Characterization of a PD-Sauvagine Analog as a New High-Affinity Radioligand for Corticotropin-Releasing Factor Studies

Supplementary Data

Supplementary Methods

Peptides and iodination.
Synthesis of PD-Svg and Tyr\(^{0}\)Glu\(^{1}\)-PD-Svg: The peptides were assembled on solid phase with Fmoc-chemistry on the CEM Liberty 1 automated microwave-assisted peptide synthesizer (CEM Corporation, Matthews, NC). Rink amide ChemMatrix resin (Pcas Biomatrix Inc., St-Jean-sur-Richelieu, Quebec, Canada) with a capacity of 0.46 mmol/g afforded an amidated C-terminus. All Fmoc-N\(^{\alpha}\)-protected amino acids with side chain protection: tert-butyl for Ser; Thr, Tyr, 2,2,4,6,7-pentamethyl-dihydro-benzofuran-5-sulfonyl for Arg; tert-butoxycarbonyl for Lys; trityl for Asn; Gln, His, Cys and tert-butyl ester for Asp, Glu (Bachem Inc., Torrance, CA; Chem Impex, Wood Dale, IL; EMD Chemicals, Novabiochem, Gibbstown, NJ; Genzyme Pharmaceuticals, Cambridge, MA; Reanal, Budapest, Hungary). Coupling reagent 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylyluronium hexafluorophosphate (HATU) (P3BioSystems, LLC, Shelbyville, KY), N,N-diisopropylethylamine (DIEA), and piperidine (Chem Impex, Wood Dale, IL) were used, and all solvents and reagents were ACS grade and were used without further purification.

Coupling reactions were performed on 0.34 g and 0.38 g of Rink amide ChemMatrix resin (~0.15 mmol and ~0.17 mmol), respectively in the presence of a 5-fold molar excess of 0.2 M Fmoc-protected amino acids dissolved in DMF (except for His in NMP) with activation by HATU:DIEA:AA (0.9:2:1) at 0 W at RT for 4 min and at 15 W with a maximum temperature of 50°C for 11 minutes. All residues were double coupled. Double coupling conditions of Arg were altered to 0 W for 25 min followed by at 15 W for 10 min with a maximum temperature of 50°C. Deprotection of the Fmoc group was performed with 20% piperidine containing 0.1 M HOBT in DMF in two stages (using a fresh reagent each time) with an initial deprotection of 2 min at 40 W followed by deprotection of 6 min at 40 W with a maximum temperature of 50°C. At the end of the syntheses, 1.03 g and 1.25 g of resin were obtained, respectively. Cleavage and simultaneous deprotection of the peptides were performed by stirring the resins with 10 ml of a reagent containing 95%TFA/2.5%water/2.5%TIS (Tri-isopropylsilane) for 3 h. Following cleavage, the peptides were precipitated and washed using ice-cold anhydrous ethyl ether, the mixtures were centrifuged, the precipitates were dissolved in 0.1% TFA/40% water/60% acetonitrile separated by filtration from the resin and lyophilized. 280 mg and 300 mg of crude peptides were acquired, respectively.

The crude, lyophilized peptides were purified by preparative HPLC on Waters PrepPak cartridge (4.7 x 30 cm) packed with Bondapak C\(_{18}\) (15-20 µm particle size) in three solvent systems TEAP (Triethylammonium phosphate) at pH 2.25, pH 6.5, and 0.1% TFA/ acetonitrile using a Waters Assoc. Prep LC/System 500A (Waters Corporation Milford, MA). The peptides eluted with a flow rate of 100 ml/min using a linear gradient of 1% B per 2 min increase from the baseline % B (eluient A = 0.25 N TEAP pH 2.25, eluent B = 60% CH\(_{3}\)CN, 40% A). A second purification step was carried out using a linear gradient of 1% B per min increase from the baseline % B (eluient A = 0.25 N TEAP pH 6.5, eluent B = 60% CH\(_{3}\)CN, 40% A). The third purification step was carried out using a linear gradient of 2% B per
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A min increase from the baseline % B (eluent A = 0.1% TFA in water, eluent B = 60% CH₃CN, 40% A) on the same cartridge. Analytical HPLC screening of the collected fractions was performed on a Vydac C₁₈ column (0.46 x 25 cm, 5 μm particle size, 300Å pore size) connected to a Rheodyne injector, two Waters pumps Model 501, System Controller Programmer, Kratos 750 UV detector, and Houston Instruments D-5000 strip chart recorder. The fractions containing the product were pooled and subjected to lyophilization. The final yields were 65 mg of PD-Svg and 37 mg of Tyr⁰Glu¹-PD-Svg, respectively.

The final purity of the peptides was determined by analytical RP-HPLC and capillary electrophoresis (CE). Quantitative RP-HPLC was performed using a GE Healthcare AKTApurifier 10 (Pittsburgh, PA) and a Phenomenex (Torrance, CA) Kinetex XB-C18 column (4.6 x 100 mm, 2.6 μm particle size, 100 Å pore size). The solvent system was comprised of eluent A = TEAP at pH 2.25, eluent B = 60% CH₃CN, 40% A. A gradient was performed from 40% B to 80% B in 30 min at a flow rate of 1.2 ml/min. Capillary electrophoresis was performed using a Groton Biosystems GPA 100 instrument. (Boxborough, MA) The electrophoresis buffer was 0.1 M sodium phosphate (15% acetonitrile), pH 2.5. Separation was accomplished by application of 20 kV to the capillary (0.75 μm x 100 cm). Detection was at 214 nm. Both analytical RP-HPLC and CE showed 99% purity for both peptides. Mass spectra (matrix associated laser desorption ionization mass spectrometry, MALDI-MS) were measured on an ABI Voyager DESTR instrument (Applied Biosystems Inc., Foster City, CA) in positive reflector mode using a saturated solution of a-cyano-4-hydroxycinnamic acid in 0.3% trifluoroacetic acid and 50% acetonitrile as matrix. The observed monoisotopic (M + H)⁺ value of 4300.43 corresponded with the calculated (M + H)⁺ value of 4300.41 for PD-Svg and the observed monoisotopic (M + H)⁺ value of 4481.5 corresponded with the calculated (M + H)⁺ value of 4481.49 for Tyr⁰Glu¹-PD-Svg.

Iodination of CRF Family Peptides.

The synthetic peptide analogs Tyr⁰Glu¹-PD-Svg, Tyr⁰Glu¹Nle¹⁷-Svg, and Tyr¹-astressin were labeled with ¹²⁵I using a chloramine T oxidation method and a 1:1:10 molar ratio of peptide:Na¹²⁵I:chloramine T in 0.25 M sodium phosphate, pH 7.2 reaction buffer. Peptides were first dissolved to 2 mg/ml in dH₂O and further diluted in reaction buffer. All other iodination reagents were prepared in reaction buffer. To a 2 mCi vial (1 nmol) of Na¹²⁵I (NEZ 033L, Perkin Elmer, Waltham MA) was added 1 nmol of peptide in 20 μl and 10 nmol of chloramine T in 5 μl and the iodination was allowed to proceed for 45 sec, at which point it was immediately quenched with 30 nmol sodium metabisulfite in 10 μl. After vortexing 30 sec, 500 μl of potassium iodide at 2 mg/ml and 50 μl of 100 mg/ml ultrapure BSA were added. The mixture was vortexed an additional 30 sec before pre-purification with a C18 silica cartridge and final purification by HPLC as described in detail for inhibin subunits [Vaughan, 1989 #125343] with the following changes. The peptide was eluted from the C18 silica cartridge using 2 ml of 75% acetonitrile-triethylammonium formate into a polypropylene tube before reduction of the fraction to ~0.1 ml using a Speed Vac concentrator (Thermo Scientific). A Vydac diphenyl column (5 μm, 0.46 x 25 cm; Grace Davison Discovery Sciences, Columbia MA) was used for HPLC purification at a flow rate of 1.5 ml/min using a trifluoroacetic acid (0.1%)-acetonitrile solvent system. After equilibration at a start concentration of 28% acetonitrile, the iodination mixture
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was applied, and a linear gradient to 40% acetonitrile in 30 min was run followed by a steep linear gradient to 80% acetonitrile in 5 min. This optimal experimentally determined gradient resulted in clearly separated and sharply defined peaks of mono- and di-iodo products.