SYNTHESIS OF NEW SULFONYLAMIDO-PENICILLANIC ACID SULFONES INHIBITORS OF β-LACTAMASES

SOPHIE VANWETSWINKEL[1], JACQUES FASTREZ[1] and JACQUELINE MARCHAND-BRYNAERT[2,*]

[1] Laboratoire de Biochimie Physique et des Biopolymères,
[2] Laboratoire de Chimie Organique de Synthèse,
Département de chimie, Université catholique de Louvain,
Place L. Pasteur, 1, B1348, Louvain-la-Neuve, Belgium

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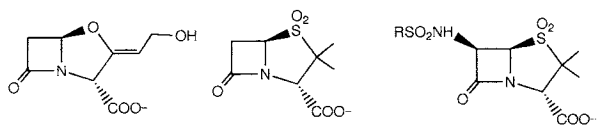
Three new sulfonylamido-penicillanic acid sulfones have been prepared by reaction of 6-aminopenicillanic esters with the monoester or monoamide derivatives obtained in nucleophilic substitution reactions by alcohol or aniline on the carboxyl chloride function of sulfoacetic dichloride followed by oxidation. These penicillin sulfones are converted to β-lactamases suicide inhibitors by removal of the C3 ester protecting group. This synthetic strategy can give access to sulfonamido-penam sulfones bearing a variety of 6-amino side chain. These inhibitors inactivate the RTEM β-lactamase rapidly. The kinetics of inactivation are consistent with the partitioning of an acyl-enzyme intermediate between two main pathways: regeneration of free enzyme and irreversible inactivation, little transient inactivation is observed. A slow inhibition by the product of enzymatic hydrolysis of the sulfones is also observed.

The discovery of clavulanic acid[1] (Scheme 1), a natural suicide inhibitor of β-lactamases, was the starting point for the development of penicillin analogues able to inactivate these enzymes. An intensive research effort, motivated by the importance of the therapeutic stakes, has led to the discovery of many compounds active on β-lactamases isolated from various microorganisms. Several penicillin sulfone derivatives are efficient inhibitors. The best known is penicillanic acid sulfone, often called sulbactam, its mechanism of action has been investigated in great detail by the group of KNOWLES[2,3]. These compounds are suicide inhibitors in that the enzyme catalyzed β-lactam ring opening to form an acyl-enzyme is the triggering event of the inhibition process. The acyl-enzyme can decompose through a normal deacylation pathway or can undergo β-elimination reactions leading reversibly to a more stable amino-acrylate derivative or to an imine that will be subject to an irreversible reaction presumably a transimination with a lysine in the active site. The easy elimination is a consequence of the oxidation of the sulfide function of the thiazoline cycle in the penicillin structure.

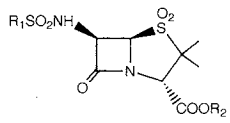
To favor inactivation over deacylation, two strategies have been followed. The deacylation has been slowed down by the introduction of bulky acyl groups on the 6-amino function, this is the case of the penicillin sulfones derived from A type substrates[4]. Alternatively, the acidity of the Hα-6 proton has been increased by introduction of a strongly electron withdrawing group like in the 6-β-sulfonamido-penam

Scheme 1.



Clavulanic acid Sulbactam 6-β-Sulfonamido-penam sulfones

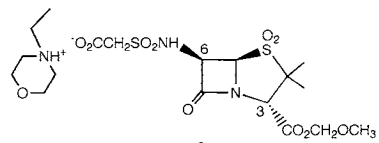
Scheme 2.



1 $R_1 = p\text{-}NO_2PhCH_2OCOCH_2$ $R_2 = MgBr$

2 $R_1 = PhNHCOCH_2$ $R_2 = MgBr$

3 $R_1 = HO_2CCH_2$ $R_2 = H$



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sulfones. These compounds have been investigated by DMITRIENKO *et al.*[5]. (R=CH_3, *p*-CH_3Ph, *p*-CH_3OPh, CF_3) and by KAZIMIERCZAK *et al.*[6] (R=CH_3, Ph, *p*-CH_3Ph). The sulfone of 6-β-trifluoromethane sulfonamidopenicillanic acid (R=CF_3) is an excellent inhibitor of the *B. cereus* 569/H and of the *E. coli* RTEM β-lactamases[5,7~10]. For this sulfone, the irreversibly inhibited form of the enzyme has been shown to be a β-aminoacrylic acyl-enzyme which has been proposed to be stabilized by a conformational change induced in the protein.

It is clear that the variety of the substituents attached to the 6 carbon of sulfonamido-penam sulfones investigated so far remains limited. We are interested in developing a strategy to give access to sulfonamido-penam sulfones bearing various substituents on the side chain. This will allow us to prepare β-lactamases labels to be used in the technique of selection of phage displayed enzymes by catalytic activity[11] or active site directed fluorescent tags. The possibility of side chain diversification will also open the way to the investigation of its interaction with the corresponding part of the enzyme and hopefully to the design of better suicide inhibitors.

In this work, we have devised a synthetic strategy to give access to a variety of sulfonamido-penam sulfones; we have prepared three new β-lactamases inhibitors (Scheme 2) and tested their inhibitory power on the *E. coli* RTEM β-lactamase.

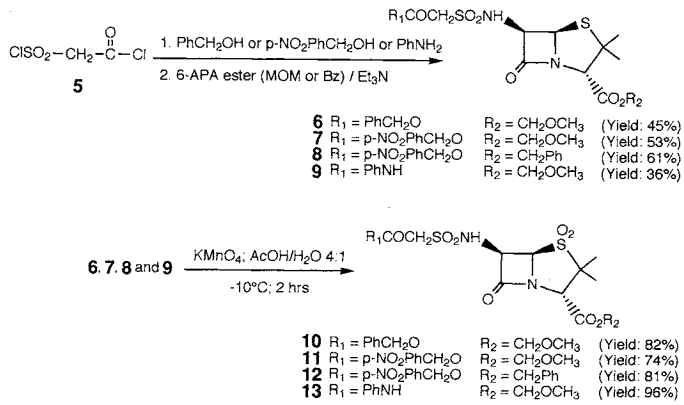
Compound **4** easily obtained in our protocol, bears a free carboxylic acid side chain and has its C3 attached carboxyl function protected as a methoxymethyl (MOM) ester. It can be functionalized at will before a MOM deprotection that is conducted under very mild conditions[12] and affords the advantage of a wide functional compatibility.

Results and Discussion

The key step in our strategy rests on the use of the dichloride of the sulfoacetic synthon; the reactivity difference between the sulfonyl chloride and the carboxyl chloride functions[13,14] can effectively be exploited so as to graft various substituents on the β-amino function of an 6-aminopenicillanic acid ester (6-APA ester) in a straightforward and simple manner.

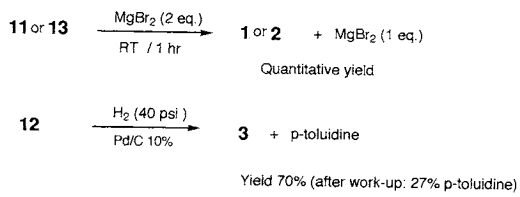
First, the sulfoacetyl dichloride **5** prepared from the corresponding diacid according to the method of HINMAN and LOCATELL[15], is reacted with a nucleophile (aniline, benzyl alcohol or *p*-nitrobenzyl alcohol) to afford the corresponding sulfonyl chloride. In the case of aniline, the presence of about 15% of double addition product is detected while in the two other cases, the selectivity of the attack in favor of the carboxyl chloride is complete. Each sulfonyl chloride is then coupled with a MOM or benzyl 6-APA ester prepared according to MANHAS *et al.*[16]; the resulting products, **6**, **7**, **8** and **9**, are then oxidized into the sulfones **10**, **11**, **12** and **13** with $KMnO_4$[5] (Scheme 3).

Scheme 3.



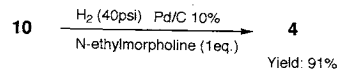
The inhibitors **1** and **2** are accessible from the sulfones **11** and **13** by cleavage of the methoxymethyl ester in the presence of magnesium bromide[12]. Catalytic hydrogenation of sulfone **12** affords a mixture of inhibitor **3** and *p*-toluidine (Scheme 4).

Scheme 4.



Compound **4** is obtained by catalytic hydrogenation of sulfone **10** (Scheme 5). This hydrogenation must be run in the presence of *N*-ethylmorpholine. In the absence of base, besides the expected product **4**, the diacid **3** is obtained as a secondary product. The emergence of the MOM deprotected product appears to be consecutive to the formation of **4** and can be explained by the high acidity of the side chain's carboxylic function (estimated pK = 2.8 ± 0.3) leading to catalysis of the acetal deprotection.

Scheme 5.



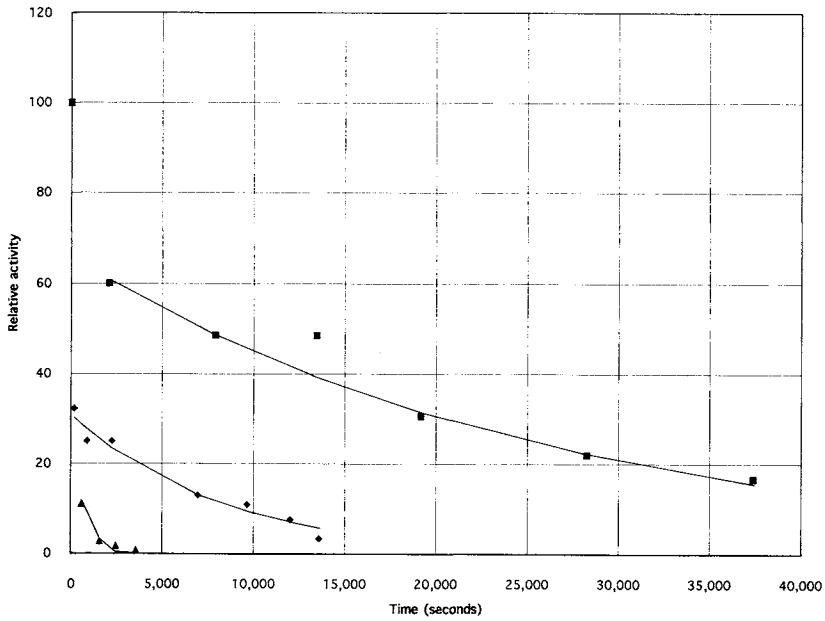
Inactivation of β-lactamase

On incubation of RTEM β-lactamase with the sulfones **1**, **2** and **3**, a time dependent irreversible inhibition is observed. The inhibition curves are biphasic with a fast reaction followed by a rather slow phase. The extent of the inhibition at the end of the fast phase depends on the ratio between the inhibitors and enzyme concentrations ([I]/[E]). The kinetics of the time dependent inactivation do not depend on the substrate (benzylpenicillin or nitrocefin) used to measure the inhibition. Representative curves are presented in Fig. 1).

The kinetics of β-lactamases inhibition by clavulanic[17], sulbactam[2,3] or other penam sulfones[4] have been interpreted by a mechanistic scheme in which the acyl-enzyme (E-Ac) formed in the first step of the interaction can decompose through at least three different pathways: deacylation with regeneration of free enzyme, stabilization to a transiently inhibited enzyme (E-Ac') and irreversible inactivation (Scheme 6).

According to this scheme, part of the inhibitor will be destroyed by the enzyme. Consequently, to lead to a complete irreversible enzyme inactivation, the [I]/[E] ratio should be larger than $(k_3+k_4+k_5)/k_4$[18]. When [I]/[E] is lower, only partial irreversible inactivation should be observed; but then, no slow

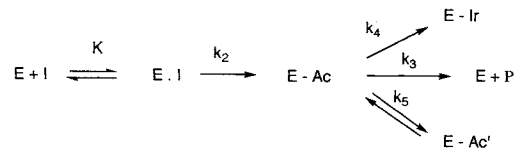
Fig. 1. Irreversible inhibition of RTEM β-lactamase by **3**.



To 95 μl of β-lactamase 2×10^{-7} M in 0.1 M acetate buffer at pH 4.5 are added 5 μl of solutions of **3** in dry DMSO to reach final [I]/[E] ratios of 4,500 (▲), 3,300 (◆) and 960 (■), respectively. 5 μl aliquots of these inhibition solutions are diluted into 300 μl of 1×10^{-3} M benzylpenicillin (▲) or into a 2.0×10^{-5} M nitrocefin solution (▲, ■) in 50 mM phosphate buffer at pH 6.86, and the remaining enzymatic activity is measured by following respectively the rate of disappearance of the substrate at 232 nm or appearance of the product at 486 nm.

phase of inactivation should appear because all the inhibitor has been consumed at the end of the first phase. On the other hand, the initial activity drop should not be sensitive to the [I]/[E] ratio if it is larger than the ratio of rate constants mentioned above; in this case, the biphase nature of the inhibition should reflect the competition between irreversible and transient inhibition and be governed by the k_4/k_5 ratio[18].

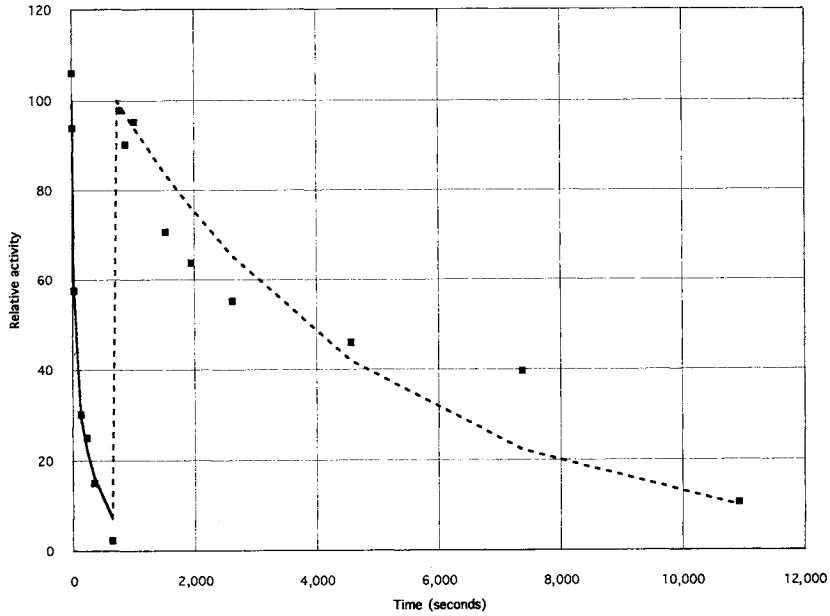
Scheme 6.



Little transient inactivation is observed with the three sulfones described here under our experimental conditions. When the kinetics of inhibition are followed, by determining the remaining activity through measurements of the 232 nm absorbance changes on benzylpenicillin solutions at concentrations 50 times above the Km, an increase in the initial absorbance change, indicative of an activity recovery through decomposition of a transiently inhibited enzyme, is observed only at high [I]/[E] ratio. Then the activity, initially close to zero, increases to only a few percents within about one minute. Similar observations are done on measurements with nitrocefin as substrate at 486 nm.

The slow phase of the inactivation appears to arise essentially from a slow inhibition by products of sulfone hydrolysis. This is shown by an experiment in which β-lactamase is added in two portions to a solution of inhibitor at a concentration about large enough to inactivate the first portion. A fast activity drop is observed immediately after addition of the first portion, on addition of the second only a slow

Fig. 2. Irreversible of RTEM β-lactamase by **3** and its enzymatic degradation products.



To 95μl of β-lactamase 1.6×10^{-7} M in 50 mM acetate buffer at pH 4.5 are added 5μl of solution of **3** in dry DMSO to reach a final [I]/[E] ratio of 4,500. The irreversible inactivation is followed for 640 seconds (——), then fresh β-lactamase is added and the activity is followed as a function of time (------).

inactivation is recorded (Fig. 2). An alternative explanation that the slow inhibition would be due to a trace of a slowly reacting impurity in the inhibitors preparations appears unlikely as the same behavior is observed with different badges of all three inhibitors. At pH 4.5, the enzyme loses its activity very slowly: after incubations of 6 or 24 hours in the presence of 5% of DMSO but in the absence of inhibitor, respectively 94% and 75% of the activity are recovered.

The number of catalytic events before inactivation is estimated by ploting the remaining activity at the end of the fast phase as a function of the [I]/[E] ratio. For measurements done at pH 4.5, the following numbers have been obtained: for inactivations by **1**: 12,000, by **2**: 55,000 and by **3**: 4,500.

The inactivation by **3** has been followed as a function of pH. The rate of inactivation is increasing when the pH is lowered: at an inhibitor concentration of 3×10^{-3} M and an [I]/[E] ratio of 20,000, the activity decays exponentially to zero with half lives of 21, 59 and 200 seconds, respectively at pH 4, 4.5 and 5. This result is consistent with observations suggesting that the presence of a negative charge on the side chain of substrates or inhibitors is not favorable for the interaction with β-lactamase[19]. At pH 5.5 and the same [I]/[E] ratio, the inactivation becomes biphasic; this suggests that the number of catalytic events before inactivation increases with pH. **3** is stable to incubation at pH 4.5 for 48 hours, as seen by the fact that the solution retains its inhibitory capacity.

The properties of the new sulfone derivatives are similar to those described for 6-β-(trifluoromethane sulfonyl)-amido-penicillanic acid sulfone except that no slow inhibition by the products was reported[7~9]. The fact that the transient inhibition pathway is negligible with these inhibitors under our conditions is favorable for the selection of phage displayed enzymes by catalytic activity. The replacement of the phenoxylacetyl amido side chain by a sulfonylamido one transforms a sulfone from a good substrate to

a relatively efficient inhibitor and this is independent of the structure of the side chain. As no increase in the transient inhibition is observed, this may not be due to the increased acidity of the C_6-H, but to the change in the hydrogen bonding interactions of this part of the substrate with residues of the cavity; Ala238 and Asn132[20].

Experimental

Material

RTEM β-lactamase was obtained from Sigma and from Boehringer and used without further purification. The active β-lactamase concentrations in solutions were determined by measuring their activity on dilution in 50 mM phosphate buffer at pH 6.86, using the known specific activity on benzylpenicillin (k_{cat}) of 2000 sec^{-1} as reference[21,22].

Synthesis of the Sulfonylchlorides

p-Nitrobenzyloxycarbonylmethylsulfonylchloride

To a solution of 1.55 g (1 eq.; 8.8 mmol) of chlorosulfonylacetyl chloride in 20 ml of anhydrous benzene are added 1.34 g (1 eq.; 8.8 mmol) of *p*-nitrobenzylic alcohol. The mixture is stirred at room temperature until complete dissolution of the *p*-nitrobenzylic alcohol (~1.5 hours). The solvent is removed *in vacuo* and the product recovered as a brown oily solid (2.58 g; quantitative yield). IR (film, cm^{-1}): 1755; 1387 and 1169; ^{1}H NMR (200 MHz, $CDCl_3$, TMS) δ 4.74 (2H, s, CH_2SO_2Cl); 5.40 (2H, s, *p*-NO_2PhCH_2); 7.57 (2H, d, J=8.7 Hz, H arom. *ortho*); 8.22 (2H, d, J=8.7 Hz, H arom. *meta*); ^{13}C NMR (50 MHz, $CDCl_3$) δ 66.83 and 67.19 (each t, J=144.2 and 150.4 Hz, CH_2); 123.89 (d, J=173.7, C arom. *ortho*); 128.68 (d, J=170.9, C arom. *meta*); 140.88 (s, C–CH_2O); 148.02 (s, C–NO_2); 159.70 (s, CO).

Anilinocarbonylmethylsulfonylchloride

To a chilled (acetone/dry ice bath) solution of 564 mg (1 eq.; 3.19 mmol) of chlorosulfonylacetyl chloride in 5 ml of anhydrous ether is added dropwise and under stirring, a solution of 581 μl (2 eq.; 6.38 mmol) of aniline in 4 ml of anhydrous ether. A white precipitate appears gradually on addition. The mixture is stirred for an additional 30 minutes at low temperature. The reaction is then stopped: the mixture is filtered and the product recovered by removing the ether under reduced pressure. The product is obtained as a pale yellow solid (750 mg); it is in fact a mixture of the expected sulfonylchloride **A** (~85%) and of the double addition compound **B** (~15%) [ratio determined by ^{1}H NMR]. This mixture is used in the following step wothout purification. IR (KBr, cm^{-1}): 1670; 1372 and 1169; ^{1}H NMR (200 MHz, $CDCl_3$, TMS) δ 4.01 (0.3H, s, CH_2 **B**), 4.66 (1.7H, s, CH_2 **A**), 7.04 to 7.47 (6.75H, m, H arom. and SO_2NH), 8.61 (0.15H, wide s, CO*NH* **B**); 8.81 (0.85H, wide s, CO*NH* **A**).

Benzyloxycarbonylmethylsulfonylchloride

To an ice-cold solution of 1.496 g (1 eq.; 8.45 mmol) of chlorosulfonylacetyl chloride in 10 ml of anhydrous benzene is added dropwise a solution of 0.9 ml (1 eq.; 8.45 mmol) of benzylic alcohol in 10 ml of anhydrous benzene. The mixture is stirred at 0°C. After one hour, it is checked that the reaction is complete (IR analysis: the COCl peak disappears while a COO peak appears). The solvent is removed *in vacuo* and the product recovered as a brown oil (2.1 g; quantitative yield) which is stored at −80°C. IR (film, cm^{-1}): 1747; 1386 and 1168; ^{1}H NMR (200 MHz, $CDCl_3$, TMS) δ 4.62 (2H, s, CH_2SO_2Cl); 5.30 (2H, s, CH_2Ph); 7.40 (5H, sharp m, H arom.); ^{13}C NMR (50 MHz, $CDCl_3$) δ 66.41 and 68.48 (each t, J=143.6 and 148.4 Hz, CH_2); 126.43 to 135.55 (m, *C*H and *C* arom.); 159.82 (s, CO).

Coupling of the Sulfonyl Chlorides with the 6-β-Aminopenicillanic Esters

General Procedure

To an ice-cold solution of 6-β-aminopenicillanic acid ester in dry methylene chloride one equivalent of the sulfonyl chloride dissolved in a minimum of methylene chloride is added then, one equivalent of triethylamine. The ice-bath is removed after 5 minutes and the mixture is stirred at room temperature for

an additional 30 minutes. The mixture is washed three times with water, dried over magnesium sulfate, and evaporated to dryness *in vacuo* to yield the crude product as a brown oil. This product is purified by silica gel chromatography (methylene chloride-ethyl acetate).

Methoxymethyl 6-β-[(Benzyloxycarbonyl)methylsulfonamido]penicillinate **6**

The general procedure is followed: quantities: Benzyloxycarbonylmethyl-sulfonylchloride: 1.7 g (1 eq.; 6.8 mmol); 6-APA MOM ester: 1.78 g (1 eq.; 6.8 mmol); Et_3N: 952 μl (1 eq.; 6.8 mmol); methylene chloride: 25 ml. Yield: 1.45 g=45%. The product is a white amorphous solid. IR (KBr, cm^{-1}): 3278; 1786; 1741; 1355 and 1164; ^1H NMR (200 MHz, $CDCl_3$, TMS) δ 1.50 and 1.57 (each 3H, s, 3-CCH_3); 3.47 (3H, s, OCH_3); 4.17~4.41 (2H, AB spectrum, J_{AB}=15.8 Hz, CH_2SO_2); 4.42 (1H, s, 3-H); 5.12 (1H, dd, J_{6-5}=4.6 Hz and $J_{6\text{-}NH}$=10.8 Hz, 6-H); 5.17 (2H, s, CH_2Ph); 5.27 (2H, sharp AB spectrum, CH_2OCH_3); 5.56 (1H, d, J_{5-6}=4.6 Hz, 5-H); 5.93 (1H, d, J_{NH-6}=10.8 Hz, NH); 7.32 (5H, sharp m, H arom.); ^{13}C NMR (50 MHz, APT, $CDCl_3$) δ 26.55 and 32.63 (3-CCH_3); 57.31 (CH_2SO_2); 58.15 (OCH_3); 62.10 (6-C); 64.71 (2-C); 67.37 (5-C); 68.08 (CH_2Ph); 70.12 (3-C); 91.70 (CH_2OCH_3); 128.34, 128.58, 128.61 and 134.34 (C arom.); 163.77, 166.88 and 172.47 (3CO).

Methoxymethyl 6-β-[(*p*-Nitrobenzyloxycarbonyl)methylsulfonamido]penicillinate **7**

The general procedure is followed: quantities: *p*-Nitrobenzyloxy-carbonylmethyl-sulfonylchloride: 569 mg (1 eq.; 1.94 mmol); 6-APA MOM ester: 500 mg (1 eq.; 1.92 mmol); Et_3N: 267 μl (1 eq.; 1.92 mmol); methylene chloride: ~7 ml. Yield: 523 mg=52.7%. The product is a white amorphous solid. IR (KBr, cm^{-1}): 3298; 1785; 1746; 1350 and 1163; ^1H NMR (200 MHz, $CDCl_3$, TMS) δ 1.58 and 1.64 (each 3H, s, 3-CCH_3); 3.54 (3H, s, OCH_3); 4.47 (1H, s, 3-H); 4.29~4.55 (2H, AB spectrum, J_{AB}=15.9 Hz, CH_2SO_2); 5.19 (1H, dd, J_{6-5}=4.6 Hz and $J_{6\text{-}NH}$=11.0 Hz, 6-H); 5.31 to 5.38 (each 2H, sharp AB spectra, CH_2OCH_3 and *p*-NO_2PhCH_2); 5.66 (1H, d, J_{5-6}=4.6 Hz, 5-H); 5.91 (1H, d, $J_{NH\text{-}6}$=11.0 Hz, NH); 7.56 (2H, d, J_{o-m}=8.9 Hz, H arom. *ortho*); 8.27 (2H, d, J_{m-o}=8.9 Hz, H arom. *meta*); ^{13}C NMR (50 MHz, APT, $CDCl_3$) δ 26.61 and 32.71 (3-CCH_3); 57.29 (CH_2SO_2); 58.21 (OCH_3); 62.08 (6-C); 64.86 (2-C); 66.64 (*p*-NO_2PhCH_2); 67.39 (5-C); 70.20 (3-C); 91.71 (CH_2OCH_3); 123.82, 128.54 and 141.59 (C arom.); 163.67, 166.83 and 172.45 (3CO).

Benzyl 6-β-[(*p*-Nitrobenzyloxycarbonyl)methylsulfonamido]penicillinate **8**

The general procedure is followed: quantities: *p*-Nitrobenzyloxy-carbonylmethyl-sulfonylchloride: 375 mg (1 eq.; 1.28 mmol); 6-APA Benzyl ester: 392 mg (1 eq.; 1.28 mmol); Et_3N: 178 μl (1 eq.; 1.28 mmol); methylene chloride: ~5 ml. Yield: 445 mg=61.8%. The product is a white amorphous solid. IR (film, cm^{-1}): 3250; 1785; 1736; 1349 and 1160; ^1H NMR (200 MHz, $CDCl_3$, TMS) δ 1.45 and 1.60 (each 3H, s, 3-CCH_3); 4.78 (1H, s, 3-H); 4.27~4.55 (2H, AB spectrum, J_{AB}=15.9 Hz, CH_2SO_2); 5.17 (1H, dd, J_{6-5}=4.6 Hz and J_{6-NH}=10.9 Hz, 6-H); 5.22 (2H, s, CH_2Ph); 5.33 (2H, s, *p*-NO_2PhCH_2); 5.65 (1H, d, J_{5-6}=4.6 Hz, 5-H); 5.89 (1H, d, J_{NH-6}=10.9 Hz, NH); 7.40 (5H, sharp m, Ph); 7.56 (2H, d, J_{o-m}=8.7 Hz, H arom. *ortho*); 8.26 (2H, d, J_{m-o}=8.7 Hz, H arom. *meta*); ^{13}C NMR (50 MHz, APT, $CDCl_3$) δ 26.57 and 32.85 (3-CCH_3); 57.29 (CH_2SO_2); 62.09 (6-C); 65.05 (2-C); 66.28 (*p*-NO_2PhCH_2); 67.37 (5-C); 67.56 (CH_2Ph); 70.15 (3-C); 123.81 to 128.77, 134.48, 141.58 and 147.95 (C arom.); 163.67, 167.02 and 172.38 (3CO).

Methoxymethyl 6-β-[(Anilinocarbonyl)methylsulfonamido]penicillinate **9**

The general procedure is followed: quantities: Anilinocarbonylmethyl-sulfonylchloride (this product contains 15% of double addition product; see above): 494 mg; 6-APA MOM ester: 550 mg (1 eq.; 2.12 mmol); Et_3N: 295 μl (1 eq.; 2.12 mmol); methylene chloride: ~7 ml. Yield: 349 mg=41.6% (Yield corrected for the presence of the double addition product in the sulfonyl chloride). The product is a white amorphous solid. IR (KBr, cm^{-1}): 3250; 1785; 1745; 1690; 1350 and 1160; ^1H NMR (200 MHz, $CDCl_3$, TMS) δ 1.51 and 1.57 (each 3H, s, 3-CCH_3); 3.59 (3H, s, OCH_3); 4.2~4.33 (2H, AB spectrum, J_{AB}=14.4 Hz, CH_2SO_2); 4.47 (1H, s, 3-H); 5.17 (1H, dd, J_{6-5}=4.5 Hz and J_{6-NH}=10.5 Hz, 6-H); 5.24 to 5.34 (2H, sharp AB spectrum, CH_2OCH_3); 5.62 (1H, d, J_{5-6}=4.5 Hz, 5-H); 6.00 (1H, d, J_{NH-6}=10.5 Hz, SO_2NH); 7.07 to 7.54 (5H, m, H arom.); 8.33 (1H, wide s, CO*NH*); ^{13}C NMR (50 MHz, APT, $CDCl_3$) δ 26.58 and 32.47 (3-CCH_3); 58.22 (OCH_3); 60.37 (CH_2SO_2); 62.05 (6-C); 64.73 (2-C); 67.69 (5-C); 70.28 (3-C); 91.67

(CH_2OCH_3); 120.20, 125.08, 128.96 and 137.13 (C arom.); 164.14, 167.04 and 173.28 (3CO).

Synthesis of the Sulfones

General Procedure

A solution of the "coupling product" in 80% aqueous acetic acid is cooled in an ice-salt bath. To this solution, a solution of potassium permanganate (2.1 to 2.5 equivalents) in water is added gradually over a period of one hour. After one hour of stirring at low temperature, the mixture is decolorized by dropwise addition of 10% aqueous hydrogen peroxide. The mixture is extracted with methylene chloride; the organic layers are combined, washed twice with water and once with $NaHCO_3$ 5%, dried over magnesium sulfate and evaporated to dryness *in vacuo* to yield the sulfone which can be purified by silica gel chromatography (methylene chloride/ethyl acetate).

Methoxymethyl 6-β-[(Benzyloxycarbonyl)methylsulfonamido]penicillinate Sulfone **10**

The general procedure is followed: quantities: Methoxymethyl 6-β-[(benzyloxycarbonyl)methylsulfonamido]-penicillinate **6**: 440 mg (1 eq.; 0.85 mmol); $KMnO_4$: 283 mg (2.1 eq.; 1.79 mmol); acetic acid - water (4 : 1): 25 ml. Yield: 390 mg = 91% (352 mg = 82% after purification). The product is a white amorphous solid. Anal. Calcd. for $C_{19}H_{24}N_2O_{10}S_2$: C 45.23, H 4.79, N 5.55. Found: C 45.17, H 5.05, N 5.42. IR (KBr, cm^{-1}): 3300; 1810; 1742; 1359; 1324; 1162 and 1117; ^1H NMR (500 MHz, $CDCl_3$) δ 1.44 and 1.63 (each 3H, s, 3-CCH_3); 3.53 (3H, s, OCH_3); 4.21 ~ 4.24 (2H, AB spectrum, J_{AB} = 15.9 Hz, CH_2SO_2); 4.48 (1H, s, 3-H); 4.81 (1H, d, J_{5-6} = 4.6 Hz, 5-H); 5.22 ~ 5.26 (2H, AB spectrum, J_{AB} = 12.1 Hz) and 5.27 ~ 5.43 (2H, AX spectrum, J_{AX} = 5.9 Hz): PhCH_2 and CH_2OCH_3; 5.46 (1H, dd, J_{6-5} = 4.6 Hz and J_{6-NH} = 10.7 Hz, 6-H); 6.51 (1H, d, J_{NH-6} = 10.7 Hz, NH); 7.36 to 7.39 (5H, sharp m, H arom.); ^{13}C NMR (125 MHz, DEPT, $CDCl_3$) δ 17.79 and 20.12 (3-CCH_3); 57.78 (CH_2SO_2); 58.52 (OCH_3); 61.55 (6-C); 63.84 (3-C); 64.63 (2-C); 65.92 (5-C); 68.43 (CH_2Ph); 92.38 (CH_2OCH_3); 128.70 to 128.89 and 134.30 (C arom.); 163.55, 166.15 and 172.98 (3CO); Mass (FAB-MNBA): $[MH]^+$ = 505; $[MH]^-$ = 503; $[M-(CH_2OCH_3)]^-$ = 459; $[M-(CH_2OCH_3)-(CH_2Ph)]^-$ = 369.

Methoxymethyl 6-β-[(*p*-Nitrobenzyloxycarbonyl)methylsulfonamido]penicillinate Sulfone **11**

The general procedure is followed: quantities: Methoxymethyl 6-β-[(*p*-Nitrobenzyloxycarbonyl)-methylsulfonamido]penicillinate **7**: 331 mg (1 eq.; 0.64 mmol); $KMnO_4$: 214 mg (2.1 eq.; 1.35 mmol); acetic acid - water (4 : 1): 21 ml. Yield: 261 mg = 75% (198 mg = 57% after purification). The product is a white amorphous solid. Anal. Calcd. for $C_{19}H_{23}N_3O_{12}S_2$: C 41.53, H 4.22, N 7.65. Found: C 41.67, H 4.32, N 7.47. IR (KBr, cm^{-1}): 3306; 1807; 1742; 1351; 1322; 1161 and 1114; ^1H NMR (500 MHz, $CDCl_3$) δ 1.44 and 1.62 (each 3H, s, 3-CCH_3); 3.52 (3H, s, OCH_3); 4.25 ~ 4.31 (2H, AB spectrum, J_{AB} = 15.9 Hz, CH_2SO_2); 4.47 (1H, s, 3-H); 4.88 (1H, d, J_{5-6} = 4.5 Hz, 5-H); 5.27 ~ 5.44 (2H, AX spectrum, J_{AX} = 5.8 Hz) and 5.31 to 5.36 (2H, sharp AB spectrum): *p*-NO_2PhCH_2 and CH_2OCH_3; 5.49 (1H, wide d, J_{6-5} = 4.5 Hz, 6-H); 6.58 (1H, wide s, NH); 7.54 (2H, d, J_{o-m} = 8.4 Hz, H arom. *ortho*); 8.24 (2H, d, J_{m-o} = 8.4 Hz, H arom. *meta*); ^{13}C NMR (125 MHz, APT, $CDCl_3$) δ 17.79 and 20.12 (3-CCH_3); 57.58 (CH_2SO_2); 58.58 (OCH_3); 61.38 (6-C); 63.88 (3-C); 64.71 (2-C); 65.80 (5-C); 66.68 (*p*-NO_2PhCH_2); 92.47 (CH_2OCH_3); 123.94, 128.89 and 141.37 (C arom.); 147.95, 163.34, 166.07 and 172.93 (4CO); Mass (FAB-MNBA): $[MH]^-$ = 548; [M-(*p*-NO_2PhCH_2O)]$^-$ = 396; $[M-(p\text{-}NO_2PhCH_2O)-(CH_2OCH_3)]^-$ = 351.

Benzyl 6-β-[(*p*-Nitrobenzyloxycarbonyl)methylsulfonamido]penicillinate Sulfone **12**

The general procedure is followed: quantities: Benzyl 6-β-[(*p*-Nitrobenzyloxycarbonyl)methylsulfonamido]penicillinate **8**: 230 mg (1 eq.; 0.41 mmol); $KMnO_4$: 147 mg (2.3 eq.; 0.93 mmol); acetic acid - water (4 : 1): 15 ml. Yield: 198 mg = 81% (after purification). The product is a white amorphous solid. Anal. Calcd. for $C_{24}H_{25}N_3O_{11}S_2$: C 48.40, H 4.23, N 7.06. Found: C 48.25, H 4.03, N 6.77. IR (KBr, cm^{-1}): 3298; 1807; 1746; 1351; 1322; 1170 and 1116; ^1H NMR (500 MHz, $CDCl_3$) δ 1.23 and 1.51 (each 3H, s, 3-CCH_3); 4.25 ~ 4.31 (2H, AB spectrum, J_{AB} = 15.9 Hz, CH_2SO_2); 4.46 (1H, s, 3-H); 4.85 (1H, d, J_{5-6} = 4.7 Hz, 5-H); 5.16 ~ 5.28 (2H, AB spectrum, J_{AB} = 11.9 Hz) and 5.31 ~ 5.33 (2H, AB spectrum, J_{AB} = 13.2 Hz): *p*-NO_2PhCH_2 and CH_2Ph; 5.49 (1H, dd, J_{6-5} = 4.7 Hz and J_{6-NH} = 11.2 Hz, 6-H); 6.61 (1H, d, J_{NH-6} = 11.2 Hz, NH); 7.34 to 7.39 (5H, sharp m, Ph); 7.53 (2H, d, J_{o-m} = 8.8 Hz, H arom. *ortho*); 8.21

(2H, d, J_{m-o}=8.8 Hz, H arom. *meta*); ^{13}C NMR (125 MHz, APT, $CDCl_3$) δ 17.71 and 19.88 (3-CCH_3); 57.48 (CH_2SO_2); 61.22 (6-C); 63.78 (3-C); 64.79 (2-C); 65.77 (5-C); 66.58 and 68.39 (*p*-NO_2PhCH_2 and CH_2Ph); 123.86 to 129.13 (C arom.); 134.12, 141.43 and 147.96 (C arom.); 163.32, 166.23 and 172.98 (3CO); Mass (FAB-MNBA): $[MH]^+$=596; $[M-(p\text{-}NO_2PhCH_2CO_2CH_2SO_2)]^+$=338; $[MH]^-$=594.

Methoxymethyl 6-β-[(Anilinocarbonyl)methylsulfonamido]penicillinate Sulfone **13**

The general procedure is followed: quantities: Methoxymethyl 6-β-[(anilinocarbonyl)methylsulfonamido]penicillinate **9**: 445 mg (1 eq.; 0.97 mmol); $KMnO_4$: 385 mg (2.5 eq.; 2.44 mmol); acetic acid-water (4:1): 30 ml. Yield: 455 mg=95.6%. The product is a white amorphous solid. Anal. Calcd. for $C_{18}H_{23}N_3O_9S_2$: C 44.17, H 4.74, N 8.58. Found: C 43.72, H 4.88, N 8.05. IR (KBr, cm^{-1}): 3300; 1805; 1750; 1690; 1540; 1355; 1330; 1165 and 1115; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.40 and 1.58 (each 3H, s, 3-CCH_3); 3.49 (3H, s, OCH_3); 4.29~4.31 (2H, AB spectrum, J_{AB}=14.9 Hz, CH_2SO_2); 4.52 (1H, s, 3-H); 4.96 (1H, d, J_{5-6}=4.2 Hz, 5-H); 5.24~5.39 (2H, AX spectrum, J_{AX}=5.8 Hz, CH_2OCH_3); 5.53 (1H, dd, J_{6-5}=4.2 Hz and J_{6-NH}=10.7 Hz, 6-H); 6.82 (1H, d, J_{NH-6}=10.7 Hz, SO_2NH); 7.05 to 7.54 (5H, m, H arom.); 8.67 (1H, wide s, CO*NH*); ^{13}C NMR (125 MHz, DM1 and DEPT, $CDCl_3$) δ 17.62 and 19.92 (3-CCH_3); 58.38 (OCH_3); 60.34 (CH_2SO_2); 61.06 (6-C); 63.68 (3-C); 64.68 (2-C); 66.21 (5-C); 92.30 (CH_2OCH_3); 120.48, 125.05, 128.91 and 137.10 (C arom.); 160.15, 166.33 and 173.71 (3CO); Mass (FAB-MNBA): $[MH]^+$=490; $[M+Na]^+$=512; $[MH]^-$=488; [dimer-H]$^-$=977.

Synthesis of the 6-β-Sulfonamidopenicillanic Acid Sulfones

Magnesium Bromide of 6-β-[(*p*-Nitrobenzyloxycarbonyl)methylsulfonamido]penicillinate Sulfone **1**

61 mg (1 eq.; 0.11 mmol) of the sulfone **11** is dissolved in a minimum of dry methylene chloride. To this solution are added 57 mg (2 eq.; 0.22 mmol) of magnesium bromide etherate; the mixture is stirred at room temperature and the reaction is monitored by TLC. After ~40 minutes, the starting material has completely disappeared. The reaction is stopped and the product is quantitatively recovered by solvent removal *in vacuo*. The product is a white solid which contains 1 eq. of magnesium bromide. IR (KBr, cm^{-1}): 3410; 1805; 1745; 1640; 1355; 1325; 1170 and 1120; ^{1}H NMR (500 MHz, D_2O) δ 1.43 and 1.58 (each 3H, s, 3-CCH_3); 4.34 (1H, s, 3-H); 5.14 (1H, d, J_{5-6}=4.2 Hz, 5-H); 5.45 (2H, s, *p*-NO_2PhCH_2); 5.69 (1H, d, J_{6-5}=4.2 Hz, 6-H); 7.70 (2H, d, J_{o-m}=8.6 Hz, H arom. *ortho*); 8.32 (2H, d, J_{m-o}=8.6 Hz, H arom. *meta*); the SO_2CH_2 signal is masked under HDO peak; Mass (FAB-MNBA): $[MH]^+$=608 and 610; $[MH]^-$=607 and 609.

Magnesium Bromide 6-β-[(Anilinocarbonyl)methylsulfonamido]penicillinate Sulfone **2**

19 mg (1 eq.; 0.04 mmol) of the sulfone **13** are dissolved in a minimum of dry methylene chloride. To this solution are added 20 mg (2 eq.; 0.08 mmol) of magnesium bromide etherate; the reaction is run as for the synthesis of **1**. The product is a white solid which contain 1 eq. of magnesium bromide. IR (KBr, cm^{-1}): 3350; 1805; 1680; 1635; 1355; 1325; 1155 and 1115; ^{1}H NMR (500 MHz, D_2O) δ 1.45 and 1.61 (each 3H, s, 3-CCH_3); 4.41 (2H, sharp AB spectrum, SO_2CH_2); 4.43 (1H, s, 3-H); 5.23 (1H, d, J_{5-6}= 4.3 Hz, 5-H); 5.72 (1H, d, J_{6-5}=4.3 Hz, 6-H); 7.30 to 7.58 (5H, m, H arom.); Mass (FAB-Glycerol): $[M-(MgBr)]^-$=444; $[M-(MgBr)-(COO^-)]$=400.

6-β-[(Carboxy)methylsulfonamido]penicillanic Acid Sulfone **3**

Hydrogenolysis of 50 mg of the sulfone **12** in a mixture of 1.25 ml of dry ethyl acetate and 5 ml of dry ethanol on a Parr hydrogenator at 40 psi over 10% palladium on charcoal (50 mg) for 1 hour, followed by filtration to remove the catalyst and solvent removal *in vacuo* gave the expected product containing 42% of *p*-toluidine. Dissolution of this product in water containing KOH; washing with methylene chloride and lyophilization gave 31 mg of a white solid; ^{1}H NMR showed that the product is still contaminated by 27% of *p*-toluidine. IR (KBr, cm^{-1}): 3306; 1808; 1739; 1697; 1355; 1325; 1164 and 1115; ^{1}H NMR (200 MHz, D_2O) δ 1.24 and 1.38 (each 2.19H, s, 3-CCH_3); 2.17 (0.81H, s, CH_3 *p*-toluidine); 3.98 to 4.12 (1.46H, sharp AB spectrum, SO_2CH_2); 4.19 (0.73H, s, 3-H); 5.00 (0.73H, d, J_{5-6}=4.4 Hz, 5-H); 5.48 (0.73H, d, J_{6-5}=4.4 Hz, 6-H); 7.05 to 7.21 (1.08H, m, H arom. *p*-toluidine); Mass (FAB-Glycerol): $[MH]^-$=369; $[M-CO_2H]$=325; $[M-2CO_2H]$=281.

Methoxymethyl 6-β-[(*N*-Ethylmorpholiniumcarboxylate)methylsulfonamido]penicillinate Sulfone **4**

Hydrogenolysis of 100 mg (1 eq.; 0.2 mmol) of the sulfone **10** in a mixture of 2 ml of dry ethyl acetate, 9 ml of dry ethanol and 25.2 μl (1 eq.; 0.2 mmol) of *N*-ethylmorpholine on a Parr hydrogenator at 40 psi over 10% palladium on charcoal (90 mg) for 1 hour, followed by filtration to remove the catalyst and removal of solvent *in vacuo* gave 96 mg (91%) of the compound **4** as a white amorphous solid. IR (KBr, cm^{-1}): 3432; 1806; 1757; 1626; 1385; 1324; 1161 and 1114; ^1H NMR (200 MHz, CD_3OCD_3, TMS): δ 1.29 (3H, t, $J_{CH_3-CH_2}=8.2$ Hz, CH_2CH_3); 1.47 and 1.61 (each 3H, s, 3-CCH_3); 3.07 (2H, q, $J_{CH_2-CH_3}=8.2$ Hz, CH_2CH_3); 3.13 (4H, m, NCH_2CH_2); 3.50 (3H, s, OCH_3); 3.85 to 3.98 (4H, m, OCH_2CH_2); 3.97~4.07 (2H, AB spectrum, $J_{AB}=16.4$ Hz, SO_2CH_2); 4.51 (1H, s, 3-H); 5.34 (1H, d, $J_{5-6}=4.7$ Hz, 5-H); 5.33~5.43 (2H, AB spectrum, $J_{AB}=6.1$ Hz, CH_2OCH_3); 5.65 (1H, d, $J_{6-5}=4.7$ Hz, 6-H); 7.70 to 8.20 (2H, wide s, 2NH); Mass (FAB-MNBA): $[MH]^+=530$; $[M-(N\text{-ethylmorpholine})]^+=415$; [*N*-ethylmorpholine]$^+$ = 116; $[M-(N\text{-ethylmorpholine}]^-=413$; [dimer of $(M-(N\text{-ethylmorpholine}))+H]^-=827$; $[M-(N\text{-ethylmorpholine})-(CH_2OCH_3)]^-=368$.

Methods

Time Dependence of β-lactamase Inactivation by the Sulfones

Samples (5 μl) from incubation mixtures of the enzyme (4×10^{-8} to 2×10^{-7} M) with a sulfone in buffer containing 5% DMSO (used to solubilize the sulfone) were mixed with 300 μl of 1 mM benzylpenicillin (Km = 20 μM[21]) or 20 μM nitrocefin (Km = 220 μM[21]). Most of the experiments were done in 50 mM acetate buffer at pH 4.5 (except when otherwise noted). The absorbance of these solutions was followed respectively at 232 nm ($\Delta\varepsilon=540\ M^{-1}\ cm^{-1}$[21]) and 486 nm ($\Delta\varepsilon=17{,}400\ M^{-1}\ cm^{-1}$) during 30 seconds or 10 minutes.

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