Chapter 25. Bacterial Resistance to β-Lactams: The β-Lactamases

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Bacteria can develop a number of ways of resisting the lethal consequences of exposure to β-lactam antibiotics, but one of the most effective is the production of an enzyme, the β-lactamase, that rapidly hydrolyzes the β-lactam to the β-amino acid derivative. These enzymes are extremely efficient catalysts, they may be constitutive or inducible, they can be coded for chromosomally or by readily transferable plasmids, and they may be largely intracellular, largely extracellular, or (for Gram-negative bacteria) predominantly periplasmic. While it is clear that the permeability of the cell's membrane to the antibiotic sharply affects β-lactam potency (for instance, it has been shown that a plasmid β-lactamase in E.coli protects the bacterium from penicillin G but not from cephaloridine, even though the enzyme hydrolyzes the latter 50% faster[1]), it appears that most commonly-encountered resistance to β-lactams derives from the presence of the β-lactamase.[2] Although the central role of the β-lactamases in resistance has been clear for some time, efforts to overcome this resistance by using β-lactamase inhibitors have not proved very fruitful because the specificity of β-lactamases is wide and broad-spectrum β-lactamase inhibitors were not available. This situation is changing however, and the tactical inclusion of β-lactamase-inhibitory function into an antibiotic, or the use of a β-lactamase inhibitor in synergy with a susceptible but effective antibiotic,[3] is becoming attractive. This short review is devoted to two topics: (a) properties of some of the better-studied β-lactamases; (b) the interaction with the E.coli RTEM β-lactamase of a number of recently-discovered β-lactams containing unusual structural features.

General - The β-lactamases (E.C. 3.5.2.6: penicillin amido-β-lactam hydrolase; E.C. 3.5.2.8: cephalosporin amido-β-lactam hydrolase) are monomeric enzymes typically of molecular weight around 30 000, that catalyze the hydrolysis of the β-lactam ring of a variety of penam (I) and cephem (II) derivatives. The enzymes are extremely efficient in catalyzing the hydrolysis of their more susceptible substrates showing k_{cat}/K_m values approaching that for a diffusion-limited reaction (e.g., 5×10^7 M^{-1} s^{-1} for the E.coli RTEM β-lactamase with benzylpenicillin). Pollock[4] has considered the evolutionary origins of the β-lactamases, bearing in mind that organisms known to have been dormant since before the clinical introduction of β-lactams have been shown to produce β-lactamase activity. He concluded that they are straightforward detoxification enzymes. β-Lactamases have been purified from a number of sources,[5] most of the detailed work having been done with the enzymes from Staphylococcus aureus, Bacillus cereus, Bacillus licheniformis, and Escherichia coli. Since it is not yet clear to what extent the characteristics of the enzyme from one source are applicable generally, we shall discuss the enzymes from these sources in turn.

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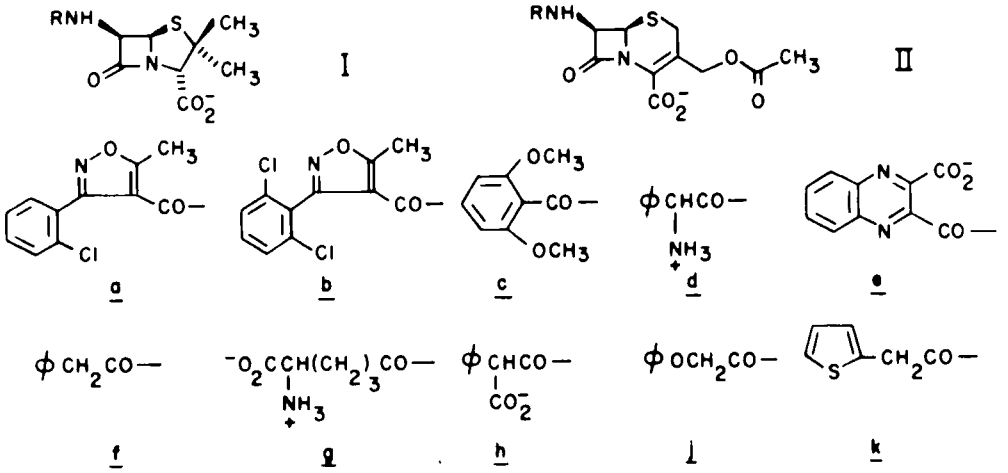
β-Lactamases from Gram-Positive Bacteria

Staphlococcus aureus - The β-lactamase from S.aureus[6] may be inducible though most studies have used a penicillinase-constitutive strain[7] which produces large amounts of the enzyme, 60% of which is extracellular.[8] The sequence of the 257 amino acids of this enzyme is known,[9] and X-ray crystallographic studies have been started.[10] The catalytic activity of this enzyme is largely destroyed by iodine and by tetranitromethane, reaction with the latter leading to preferential nitration of tyrosine-82.[11] On treatment with guanidine hydrochloride, the enzyme is reversibly denatured via a definable partially unfolded state.[12] S.aureus β-lactamase is evidently a rather 'floppy' molecule. The S.aureus enzyme is a better penicillinase than cephalosporinase and the activity towards a number of



6-acylaminopenicillanic acids (I) depends unsurprisingly on the nature of the 6-acyl substituent. It has been suggested that the inhibition of the enzyme by resistant β-lactams such as dicloxacillin (I, R=b), cephaloglycin (II, R=d) and 7-aminocephalosporanic acid (II, R=H) is a function of the preincubation time of enzyme and inhibitor.[13,14]

Two detailed mechanistic studies have been made with the S.aureus β-lactamase. First, Halford (see ref. 10) has studied the hydrolysis of a chromogenic cephalosporin substrate by fast kinetic methods, and has concluded from the absence of a D_2O solvent kinetic isotope effect that the catalytic step (which involves proton release) may also include a rate-limiting enzyme conformational change that is insensitive to solvent isotope. In the second study, Virden, et al.[15] allowed the S.aureus enzyme to hydrolyze the poor substrate quinacillin (I, R=e), and quenched the reaction from the steady state using denaturants. They concluded that 0.3-0.7 moles of quinacillin become covalently linked to the enzyme in the steady-state. Although the kinetic competence of the complex was not established, the authors suggested that this was an acyl-enzyme intermediate in the normal catalytic cycle of this β-lactamase. One other intriguing observation on the S.aureus enzyme should be mentioned. Some preparations

of this β-lactamase have been found to contain what appears to be two moles of non-covalently bound cell wall peptide. While this may be fortuitous, it is tempting to suggest that there may be some steric analogy between this peptide and penicillin, equivalent to the relationship between penicillin and the cell wall peptide originally suggested by Tipper and Strominger[16] and recently discussed by Boyd.[17]

Bacillus cereus - B.cereus produces two β-lactamases[18,19] which are coded for by separate genes and which do not cross-react immunologically. β-lactamase I is rather similar to the enzymes from other Gram-positive organisms, and its specificity is similar to the enzymes from S.aureus and B.licheniformis. β-Lactamase II is, in contrast, a zinc metalloenzyme[20] of 22 000 daltons, which shows a broader specificity including high activity towards cephalosporins. The B.cereus enzyme I has been largely sequenced[21] and it is homologous with the enzymes from B.licheniformis (60% homology) and S.aureus (40% homology). A small-scale purification using an affinity column has been reported.[22] Several attempts have been made to identify catalytically essential amino acids, using both amino acid-selective reagents (e.g., iodine, tetranitromethane, carbodiimide, triethyloxonium fluoroborate, N-bromosuccinimide, diethylpyrocarbonate) and enzyme specific reagents (e.g., the diazotization products of 6-aminopenicillanic acid (I, R=H) or of ampicillin (I, R=d).[23-27] Until the modified proteins from such studies have been properly characterized, only circumstantial statements can be made about the essentiality of particular amino acids.

In the presence of poor substrates that contain large aromatic 6-acyl side chains, B.cereus β-lactamase I becomes more susceptible to inactivation by such diverse treatments as heat, urea, or iodine.[5] This has led to the view that the β-lactamase is a 'floppy' enzyme, and the phenomenon has been studied by hydrogen-exchange.[28] For instance, the poor substrates methicillin (I, R=c) or cloxacillin (I, R=a) accelerate the rate of exchange of the enzyme's amide protons with those of the medium, which suggests that the protein conformation may be altered by the binding of these ligands. This conformational alteration apparently has kinetic consequences, too, and Samuni and Citri[29] have shown that while the hydrolysis rate of the 'natural' substrate benzylpenicillin (I, R=f) is linear from t=0, substrates such as methicillin (I, R=c) or cloxacillin (I, R=a) show biphasic "burst" kinetics. On the basis of this work it has been suggested that the enzyme conformation adjusts to accommodate the more sterically-hindered substrates giving a lower K_m value, and in doing so some catalytic capability is sacrificed by the enzyme.[30] The molecular details of these changes are unknown. Finally, some substrate-analogue competitive inhibitors have been investigated, and the pH-dependence of the steady-state parameters for benzylpenicillin (I, R=f) and for ampicillin (I, R=d) have been reported.[26,31]

Bacillus licheniformis - There are two β-lactamases from B.licheniformis, one of which is extracellular and the other a membrane-bound enzyme.[32] The extracellular enzyme has been sequenced.[33] The membrane-bound form has been shown to be a precursor of the extracellular enzyme and is a

phospholipoprotein having an extra N-terminal peptide of 24 amino acids ending in phosphatidylserine.[34] While trypsin cleaves 25 amino acids from the N-terminus of the membrane enzyme (leaving an enzyme that differs only from the exo-enzyme in that it lacks N-terminal Lys), Aiyappa and Lampen[35] have isolated a 'penicillinase-releasing protease' that removes the 24 N-terminal amino acids. The B.licheniformis enzyme, like many other Gram-positive β-lactamases, has low activity against cephalosporin derivatives. This has been exploited in the use of Sepharose-bound cephalosporin C (II, R=g) as an affinity matrix for the purification of this enzyme.[36]

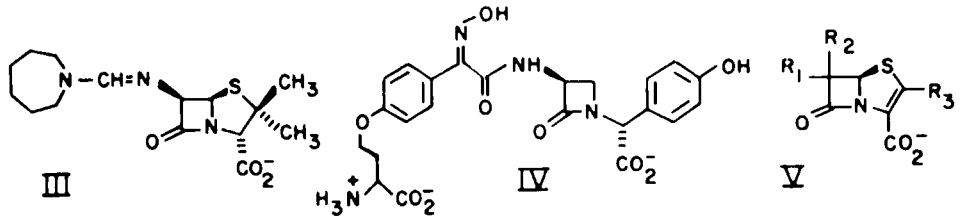
β-Lactamases from Gram-Negative Bacteria

β-Lactamases from Gram-negative organisms are generally constitutive, plasmid-coded, and are usually more effective in the hydrolysis of cephalosporins than are their Gram-positive counterparts.[37,38] Several papers dealing with β-lactamases in the Enterobacter,[39,40] Acinetobacter,[41] and Klebsiella groups[42] have appeared. The Pseudomonas are particularly opportunistic strains that are generally susceptible to carbenicillin (I, R=h), though P.aeruginosa strains containing the RTEM enzyme[43] or penicillinases that efficiently hydrolyze carbenicillin[44,45] are well-known. Labia and co-workers have suggested that the inhibition of a number of Gram-negative cephalosporinases by carbenicillin results from the formation of a rather stable non-covalent complex (K_i = 0.01 μM).[46]

Escherichia coli - Although some chromosomal enzymes are known from E.coli[47] most of the β-lactamases from Gram-negative organisms are encoded by plasmids. The enzyme from E.coli W3310 carrying the plasmid R6K (also called the TEM R-factor) has been purified,[48] and the almost complete amino acid sequence[49] has been favorably compared with the complete sequence of the structural gene from the plasmid pBR322.[50] There is some homology with the enzymes from Gram-positive species. The enzyme has been crystallized, and an X-ray map at 5.5 Å has been published.[51] Of the simple N-acyl penams benzylpenicillin (I, R=f) is the best substrate (relative V_{max} = 1.00; K_m = 0.02 mM) followed by ampicillin (I, R=d) (0.95; 0.02 mM), 6-aminopenicillanic acid (I, R=H) (0.79, 0.19 mM), phenoxymethylpenicillin (I, R=j) (0.50, 0.02 mM) and carbenicillin (I, R=h) (0.15, 0.05 mM). The cephalosporins suffer in terms of both V_{max} and K_m [cephaloglycin (II, R=d) (0.11, 0.46 mM), cephalothin (II, R=k) (0.06, 0.15 mM), and cephalosporin C (II, R=g)(0.014, 0.68 mM)]. In general, the RTEM enzyme is much better behaved kinetically than the B.cereus enzyme. The criterion used by Citri and co-workers[30] to identify kinetically anomalous penicillins (susceptibility relative to 6-aminopenicillanic acid) is not applicable to the RTEM β-lactamase, as all but two of the penams and cephems listed above are hydrolyzed more slowly than 6-aminopenicillanic acid,without any observable deviation from Michaelis-Menten kinetics. Experiments involving the sequential addition of different substrates indicate a greater conformational stability for the RTEM enzyme. Protein modification has been achieved using tetranitromethane, photooxidation, iodoacetic acid, and the C-3 isocyanate of benzylpenicillin.[52-54]

New Classes of β-Lactam and Their Interaction with the RTEM β-Lactamase

The recent discovery of a number of novel β-lactam-containing antibiotics[55] and the development of structures that both inhibit bacterial cell wall synthesis and inhibit β-lactamases, suggest that the interaction of these new structures with β-lactamases should be investigated at the molecular level. Interaction of these compounds with the RTEM β-lactamase is discussed below. The choice of the enzyme is dictated by the following: (a) By virtue of its plasmid origin, this is now the most widely distributed β-lactamase among the enteric Gram-negative bacteria. (b) The plasmid has the disturbing capability for transfer into previously susceptible species (e.g., N.gonorrhoeae and H.influenzae). (c) Infections derived from Gram-negative bacteria are becoming increasingly important clinically.



Mecillinam - The 6β-imidopenicillanic acids, exemplified by mecillinam (III), were the first class of β-lactams recognized to possess antibiotic activity in the absence of an N-acyl side chain.[56] The 6β-imidopenicillins are also unusual in terms of their bacteriocidal mechanism: they are lethal to certain Gram-negative bacteria (particularly E.coli) where the morphological change they elicit is the conversion of the cell rod to an ovoid.[57] Filamentation, which is the normal initial development in the expression of most other β-lactams, is not observed. Mecillinam binds specifically to a single penicillin binding protein (PBP2) of the inner membrane of E.coli,[58] and there is no significant binding to the other PBP species.[59,60] Mecillinam is 500 times more potent than ampicillin (I, R=d) against ampicillin-resistant (i.e., β-lactamase-producing) strains of E.coli, and has been shown to act synergistically with other β-lactam antibiotics.[61-63] Curiously, mecillinam is hydrolyzed very rapidly by β-lactamase, having the highest k_{cat} for any substrate yet discovered for this enzyme. The susceptibility of β-lactamase-producing strains of E.coli to this β-lactam must be dependent on factors other than the β-lactamase, and the killing locus (or loci) must scavenge the antibiotic very effectively before it is destroyed by the enzyme.

Nocardicin A - Nocardicin A (IV) is singular among β-lactam antibiotics in retaining antibiotic activity while having the relatively unreactive monocyclic β-lactam system. An account of its discovery, a summary of its antibiotic properties, and a total synthesis, has recently been presented by Kamiya.[64] This compound is particularly effective in vitro[65,66] and in vivo[67] (subcutaneous injection) against P.aeruginosa, the Proteus (with the exception of P.morganii) and the Neisseria groups, in comparison with carbenicillin (I, R=h). The in vitro inhibition behavior generally underestimates the efficacy of nocardicin A in vivo, and this is believed

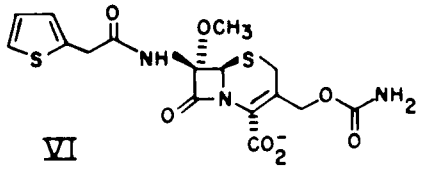
to be a consequence of the ability of nocardicin A to modify the cell surface structure and facilitate recognition and ingestion of the bacteria by polymorphonuclear leukocytes. From the antibacterial potency of a number of nocardicin derivatives it appears that the oxime in a syn relationship to the acylamino group, and the 3-amino-3-carboxypropoxy side chain are both necessary for optimal activity.[68,69]

The β-lactam ring of nocardicin A is rather resistant to β-lactamases, and only the chromosomal cephalosporinase from P.vulgaris and the E.coli RTEM β-lactamase show appreciable activity towards it.[65] As a substrate for the RTEM enzyme, nocardicin A has a V_{max} of 0.02 relative to benzylpenicillin. The product from the enzymatic action has not been fully characterized, although its properties are consistent with a normal hydrolytic cleavage of the β-lactam.

Penems - The total synthesis of a number of penems substituted at C-2 and C-6 (V) has been accomplished by Ernest, et al.[70] The rationale for their synthesis was to incorporate the Δ^3 double bond of the cephalosporins into the penicillin nucleus. These penems exhibit antibacterial activity. The observation that penems fragment in dilute acid to the ketene and the thiazole,[71] rather than suffer the normal β-lactam hydrolysis, suggested them as possible enzyme inactivators. It appears however, that the parent penem (V, $R_1=R_2=R_3=H$) is a normal substrate for the RTEM β-lactamase. This β-lactam is hydrolyzed at a relative V_{max} of 0.36, with a K_m of 310 μM. No loss in β-lactamase activity is detectable, even after 50,000 turnovers. The products from the enzymatic reaction have not yet been identified.

Cefoxitin - The finding that the addition of a 7α-methoxyl substituent enhanced cephalosporin antibacterial activity (whereas 6α-methoxy-penam is less effective than the parent penam), culminated in the synthesis of cefoxitin (VI). This β-lactam possesses an unusually broad activity spectrum, partly due to its exceptional resistance to hydrolysis by the β-lactamases.[72-74] Introduction of the 7α-methoxyl group depresses the rate of enzymatic hydrolysis by a factor of 3×10^4 for cefoxitin (VI) relative to cephalothin (II, R=k). However, the effects of the methoxyl substituent extend beyond a simple reduction in V_{max}, and the characteristics of the hydrolytic pathway are changed. Compare cefoxitin and 7-aminodesacetoxy cephalosporanic acid (7-ADCA), which shows an almost identical k_{cat}. If a portion of enzyme solution catalyzing 7-ADCA hydrolysis is diluted into a solution containing phenoxymethylpenicillin (I, R=j), the new substrate is hydrolyzed with a linear time course, at the predicted rate. If, however, cefoxitin replaces 7-ADCA as the first enzyme substrate, the rate of phenoxymethylpenicillin hydrolysis becomes biphasic, and accelerates over about 15 minutes to a new limiting velocity. No irreversible inactivation of the enzyme is detectable. This kinetic behavior is reminiscent of the observations of Citri and co-workers[30] with the B.cereus β-lactamase, which have been interpreted in terms of a substrate-induced conformational change of the enzyme. An alternative explanation may be offered, however, that is consistent with the kinetic behavior of clavulanic acid (VII, $R_1=CH_2OH$, $R_2=H$). The biphasic kinetics

induced in the hydrolysis of the better substrate (phenoxymethylpenicillin) by preincubation with cefoxitin may derive from the slow decomposition of a transiently-inhibited form of the enzyme (see below).



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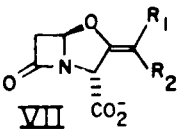

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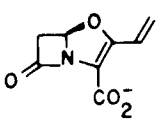


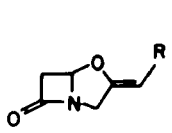
VIII

Clavulanic Acid - This unusual compound (VII, R_1=CH_2OH, R_2=H) was the first natural β-lactam shown to have specific, irreversible β-lactamase inhibitory activity.[75-79] A moderately active antibiotic itself, clavulanate acts synergistically at concentrations as low as 5 μg/mL to lower the MIC for a number of test organisms by as much as 10-fold (e.g., carbenicillin with P.aeruginosa Daglaish) to 10^3-fold (e.g., ampicillin with S.aureus Russel). There are three events that characterize the interaction of clavulanate with the RTEM β-lactamase. First, clavulanate is destroyed by the enzyme in what appears to be a normal hydrolytic reaction of the β-lactam. Secondly, two catalytically inactive forms are produced, one of which is an irreversibly inactivated species, and the other is only transiently inhibited and slowly decomposes to active enzyme. The transient complex is formed from the Michaelis complex at a rate which is some 3-fold faster than the rate of formation of the irreversibly inactivated complex. The transient complex is, therefore, the principle enzyme form present after short time periods. In the presence of excess clavulanate, however, all the enzyme accumulates into the irreversibly inactivated form. The number of clavulanate turnovers that occur before complete enzyme inactivation is 115. If the enzyme is totally irreversibly inactivated by incubation with a large (≥ 300-fold) molar excess of clavulanate, three different species of inactive enzyme are discerned on isoelectric focusing. Incubation of this mixture with hydroxylamine results in the recovery of about one-third of the catalytic activity, and one of the three bands on isoelectric focusing now runs as native enzyme. It seems clear that clavulanic acid is a 'suicide reagent' and that the enzyme generates from it, one or more acylating or alkylating agents that result in enzyme inactivation.[80,81]

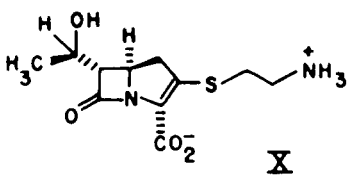
Clavulanic Acid Derivatives - Although only a single naturally-occurring derivative of clavulanate (VII, R_1=CH_2OH, R_2=H) is known (VII, R_1=$CH_2OCOCH_2CH_2OH$, R_2=H),[82] a large number of derivatives have been prepared either by direct chemical functionalization of clavulanate or by total synthesis. The dihydro[83] and desoxy[84] (VII, R_1=CH_3, R_2=H) clavulanates have been prepared, as well as the sulfated[85] (VII, R_1=CH_2OSO_3H, R_2=H) and esterified (VII, R_1=CH_2OCOR, R_2=H) derivatives.[86] The allylic oxygen has been replaced with nitrogen[87] and with sulfur.[88] The double bond of clavulanate has been both photoisomerized[89,90] and cleaved with ozone.[91] Oxidation provides either the conjugated aldehyde or the unstable hept-2-ene (VIII).[92] Additionally, a number of other 7-oxo-4-oxa-1-azabicyclo-[3.2.0]hept-2-ane and hept-2-ene derivatives (e.g., IX) have been

synthesized by the Beecham group.[93-100] Virtually every derivative mentioned is claimed to be a β-lactamase inhibitor and to act synergistically with other β-lactam antibiotics. A quantitative assessment of their efficacy is awaited. A procedure to hydrolyze clavulanate 3-esters to the free carboxylates in 0.1 M base[101] appears to belie earlier estimates of the instability of oxygen analogs of penicillins.[102]

Thienamycin - Of all of the novel β-lactams recently isolated, the carbocyclic β-lactams thienamycin[103] (X) and olivanic acid (XI, R_1=R_2=H) emerge as the β-lactam structures most responsible for rescuing this field from the quiescent state it had lapsed into in the years following the discovery of the cephalosporins. Thienamycin is truly a broad spectrum antibiotic, active against both Gram-negative and Gram-positive bacteria at MIC values lower than carbenicillin, ampicillin or the aminoglycosides. Thienamycin possesses a high affinity for all the PBPs of the E.coli inner membrane, causing ovoid formation at low concentrations and cell lysis at 0.6 μg/mL and above.[78] The 6α-configuration of the R-hydroxyethyl side chain is curious, and the suggestion has been made that the backbone mimics the 7α-methoxyl of the cephamycins while positioning the hydroxyl in the site normally occupied by the 6β-amide.[104] While this interpretation is supported by the lack of bacteriocidal activity in O-methylated thienamycin and in the N-methylated penicillins, it can only be part of the answer. Both the cis and trans hydroxyethyl penicillins and cephalosporins have been synthesized: all are very weak antibiotics, though interestingly, the cis compounds are the more active.[105]



IX



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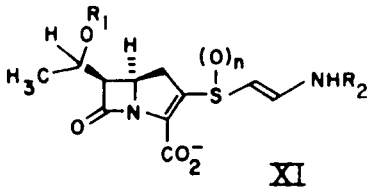

XI

Olivanic Acid - The structures of the β-lactam antibiotics and β-lactamase inhibitors MM13902 (XI, R_1=SO_3H, R_2=$COCH_3$, n=0) and MM4550 (XI, R_1=SO_3H, R_2=$COCH_3$, n=1) also known as MC696-SY2-A) have been simultaneously disclosed by two groups.[106,107] These antibiotics are present in several Streptomyces strains (but isolated from S.olivaceus and S.fuloviridis), and the trivial name olivanic acid has been suggested for the parent compound (XI, R_1=R_2=H, n=0). Their structural similarity to thienamycin is apparent, and a dihydroolivanate derivative (MM17880) has also been isolated.[108] The only structural difference of consequence between the two classes is the stereochemistry at C-6, with olivanic acid possessing the accustomed 6β-configuration. MM4550 and MM13902 are several-fold better than clavulanic acid as antibiotics for most test organisms (but still several-fold poorer than thienamycin). In synergy with ampicillin, MM4550 is equipotent with clavulanate towards K.aerogenes A, while clavulanate is marginally better than MM4550 in ampicillin synergy with S.aureus Russel.[109]

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