Efficient Solid-Phase Lipopeptide Synthesis Employing the Ellman Sulfonamide Linker

Jose M. Palomo, Maria Lumbierres, and Herbert Waldmann*

Reagents and chemicals were purchased from Novabiochem, Fluka, Aldrich, Advanced Chemtech, and Biosolve were used without further purification. Electrospray mass spectroscopy (ESI-MS) was performed in the positive mode on an Agilent 1100 series instrument. Analytical reverse phase HPLC separation was performed using a C4 column using isocratic elution with 70% acetonitrile/29% water/1% TFA for 30 min and a Finnigan Thermoquest LCQ. 1H data were recorded on a Varian Mercury 400 spectrometer. Optical rotations were measured with a Perkin-Elmer Polarimeter 341. MALDI MS spectra were measured using 2,5-dihydroxybenzoic acid (DHB) as matrix.

General conditions for the synthesis of lipidated and labeled peptides with the alkanesulfonamide linker

For all reactions commercially available 4-sulfamoylbutyryl-aminomethyl resin (loading 1.1 mmol/g) from Novabiochem was used. All solid-phase reactions were carried out in a 50 mL solid phase peptide synthesis reactor. Agitation was achieved with an orbital shaker. The yield and scale of the solid-phase reactions are given with regard to the amount of the first amino acid coupled to the resin. The resin loading was determined by measuring the Fmoc groups remaining on the resin by the established UV method. To this end, a small amount of resin (~3 mg) was treated with 20% piperidine/DMF solution (10 mL) for 30 min and the UV absorption of the solution at 301 nm (ε = 7800 M⁻¹cm⁻¹) was determined.
Conditions for coupling of the first amino acid to the sulfonamide resin.

Fmoc-Cys (Far)-OH was coupled to the resin using TFFH and DIPEA (1:1:2) for 1.5 h at room temperature in DMF:CH₂Cl₂ (1:1) and Fmoc-Cys(GerMant)-OH was coupled using 1.5 equivalent for 3 hours (1.5:1:2). This procedure was repeated three times giving 65% yield for both building blocks. Subsequently the remaining amino groups on the resin were capped by treatment with Ac₂O (3 eq) and pyridine (3 eq).

After the first amino acid had been coupled, all protected amino acids and NBD-labeled Lys were coupled by using HBTU /HOBt chemistry. Typically, the amino acid (AA) (5 equiv) was treated for 2 minutes with HBTU (5 equiv), HOBt (5 equiv) and DIPEA (10 equiv) in DMF. The solution was added to the resin which was then agitated for 2 hours at room temperature. After coupling and Fmoc-deprotection with DMF the resin was washed (5 x 2 min). Every step was carried out under an argon atmosphere except for the washings.

The Fmoc-Cys(Pal)-OH building block was coupled using reactivation with HBTU/HOBt/TMP in CH₂Cl₂/DMF (1:1) using 4 equivalents for 5 hours.

After incorporation of Fmoc-Cys(Pal)-OH, the Fmoc group was cleaved by treatment with 1% DBU/DMF (1 mL/0.1 g of resin, 2 x 30 seconds). In order to avoid an S,N-shift, 5 eq HATU in CH₂Cl₂/DMF (7:1) and 20 eq DIPEA was used for preactivation of the subsequent coupling of amino acid. For the longest peptides, after 8 amino acids had been coupled to the resin, the coupling time was increased up to 3-4 h and HATU (5 eq) and DIPEA (10 eq) in DMF were used.

General procedure for alkylation and activation with iodoacetonitrile.

The resin-bound N-acylsulfonamide (150 mg) was washed three times with NMP (3 x 5 mL). After filtration through an alumina basic plug prior to use, a mixture of iodoacetonitrile (20 eq) and DIPEA (10 eq) in NMP (4 mL) was added to the resin in an argon atmosphere and the reaction flask was shielded from light. The resin was agitated for 24 h, filtered, and
successively washed with dry NMP (5 x 5 mL, 10 min each time), CH$_2$Cl$_2$ (5x 5 mL, 10 min) and THF (3x 5 mL, 5 min).

**Cleavage of peptides from the solid support.**

The activated resin was treated with a solution of nucleophile (dry MeOH or H$_2$O) (20-40 eq) and DMAP (0.8 eq) in dry THF (3 mL/0.05 g of resin) for 24 hours under an argon atmosphere. The resin was filtered and washed with THF and CH$_2$Cl$_2$ (3x 5 mL each). The solvents were evaporated under reduced pressure, the crude residue was dissolved in CH$_2$Cl$_2$ and the solution was extracted with water containing 0.5% TFA. After re-extraction of the water phase with CH$_2$Cl$_2$ the combined organic solvents were dried with MgSO$_4$ and the solvent was evaporated. The crude was washed several times with cold ether (5 x 2 mL) and dried *in vacuo*.

**Removal of the protecting groups**

The protecting groups were removed by treatment with a cocktail consisting of TFA/TIS/H$_2$O/DTE (94:1:2.5:2.5) (v/v/v/v) for 2 h at room temperature. A solution of 1% TFA, 2% triethylsilane in CH$_2$Cl$_2$ (2 mL/0.10 mg g of peptide) was added to the farnesylated cysteine containing peptides for 2 h at r.t. to remove triphenylmethane (Trt) or 4-methoxytriphenylmethane (Mtt) blocking groups. The solvent was evaporated under reduced pressure and the crude residue was washed 3 times with toluene followed by evaporation to dryness. The residue was transferred to a sinter funnel and washed twice with pentane.

**MIC-Gly-Cys(Pal)-Met-Gly-Leu-Pro-Cys(Far)-OMe, 10**

Yield: 66 %; [α]$_D^{20}$ = -17.1° (c = 0.4 in CHCl$_3$). $^1$H-NMR (400 MHz, CDCl$_3$): δ = 6.62 (s, 2H, MIC), 5.12 (t, $J$ = 7.2 Hz, 1H, Far), 5.02 (m, 2H, Far), 4.52 (m, 1H) 4.33 (t, $J$ = 7.0 Hz, 1H), 4.21 (t, $J$ = 7.0 Hz, 1H), 3.99 (m, 2H), 3.78-3.44 (m, 7H, distinguished singlet at 3.68, OMe), 3.12-3.23 (m, 3H), 2.89-3.04 (m, 2H), 2.65-2.70 (m, 1H), 2.54 (t, $J$ = 7.2 Hz, 2H, Pal),
2.46 (t, J = 7.2 Hz, 2H), 2.19-1.95 (m, 19H), 1.66-1.53 (m, 17H), 1.35-1.15 (m, 30 H), 0.92-0.79 (m, 9H, Leu, Pal). MS (ESI) +m/z: calcld for C_{68}H_{112}N_{8}O_{12}S_{3} [M+H]^+ 1329.80; found 1329.44.

**Ac-Lys(NBD)-Ser-Gly-Ser-Gly-Gly-Cys(Pal)-Cys(Far)-OMe, 14**

Acetylation of the N-terminus: after cleavage of the N-terminal Fmoc-group from the Lys residue, the N-terminus was acetylated using a cocktail of 5 equivalents of acetic acid/HBTU/HOBt/DIPEA in CH_2Cl_2/DMF (1:1) for 8 hours. After removal of the protecting groups the crude residue was filtered through a short silica cartridge (2 mL) using ethyl acetate and 10% methanol as the eluent. Yield: 44%; [α]_D^{20} = 15° (c = 0.10 in CHCl_3/2,2,2-trifluoroethanol 3:1, v/v). 1^H-NMR (400 MHz, CDCl_3): δ= 8.1 (d, J = 8.2, 1H, NBD), 6.6 (d, J = 8.2, 1H, NBD), 5.5-5.3 (m, 3H, Far), 4.05-3.90 (m, 4H), 3.5 (s, 3H, OMe), 3.4-3.3 (m, 3H), 2.89-3.04 (m, 2H), 2.48 (m, 2H), 2.2-1.91 (m, 13H, CH_2 Far), 1.90-1.50 (m, 19H, 4x CH_3 Far, CH_2 Lys, CH_2 Pal), 1.40-1.15 (m, 28 H, Pal, 2 x CH_2 Lys), 0.95 (m, 3H, Pal). MS (ESI) +m/z: calcld for C_{64}H_{102}N_{12}O_{16}S_{2} [M+H]^+ = 1359.70, found 1359.38.


After cleavage and deprotection, the oily residue was filtered through a short silica gel column (2 mL) using ethyl acetate and 5% methanol as the eluent. Yield: 39%; [α]_D^{20} = -6.3° (c = 0.24 in CHCl_3/2,2,2-trifluoroethanol 3:1, v/v). 1^H-NMR (400 MHz, CD_3OD): (characteristic signals): δ= 8.1 (d, J = 7.9, 1H, NBD), 7.1-6.8 (m, 10H, Trp, Tyr), 6.6 (m, 1H, NBD), 5.2 (s, 1H, OH Tyr), 3.85 (m, 4H), 2.90-2.65 (m,10H), 2.4 (t, J = 7.0 Hz, 4H, Pal), 2.3-2.1 (m, 8H), 1.85-1.5 (m, 34H, 2 CH_2 biotin, 2 x CH_2 Aca, 2 x CH Leu, 2 x CH_2 Leu, 4 x CH_2 Arg, 2 x CH_2 Lys, SH, CH_3 ala, 2x CH_2 Pal), 1.40-1.24 (m, 54H, 24 x CH_2 Pal, CH_2 Lys, CH_2 Aca,
CH₂ biotin), 0.96-0.80 (m, 16H, 4 xCH₃ Leu, 2x CH₃ Pal ). MS (ESI) +m/z: calcd for C₁₄⁷H₂₂₉N₃₉O₃₀S₄ (3122.88) [M+2Na+H]⁴⁺ = 1055.88, found 1055.67.


After cleavage the resin was washed with CHCl₃ and CHCl₃/2,2,2-trifluoroethanol (3x 5 mL). The solvent was removed under reduced pressure and the peptide was sequentially washed with CH₃CN, MeOH, and CH₂Cl₂. The target peptide is insoluble in these solvents. The residue was dissolved in a mixture of CF₃CO₂H/ethanedithiol/water/TIS 94:2.5:2.5:1 (v/v/v/v) and shaken at 20°C for 60 min. The solvent was evaporated under reduced pressure and the residue was washed twice with toluene followed by evaporation to dryness. The solid residue was washed twice with pentane and then with cold ether. The lipidated peptide is soluble in CHCl₃/2,2,2-trifluoroethanol 3:1 (v/v) containing 1% TFA, (completely soluble); Yield 24%; [α]D²⁰ = -17.5° (c = 0.10 in CHCl₃/2,2,2-trifluoroethanol 3:1 v/v). ¹H-NMR (500 MHz, [D₆]-DMSO): δ = 8.20-7.70 (m, 21 H; CONH), 5.6 (s, 1 H; CONH), 4.54-4.06 (m, 18 H; CH), 3.80-3.40 (m, 26 H), 3.05 (m, 4 H; 2 Cys CH₂), 2.75 (br s, 2 H; CH₂ Lys), 2.4 (br s, 4 H; 2 x CH₂ Pal), 2.34-2.30 (m, 4 H; CH₂ Gln, CH₂ Glu), 2.10-1.98 (m, 5 H; CH₂ Myr, CH₂ Asn, CH Val), 1.70-1.4 (m, 48 H; β-CH₂ Gln, β-CH₂ Glu, 3 x β-CH₂ Pro, 3 x γ-CH₂ Pro,β-CH₂ Lys, Myr β-CH₂, Myr γ-CH₂, 2 x CH₂ Pal, 6 x γ-CH Leu, 6 x β-CH₂ Leu, δ-CH₂ Lys, γ-CH₂ Lys), 1.24 (br s, 66 H; 9 x CH₂ Myr, 24 x CH₂ Pal), 0.90-0.80 (m, 51 H; CH₃ Myr, 2 x CH₃ Pal, 12 x ω-CH₃ Leu, 2 x ω-CH₃ Val); MS (ESI-MS): m/z: calcd for C₁₄₉H₂₅₉N₂₉O₃₅S₂ (3078.88) [M+H+K]²⁺ 1559.44; found: 1559.50.

**Figure 1.** RP-HPLC trace of crude lapidated peptide 15. For reverse phase HPLC separation a C₄ column using isocratic elution with 70% acetonitrile/29% water/1% TFA and a Finnigan Thermoquest LCQ were used.