A Robust Strategy for the Preparation of Libraries of Metallopeptides. A New Paradigm for the Discovery of Targeted Molecular Imaging and Therapy Agents

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Large Scale Preparation of Fmoc-SAAC (1)

Fmoc-L-lysine (30.0 g, 81.5 mmol) and $Na[BH(OAc)_3]$ (45.0 g, 212 mmol) were combined in anhydrous 1,2-dichloroethane (400 mL) under an inert (argon) atmosphere and the resulting suspension was cooled to 0^oC. A solution of 2- pyridine-carboxaldehyde (18 mL, 188.55 mmol) in 1,2-dichloroethane (60 mL) was added to the reaction mixture over 45 min. The suspension was stirred at 22°C for 1.5h, then cooled to 0°C prior to the slow addition of water (300 mL) and chloroform (300 mL). The organic layer was separated and washed with water (200 mL), brine (200 mL) and dried over Na_2SO_4. The mixture was filtered, the solid material washed with $CHCl_3$ (100 mL) and the filtrate evaporated to dryness. The residue was purified by flash chromatography on a silica column (4′ x 8′) eluted with CH_2Cl_2:CH_3OH (5:1, approx. 8L). The appropriate fractions were combined and evaporated to dryness; the resulting solid was dried overnight under high vacuum to yield **1** (36.79 g, 82%). The characterization data were consistent with those reported in the literature.[1]

Preparation of $[Re(CO)_3(SAAC)]Br$ (3)

Fmoc-SAAC (**1**) (2.77 g, 5.02 mmol) was combined with $[Re(CO)_3(H_2O)_3]Br$[2] (2.45 g, 6.06 mmol) in a 20 mL Emery microwave vial. The solids were dissolved in a mixture of acetonitrile (10 mL) and water (4 mL) and the resulting solution heated in a Biotage Initiator 60 microwave reactor at 110 °C for 11 min. The solvent was removed by rotary evaporator, the residue dissolved in dichloromethane (50 mL) and washed with water (3 x 30 mL). The organic phase was dried over sodium sulfate prior to removal of the solvent by rotary evaporator. Compound **3** was isolated as a pale yellow powder (4.02

g, 89 %). Spectroscopic data were consistent with those reported in the literature.[3] Note that the use of CH_3OH in this synthesis should be avoided, as it can lead to formation of the methyl ester of **3**.

Preparation of $[Re(CO)_3(SAACQ)]Br$ (4)

Fmoc-SAACQ (**2**) (0.390 g, 0.649 mmol) was combined with $[Re(CO)_3(H_2O)_3]Br$ (0.323 g, 0.799 mmol) in a 5 mL Emery microwave vial. The solids were dissolved in a mixture of acetonitrile (3 mL) and water (1 mL) and the resulting solution was heated in a Biotage Initiator 60 microwave reactor at 110 °C for 11 min. The solvent was removed from the reaction mixture by rotary evaporator, the residue dissolved in dichloromethane (30 mL) and washed with water (3 x 25 mL). The organic phase was dried over sodium sulfate prior to removal of the solvent by rotary evaporator; compound **4** was isolated as a pale yellow powder (0.401 g, 62 %). Spectroscopic data were consistent with those reported in the literature.[4] The purity of **4** was increased to > 99.9 % by automated reverse phase chromatography using a Biotage SP1 autopurification system with a C18 column and a gradient of 20-80% CH_3CN *vs.* H_2O. Note that the use of CH_3OH in this synthesis should be avoided, as it can lead to formation of the methyl ester of **4**.

General Procedure for Construction of Peptide Libraries

A Four Season Shaker (Figure 1) with up to 20 ISOLUTE SPE columns (25 mL, Biotage) encased in aluminum sheathes was employed for library construction. Each vessel was fitted with a fine porosity frit, a drainage tube and a cap to allow for filtration by pressurizing the top of the tube with air. Peptide libraries were constructed using

TentaGel S NH_2 (Fluka) resin (0.26 mmol/g, 90 μm) using standard Fmoc solid phase peptide synthesis techniques. Fmoc-amino acids with appropriate acid-labile side chain protecting groups were obtained from Advanced ChemTech.

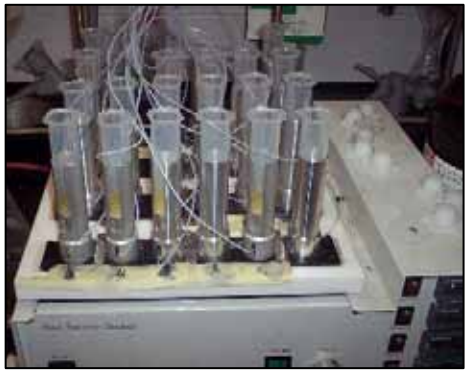

Figure 1 – Apparatus used in the construction of peptide libraries.

In a typical experiment, an appropriate amount of resin was swelled in DMF for 18 h prior to the commencement of the synthesis, then washed thoroughly with DMF, CH_3OH and DMF, and re-suspended in an isotonic DMF/CH_2Cl_2 mixture (2:1 v/v, 20 ml). An equal amount (1 mL) of the resin suspension was transferred to each of the 19 reaction vessels; the remaining suspension of beads (1 mL) was diluted to 20 mL (DMF/ CH_2Cl_2) and the distributed process repeated until essentially no resin beads remained in the original suspension.

A threefold excess (based on the resin loading) of each amino acid was combined with equimolar amounts of hydroxybenzotriazole (HOBt) and N,N'-diisopropylcarbo-diimide (DIC, Aldrich) and dissolved DMF (Aldrich, HPLC grade). These solutions were added to the appropriate reaction vessels which were shaken gently for the duration of the coupling reaction (60 min). To confirm that the coupling reaction was complete, two small samples of beads (2-6) were removed from the reaction vessel and washed with

CH_3OH: one was treated with a solution of bromophenol blue (1 % in CH_3CN),[5] the other with freshly prepared Kaiser Reagent.[6] The observed yellow colour of the beads indicated the absence of free amines and hence the completeness of the coupling step.

The resin in the reaction vessels was washed thoroughly with DMF, CH_3OH and again with DMF, then recombined into a single vessel and treated with piperidine (20 % in DMF, 10 mL, 2 x 15 min) to effect removal of the Fmoc group. Again, two small samples of the resin were washed and subjected to bromophenol blue[5] and Kaiser[6] tests, respectively; if yellow beads remained, the deprotection reaction was extended until the beads were uniformly dark blue due to the presence of a free amine at the *N*-terminus of the peptide. After thorough washing, the resin beads were redistributed into the 19 reaction vessels (as described above) prior to the addition of the next amino acids in the sequence.

At the conclusion of the synthesis, the resin beads were treated with a cleavage cocktail (30 mL) composed of phenol (7.5 % w/v), thioanisole (5 % v/v), water (5 % v/v), 1,2-ethanedithiol (2.5 % w/v) and trifluoroacetic acid to remove the side chain protecting groups. After 2.5 h, the suspension was filtered and the resin beads were washed thoroughly with DMF, CH_3OH, CH_2Cl_2, DMF, aqueous DMF (70 %, then 40 %), water, and finally phosphate buffered saline (PBS, pH 7.4). The beads were suspended in PBS in the presence of NaN_3 (0.05 %) and stored at 4 ºC.

Infrared Spectroscopy

The resin beads were ground and pressed into KBr pellets for analysis; Infrared spectra were obtained using a Bio-Rad FTS-40 FTIR spectrometer. The beads displayed

strong CO stretching absorptions characteristic of the $[Re(CO)_3]^+$ moiety at υ(CO) 1910 and 2028 cm^{-1}.

Screening of Resin-bound Peptides Using a COPAS System

Beads of Tentagel S resin loaded with the uPAR-binding peptide[7] (D-Glu)-Cha-Phe-(D-Ser)-(D-Tyr)-Tyr-Leu-Trp-Ser were washed twice with each of the following: water, Tris buffered saline (Tris = trishydroxymethylaminomethane, TBS; 50 mM Tris-HCl, 150 mM NaCl), and incubation buffer (50 mM Tris, 0.25 M NaCl, 0.1 % (w/v) BSA, 0.05 % (v/v) Tween-20, 0.02 % (w/v) NaN_3), then incubated in incubation buffer at 22 °C for 30 min. The beads were resuspended in recombinant human soluble uPAR (0.1μ M, 807-UK, R&D Systems) and incubated, rotating constantly, for 60 min at 22 °C. The beads were washed with incubation buffer supernatant (3 x) then resuspended in biotinylated anti-uPAR antibody (5 μg/ml, BAF807, R&D Systems) and incubated, rotating constantly, at 22 °C for 60 min. The resin beads were washed in incubation buffer (3 x), then resuspended in fluorescein isothiocyanate (FITC) conjugated streptavidin (0.02 mg/ml, ab7238, Abcam Inc.) and incubated, rotating constantly, at 22 °C for 60 min. The resin beads were then washed again in incubation buffer (3 x) and the stained beads analyzed using a COPAS™ Select large particle sorter.

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