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Synthesis of Bulky β-Lactams for Inhibition of Cell Surface β-Lactamase Activity†

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Procedures are described for the preparation of a series of compounds consisting of methicillin linked to β-cyclodextrin through variable hydrophilic linkers. β-Cyclodextrin was coupled to the antibiotic methicillin to prevent the antibiotic from permeating the outer membranes of bacteria. Stoichiometric oxidation of the β-cyclodextrin with sodium metaperiodate provided a functional group for coupling to the linker. Methicillin was coupled to the linker via its carboxyl group. These compounds were tested for activity toward purified β-lactamase. The length of the spacer arm between β-cyclodextrin and methicillin was crucial in binding β-lactamase and inhibiting activity. Compounds with longer spacers were effective inhibitors of β-lactamase. We have deduced that the length of the spacer should be greater than 16 Å for optimum inhibition of β-lactamase.

INTRODUCTION

The biochemical targets of β-lactam molecules (the penicillin-binding proteins) are located on the cytoplasmic membrane. In Gram-negative bacteria the antibiotic molecules must first permeate the barrier formed by the outer membrane. The penetration of the bacterial surface by β-lactam molecules occurs by diffusion through the water-filled channels of porins (Hancock and Bell, 1988). These channels are just a little larger than the β-lactams themselves, and uptake is influenced by frictional, steric, and charge interactions (Yoshimura and Nikaido, 1985). The permeability of the outer membrane is known to affect the antibacterial effectiveness (MIC value). An accurate method for the measurement of diffusion rates of β-lactams across the outer membrane has been developed utilizing a periplasmic β-lactamase (Bellido et al., 1991). As long as the cells are not "leaky" and the β-lactamase molecules remain in the periplasmic space, the in vivo rate of hydrolysis of various β-lactams at equilibrium is diffusion limited and thus may be used to calculate the rate of diffusion of these molecules through the outer membrane (Zimmermann and Rosselet, 1977). This assay is not accurate when the cells are leaky (Lei et al., 1991), or when the bacterial strains express surface β-lactamase activity (Liu and Nikaido, 1991). Therefore the synthesis of a β-lactamase inhibitor which could not penetrate the outer membrane and interfere with periplasmic β-lactamase was attempted. It was necessary that the inhibitor was water-soluble to permit free access to surface-bound β-lactamase. The bulky water soluble carbohydrate β-cyclodextrin was used as the soluble support to which the antibiotic methicillin was linked through varying lengths of spacers.

EXPERIMENTAL PROCEDURES

Chemicals. Methicillin, ethylenediamine, diethylenetriamine, triethylenetetraamine, β-cyclodextrin, and sodium metaperiodate were purchased from Sigma; succinic anhydride was purchased from Aldrich; 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride was purchased from Bio-Rad; sodium borohydride, and DMSO were purchased from BDH. Pyridine was dried over molecular seives. HPLC-grade methanol was purchased from Fisher Scientific. Nitrocefin was kindly provided by C. O'Callaghan (Glaxo Research Groups Ltd., Middlesex, England).

Chromatography and Spectrometric Techniques. Gel-permeation chromatography was performed on a Bio-Gel P4 –400 mesh column (1.5 × 27 cm) irrigated with 0.02% sodium azide in water or a Bio-Gel P30 column (2.5 × 100 cm) irrigated with 1 M acetic acid adjusted to pH 5.0 with pyridine.

HPLC was performed on a Beckman System Gold instrument with a dual-pump programmable solvent module 126 and programmable UV-detector module 166. An Ultrasphere C_{18} ion-pair reverse-phase analytical column (5 μm, 4.6 mm (i.d.) × 15 cm) was used. The solvents used were as follows: (A) 10 mM sodium dihydrogen phosphate buffer, pH 2.5, and (B) methanol. Samples were run isocratically in 65% buffer A and 35% B or a solvent gradient programmed at 15% B for 5 min, increasing from 15 to 50% (B) in 20 min, then 50% B for 5 min, and back to 15% B in 5 min. Elution of sample was monitored at a wavelength of 225 nm. The flow rate was 1 mL/min, at a pressure of 1.8 kpsi.

Ion-exchange chromatography was performed on a column (1.2 × 20 cm) of Amberlite IR-120 (H^+) analytical-grade resin in the H^+ form in water. Samples were loaded and eluted by washing continuously with water, until two bed volumes were collected (approximately 40 mL) and then lyophilized.

Thin-layer chromatography was performed on silica gel 60 F_{254} precoated aluminium sheets (layer thickness, 0.2 mm) from E. Merck, Darmstadt, Germany. The plates were developed in 2-propanol (30%)/methanol (70%) solvent. The compounds were visualized by UV light (254 nm).

^{1}H-NMR spectra were recorded at 400 MHz on a Brucker WH 400 spectrometer. Compounds were dissolved in deuterated water. TMS was used as the external standard.

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† Abbreviations used: DMSO = dimethyl sulfoxide. EDC = 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride. HPLC = high-pressure liquid chromatography. LSIMS = liquid secondary ion mass spectrometry. MIC = minimum inhibitory concentration. NMR = nuclear magnetic resonance spectroscopy. TMS = tetramethylsilane

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Liquid secondary ion mass spectrometry (LSIMS) was performed on a Kratos concept II HQ mass spectrometer using a Cs^+ ion gun of 12 kV.

Synthetic Methodology. Oxidation of β-Cyclodextrin. To β-cyclodextrin (2.0 g,1.76 mmol) was added 0.05 M $NaIO_4$ at pH 6.0 (7.0 mL, 3.5 mmol). The solution was mixed and left at 4 °C in the dark. The optical density was measured at 223 nm to monitor uptake of periodate (Hay et al., 1965). The reaction was allowed to run for 60 h and then quenched with ethylene glycol (0.5 mL), and the products were dialyzed and lyophilized to yield 2.150 g of the oxidized product. ^1H NMR (D_2O) δ 9.3 (s, 0.7H), 5.4 (br signal 2.4H), 5.0 (d, 6H, $J = 4$ Hz anomeric), 3.5–4.0 (series of m, 43H, ring protons).

Refer to Scheme I for the general reaction pathways of the following reactions.

Coupling Ethylenediamine to Oxidized β-Cyclodextrin To Synthesize Compound 1. To the oxidized β-cyclodextrin (60 mg, 0.05 mmol) in 5 mL of water was added ethylenediamine (33 μL, 0.5 mmol) and the mixture stirred for 15 h at 4 °C in a sealed vial (Jeffrey et al., 1975). Subsequently sodium borohydride (12 mg, 0.33 mmol) was added and the mixture stirred for 16 h in a vented flask. It was then diluted with 4 mL of water, and glacial acetic acid was added dropwise until there was no more evolution of gas. Borate was removed by coevaporation with methanol.

Coupling of Methicillin to Diamino Compounds: Synthesis of Compounds 2–4 and P1. To methicillin in water (4 mL) were added an equimolar proportion of the corresponding amine-containing compound and an equimolar amount of EDC [1-ethyl-3-[3-(dimethyl amino)-propyl]carbodiimide hydrochloride] as follows: (i) methicillin (201 mg, 0.5 mmol), ethylenediamine (33 μL, 0.5 mmol), EDC (96 mg, 0.5 mmol); (ii) methicillin (201 mg, 0.5 mmol), diethylenetriamine (60 μL, 0.5 mmol), EDC (96 mg, 0.5 mmol); (iii) methicillin (201 mg, 0.5 mmol), triethylenetetraamine (70 mg, 0.5 mmol), EDC (96 mg, 0.5 mmol); (iv) methicillin (40 mg, 0.1 mmol), compound 1 (70 mg, 0.05 mmol), EDC (42 mg, 0.22 mmol). The pH was maintained at 6.0 with 2 M HCl for a period of 4 h in an ice bath. The reaction was allowed to proceed overnight at 4 °C. The final reaction products **2–4** and **P1** were lyophilized. The compounds **2–4** and P1 were run isocratically on HPLC. Their retention times were 2.9, 2.64, 2.8, and 1.99 min, respectively. The retention times of methicillin, oxidized β-cyclodextrin, compound **2**, ethylenediamine, diethylenetriamine, and triethylenetetraamine were 26.44, 1.77, 2.92, 2.2, 2.46, and 1.69 min, respectively. All compounds did not contain free methicillin or any unreacted reactants. **P1** (in 2 mL water) was loaded on Bio-Gel P30. **P1** eluted as a sharp peak (elution volume of 80 mL) very close to, but before, the elution volume of free β-cyclodextrin (90 mL). A second broad peak (which did not inhibit β-lactamase) eluted immediately thereafter. The yield of **P1** was 68 mg. R_f values of 0.56, 0.56, and 0.40 were obtained for TLC of compounds **2**, **3**, and **4**, while methicillin had a R_f value of 0.60. The three compounds **2**, **3**, and **4** were not purified further. Their crude yields were 337, 342, and 335 mg, respectively.

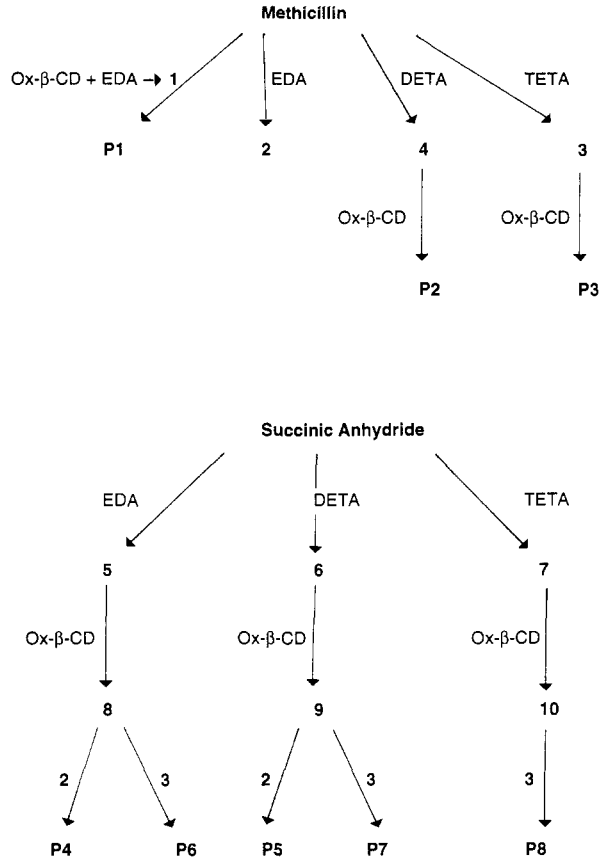
Formation of *N*-(Aminoethyl)succinamic Acid Compounds 5–7. To succinic anhydride (100 mg, 1 mmol) in dry pyridine (5 mL) was added either ethylenediamine (67 μL, 1 mmol), diethylenetriamine (100 μL, 1 mmol), or triethylenetetraamine (146 mg, 1 mmol) and the mixture stirred for 22 h. Pyridine was removed by repeated coevaporation with water, and the product **5**, **6**, or **7** were respectively lyophilized. The crude yields of the compounds were 178, 182, and 240 mg, respectively.

Linking of *N*-(Aminoethyl)succinamic Acid to Oxidized β-Cyclodextrin: Synthesis of 8–10. To oxidized β-cyclodextrin (500 mg, 0.44 mmol) in 8 mL water was added *N*-(aminoethyl)succinamic acid [69 mg (0.44 mmol) of compound **5**] and the pH adjusted to 10.0 with 2 M NaOH. Similarly, to oxidized β-cyclodextrin (425 mg, 0.37 mmol) was added either 75 mg (0.37 mmol) of compound **6** or 91 mg (0.37 mmol) of compound **7** and the pH was increased to pH 10 by the addition of 6 or 7 drops of 2 M NaOH. The solutions were stirred for 18 h at 4 °C. Sodium borohydride was added to the former (60 mg, 1.5 mmol) and to the two latter vessels (40 mg, 1 mmol) and allowed to react at room temperature for 24 h. Excess borohydride was neutralized with 2 N HCl and the entire sample passed through a column of IR-120 (H^+) cation-exchange resin. The resulting yellow solution was concentrated by rotary evaporation and coevaporated with acidified methanol to remove borate. The products obtained were lyophilized to yield **8** (603 mg), **9** (530 mg), or **10** (544 mg), respectively.

Synthesis of β-Cyclodextrin Coupled Methicillin Derivatives. (a) Synthesis of P2 and P3. To the amino-spacer-linked methicillin compounds [0.05 mmol of **4** (23 mg) or **3** (25 mg)] in water was added oxidized β-cyclodextrin (56 mg, 0.05 mmol) and the pH was adjusted to 10 with 3 drops of 2 M NaOH. After allowing the reaction to proceed at room temperature for 24 h, sodium borohydride (10 mg, 0.25 mmol) was added and stirring continued at room temperature for a further 20 h. The solution was neutralized with 2 M HCl and passed through a column of IR-120 (H^+) resin. The eluate was concentrated and coevaporated with acidified methanol to remove borate and yield compound **P2** (83 mg) or **P3** (89 mg). **P2** appeared as a sharp peak at a retention time of 1.91 min and **P3** at 1.84 min when subjected to HPLC. Peaks due to free β-cyclodextrin, compound **4**, or compound **3** at retention times of 1.77, 2.8, or 2.64 min, respectively, were absent.

(b) Synthesis of P4-P8. To an aqueous solution (10 mL) of β-cyclodextrin linked to *N*-(aminoethyl)succinamide (**8**, **9**, or **10**) was added an amino-spacer-linked methicillin compound (**2** or **3**) as follows: (i) compound **8** (102 mg, 0.08 mmol) and compound **2** (34 mg, 0.08 mmol); (ii) compound **9** (200 mg, 0.15 mmol) and compound **2** (63 mg, 0.15 mmol); (iii) compound **8** (255 mg, 0.20 mmol) and compound **3** (101 mg, 0.20 mmol); (iv) compound **9** (200 mg, 0.15 mmol) and compound **3** (76 mg, 0.15 mmol); (v) compound **10** (215 mg, 0.15 mmol) and compound **3** (76 mg, 0.15 mmol). The solutions were kept in an ice bath and the pH was adjusted to 6 with 1 M NaOH where necessary. After addition of EDC (equimolar amounts to each) the pH was maintained at 5.5–6.0 with the addition of 0.1 M HCl over a period of 4h and thereafter stirred at 4 °C for a further 20 h. The solutions were lyophilized to yield **P4**, **P5**, **P6**, **P7**, and **P8**, respectively. All products [**P4-P8** in a and b above] were purified on a Bio-Gel P4 –400 mesh column in 0.02% sodium azide. The eluates were monitored by the phenol sulfuric method for carbohydrates (Dubois et al., 1956). The modified compounds eluted before unmodified β-cyclodextrin and were well-resolved from free methicillin. The major peak appearing in the elution region of 30–40 mL was pooled and lyophilized to give **P4** (41 mg), **P5** (210 mg), **P6** (335 mg), **P7** (244 mg), or **P8** (143 mg). Minor peaks observed after this peak were also pooled and tested for β-lactamase inhibitory activity. Only the major peak was an active

Scheme I



General reaction pathways—see text for details. Abbreviations: EDA = ethylenediamine, DETA = diethylenetriamine, TETA = triethylenetetraamine, Ox-β-CD = oxidized β-cyclodextrin.

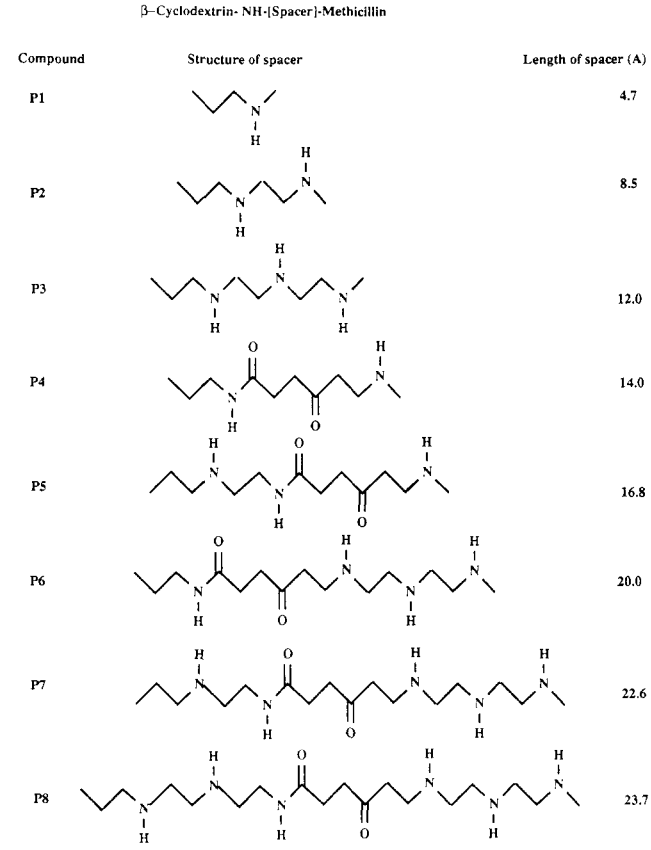


Figure 1. The structures of the different spacers in the compounds **P1-P8** formed between β-cyclodextrin and methicillin are shown here. The spacer consists of two amino termini, one of which is linked to the aldehyde generated in β-cyclodextrin (not drawn in the model), the other to the carboxyl group of methicillin. The lengths of the spacers were calculated from molecular models generated by using the Alchemy program.

inhibitor. Therefore the later-eluting minor peaks were not characterized further. P4 was observed at 1.99 min by isocratic elution on HPLC. Alternatively, purification of P4 was also performed by HPLC by repeated injection of 50 μL of a 15 mg/mL soluton at each run and collection of eluant between the retention times of 1.8 and 2.5 min. Since this method was not suitable for large-scale purification, Bio-Gel P4 chromatography was utilized to purify **P4** successfully. **P5** and **P6** were observed at retention times of 1.89 and 1.85 min, respectively, when HPLC was performed using the solvent gradient program. Methicillin eluted at 25.8 min on this program.

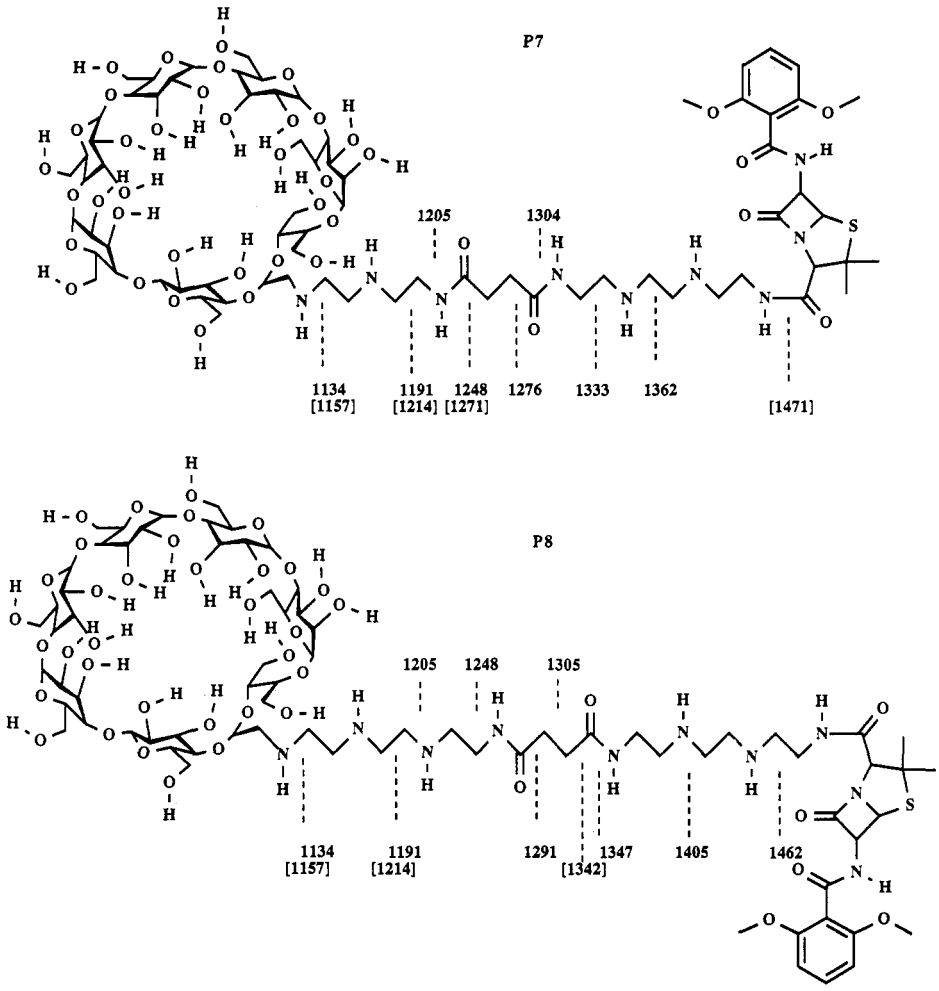
P7 ^{1}H NMR (D_2O): δ 7.4 (t, 1H, J = 6 Hz, aromatic H-4), 6.7 (d, 2H, J = 6 Hz, aromatic H-3,5), 5.4 (d, 1H, J = 4 Hz, anomeric), 5.0 (d, 6H, J = 4 Hz, anomeric), 3.5 - 4.0 (series of m, 51H, ring protons, aromatic OCH_3, methicillin ring), 3.2 (m, 12H, spacer), 2.9 (m, 12H, spacer), 1.6 (s, 3H, methicillin CH_3), 1.5 (s, 3H, methicillin CH_3). Mass spectrum (thioglycerol): m/z 1780 $(M - OMe)^+$. **P8** ^{1}H NMR (D_2O): δ 7.4 (t, 1H, J = 6 Hz, aromatic H-4), 6.7 (d, 2H, J = 6 Hz, aromatic H-3,5), 5.4 (d, 1H, J = 4 Hz, anomeric), 5.0 (d, 6H, J = 4 Hz, anomeric), 3.5–4.0 (series of m, 51H, ring protons, aromatic OCH_3, methicillin ring), 3.2 (m, 16H, spacer), 2.9 (m, 12H, spacer), 1.6 (s, 3H, methicillin CH_3), 1.5 (s, 3H, methicillin CH_3). Mass spectrum (thioglycerol); m/z 1805 $(M - H_2O - OCH_3)^+$. Biological assays demonstrated that these compounds were stable in lyophilized state for more than 6–8 weeks; however, in solution their ability to inhibit β-lactamase deteriorated within days.

Biological Methods. β-Lactamase Assays. β-Lactamase assays were performed (Angus et al., 1982) using the chromogenic cephalosporin nitrocefin (O'Callaghan et al., 1972). *Pseudomonas aeruginosa* PAO1 strain H103 containing the TEM-2 β-lactamase encoded by plasmid RP1 was grown to an optical density at 660 nm (OD_{660}) of 0.5–0.8 in Luria broth. The cells were then centrifuged and washed and resuspended in 5 mM sodium Hepes, pH 7.2, to OD_{600} = 0.5. Assays were performed on whole cells which were left at 23 °C, or incubated on ice for 10 min to release high levels of β-lactamase. Cells were then preincubated with the test compounds for 10 min before performing a nitrocefin hydrolysis assay. One hundred microliters of cells was added to 0.65 ml of nitrocefin solution (0.1 mg/ml in 5 mM Hepes) in a semimicrocuvette, and the kinetics of nitrocefin hydrolysis was monitored at OD_{495} using a Perkin-Elmer (lambda 3) dual-beam spectrophotometer coupled to a Perkin-Elmer 561 chart recorder. Initially compounds were tested for inhibition of activity of a crude extract of β-lactamase from *Enterobacter cloacae* rather than whole cells. Compounds **P1** and **P2** were found to be inactive and therefore discontinued in the study of the effect of the compounds on whole cells.

RESULTS AND DISCUSSION

Outer membrane permeability in intact cells is usually measured by the Zimmermann–Rosselet (1977) approach in which an equilibrium is established between the rate of diffusion across the outer membrane and the rate of hydrolysis of β-lactams by intact cells. Thus the rate of diffusion can be estimated as the rate of cleavage of the β-lactam ring by a β-lactamase enzyme present in the

Scheme II



Fragmentation pattern obtained for the compounds **P7** and **P8** and the *m/z* values for major fragments observed in the negative LSIMS spectra are indicated. The fragments observed in the positive LSIMS spectra showing the addition of Na^+ are indicated in square brackets. Ions due to sequential loss of sugar units (162 mass units) were also observed. Mass ions at *m/z* 1287 and 1229 are due to loss of a water molecule from the fragments of mass 1305 and 1247 in **P8**. **P7** shows an ion at *m/z* 1285 due to loss of water and H from the fragment of mass 1304. The peak at *m/z* 1169 in both spectra arises from the loss of two water molecules from the fragment of mass 1205.

periplasm (the cellular compartment that is immediately internal to the outer membrane) (Liu and Nikaido, 1991) or the rate of disappearance of β-lactam from the extracellular space (Bellido et al., 1991). Since the rate of hydrolysis of β-lactam by β-lactamase sequestered in the periplasm is often only 1–5% of the rate of hydrolysis that would be observed if this β-lactamase was in free solution, any leakage of β-lactamase into the supernatant (Angus et al., 1982) or to the cell surface (Liu and Nikaido, 1991) can result in considerable errors in estimations of diffusion rate.

Therefore we sought here to produce a class of high molecular weight inhibitors that could not cross the outer membrane, but instead inhibited only external β-lactamase. We chose to investigate compounds that had previously been conjugated to beads as a means of purifying β-lactamases. Preliminary experiments, utilizing aminoboronic acid or 6-aminopenicillanic acid as the conjugated compound, revealed a complete absence of inhibition in the former case and, in the latter case, inhibition of a cephalosporinase-type β-lactamase (the chromosomal β-lactamase of *Enterobacter cloacae*; Bellido et al., 1991) but no inhibition of the penicillinase-type β-lactamase TEM-2. Therefore, we concentrated these studies on methicillin as the conjugated compound since preliminary studies demonstrated that conjugates inhibited both classes of enzymes. Since TEM-2 β-lactamase is expressed from the plasmid RP1, which is a broad host range plasmid capable of being delivered by conjugation to virtually any Gram-negative bacterium, the methicillin conjugates would be broadly useful in outer membrane permeability studies.

Methicillin has been previously demonstrated to act as a β-lactamase inhibitor (Sutherland and Batchelor, 1964). In addition it has been coupled to ethylenediamine and 1,6 diaminohexane bound to Sepharose and used effectively as a matrix for purification of penicillinase (Coombe and George, 1976). The method used by these authors for coupling methicillin was similar to that reported in this paper, in that carbodiimide was utilized for linking the carboxyl group of methicillin to the free amino group of the matrix-bound diaminoalkane. Therefore a series of compounds were synthesized using β-cyclodextrin (oxidized to introduce an aldehyde functional group) as the soluble matrix and the antibiotic methicillin (Scheme I). The carboxyl group on methicillin was linked to form an amide bond with one amino end of diamino alkyl derivatives, leaving the other end free to link to oxidized β-cyclodextrin. The length of the spacer was gradually increased by coupling the free amino group to succinic anhydride and then to a second diamino alkyl derivative

to produce hydrophilic spacers. The compounds synthesized contained spacers ranging from 4 to 24 Å (Figure 1).

The final products were pure as judged by HPLC, TLC, and, in selected instances, NMR. The active compounds with the longest spacers (**P7** and **P8**) were characterized by NMR and cesium ion ionization mass spectrometry. Proton NMR spectra indicated that only a single spacer and a single methicillin moeity were attached to one oxidized β-cyclodextrin molecule. The ion obtained by cleavage of the side arm was prominent in negative-ion LSIMS (oxidized β-cyclodextrin with loss of a proton, m/z 1133) and in positive-ion LSIMS (oxidized β-cyclodextrin + Na^+, m/z 1157]). Ions due to fragmentation as shown in Scheme II were also observed.

β-Cyclodextrin was chosen as the bulky group in these studies because of its solubility in water and the ease of modification of its sugars. The bulky cyclic nature of the molecule would prevent its entry across any channels in the bacterial outer membrane (Hancock and Bell, 1988). β-Lactamase has a binding site for β-lactams which is located below its surface, and therefore when the spacer arm between β-cyclodextrin and the β-lactam is not long enough, the bulky β-cyclodextrin would not allow the antibiotic to fit into the binding site on the β-lactamase molecule. The activity of β-lactamase was measured by using the nitrocefin assay (Angus et al., 1982). When β-lactamase was inhibited by the compound, nitrocefin hydrolysis did not occur. Initially the inhibitory activity was measured using intact washed *P. aeruginosa* cells that had been kept at 23 °C. Although the amount of β-lactamase activity was low, it was suppressed nearly 3-fold by compounds **P4-P8** at a concentration of 1 mM (Figure 2A). In contrast, **P3** had virtually no inhibitory activity, whereas free methicillin suppressed both excreted and periplasmic (cell-associated) β-lactamase activity. A control experiment to show that cyclodextrin and linker without methicillin would not inhibit β-lactamase was not performed. However, lack of inhibitory activity of **P1-P3** are an equivalent negative control. All compounds with methicillin and spacer alone were effective inhibitors of the enzyme. Methicillin inhibited β-lactamase at 0.1 and 1.0 mM concentrations, but did not inhibit at 0.01 mM concentration. Therefore our compounds were tested at concentrations between 0.1 and 1.0 mM. There was no inhibition at concentrations below 0.5 mM, and therefore the tests were performed at 1.0 mM concentration. To improve the sensitivity of this assay, cells were incubated for 10 min at 4 °C. This led to a 7.6-fold increase in β-lactamase leakage (Figure 2B), consistent with the observations of Lei et al. (1991). Under these more extreme conditions, the extent of inhibition of external β-lactamase activity varied with the length of the cross-linker when all compounds were present at concentrations of 1 mM. Thus, the extents of inhibition of surface and secreted β-lactamase were 6% using **P3**, 20% using **P4**, 43% using **P5**, 77% using **P6**, 91% using **P7**, and 100% using **P8** (see Figure 2 legend for actual rates of hydrolysis) at a nitrocefin substrate concentration of 71 μg/mL. Therefore we consider both **P7** and **P8** to be adequate for suppressing external β-lactamase activity, with the latter being more reliable under extreme conditions. As expected, methicillin at 1 mM concentration completely suppressed whole cell β-lactamase activity, suppressing surface-associated and periplasmic β-lactamase. By performing similar experiments at different concentrations of compounds, it was determined that free methicillin was about 10-fold more effective (on a molar basis) at inhibiting β-lactamase than, for example, **P8**, presumably due to the influence of

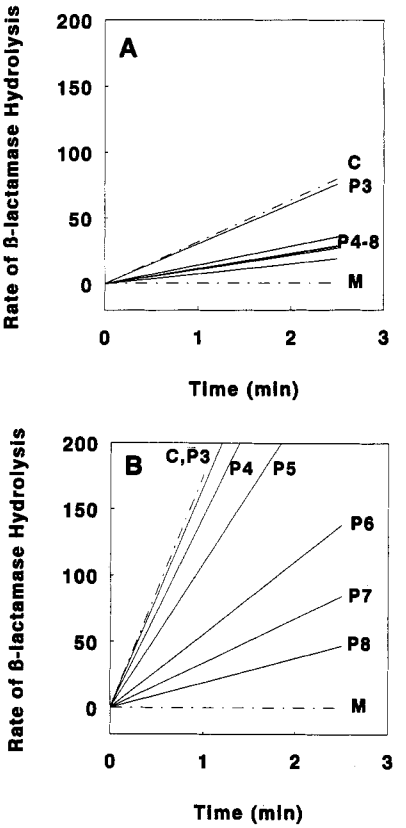


Figure 2. Inhibition of β-lactamase activity on whole cells preincubated with test compounds. (A) Cells at 23 °C. Dashed lines represent the controls with buffer only (C; β-lactamase activity 17.1 pmol of nitrocefin hydrolyzed/mg of cells per min) and using free methicillin as an inhibitor (M; 0 pmol/mg of cells per min). Solid lines represent β-lactamase activity in the presence of **P3**, **P4**, **P5**, **P6**, **P7**, and **P8** (respectively 16.25, 7.6, 6.0, 6.2, 4.0, and 6.0 pmol/mg of cells per min). (B) Cells pretreated 10 min at 4 °C. See 2A for legend. β-Lactamase activities were (in pmol of nitrocefin hydrolyzed/mg of cells per min) C = 94.5, M = 0, **P3** = 89.8, **P4** = 77.5, **P5** = 58.3, **P6** = 29.7, **P7** = 18.1, P8 = 10.0.

derivatization of the carboxyl group. Nevertheless, **P8** (under ice treatment conditions) and **P4-P8** (at room temperature) were able to suppress surface-associated and secreted β-lactamase. The residual unsuppressed β-lactamase activity provided an estimate of the actual rate of diffusion of nitrocefin into *P. aeruginosa* cells. At this substrate concentration, it was estimated as 6.6 ± 2.0 pmol of nitrocefin/mg of cells per min. Since it is well-known that the β-lactams do not bind to the interior of nonspecific porin channels (Bellido et al., 1991; Yoshimura and Nikaido, 1985), we are confident that, even at 1.0 mM compound concentration, porin diffusion would not be affected. We conclude therefore that the described compounds will be useful for measuring outer membrane permeability in diverse Gram-negative bacteria, to prevent substantial errors introduced by leakage of β-lactamase out of cells (Bellido et al., 1991; Lei et al., 1991; Liu and Nikaido, 1991).

From these data it appears that the minimum length of the spacer should be at least 16 Å (Figure 1) to provide maximum interaction between the β-lactam (methicillin) and its binding site within the β-lactamase molecule. This does not imply that the binding site is 16 Å below the surface of the β-lactamase molecule, because X-ray studies conducted with antibiotics bound to these enzymes showed that the β-lactam is oriented such that the carboxyl group is directed partially into the interior of the binding cavity (Ghuysen, 1991). Since these compounds all contain

spacers linked to the carboxyl group, the spacer may span the entire length of the binding cavity.

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