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β-Lactamase-Dependent Prodrugs—Recent Developments

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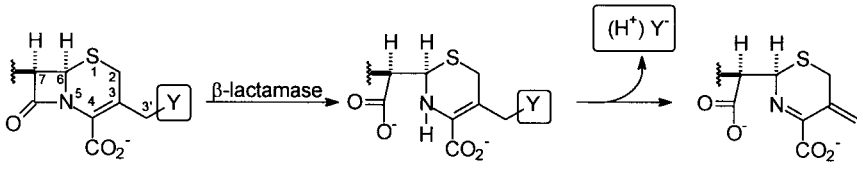
Abstract—Penicillins and cephalosporins bearing an *S*-aminosulfenimine side chain at the 6- and 7-positions, respectively, are prototypic examples of novel classes of β-lactamase-dependent prodrugs wherein enzyme-catalyzed cleavage of the β-lactam ring triggers the rapid expulsion of the *S*-amino moiety. The cephalosporin structure behaves as a dual-release prodrug as here release of both the *S*-amino moiety and of the 3′-substituent occurs sequentially following cleavage of the β-lactam ring. This reaction pattern constitutes an enabling technology at the molecular level and has potential application in antibody-directed enzyme prodrug therapy (ADEPT) and in the further development of β-lactamase-dependent prodrugs for use as antibiotics.

Introduction

β-Lactamase enzymes are highly efficient catalysts for a very specific reaction, viz. the hydrolytic cleavage of the β-lactam ring in penicillins, cephalosporins and some closely related structures. This process renders such materials useless as antibiotics and is a primary defense mechanism of many bacteria, notably those of the gram-negative type.[1] Compounds that behave as substrates of β-lactamase enzymes but which incorporate a hidden, or latent, chemical reactivity which is triggered uniquely by cleavage of the β-lactam ring are of special interest. In particular, structures wherein the triggered reactivity involves release of a well-defined, and variable, part of the original compound have the potential to be used as β-lactamase-dependent prodrugs. Such materials offer the possibility of exploiting the presence of β-lactamase enzymes in a variety of settings: the presence of these enzymes in pathogenic bacteria is a naturally occurring instance, whereas their use in antibody-directed enzyme prodrug therapy (ADEPT)—a key emerging anticancer therapy—is a man-made one.[2]

A number of naturally occurring β-lactams embody an inherent reactivity pattern which is activated by enzyme-catalyzed hydrolysis of the β-lactam ring: this event triggers elimination of the 3′-substituent in cephalosporins[3]—the reverse of 1,4-addition to a conjugated imine (Scheme 1), and cleavage of the non-lactam ring in clavulanic acid;[4] this is likewise an elimination process—the reverse of 1,2 addition to an imine. In the case of naturally occurring cephalosporins no direct biological effect has been associated with elimination of the 3′-acetate substituent although the feature of a releasable element is key to a β-lactamase-dependent prodrug strategy. Man-made elaboration of this intrinsic reactivity pattern has been made with the incorporation of fluoroquinolones as the displaceable 3′-substituent being the most common modification.[5] Carbacephalosporins, carbapenems and penems all contain the required structural motif for an equivalent elimination and these materials have also been the subject of similar modification.[6]

In contrast, penicillin does not possess the same structural motif and consequently this archetypal penam is not endowed with an intrinsic, hidden, reactivity pattern. We recently prepared a penicillin bearing a unique sidechain, an *S*-aminosulfenimine R(R′)NSN=, at the 6-amino position and we established that this modification engendered a reaction pattern not previously encountered in penicillin



Scheme 1.

Keywords: β-lactam; NMR; antibody-directed enzyme prodrug therapy.
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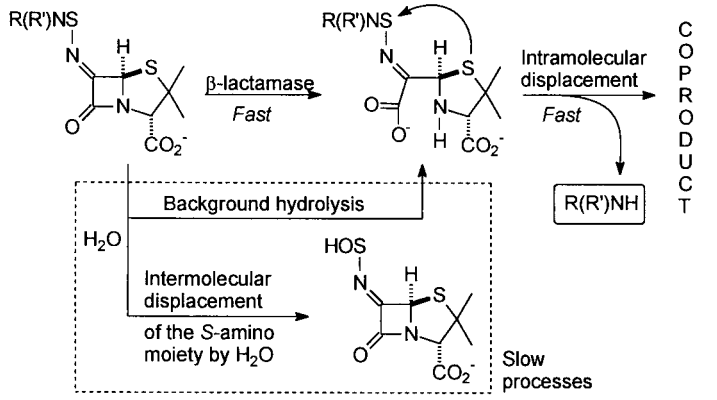
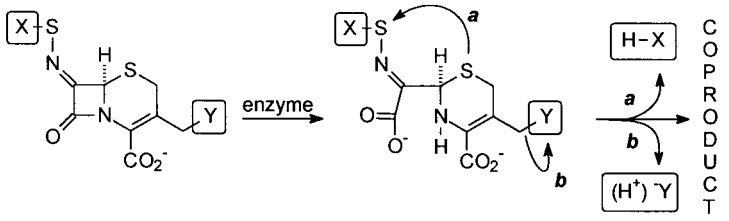


Scheme 2.



Scheme 3.

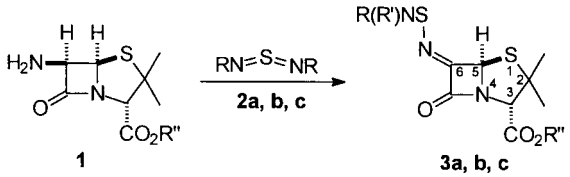
chemistry. Whereas the intact structure was quite stable, cleavage of the β-lactam ring led to the rapid and quantitative release of the sulfur-attached moiety as R(R′)NH; the key to this reactivity pattern is the unleashing of a rapid intramolecular nucleophilic displacement consequent on cleavage of the β-lactam ring[7a] (Scheme 2).† We also demonstrated that a standard cephalosporin which incorporated the same *S*-aminosulfenimine side chain at the 7-position behaved as a dual-release prodrug (Scheme 3, X= R(R′)N, Y=OAc)—here displacement of the sulfur-attached moiety and elimination of the 3′-substituent occurred sequentially following enzyme-catalyzed hydrolysis of the β-lactam ring.[7b]

In this paper the chemistry underlying the novel reaction pattern is summarized and the potential to exploit this is commented on with reference to work carried out by others on cephalosporin–quinolone conjugates in the antibiotic area and cephalosporin–cytotoxic conjugates in the area of ADEPT.

Results and Discussion

Penicillin-based β-lactamase-dependent prodrugs

Reaction of a selected set of sulfur diimides **2a**, **b**, **c** with 6-aminopenicillanic acid ester **1** led directly to the rapid formation of the corresponding *S*-aminosulfenimines **3a**, **b**, **c** (Scheme 4) which were fully characterized.‡ This reaction in itself is novel and had not previously been reported for sulfur diimides—mechanistic details have been given elsewhere.[7a] Variable temperature ^{1}H NMR clearly indicated that the *cis* and *trans* isomers about the exocyclic imino bond were in rapid equilibrium at room temperature in solution. In the case of a related structure—a penicillin bearing a sulfenimine sidechain (RSN=) prepared by Gordon and co-workers—X-ray analysis showed only the *trans* isomer (i.e. with the sidechain sulfur away from the lactam carbonyl) indicating that this was the more stable form.[8] Treatment of **3b** and **3c** with methyl iodide in the presence of Proton Sponge gave the *N*-methylated species **3bm** and **3cm**, respectively. Study of the esters **3a**, **b**, **bm** and **cm** in weakly basic methanol followed by examination of the salt **4bm** in aqueous solution—both in the presence and absence of a β-lactamase enzyme—were the critical steps in revealing the novel, hidden, reactivity pattern of these structures.

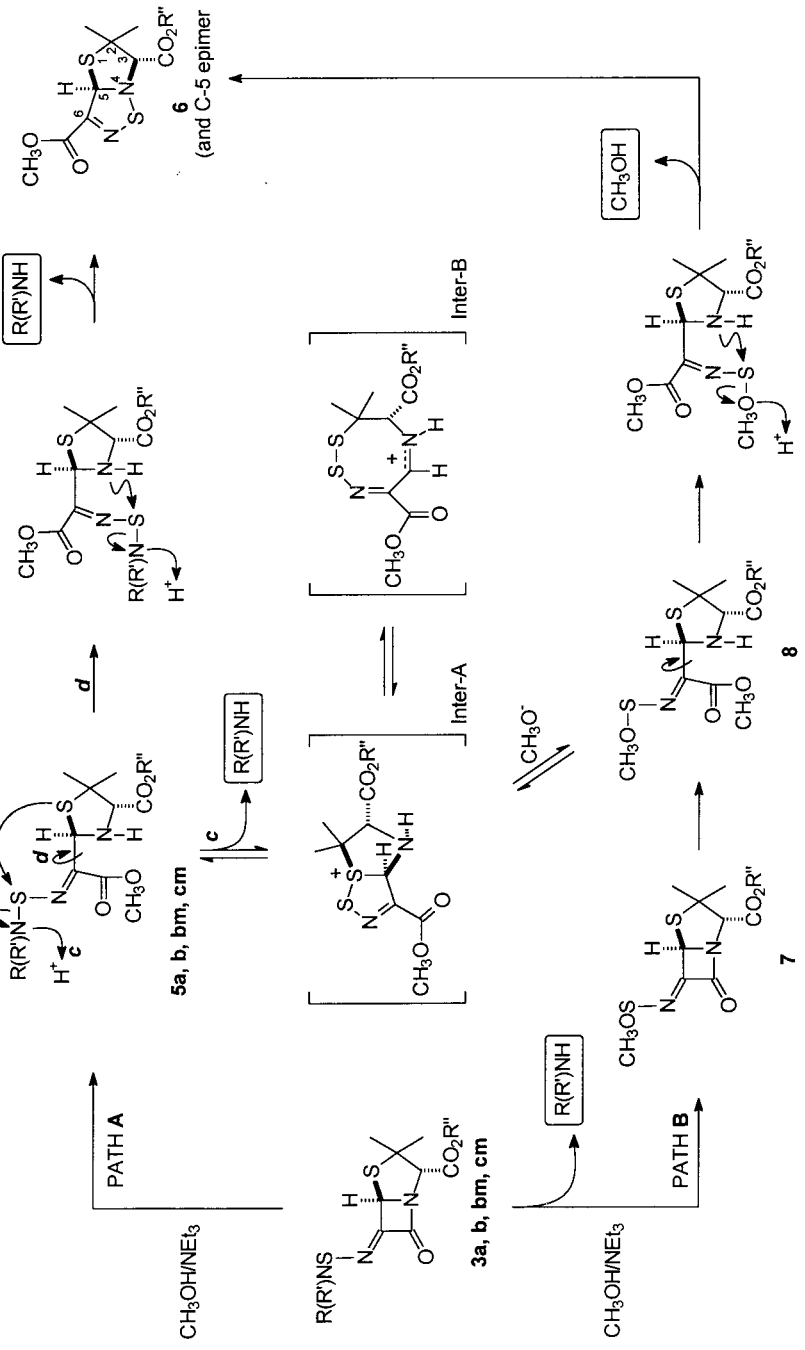


R"		R'	R
$CH(C_6H_5)_2$	Na^+		
3a	**4a**	H	EtOC(O)
3b	**4b**	H	*p*-$CH_3C_6H_4SO_2$
3c	**4c**	H	*p*-$ClC_6H_4SO_2$
3bm	**4bm**	CH_3	*p*-$CH_3C_6H_4SO_2$
3cm	**4cm**	CH_3	*p*-$ClC_6H_4SO_2$

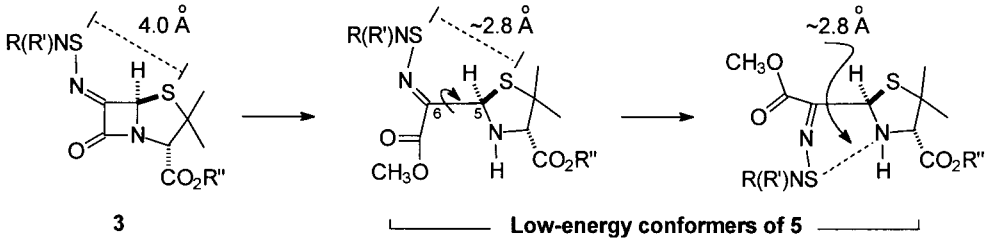
Scheme 4.

† Schemes 2 and 9 are reprinted with permission from Smyth et al.[7b] Copyright 1999 American Chemical Society. Scheme 5 is reprinted with permission from Smyth et al.[7a] Copyright 1998 American Chemical Society.

‡ Full details on the synthesis and characterization of the penicillin and cephalosporin compounds referred to here have been published elsewhere: see Refs. 7a,b.


PATH A
PATH B
CH3OH/NEt3
R(R')NS
3a, b, bm, cm
R(R')NH
5a, b, bm, cm
Inter-A
Inter-B
CH3O-
CH3OH
6
(and C-5 epimer
7
8

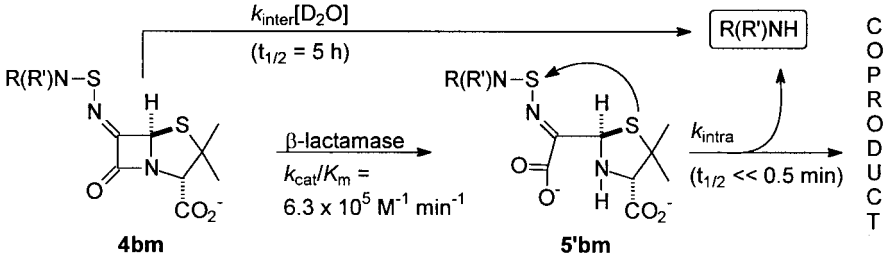
Scheme 5.



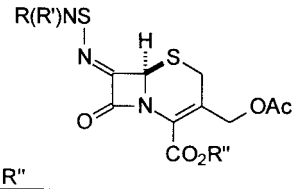
Scheme 6.

The β-lactam-ring-opened product **5a** was readily obtained (Scheme 5) as the unique product on treatment of ester **3a** in methanol containing 35 mM triethylamine; key characterization of the ring-opened structure was by the upfield shift of the α-CH_3 and also of H-3.[9] This ring-opened structure remained unchanged over a period of several hours in $CDCl_3$ at room temperature. In contrast, the ring-opened structure **5b**, obtained from **3b**, was found to react further yielding *p*-toluenesulfonamide, (R(R′)NH), and the bicyclic structure **6** quantitatively as the sole products after 6 h in $CDCl_3$, in the absence of added bases or nucleophiles at room temperature (the bicyclic structure **6** was isolated and characterized).[7a] In the case of the *N*-methylated structure **3bm** cleavage of the β-lactam ring led directly to the formation of **6** and liberation of *N*-methyl-*p*-toluenesulfonamide, i.e. cleavage of the β-lactam ring was the rate determining step. (^{1}H NMR spectroscopy was used to follow reaction progress; one aspect which was very specifically diagnostic for the liberation of *N*-methyl-*p*-toluenesulfonamide was the change in chemical shift of the *N*-methyl group—in **3bm** this occurred as a singlet at 3.25 ppm whereas in *N*-methyl-*p*-toluenesulfonamide this occurred as a doublet at 2.63 ppm.) It was also the case that reaction path **A** (Scheme 5) predominated for **3bm**, i.e. intermolecular displacement of *S*-amino group to form **7** was slower than cleavage of the β-lactam ring, nonetheless, a small amount of **7** was formed. The better *S*-amino leaving group in **3cm** caused a greater degree of intermolecular displacement leading to **7** to occur in competition with cleavage of the β-lactam ring. Compound **7**, thus formed in situ from **3cm**, was observed to react in the MeOH/Et_3N system more slowly than **3cm** itself but led directly to the formation of the bicyclic structure **6**—the ring-opened structure **8** was not detected. *Thus **3bm** and **7** are prototypic penicillin structures wherein cleavage of the β-lactam ring triggers rapid release of the sulfur-attached moiety; the finding that **7** followed this reaction pattern indicates the generality and efficiency of the intramolecular displacement process and that variation of the sulfur-attached group is feasible.*

The change in the internuclear separation of the sidechain and thiazolidine-ring sulfur atoms, on going from the intact to the β-lactam-ring-opened structure, is the controlling feature of the latent reactivity of the above penicillin compounds (Scheme 6). In the intact structure this distance is 4.0 Å,[10] which is marginally outside the sum of the van der Waals radii for two sulfur atoms (3.70 Å), while the fused ring system prevents closer approach of these atoms. This constraint is released on cleavage of the β-lactam ring and, as a result of free rotation about the C5–C6 bond in the β-lactam-ring-opened structure, the sulfur atoms can approach to a distance which is well inside the sum of the van der Waals radii:[11] semi-empirical molecular modeling of the ring-opened structure indicated that an internuclear separation of approximately 2.8 Å occurred for a low-energy—hence, well populated—conformer. The reaction pathway **5bm→Inter-A→8** (Scheme 5) illustrates the operation of an intramolecular displacement process that has a well established counterpart in the chemistry of sulfur mustards.[12] The bicyclic structure **6** was the thermodynamically stable end product isolated and resulted from action of the thiazolidine-ring nitrogen as a nucleophile on the sidechain sulfur group: our semi-empirical modelling study showed that the sidechain sulfur and thiazolidine-ring nitrogen in the β-lactam-ring-opened structure (Scheme 6) could also approach to a distance inside the sum of the van der Waals radii for this atom pair (3.35 Å). The two intramolecular sequences shown in Scheme 5 leading to **6** from the conformers of **5bm** and of **8,** have well-established counterparts.[13] Our finding that **6** was formed as a pair of stereoisomers—one with H5 and H3 *cis* and the other with these hydrogens *trans* to each other—but that exchange of neither of these two hydrogen atoms took place during the reaction, implied that rapid equilibration of **Inter-A** and **Inter-B** must have occurred.[14] This result provided the clearest evidence for the role of the thiazolidine-ring sulfur atom as the primary nucleophile, as the formation of **6** directly from **5bm** via reaction of the thiazolidine-ring nitrogen could not have accounted for the



Scheme 7.



R"			
$CH(C_6H_5)_2$	Na^+	R'	R
10	**11**	H	p-$CH_3C_6H_4SO_2$
10m	**11m**	CH_3	p-$CH_3C_6H_4SO_2$

Scheme 8.

formation of **6** as a pair of epimers, given that exchange of a labile hydrogen did not occur under our reaction conditions.

Enzyme-catalyzed reactivity pattern

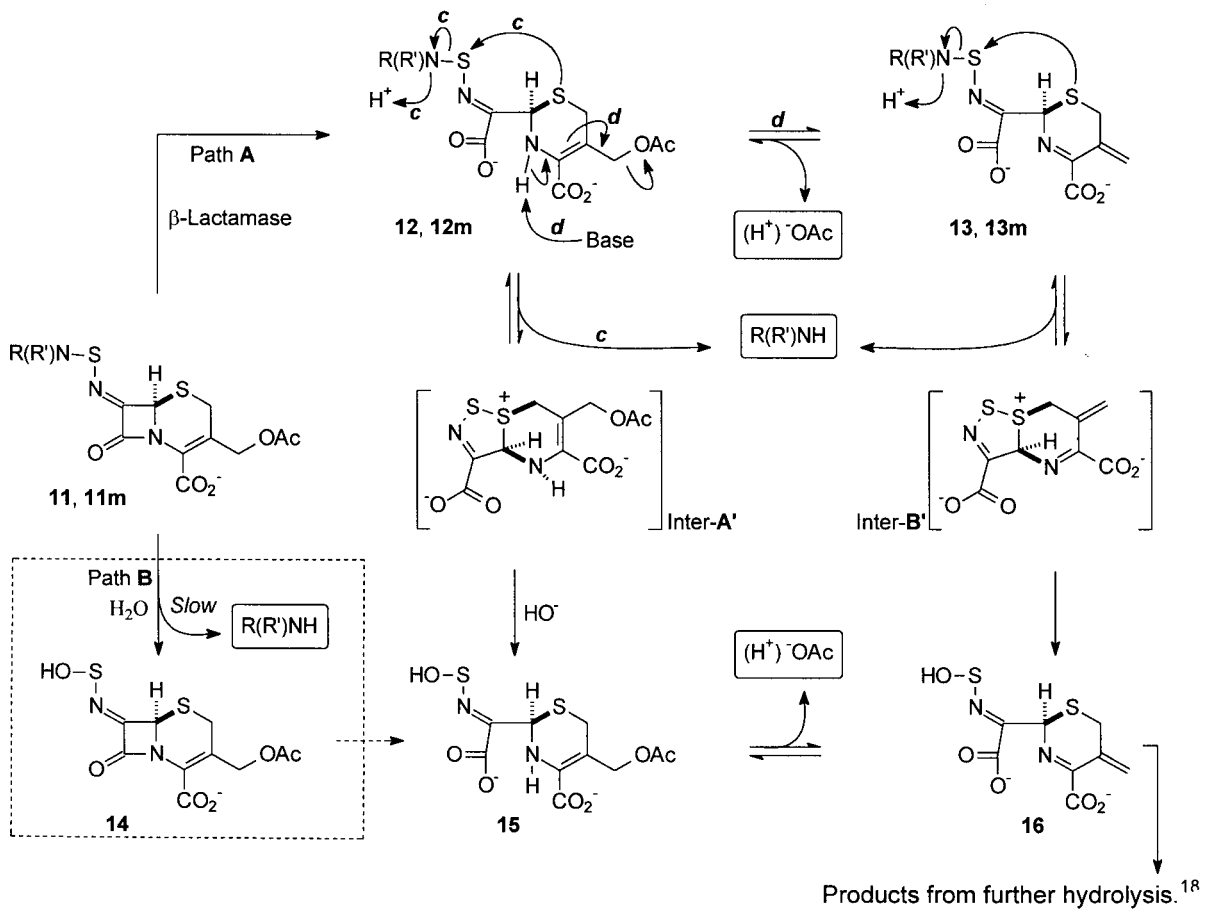
Having established the inherent reactivity pattern of the ester **3bm** in MeOH/Et_3N we next examined the behavior of the salt **4bm** in an aqueous buffer both in the presence and absence of a β-lactamase enzyme; it was established that **4bm** behaved as a modest substrate for β-lactamase type 1 from *B. cereus*—a value of $6.3\times10^5\ M^{-1}\ min^{-1}$ was obtained for k_{cat}/K_m.[15] The key questions to answer were: (i) how stable was **4bm** in an aqueous buffer; and (ii) would enzyme-catalyzed hydrolysis of the β-lactam ring lead to the rapid and quantitative release of the *S*-amino moiety as *N*-methyl-*p*-toluenesulfonamide? The essential findings are summarized in Scheme 2 and are given in more detail in Scheme 7. The hydrolysis reactions were monitored directly in a D_2O buffer (0.1 M phosphate, pD 7.2 at 25°C) using ^1H NMR spectroscopy: the change in chemical shift of the *N*-methyl peak at 3.33 in **4bm** to 2.55 ppm in *N*-methyl-*p*-toluenesulfonamide, in this solvent system, was a key diagnostic feature. In the absence of β-lactamase enzyme the half life for disappearance of **4bm** was 5 h in the above buffer system: *N*-methyl-*p*-toluenesulfonamide was formed as a product of the hydrolysis processes concomitant with loss of **4bm**—the identity of the coproduct was not ascertainable directly from the NMR data in this aqueous buffer system.[16] In a parallel run a small sample of β-lactamase (type I from *B. cereus*) was added to effect immediate and complete hydrolysis of the β-lactam ring and a spectrum was accumulated as rapidly as possible thereafter. The NMR data showed, quite unambiguously, that **4bm** had disappeared fully and that the formation of *N*-methyl-*p*-toluenesulfonamide was complete at the shortest observation time that we achieved which was 5 min after addition of the enzyme. A half-life value of 0.5 min for expulsion of *N*-methyl-*p*-toluenesulfonamide from the β-lactam-ring-opened structure **5′bm** was estimated on the basis that the 5 min reaction time, showing complete release of the *S*-amino moiety, corresponded to at least 10 half-lives—this can only be taken as an upper limit as the NMR technique did not allow a shorter observation time under our reaction conditions in the D_2O buffer. The ratio of the two half-lives is 600 and this gives a measure of the kinetic advantage pertaining to the intramolecular displacement process in aqueous solution[7a] (Scheme 7). *Overall, the above results identified **4bm** as a prototypic penicillin-based β-lactamase-dependent prodrug structure*; the potential to exploit this is elaborated later.

Cephalosporins as dual-release β-lactamase-dependent prodrugs

Reaction of the sulfur diimide **2b** with the diphenylmethyl ester of 7-aminocephalosporonic acid (**9**) yielded the *S*-aminosulfeniminocephalosporin **10** (Scheme 8); treatment of **10** with diazomethane yielded the *N*-methylated structure **10m**.[17‡] Our prime interest in these structures lay in ascertaining if cleavage of the β-lactam ring would trigger the rapid expulsion of both the 3′-acetoxy group and the *S*-amino moiety. Two distinct pathways are shown in Scheme 9[18] which lead to the sequential expulsion of these side-chains: in path ***c*** expulsion of the *S*-amino moiety precedes expulsion of the 3′-acetoxy group whereas in path ***d*** the sequence is reversed. The salts **11** and **11m** were found to be moderate substrates of β-lactamase type I from *B. cereus*: values of 1.15×10^5 and $1.85\times10^5\ M^{-1}\ s^{-1}$, respectively, were obtained for k_{cat}/K_m. The hydrolysis of these salts was then monitored directly using ^1H NMR spectroscopy in the same D_2O buffer as before, in the presence and absence of this enzyme. The release of acetate was readily identified by the change in chemical shift of the methyl group—in acetate ion this occurs at 1.89 ppm which is quite distinct from that in acetate esters (2.11 ppm) such as **11** and **11m**. Likewise, the expulsion of *N*-methyl-*p*-toluenesulfonamide was identifiable by the change in chemical shift of the *N*-methyl group from 3.37 in **11m** to 2.55 ppm in *N*-methyl-*p*-toluenesulfonamide; in the case of **11** the change in chemical shift of the aromatic hydrogens on release of *p*-toluenesulfonamide was quite small but, nonetheless, observable. The half-lives for hydrolysis of **11** and **11m**, in the absence of enzyme were determined to be 5 and 20 h, respectively; the appearance of the arylsulfonamides and of acetate closely paralleled the disappearance of the intact cephalosporins in each case. Addition of the β-lactamase enzyme led to the rapid hydrolysis of the intact structures and to the rapid release of both acetate and the *S*-amino moiety—in each case the rate of release of these groups was considerably faster than the rate of background hydrolysis in the absence of enzyme.[7b] *Thus the S-aminosulfeniminocephalosporin structure is a prototypic β-lactamase-dependent dual-release prodrug. The dual-release feature brings a singular enhancement to the capacity of a cephalosporin as a prodrug nucleus as it should allow co-release of two distinct molecular fragments which can be designed to act synergistically* (Scheme 3).

Application of β-lactamase-dependent prodrugs—antibiotic resistance

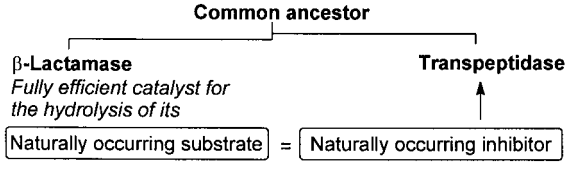
Of the known mechanisms of resistance, that which is β-lactamase-based offers the clearest opportunity of being turned around or exploited as these enzymes are highly efficient catalysts for a very specific reaction, viz. hydrolysis of the β-lactam ring in penicillins and cephalosporins. Derivatives of these materials, therefore, which release a well defined molecular fragment consequent on cleavage of the β-lactam ring, and wherein the released fragment is bactericidal, could make the presence of β-lactamase enzymes a liability for pathogenic bacteria rather than a superior



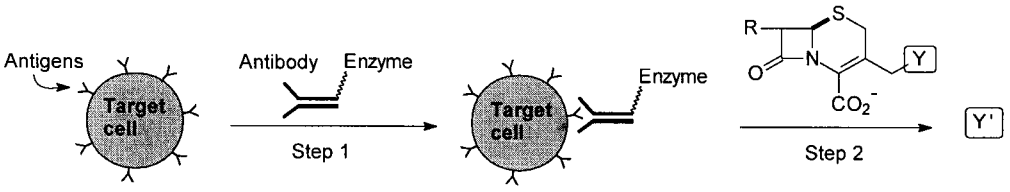
Scheme 9.

defense mechanism. Elaborating such structures to be ideal substrates for a β-lactamase enzyme in the first instance, should result in the intact β-lactam structure also having inhibitor characteristics for a transpeptidase enzyme, at least in some cases, as it is well established that a number of β-lactamases are 'fully efficient' enzymes, which means that they have evolved to be perfect catalysts for their naturally occurring substrates—the naturally occurring inhibitors of transpeptidase enzymes (Scheme 10).[19] Use of such a strategy, in the long term, should redress the balance in favor of non-β-lactamase producers in the general population of pathogenic bacteria.

To date, the inherent, hidden reactivity of cephalosporins and closely related structures such as carbapenems and penems has been explored by a number of pharmaceutical companies. In 1976 O'Callaghan and co-workers reported on the incorporation of a topical biocide, 2-mercaptopyridine-*N*-oxide (omadine), as the 3′-component in a cephalosporin;[20a] although the validity of the approach was demonstrated it was concluded that omadine was inherently too toxic for therapeutic use.[20b] Later, Mobashery and Johnson reported on the incorporation of a halogenated dipeptide at the 3′-position; on β-lactamase-catalyzed release the dipeptide could subsequently be transported and cleaved to generate β-Cl-L-alanine, a potent inhibitor of alanine racemase, which is a critical enzyme in the formation of cell wall components.[21] The incorporation of an established antibiotic, a fluoroquinolone, as the 3′-component was the approach taken subsequently by a number of others: such compounds may be termed β-lactam-quinolone conjugates. In many instances the intact conjugates presented two pharmacophores, viz. that associated with the β-lactam moiety and that of the quinolone moiety. This was generally the case where the essential carboxylic acid group at the 3-position of the quinolone was not blocked, i.e. where this acid group was not part of an ester linkage to the cephalosporin 3′-position. These dual-phamacophore conjugates typically showed broad spectrum-type activity both in vitro[5,6] and in vivo[22] with the activity associated with the quinolone part, frequently, being dominant. Conjugates wherein the quinolone pharmacophore was largely masked—by use of the essential quinolone-carboxylic acid in an ester linkage—strongly supported the β-lactamase-dependent approach when these materials were used on bacterial strains which were known to be over-producers of β-lactamase enzymes.[5a,c] Despite the large



Scheme 10.



Scheme 11.

amount of work which has been carried out with the β-lactam-quinolone conjugates, to date, no such material has been launched as a therapeutic agent. Some pharmacokinetic and pharmacodynamic differences between β-lactams and quinolones may contribute to making their combination as drug-prodrug conjugates unfavorable. In vivo, the bactericidal efficacy of β-lactams correlates with the dosage time span during which the β-lactam concentration is somewhat higher than the MIC, whereas, in the case of quinolones the bactericidal efficacy closely correlates with their concentration.[23] Furthermore, the rate of clearance from the blood stream of a β-lactam-quinolone conjugate was shown to be faster than clearance of the released quinolone;[24] this feature could result in accumulation of the quinolone component to toxic levels[25] during repeated dosage of the conjugate itself. The fact that gram-positive bacteria express β-lactamase enzymes extracellularly whereas gram-negative bacteria express these within the periplasmic space also brings a complicating feature to the use of broad-spectrum β-lactamase-dependent prodrugs.

Notwithstanding the above observations, the strategy of developing β-lactamase-dependent prodrugs for use as antibiotics remains valid. Bacterial strains which are overproducers of β-lactamases, or produce multiple variants of β-lactamases (e.g. serine-based and zinc-dependent metalloenzymes), or produce inhibitor-resistant β-lactamases[1,26] are situations where this strategy should have particular application. The development of a rapid diagnostic methodology must be an integral part of an overall approach to containing resistance, and this should allow appropriate use of specific, narrow-spectrum materials, some of which may include β-lactamase-dependent prodrugs. The dual-release feature of the cephalosporins reported here allows for the possibility of establishing synergy between the released components; in the field of antibiotics there are currently a number of well established examples of the use of synergistic combinations.[27]

Application of β-lactamase-dependent prodrugs—ADEPT

The primary objective of ADEPT[2] (Scheme 11) is to eliminate secondary effects associated with systemic delivery of cytotoxic agents by effecting site-specific release of the cytotoxic entity from a relatively non-toxic prodrug precursor. The strategy depends on the use of an antibody-enzyme conjugate and a substrate of the relevant enzyme as the prodrug entity. β-Lactamase enzymes have been used,[2a] amongst others, as they are not endogenous to humans and the inherent reactivity of cephalosporins—described in the foregoing sections—is highly applicable in that the cytotoxic component can be attached at the 3′-position from where it can be released by enzyme-catalyzed cleavage of the β-lactam ring; it is imperative that its cytotoxicity while attached to the cephalosporin nucleus be considerably less than when released as a free component. In the first step of the process the antibody-enzyme conjugate is administered systemically and allowed to localize on the target cell and, after clearance of any non-complexed conjugate from the body, the cephalosporin prodrug is then administered systemically; release of the cytotoxic component (Y′ in Scheme 11) is catalyzed by the enzyme, which is now localized on the surface of the target cell, and so the concentration of the cytotoxic agent is high, primarily, in the vicinity of the targeted cell. A cephalosporin prodrug which would co-release two distinct molecular fragments at this point (see Scheme 3) should bring a singular enhancement to their use in ADEPT in that the co-released fragments could be chosen to act synergistically on the target cell. There are numerous examples of synergestic interactions in the field of cancer chemotherapy[28] and those that involve use of a second component to disrupt resistance mechanisms—such as efflux pumps and repair enzymes[28e]—are of particular importance. Multidrug resistance (MDR) inhibitors disrupt the action of efflux pumps and can serve to augment the intracellular concentration of a structurally diverse set of anticancer agents,[28] whereas more specific synergy can be realized through use of inhibitors of a repair enzyme in concert with use of alkylating agents for example.[28e,f] The site-specific co-release of the potentiating agent would be advantageous as many of the currently identified active materials are not well suited to systemic delivery.[28g]

The chemistry associated with varying the 3′-substituent in cephalosporins is well established and considerable diversity is tolerated in terms of substrate fitness with respect to specific β-lactamase enzymes, whereas variation in the sulfur-attached group has yet to be explored. The process of directed evolution of enzymes[29] can be applied to produce a β-lactamase enzyme that is tailored to the required substrate[30] and, as one has control over the β-lactamase variant used in ADEPT, this process may be pertinent in optimizing the use of a cephalosporin-based, dual-release, prodrug in this approach to cancer chemotherapy.

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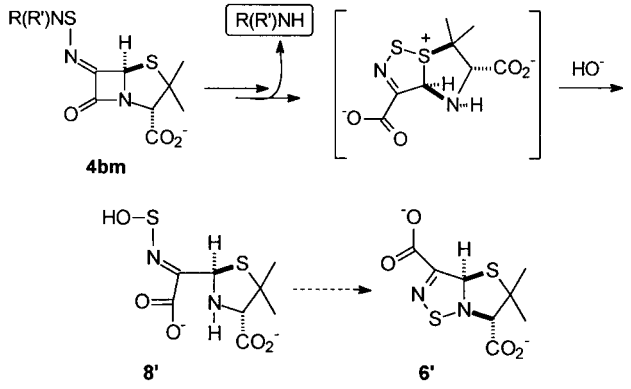

17. The acidity of the side chain NH in **10** is such that methylation occurred readily with CH_2N_2—this process avoided base catalysed Δ^3/Δ^2 isomerisation which was found to occur when using CH_3I with Proton Sponge.

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