 β-lactamase was studied using varying molar ratios of izumenolide to enzyme (Fig. 5). At low inhibitor levels a possible reversal of inhibition was observed with time. At inhibitor ratios of 4.0 or greater a rapid initial loss of activity was followed by a slower rate of inhibition. Complete loss of enzymatic activity required at least a ten-fold excess of inhibitor. This loss of activity was irreversible, as no activity could be regained after overnight dialysis of the enzyme-izumenolide reaction mixture; passage of an inhibited enzyme-izumenolide complex through Sephadex G-25 again resulted in no recovery of activity.

When TEM-2 β-lactamase was inactivated by izumenolide, treatment with 20 mM hydroxylamine for 20 minutes at pH 7.0 did not restore enzymatic activity. Also, no free enzyme band appeared in isoelectric focusing experiments after inactivated enzyme had been incubated with hydroxylamine. Therefore, the izumenolide inactivated β-lactamase must not involve acyl enzyme formation as seen, for example, with clavulanic acid[8,9].

Fig. 4. Protection of TEM-2 β-lactamase by carbenicillin against inactivation by izumenolide.

Enzyme (0.023 μM) was incubated in 15.0 ml buffer or 1.0 mM carbenicillin for 1.0 minute at 25°C before addition of 30 μl izumenolide (0.21 μM) at t=0. Aliquots of 1.0 ml were mixed with 50 μl of cephaloridine (1.0 mM) at the times indicated.

A control for each reaction mixture was prepared by adding buffer in place of izumenolide. The carbenicillin control after 6.5 minutes exhibited 91% of the buffer control activity.

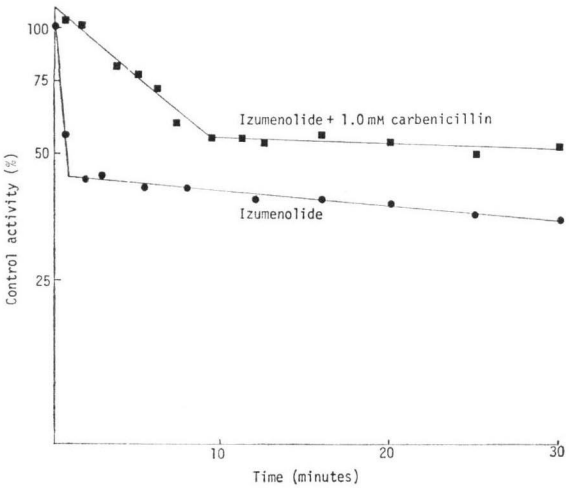
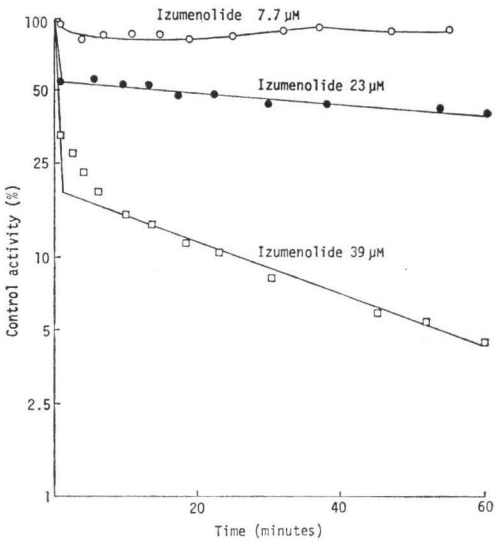


Fig. 5. Inactivation of TEM-2 β-lactamase by izumenolide.

Enzyme (5 μl, 3.8 μM) was incubated at 25°C with 25 μl izumenolide (7.7 μM, 23 μM, 39 μM). Aliquots of 2 μl were added to 3.0 ml benzylpenicillin (1.0 mM) at the times indicated for assay.

Controls containing buffer in place of izumenolide retained 92~98% of their original activity for 1.0 hour.



After the izumenolide inactivated TEM-2 β-lactamase had been subjected to SDS-polyacrylamide gel electrophoresis, a band with the same mobility as native enzyme was observed. Therefore, covalent binding at multiple sites is probably not involved, as a molecular weight difference of approximately 2,000 (representing two moles of izumenolide per mole of enzyme) would be readily detected in this system.

Izumenolide (100 μg/ml), showed no inhibition of *Streptomyces* R61 DD-carboxypeptidase/transpeptidase, horse liver alcohol dehydrogenase and α-chymotrypsin.

Susceptibility-Synergy Testing

Izumenolide exhibited limited antibiotic activity against a few Gram-negative bacteria (Table 3). Poor activity was partially due to media antagonism as can be seen from the generally inferior MIC values found when MH was employed as opposed to YEA. A similar antagonism was observed when izumenolide inhibition of TEM-2 β-lactamase was monitored in the presence of 1% MH Broth or 1% YE Broth: the I_{50} value was increased fivefold in MH from the value observed either in YE or in the control. An increase in I_{50} values for TEM-2 β-lactamase was also observed if additional proteins such as gelatin or serum albumin were added to the assay mixtures, therefore suggesting that izumenolide may bind to proteinaceous material. Because yeast extract broth did not interfere with β-lactamase inhibitory activity, subsequent microbiological testing was done on YEA.

Antibiotic activity was also limited due to poor access of izumenolide into the bacteria. Wild-type *E. coli* SC 8294 was not affected at 500 μg/ml while *E. coli* outer membrane permeability mutants

Table 3. *In vitro* antibacterial activity of izumenolide.

Organism	SC #	Minimum inhibitory concentration (μg/ml)*		Organism	SC #	Minimum inhibitory concentration (μg/ml)*	
		Yeast extract agar	MUELLER-HINTON agar			Yeast extract agar	MUELLER-HINTON agar
Staphylococcus aureus	1276	>500	>500	*Proteus mirabilis*	3855	>500	>500
Staphylococcus aureus	2399	>500	>500	*Proteus rettgeri*	8479	250	250
Staphylococcus aureus	2400	>500	>500	*Proteus vulgaris*	9416	500	250
Staphylococcus aureus	10165	>500	>500	*Salmonella typhosa*	1195	>500	>500
Streptococcus faecalis	9011	>500	>500	*Shigella sonnei*	8449	>500	>500
Micrococcus luteus	2495	>500	>500	*Enterobacter cloacae*	8236	>500	>500
Escherichia coli	8294	>500	>500	*Citrobacter freundii*	9518	>500	>500
Escherichia coli	10857	62.5	500	*Serratia marcescens*	9783	>500	>500
Escherichia coli	10896	250	250	*Pseudomonas aeruginosa*	9545	125	500
Escherichia coli	10909	62.5	500	*Pseudomonas aeruginosa*	8329	>500	>500
Klebsiella pneumoniae	9527	>500	250	*Acinetobacter calcoaceticus*	8333	500	>500

* 10^4 colony-forming units

Table 4. Activity of ampicillin and cephaloridine in the presence of izumenolide.

Organism	SC #	Enzyme	Minimum inhibitory concentration (μg/ml)*				
			Izumenolide alone	Ampicillin alone	Ampicillin +10 μg/ml Izumenolide	Cephaloridine alone	Cephaloridine +10 μg/ml Izumenolide
Staphylococcus aureus	2400	PENase	>100	100	25	1.6	1.6
Enterobacter cloacae	10435	P-99	>100	>100	>100	>100	>100
Klebsiella aerogenes	10436	K-1	>100	>100	>100	>100	>100
Escherichia coli (DC 0)	10858	—	>100	3.1	3.1	3.1	3.1
Escherichia coli	10404	TEM-1	>100	>100	>100	100	12.5
Escherichia coli	10979	TEM-2	>100	>100	>100	>100	100
Escherichia coli (DC 2)	10857	—	100	0.8	0.4	1.6	0.8
Escherichia coli (DC 2)	10990	TEM-1	100	>100	>100	25	3.1
Escherichia coli (DC 2)	10991	TEM-2	100	>100	>100	100	6.3

* 10^6 colony-forming units

SC 10857, SC 10896 and SC 10909 were inhibited at lower levels.

When a low level of izumenolide was added to β-lactamase labile compounds some protection of these compounds was afforded against certain β-lactamase producing organisms (Table 4). Increases in activity were seen with ampicillin against a penicillinase-producing *Staph. aureus* and cephaloridine against a TEM-1 or TEM-2 producing *E. coli* when the tests were run with the addition of izumenolide. Protection of ampicillin or cephaloridine by izumenolide against the P-99 β-lactamase-producing *Enterobacter* or the K-1 β-lactamase-producing *Klebsiella* was not seen, perhaps reflecting entry problems of izumenolide into these bacteria.

The permeability of izumenolide into bacteria was further studied using the *E. coli* DC-0 wild type (parent) and DC-2 outer membrane permeability mutants[19,20]. As seen in Table 4 from its MIC and protection of cephaloridine, izumenolide does penetrate the DC-2 mutants better than the DC-0 parent; however, access is still somewhat restricted in that the MIC's of the DC-2/TEM-1 and DC-2/TEM-2 mutants to cephaloridine+izumenolide are still higher than the MIC of the non-lactamase producing DC-2 to the mixture. If access were not restricted it would be expected that the MIC values should be equivalent in light of the potent inhibitory action of izumenolide against the TEM-1 and TEM-2 enzymes.

Toxicity

The acute (s.c.) LD_{50} of izumenolide for mice was 2 mg/kg.

Acknowledgements

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