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Supplementary information Solid- and solution-phase-based library of 2β-methyl substituted penicillin derivatives. Effects on growth inhibition of tumor cell lines

Dora B. Boggián,[a] Patricia G. Cornier,[a] Ernesto G. Mata,[a] Viviana C. Blank,[b] Mariano G. Cárdenas,[b] and Leonor P. Roguin[b]**

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Experimental

1. Chemistry

Chemical reagents were purchased from commercial sources and were used without further purification unless noted otherwise. Solvents were analytical grade or were purified by standard procedures prior to use. Resins were purchased from Aldrich. ^{1}H NMR spectra were recorded in a Bruker Avance spectrometer at 300 MHz in $CDCl_3$ with tetramethylsilane (TMS) as internal standard. ^{13}C NMR spectra were recorded on the same apparatus at 75 MHz with $CDCl_3$ as solvent and reference (76.9 ppm). The chemical shifts (δ) are reported in ppm downfield from TMS and coupling constants (*J*) are expressed in hertz. Flash column chromatography was performed using Merck silica gel 60 (230-400 mesh). Elution was carried out with hexane-EtOAc mixtures, under positive pressure and employing gradient of solvent polarity techniques. Solid-phase reactions were carried out in polypropylene cartridges equipped with a frit (Supelco, Bellefonte, PA), unless reflux conditions were required, in that cases standard glassware was used. All solid-phase reaction mixtures were stirred at the slowest rate. Culture medium and fetal bovine serum were purchased from Gibco BRL Gaithersburg, MD, USA.

2. In vitro Assay

2.1 Cell lines and culture conditions

HeLa (ATCC CCL-2) were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin. NIH-3T3 cell line (ATCC CRL-1658) was cultured in Dubecco´s modified Eagle´s Medium (DMEM) with 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10% FBS, 4 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin. In addition, a supplement of 1 mM sodium piruvate and 0.1 mM non-essential amino acids was also included to maintain MCF-7 cells (ATCC HTB-22). B16-F0 cell line, kindly provided by the Laboratory of Molecular Oncology (Quilmes National University, Buenos Aires, Argentina), was grown in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin. NMuMG (ATCC CRL-1636) and LM3 cells, gently provided by the Institute of Oncology Angel H. Roffo (Buenos Aires, Argentina), were cultured in DMEM-F12 containing 10% FBS, 2 mM L-glutamine, 0.6% HEPES, 50 U/mL penicillin and 50 µg/mL streptomycin.

2.2 Cell proliferation assay

Cells were placed in 96-well microplates at a density of $1x10^4$ cells/well (B16-F0) or $2x10^4$ cells/well (HeLa, MCF-7, LM3, NMuMG, NIH-3T3) and incubated for 72 h at 37ºC in the presence or absence of 20 µM of the different compounds in a total volume of 0.2 ml of culture medium. Compounds were dissolved in dimethyl sulfoxide (DMSO) as 10 mM stock solutions and stored at -70°C. Prior to use, the compounds were diluted 1:10 in ethanol and added at the indicated concentrations to the culture medium. Control cells were treated under similar conditions and a final concentration of 20 µl vehicle/ml of culture medium was used in all experiments. Total cell number was evaluated by colorimetric determination of hexosaminidase levels, an ubiquitous lysosomal enzyme.[1] Briefly, cells were washed twice with phosphate-buffered saline and then incubated at 37ºC with 60 µl of 3.25 mM *p*-nitrophenol-*N*-acetyl-β-D-glucosaminide dissolved in 50 mM citrate buffer, pH 5, 0.25% Triton X-100. After 45-120 min, the color reaction was developed and enzyme activity was blocked by adding 90 µl of 50 mM glycine buffer, pH 10.4, containing 5 mM EDTA. Absorbance values were measured at 405 nm in a visible plate reader (Biotrak II, Amersham Biosciences, USA). The molar drug concentrations required to cause 50% growth inhibition (IC_{50}) were determined from dose-response curves ranging from 1.25- 80 µM.

3. Synthesis

3.1 General procedure for the solid-phase synthesis of methyl 6,6-dihalo-, 6α-halo and 6,6-dihydro-2β-methyl substituted penicillanates.

Merrifield resin (0.5 g, ~0.8 mmol/g) was swelled by gentle stirring in DMF (3 mL) and then the corresponding penicillanic acid (0.6 mmol) in DMF (1 mL) and potassium fluoride (0.07 g, 0.7 mmol) were added. This suspension was stirred at 60ºC for 24 h. After filtration, the resin was sequentially washed with EtOH (3x), water (3x), MeOH (3x) and CH_2Cl_2 (3x) and finally dried under high vacuum to afford resin **1a-c** which was taken onto the next step. Immobilized penicillin **1a-c** (478 mg, 0.62 mmol/g) was swollen in CH_2Cl_2 (4 mL) and *m*-chloroperbenzoic acid (MCPBA, 66 mg, 0.379 mmol, 1.5 equiv) in CH_2Cl_2 (1 mL) was added at 0°C. The reaction mixture was stirred overnight at the same temperature, after which the resin sulfoxide **2a-c** was filtered, washed with CH_2Cl_2(3x), EtOAc (3x), MeOH (3x), CH_2Cl_2 (3x), and finally dried in vacuo. An aliquot of resin **2a-c** (241 mg, 0.153 mmol) in benzene (5 mL) was treated with 2-mercaptobenzothiazole (2-MBT, 38 mg, 0.23 mmol, 1.5 equiv.) and stirred at reflux for 12 h. After filtration, the resin was sequentially washed with CH_2Cl_2 (3x), EtOAc (3x), MeOH (3x), CH_2Cl_2 (3x), and finally dried under high vacuum to afford resin **3e** which was taken onto the next step. Resin **3e** (99 mg, 0.06 mmol) was swelled in CH_2Cl_2 (1.3 mL) and treated with sulfuryl chloride (9 mg, 0.067 mmol) at -40°C for 1h and then quenched by adding 5% $NaHCO_3$. The obtained resin was filtered, washed with H_2O (3x), MeOH (3x), AcOEt (3x), CH_2Cl_2 (3x) and drying in vacuo to afford the immobilized 2β-choromethyl penicillin derivatives (**4a-c**). This resin was then cooled at 0ºC and treated with aluminum chloride as previously reported[2] and finally treated with diazomethane in ethyl ether to give the crude product that was purified by flash column chromatography (silica gel, AcOEt/hexane) to afford the desired penicillin derivatives (**5b-c**). On the other hand, another aliquot of resin **2a-c** (435 mg, 0.274 mmol) in toluene (8 mL) was treated with the corresponding heterocyclic thiol (4 equiv.) in the presence of catalytic amounts of *p*-TsOH and stirred at reflux for 4h. After that, the suspension was filtered, washed with MeOH (3x), EtOAc (3x), CH_2Cl_2 (3x) and drying in vacuo to give the corresponding immobilized penicillins (**8ae-bk**) that was then subjected to the aluminum chloride / diazomethane procedure to yield the 2β-(heterocyclyl)thiomethyl penicillins (**9ae-bk**).

Compounds **5b**, **5c**, **7a**, **9ae**, **9af,** **9ah**, **9be**, **9bf**, **9bh** and **9bj** have been previously characterized.[3]

3.1.1 (2*S*,3*R*,5*R*)-Methyl 6,6-dibromo-3-methyl-7-oxo-3-((1-phenyl-1*H*-tetrazol-5-ylthio)methyl)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (9ag)

According to the general procedure, **9ag** was obtained in 43% overall yield based on the manufacturer's loading of Merrifield resin. IR (film) 1798 (β-lactam), 1747 (ester) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 1.57 (s, 3 H, Me-α), 3.99 (d, AB system, *J* = 12 Hz, 1 H, CH_2S-Het), 3.82 (d, AB system, *J* = 12 Hz, 1 H, CH_2S-Het), 3.81 (s, 3 H, Me), 4.85 (s, 1 H, 2-H), 5.83 (s, 1 H, 5-H), 7.58 (s, 5 H, Ar). ^{13}C NMR (75 MHz, $CDCl_3$): δ 22.69, 46.26, 52.82, 58.26, 66.97, 69.45, 80.53, 124.00, 129.99, 130.47, 133.33, 153.62, 163.679, 166.44.

3.1.2 (2*S*,3*R*,5*R*)-Methyl 3-((1*H*-tetrazol-5-ylthio)methyl)-6,6-dibromo-3-methyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (9ai)

According to the general procedure, **9ai** was obtained in 52% overall yield based on the manufacturer's loading of Merrifield resin. IR (film) 1798 (β-lactam), 1732 cm^{-1} (ester). ^1H NMR (300 MHz, $CDCl_3$): δ 1.55 (s, 3 H, Me-α), 3.78 (d, AB system, *J*=14 Hz, 1 H, CH_2S-Het), 3.98 (s, 3H, Me), 3.93 (d, AB system, *J*=14 Hz, 1 H, CH_2S-Het), 4.84 (s, 1 H, 2-H), 5.84 (s, 1 H, 5-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 22.6, 33.4, 46.1, 52.7, 58.1, 66.7, 69.4, 163.7, 163.7, 166.3.

3.1.3 (2*S*,3*R*,5R,6*S*)-Methyl 6-chloro-3-((4,5-dihydrothiazol-2-ylthio)methyl)-3-methyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (9bk)

According to the general procedure, **9bk** was obtained in 48% overall yield based on the manufacturer's loading of Merrifield resin. IR (film): 1790 (β-lactam), 1748 cm^{-1} (ester). ^1H NMR (300 MHz, $CDCl_3$): δ 1.49 (s, 3 H, Me-α), 3.40 (d, AB system, *J* = 14 Hz, 1 H, CH_2S-Het), 3.48 (d, AB system, *J* = 14 Hz, 1 H, CH_2S-Het), 3,64 (t, 2 H, dihydrothiazole), 3.81 (s, 3 H, Me), 4.18 (t, 2 H, dihydrothiazole), 4.85 (s, 1H, 2-H), 5.15 (d, *J* = 3 Hz, 1 H, 6-H), 5.35 (d, *J*= 3 Hz, 1 H, 5-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 22.31, 35.93, 44.74, 52.54, 58.61, 63.56, 66.56, 69.19, 80.26, 163.41, 164.18, 166.66.

3.1.4 (2*S*,3*R*,5*R*)-Methyl 3-((4,5-diphenyloxazol-2-ylthio)methyl)-3-methyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (9df)

Compound **9bf**, obtaining according to the general procedure, was treated with tri-butyl phosphine (1.5 equiv.) in methanol at 0°C for 30 min. Then, methanol was evaporated, and the residue was purified by column chromatography to afford **9df** in 32% yield. IR (film) 1790 (β-lactam), 1750 cm^{-1} (ester). ^1H NMR ($CDCl_3$): δ 1.61 (s, 3 H, Me-α), 3.17 (dd, *J*= 16, 1.7 Hz, 1 H, 6-H), 3.58 (dd, *J*= 16, 4.1 Hz, 1 H, 6-H), 3.75 (d, *J*= 14 Hz, 1 H, CH_2S-Het), 3.79 (s, 3 H, Me), 3.88 (d, *J*= 14 Hz, 1 H, CH_2S-Het), 4.87 (s, 1 H, 2-H), 5.36 (dd, *J*= 4, 1.6 Hz, 1 H, 5-H), 7.68-7.31 (m, 10 H, Ar). ^{13}C NMR ($CDCl_3$): δ 23.9, 45.6, 47.5, 52.4, 61.0, 67.3, 70.3, 126.4, 127.8, 128.3, 128.5, 128.6, 147.5, 158.0, 168.0, 171.4.

3.1.5 (2*S*,3*R*,5*R*,6*S*)-Methyl 6-chloro-3-((6-ethylbenzo[*d*]thiazol-2-ylsulfonyl) methyl)-3-methyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate 4,4-dioxide (10bj)

Compound **9bj**, obtaining according to the general procedure, was oxidized by treatment with *m*-CPBA (3 equiv.) in DCM at 0°C for 1h. The reaction mixture allowed to reach room temperature. After completion of reaction, monitored using tlc, organic layer was washed with $NaHCO_3$, H_2O and dried over anhydrous Na_2SO_4, solvent was evaporated under reduced pressure to yield a crude product which was purified via column chromatography to give **10bj** in 34% yield. IR (film) 1810 (β-lactam), 1755 cm^{-1} (ester). ^1H NMR ($CDCl_3$): δ 1.49 (t, *J*= 7Hz, 3 H, CH_2-CH_3), 1.64 (s, 3 H, Me-α), 3.77(d, *J*= 14Hz, 1 H, CH_2S-Het), 3.89 (s, 3 H, OCH_3), 4.03 (d, *J*= 14Hz, 1 H, CH_2S-Het), 4.14 (c, *J*= 7Hz, 2 H, CH_2-CH_3), 4.61 (s, 1 H, 2-H), 5.06 (d, *J*= 1.4 Hz, 1 H, 6-H), 5.14 (d, *J*= 1.4 Hz, 1 H, 5-H), 7.18 (dd, *J*= 9, 2.4 Hz, 1 H, Ar), 7.42 (d, *J*= 2.2Hz, 1 H, Ar), 7.96 (d, *J*= 9 Hz, 1 H; Ar). ^{13}C NMR ($CDCl_3$): δ 14.7. 53.7, 57.5, 64.3, 65.3, 74.7, 79.7, 104.6, 117.8, 124.7, 147.9, 158.2, 165.3, 167.2.

3.2 General procedure for the synthesis of benzyl 6,6-dihalo- and 6,6-dihydro-2β-methyl substituted penicillanates in homogeneous phase.

6.6-Dibromopenicillanic acid (600 mg, 1.67 mmol), tetrabutylammonium iodide (122 mg, 0.33 mmol) and catalytic amounts of DMAP were dissolved in anhydrous $CHCl_3$ (10 mL) under nitrogen atmosphere. Then, triethylamine (349 µL, 2.5 mmol) and benzyl chloride (287 µL, 2.5 mmol) were added dropwise. The reaction mixture was heated at reflux for 24 h and then filtered on silica gel. Final purification was performed by column chromatography to obtain the benzyl 6,6-dibromopenicillanate in 89% yield (688 mg, 1.48 mmol). This product was then dissolved in anhydrous DCM and 1 equiv. of m-CPBA in DCM was added dropwise at 0° C. Stirring was continued at 0° C until reaction completion monitored by TLC (35 min). The crude was washed with $NaHCO_3$ solution and water. The organic layer was dried with Na_2SO_4 and solvent removed in vacuo. Finally, purification was achieved by column chromatography yielding the benzyl 6,6-dibromopenicillanate sulfoxide **11a** (493 mg, 70% yield). Sulfoxide **11a** (493 mg, 1.09 mmol) was dissolved in toluene (10mL) and treated with the corresponding heterocyclic thiol (1.09 mmol, 1 equiv.) in the presence of catalytic amounts of p-TsOH (0.054 mmol, 10.3 mg) and stirred at reflux temperature for 5 h. Solvent was removed by evaporation and the crude was purified by column chromatography to give the corresponding 2β-methyl substituted penicillanates **12ae-ak**.

3.2.1 (2*S*,3*R*,5*R*)-Benzyl 3-((benzo[*d*]thiazol-2-ylthio)methyl)-3-methyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (13de)

Compound **12ae**, obtaining according to the general procedure, was treated with tri-butyl phosphine (1.5 equiv.) in methanol at 0°C for 30 min. Then, methanol was evaporated, and the residue was purified by column chromatography to afford **13de** in 46% yield. IR (film) 1782 (β-lactam), 1750 cm^{-1} (ester). ^1H NMR ($CDCl_3$): δ 1.52 (s, 3 H, Me-α). 3.19 (dd, *J*= 15.9, 1.8 Hz, 1 H, 6-H), 3.58 (dd, *J*= 15.9, 4.1 Hz, 1H, 6-H), 3.87 (d, *J*= 13.8 Hz, 1H, CH_2S-Het), 4.05 (d, *J*= 13.8 Hz, 1 H, CH_2S-Het), 4.88 (s, 1H, 2-H), 5.21 (s, 2 H, CH_2Bn), 5.36 (dd, *J*= 4, 1.7 Hz, 1 H, 5-H), 7.46-7.29 (m, 7 H, Ar), 7.76 (d, *J*= 7.7 Hz, 1 H, Ar), 7.85 (d, *J*= 7.7 Hz, 1 H, Ar). ^{13}C NMR ($CDCl_3$): δ 24.0, 45.7, 47.6, 61.1, 67.5, 70.6, 121.0, 121.6, 124.5, 126.1, 128.7, 134.6, 135.3, 152.6, 165.5, 167.4, 171.4.

3.2.2 (2*S*,3*R*,5*R*)-Benzyl 3-((4,5-diphenyloxazol-2-ylthio)methyl)-3-methyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (13df)

Compound **12af**, obtaining according to the general procedure, was treated with tri-butyl phosphine (1.5 equiv.) in methanol at 0°C for 30 min. Then, methanol was evaporated, and the residue was purified by column chromatography to afford **13df** in 17% yield. IR (film) 1790 (β-lactam), 1748 cm^{-1} (ester). ^1H NMR ($CDCl_3$): δ 1.54 (s, 3 H, Me-α). 3.17 (dd, *J*= 16, 1.7 Hz, 1H, 6-H), 3.55 (dd, *J*= 16, 4.1 Hz, 1H, 6-H), 3.72 (d, *J*= 14 Hz, 1H, CH_2S-Het), 3.85 (d, *J*= 14 Hz, 1 H, CH_2S-Het), 4.90 (s, 1 H, 2-H), 5.22 (s, 2 H, CH_2Bn), 5.36 (dd, *J*=4, 1.6 Hz, 1 H, 5-H), 7.37 (m, 11 H, Ar), 7.62 (m, 4 H, Ar). ^{13}C NMR ($CDCl_3$): δ 23.8. 45.7, 47.6, 61.1, 67.3, 67.5, 70.4, 126.4, 127.8, 128.5, 128.6, 131.9, 134.6, 136.3, 147.5, 158.0, 167.4, 171.4.

3.2.3 (2*S*,3*R*,5*R*)-Benzyl 3-((4,5-dihydrothiazol-2-ylthio)methyl)-3-methyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (13dk)

Compound **12ak**, obtaining according to the general procedure, was treated with tri-butyl phosphine (1.5 equiv.) in methanol at 0°C for 30 min. Then, methanol was evaporated, and the residue was purified by column chromatography to afford **13dk** in 19% yield. IR (film) 1785 (β-lactam), 1755 cm^{-1} (ester). ^1H NMR ($CDCl_3$): δ 1.45 (s, 3 H, Me-α), 3.16 (dd, *J*= 16, 1.7 Hz, 1H, 6-H), 3.41 (t, *J*= 8Hz, 2H, CH_2), 3.55 (dd, *J*= 16, 4 Hz, 1H, 6-H), 3.62 (d, *J*= 14 Hz, 1 H, CH_2S-Het), 3.71 (d, *J*= 14 Hz, 1 H, CH_2S-Het), 4.17 (t, *J*= 8Hz, 2H, CH_2), 4.78 (s, 1 H, 2-H), 5.20 (s, 2 H, CH_2Bn), 5.36 (dd, *J*= 4, 1.7 Hz, 1 H, 5-H), 7.38 (m, 5 H, Ar). ^{13}C NMR ($CDCl_3$): δ 23.8, 35.8, 44.9, 47.5, 60.9, 63.7, 67.4, 70.3, 128.6, 134.7, 167.5, 177.3.

3.2.4 (2*S*,3*R*,5*R*)-Benzyl-3-((benzo[d]thiazol-2-ylthio)methyl)-3-methyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate 4,4-dioxide (14de)

Compound **13de**, obtaining according to procedure described in 4.3.2.2, was oxidized by treatment with *m*-CPBA (3 equiv.) in DCM at 0°C for 1h. The reaction mixture allowed to reach room temperature. After completion of reaction, monitored using tlc, organic layer was washed with $NaHCO_3$, H_2O and dried over anhydrous Na_2SO_4, solvent was evaporated under reduced pressure to yield a crude product which was purified via column chromatography to give **14de** in 11% yield. IR (film) 1805 (β-lactam), 1752 cm^{-1} (ester). ^1H NMR ($CDCl_3$): δ 1.27 (s, 3 H, Me-α), 3.37 (m, 2 H, 6-H), 3.98 (d, *J*= 14.5 Hz, 1H, CH_2S-Het), 4.44 (d, *J*= 14.5 Hz, 1H, CH_2S-Het), 4.71 (s, 1H, 2-H), 4.93 (dd, *J*= 3.6, 2.8 Hz, 1H, 5-H), 5.29 (s, 2 H, CH_2Bn), 7.49-7.29 (m, 7 H, Ar), 7.77 (m, 2 H, Ar), ^{13}C NMR ($CDCl_3$): δ 14.8, 34.9, 36.4, 65.0, 68.3, 70.6, 79.7, 121.1, 121.7, 124.7, 126.2, 128.8, 128.9, 134.4, 135.6, 152.6, 164.8, 167.6, 170.1.

3.2.5 (2S,3R,5R)-Benzyl 6,6-dibromo-3-(chloromethyl)-3-methyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (15a)

According to the general procedure, **11a**[4] was obtained in 32% yield (based on compound **11a**). IR (film) 1790 (β-lactam), 1752 cm^{-1} (ester). ^1H NMR (300 MHz, $CDCl_3$): δ 1.44 (s,3H, Me-α), 3.56 (d, 1H, AB system, *J*=11 Hz, CH_2Ph), 3.64 (d, 1H, AB system, *J*=11 Hz, CH_2Ph), 5.09 (s, 1H, 2-H), 5.23 (d, 2H, *J*= 2 Hz, CH_2Cl), 5.82 (s, 1H, 5-H), 7.38 (s, 5H, Ar). ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.1, 52.1, 58.4, 64.3, 67.9, 68.6, 80.2, 128.7. 128.8, 128.9, 134.4, 163.7, 166.1.

Notes and references

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