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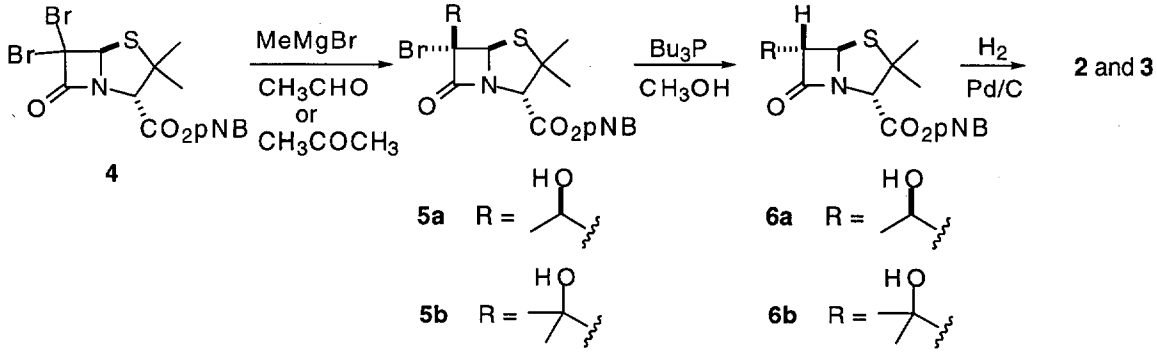
Compounds **2** and **3** were made according to Scheme 1.

Scheme 1



Syntheses. Compound **4** was synthesized as described previously.[13]

***p*-Nitrobenzyl 6α-bromo-6β-(1*R*-hydroxyethyl)penicillanate (5a).** A solution of methylmagnesium bromide in ether (3 M, 3.4 mL, 10.1 mmol) was added dropwise to a solution of *p*-nitrobenzyl 6,6-dibromopenicillanate (**4**, 5.0 g, 10.1 mmol) in dry THF (200 mL) at -78 °C, and the reaction mixture was stirred an additional 30 minutes after the completion of the addition at the same temperature. After the addition was completed, a 10% solution of acetaldehyde in THF (15.5 mL, 50.5 mmol of acetaldehyde) was added dropwise to the above reaction mixture, and the mixture was stirred for 2 hr at -78 ° C. Subsequent to the addition of a saturated NH_4Cl solution (ca. 3 mL), the reaction mixture was warmed to room temperature and the solvent was evaporated to near dryness *in vacuo*. Both water and ethyl acetate were added to the residue and the aqueous layer was further washed with additional portions of ethyl acetate (50 mL, 3x). The combined organic layer was washed with water, saturated NaCl, dried over $MgSO_4$, and was concentrated *in vacuo* to afford a brown oil. The crude product was purified by silica-gel column chromatography (hexane:ethyl acetate,

2/1) to afford the desired pure product as a yellow oil (1.72 g, 37%), in addition to a mixture of all four possible isomers which were combined together (0.96 g, 21%; 6β, 1*R* : 6α, 1*R* : 6β, 1*S* : 6α, 1*S*, approx. 3/6/2/22). IR (film) 3490, 1782, 1745, 1605, 1518, 1344 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 1.26 (3H, d, *J* = 6.3 Hz, side-chain methyl), 1.41 (3H, s, C_2 methyl), 1.64 (3H, s, C_2 methyl), 2.58 (1H, br s, OH), 4.21 (1H, q, *J* = 6.3 Hz, CHOH), 4.58 (1H, s, C_5 methine), 5.23-5.36 (2H, unresolved AB type, benzylic methylene), 5.56 (1H, s, C_5 methine); ^{13}C-NMR ($CDCl_3$): δ 18.02, 25.90, 33.22, 64.61, 65.93, 67.10, 68.01, 71.96, 74.51, 123.99, 128.93, 141.66, 166.55, 169.15; EI HRMS 458.0156 (M^+, calcd for $C_{17}H_{19}BrN_2O_6S$: 458.0147).

***p*-Nitrobenzyl 6α-bromo-6β-(1-hydroxy-1-methylethyl)penicillanate (5b)** . Prepared from **4** as described for **5a**, except acetone substituted acetaldehyde in the procedure to afford 1.90 g of the title compound. Yield, 28%, after silica-gel column chromatography (hexane:ethyl acetate, 5/3); IR (film) 3451, 1786, 1746, 1606, 1521, 1345 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 1.44 (3H, s, C_2 methyl), 1.68 (3H, s, C_2 methyl), 1.45 (3H, s, side-chain methyl), 1.56 (3H, s, side-chain methyl), 3.70 (1H, br s, OH), 4.58 (1H, s, C_3 methine), 5.29 (2H, s, benzylic methylene), 5.67 (1H, s, C_5 methine), 7.55 (2H, d, *J* = 8.7 Hz, aromatic), 8.23 (2H, d, *J* = 8.7 Hz, aromatic); ^{13}C-NMR ($CDCl_3$): δ 25.24, 26.18, 27.92, 32.79, 65.87, 66.00, 67.54, 71.52, 74.80, 75.34, 123.98, 128.90, 141.69, 148.00, 166.71, 167.36; CI MS 473, 475 (M^+ +H, 0.6%, 0.6%).

***p*-Nitrobenzyl 6α-(1 *R*-hydroxyethyl)penicillanate (6a).** Tributylphosphine (0.43 mL, 1.64 mmol) was added to a solution of **5a** (500 mg, 1.09 mmol) in methanol (20 mL) and the reaction mixture was stirred for 1 hr. After concentration under reduced pressure, the residual oil was purified by silica-gel column

chromatography (hexane:ethyl acetate, 2/1) to afford the title compound (380 mg, 95%) as a colorless syrup. IR (film) 3468, 1773, 1752, 1602, 1525, 1342 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 1.32 (3H, d, *J* = 6.0 Hz, side-chain methyl), 1.39 (3H, s, C_2 methyl), 1.61 (3H, s, C_2 methyl), 2.57 (1H, br s, OH), 3.33 (1H, dd, *J* = 1.2, 6.0 Hz, CHOH), 4.52 (1H, s, C_3 methine), 5.21-5.33 (2H, unresolved AB type, benzylic methylene), 5.30 (1H, d, *J* = 1.2 Hz, C_5 methine), 7.55 (2H, d, *J* = 9.3 Hz, aromatic), 8.23 (2H, d, *J* = 9.3 Hz, aromatic); ^{13}C-NMR ($CDCl_3$): δ 21.71, 26.28, 32.44, 63.56, 65.08, 65.24, 65.66, 68.47, 69.65, 123.93, 128.80, 142.01, 147.89, 167.57, 172.56; EI HRMS 380.1049 (M^+, calcd for $C_{17}H_{20}N_2O_6S$: 380.1042).

***p*-Nitrobenzyl 6α-(1-hydroxy-1-methylethyl)penicillanate (6b).** The title compound was prepared from **5b** as desribed for **6a**. Purification by silica-gel column chromatography (hexane:ethyl acetate, 5/2) gave the desired product as the main component (86%), along with some of the β-product (14%)(407 mg; yield, 84%). IR (film): 3478, 1772, 1747, 1605, 1521, 1343 cm^{-1}; ^{1}H-NMR ($CDCl_3$): 1.30 (3H, s, side-chain methyl), 1.37 (6H, s, side-chain methyl and one C_2 methyl), 1.58 (3H, s, C_2 methyl), 2.48 (1H, br s, OH), 3.33 (1H, d, *J* =1.5 Hz, C_6 methine), 4.52 (1H, s, C_3 methine), 5.20-5.30 (2H, unresolved AB type, benzylic methylene), 5.27 (1H, d, *J* = 1.5 Hz, C_5 methine), 7.53 (2H, d, *J* = 8.4 Hz, aromatic), 8.19 (2H, d, *J* = 8.4 Hz, aromatic); ^{13}C-NMR ($CDCl_3$): δ 29.20, 27.32, 28.39, 32.79, 63.45, 65.15, 65.58, 69.07, 69.56, 72.20, 123.87, 128.73, 142.11, 147.83, 167.55, 172.42; EI HRMS 394.1196 (M^+, calcd for $C_{18}H_{22}N_2O_6S_1$: 394.1199

6α-(1 *R*-hydroxyethyl)penicillanic Acid (2). A solution of **6a** (280 mg, 0.737 mmol) in methanol (8 mL), 0.1 M sodium phosphate buffer, pH 7.0 (1 mL) and

water (2 mL) was added to a suspension of 10% Pd-C (200 mg) in methanol (5 mL) which had been stirred under an atmosphere of hydrogen for 3 hr; the resultant suspension was stirred for 30 min under hydrogen. After removal of the catalyst by filtration through celite, methanol was removed *in vacuo*. Saturated $NaHCO_3$ was added to the residue and the aqueous phase was washed with ethyl acetate and then was acidified (approx. pH 2) with 2.5 M HCl. After washes with ethyl acetate (3x), the combined organic layer was washed with water and saturated NaCl, dried over $MgSO_4$ and was concentrated *in vacuo* to afford the title compound (150 mg, 80%) as a white powder, mp 140-142°C (decomp.); IR (KBr): 3390, 1773, 1721 cm^{-1}; ^{1}H-NMR (acetone-d_6): δ 1.24 (3H, d, J = 6.3 Hz, side-chain methyl), 1.50 (3H, s, C_2 methyl), 1.61 (3H, s, C_2 methyl), 3.21 (1H, dd, J = 1.8, 7.2 Hz, C_6 methine), 4.10 (1H, m, C<u>H</u>OH), 4.35 (1H, s, C_3 methine), 5.26 (1H, d, J = 1.8 Hz, C_5 methine); ^{13}C-NMR (acetone-d_6): δ 21.26, 25.86, 31.32, 63.49, 64.78, 64.91, 68.47, 69.66; EI HRMS 245.0716 (M^+, calcd. for $C_{10}H_{15}NO_4S$: 245.0722).

6α-(1-Hydroxy-1-methylethyl)penicillanic Acid (3). Prepared from **6b** as described for **2**. The product was isolated as a mixture of α- and β-isomers (255 mg, 85%; ca 6 α : 1 β). Fractional crystallization from ethyl acetate-chloroform afforded the title compound as colorless crystals (99 mg, 38%). mp 74 °C (sintered), 81-83 °C (decomp.); IR (KBr): 3393, 1772, 1740 cm^{-1}; ^{1}H-NMR (acetone-d_6): δ 1.27 (3H, s, side-chain methyl), 1.32 (3H, s, side-chain methyl), 1.50 (3H, s, C_2 methyl), 1.60 (3H, s, C_2 methyl), 3.31 (1H, d, J = 1.8 Hz, C_6 methine), 4.36 (1H, s, C_3 methine), 5.30 (1H, d, J = 1.8 Hz, C_5 methine); ^{13}C-NMR (acetone-d_6): δ 25.77, 26.33, 28.18, 31.64, 62.95,

64.57, 68.02, 69.59, 72.15, 168.42, 172.22; EI HRMS 259.0883 (M^+, calcd. for $C_{11}H_{17}NO_4S$: 259.0878).

Kinetic Determinations. The kinetics parameters for turnover (K_m and k_{cat}) for the NMC-A substrates were determined by non-linear regression of the equation for Michaelis-Menten kinetics. All the experiments were carried out in 50 mM sodium phosphate, pH 7.0, at 20 °C. The typical assay volume was 500 mL. The concentration range for various substrates were as follows: ampicillin, 1 to 50 mM; imipenem, 20 to 100 μM; **1**, 20 to 100 μM; **2**, 0.2 to 1.4 mM. A portion of the enzyme was added to a solution of substrate to achieve a final enzyme concentration of 34.5 nM for ampicillin, imipenem and **1**; and 34.5 μM for **2**. Substrate hydrolysis was monitored at 235 nm for ampicillin ($\Delta\varepsilon_{235}$=775 $M^{-1}cm^{-1}$); 240 nm for imipenem ($\Delta\varepsilon_{240}$ = 1112 $M^{-1}cm^{-1}$); 220 nm for **1** and **2** ($\Delta\varepsilon_{220}$ = 783 $M^{-1}cm^{-1}$ and $\Delta\varepsilon_{220}$ = 643 $M^{-1}cm^{-1}$, respectively).

Inactivation experiments were performed according to the method of Bush *et al.*[21] An aliquot of the stock solution of **3** (50 to 500 μM final concentrations) was added to the NMC-A β-lactamase (3.3 μM) in 100 mM sodium phosphate, pH 7.0, at 4 °C. Portions (10 μL) were removed from the mixture at time intervals, and were diluted 100-fold into the assay mixture containing 0.5-mM cephaloridine in 1 mL total assay volume. The enzyme activity was monitored until cephaloridine had been entirely consumed. The remaining enzyme activity was calculated from the initial linear portion of the hydrolysis curve.

Data Collection and Processing. Crystals were transferred from their crystallization drops into 2 μL of a freshly prepared solution of the inhibitor (21.5 % PEG 1500, 200 mM MES, pH 5.25). Crystals were then mounted in cryoloops and were flash-

frozen in a stream of nitrogen gas cooled to 120 K, immediately prior to data collection. The quality of the crystals of the inhibited enzyme was found to be critically dependent on the concentration of the inhibitor and on the duration of the soaking process. The best results were obtained with 5 mM inhibitor and a soaking time of 5 min. At this concentration, and taking into account the crystal size, the molecular ratio between inhibitor and protein is about 200.

A 1.9 Å data set was collected on the W32 wiggler beam line at LURE-DCI (Orsay, France), tuned at a wavelength of 0.97 Å and equipped with a large Mar scanner. The crystal to detector distance was set to 260 mm and the crystal temperature was maintained at 120 K. A total of 63 frames (1.5° oscillation per frame and 9 min exposure) was collected from a single NMC-A crystal ($200 \times 100 \times 80\ \mu m^3$). Data were processed with MOSFLM[22] and CCP4[23] packages (Table 2).

Crystal Structure Refinement. The structure was refined with the program X-PLOR, version 3.1,[24] applying a bulk solvent correction. A total of 7.5% of the reflections were randomly selected in the entire dataset in order to provide a test set for the R_{free} calculations.[25] These reflections were omitted during refinement, but were included in the electron density maps calculations. In each refinement cycle, simulated-annealing from 3000 to 300 K, followed by conventional energy minimization, and individual *B* factors refinement were applied. Models and electron density maps were displayed with the program TURBO FRODO.[26]

The 1.64 Å refined NMC-A β-lactamase structure,[27] truncated as a polyalanine, was used as a starting model for molecular replacement calculations carried out with the AMoRe program[28] to account for the 1.2 Å variation of the cell parameters along the

crystallographic *a* axis. Rigid-body refinement was then performed between 10.0 and 3.0 Å (R_{factor} = 0.42), and the initial model was refined to 2.5 Å resolution. The resolution was then extended to 1.9 Å. Solvent molecules were added as neutral oxygen atoms when they appeared as positive peaks above 4.0σ in the $(F_{obs}-F_{calc})\exp(i\alpha_{calc})$ map, and displayed acceptable hydrogen-bonding geometry. Hereafter, the simulated annealing step was performed from 500 K. The starting geometry of the inhibitor was derived from the crystal structure of 6α-(hydromethyl)penicillanate in complex with the TEM-1 β-lactamase,[14] and the coordinates and the X-PLOR dictionary files for the MES buffer chemical structure were taken from the Hetero-compound Information Centre (G.J. Kleywegt, Uppsala, Sweden).

Table 2. Data processing statistics given for the entire resolution range and for the highest resolution shell.

	18.63 – 1.895 Å	1.96 – 1.895 Å
Number of measurements	81088	6594
Number of unique reflections	22025	2019
Completeness (%)	98.6	95.0
R_{sym} (%)	4.6	9.8
$\langle I/\mathrm{sd}(I)\rangle$	9.0	5.9

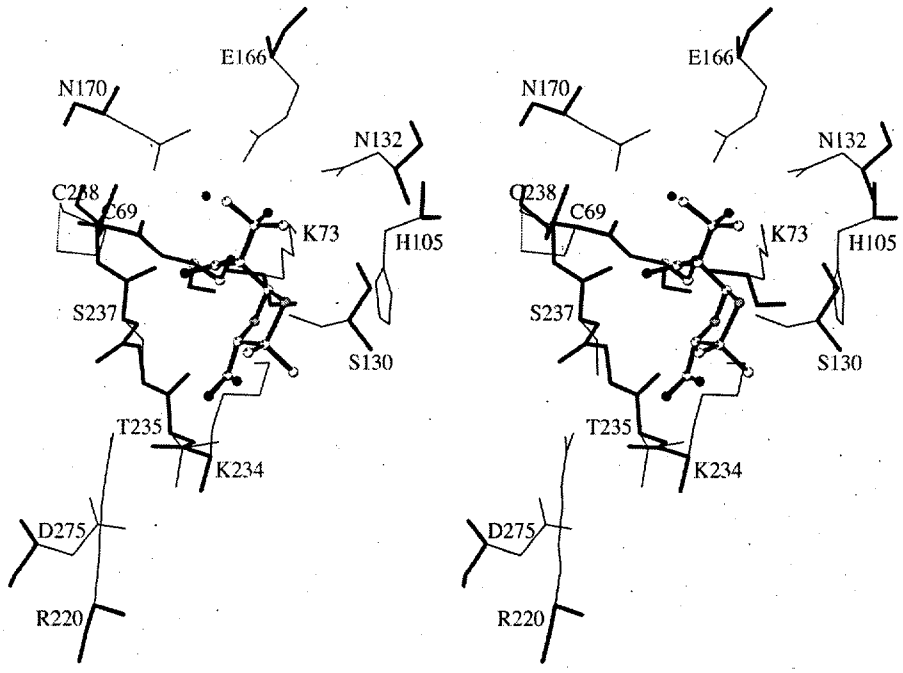


Figure 2. The image in Figure 1 without the electron density.

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