Supplementary Material (ESI) for Chemical Communications

Supporting Information.

Phage display selection of miniprotein binders of the Estrogen Receptor

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Miniprotein synthesis.

General information peptide synthesis. Rink Amide MBHA resin with an initial loading of 0.72 mmol/g was purchased from Novabiochem. Fmoc-protected amino acids were purchased from MultiSyntech and Novabiochem in their appropriately protected forms. All other reagents were purchased from Aldrich-Sigma, Fluka, and Acros. All automated peptide syntheses were performed on a Syro II automated peptide synthesizer (MultiSynTech GmbH), using standard solid phase Fmoc-chemistry.

Analytical LC-MS experiments were performed on an Agilent 1100 series HPLC system connected to a Thermo LCQ Advantage mass spectrometer equipped with an electrospray ion source. Analytical chromatography separations were performed using a C18 Nucleodur gravity column (125 x 4 mm, 3 μm particle size, Macherey-Nagel). Material was eluted using a gradient system of acetonitrile and water containing 0.1% formic acid and a flow rate of 1 ml/min.

Preparative HPLC was performed on a Agilent Series 1100 system equipped with a C18 Nucleodur gravity column (125 x 21 mm, 5 μm particle size, Macherey-Nagel) using a gradient system of acetonitrile and water each containing 0.1 % trifluoroacetic acid and a flow rate of 25 ml/min.

Peptide synthesis. All sequences were synthesized from *C*- to *N*-terminus on solid support, using an automatic solid phase synthesizer on a 144 μmol scale (200 mg of resin, loading of 0.72 mmol/g). The coupling of amino acids was carried out following standard Fmoc-chemistry, using HOB*t*/DIPEA (4 eq.) as amino acid activation, DMF as solvent and 4 eq. of the protected Fmoc-amino acid. The resin was first swollen in DMF (1 x 30 min.) and the Fmoc protecting group was removed by treatment with piperidine/DMF (2/3, 1 x 3 min.; 1/4 1 x 10 min.), then washed with DMF (6 x 1 min.). One cycle of peptide elongation consisted of the following steps. First, the deprotected resin was treated for 50 min. with a cocktail containing the appropriate amino acid (4 eq., solution 0.3 M in DMF) with an equimolar addition of HOB*t*, DIC (4 eq., solution 0.3 M in DMF) and DIPEA (4 eq.). After washing the resin with DMF (4 x 1 min.), the Fmoc protecting group was removed by treatment with piperidine/DMF (2/3, 1 x 3 min.; 1/4, 1 x 10 min.). After deprotection, the resin was again washed with DMF (6 x 1 min). These steps were repeated until the desired peptide sequence was complete. After the completion of the

sequence, the resin was subsequently washed with DMF (5 x 30 s), CH_2Cl_2 (5 x 30 s) and Et_2O (5 x 30 s) and dried under vacuum for 2-3 h.

Cleavage and side chain deprotection was carried out by treatment of the resin for 2 h with a cleavage cocktail containing TFA/H_2O/EDT/TIS (96:2:1:1). The cleaved resin was washed with TFA (2 x 2 ml) and the cleaved peptide was collected, concentrated into less than 1 ml solution and precipitated by addition of cold Et_2O (30 ml). The mixture was cooled, centrifuged (4000 rpm, 5 min, 4 °C) and the Et_2O was decanted from the pellet. Cold Et_2O was added again and the procedure was repeated twice. The crude peptide obtained was dissolved in H_2O/CH_3CN and lyophilized to dryness.

Peptide purification. After the syntheses of the mini-proteins on the automated peptide synthesizer and lyophilization, all mini-proteins were inserted in the following step of oxidation. The crude peptide was dissolved in a 2 : 1 mixture of sodium phosphate buffer (100 mM NaH_2PO_4/Na_2HPO_4, 1 : 17.8, pH 8) and trifluorethanol. To this solution 10 % DMSO was added and stirring was performed at room temperature. The end of the oxidation step was determined by LC-MS. The crude material was then lyophilized and purified by preparative HPLC on a Nucleodur C18 Gravity column with a gradient of A (0.1 % TFA in H_20) and B (0.1 % TFA in CH_3CN) with a flow rate of 25 ml/min. After purification, all peptides had a purity > 90%, were lyophilized and kept at – 20 °C.

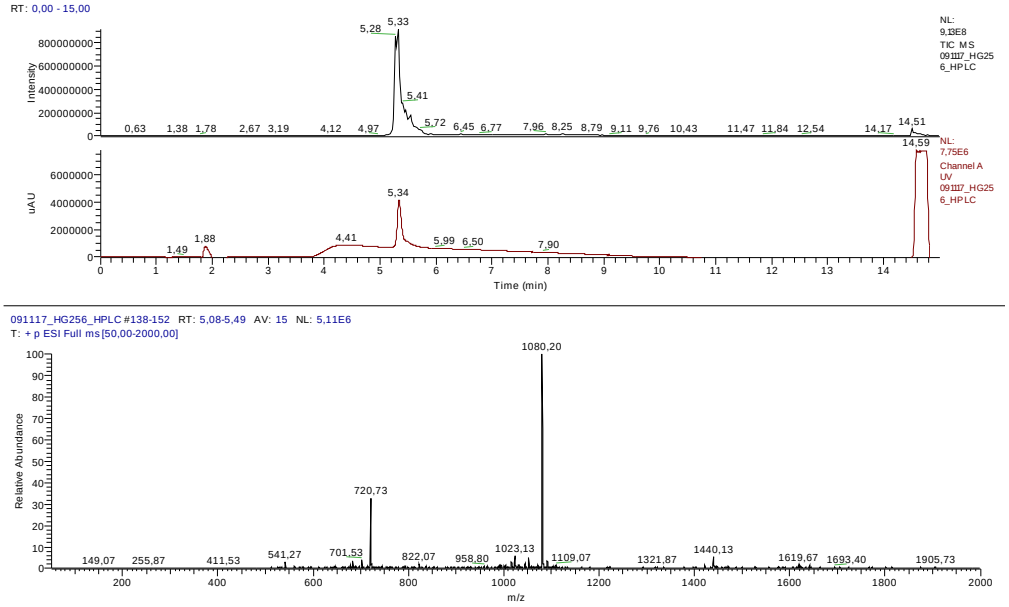


Figure S1. LC-MS trace and spectrum of purified **ER-apamin 1** Mass Calc.: 2159.0 Da; found: 2159.1. (m/z 720.7 $(M)^{3+}$; 1080.2 $(M)^{2+}$; 1440.1 $(2M)^{3+}$)

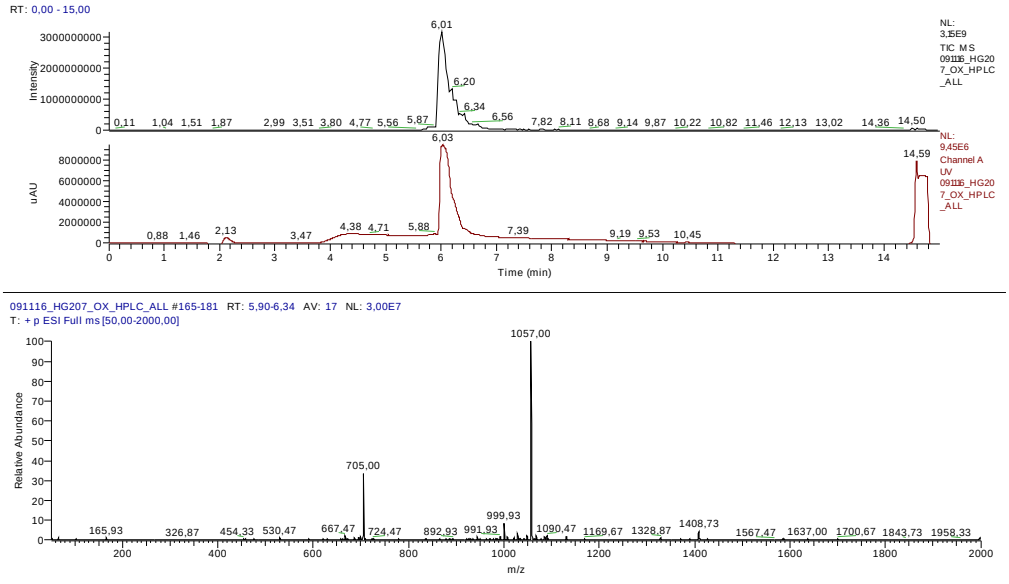


Figure S2. LC-MS trace and spectrum of purified **ER-apamin 2** Mass Calc.: 2112.0 Da; found: 2112.0. (m/z 705.0 $(M)^{3+}$; 1057.0 $(M)^{2+}$; 1408.7 $(2M)^{3+}$)

L**abeling of Src1Box2 reference peptide.** The peptide sequence corresponding to the Src1Box2 sequence was synthesized featuring an N-terminal cysteine (CQLLTERHKILHRLLQEGSPSD) for fluorescein labeling. Labeling with fluorescein was performed in potassium phosphate buffer (1 M, pH 8, previously degassed by argon) and TCEP·HCl (5 equiv.). The resulting clear solution was stirred for 1h under argon. After this, a solution of 4(5)-(Iodoacetamido)fluorescein (5 equiv.) in DMSO (10 mg/mL) was added to the reaction mixture and the homogenous mixture was stirred at room temperature. The course of the reaction was followed by LC-MS. After completion of the reaction, quenching of the excess of fluorophore was performed 2-mercaptoethanol. The resulting mixture was directly lyophilized. The labeled peptide was purified by RP-HPLC and FPLC with a size exclusion column (Superdex 75 10/300 GL, Amersham Bioscience) in the running buffer (20 mM HEPES, 50 mM NaCl, pH 7.9).

Construction, expression and purification of ER LBDs (Strep-His-ERα-LBD and Strep-His-ERβ-LBD).

Plasmid pHT401 was constructed by introducing the DNA encoding Strep-tag using two complementary nucleotides ON001, 5′-catggcaagctggagccacccgcagttcgaaaagggatccgagctcgaattcggcc-3′ and ON002, 5′-tcgaggccgaattcgagctcggatccctttttcgaactgcgggtggctccagcttgc-3′ into plasmid pTriEx4 Neo (Novagen) at *Nco*I and *Xho*I. The plasmid pET15b-ERβ-LBD (gifts from Bayer-Schering Pharma AG) encoding the LBD of the human ERβ was used as template for PCR amplification using the primers ON151, 5′-ccggatggatccatgggcagcagccatcatcat-3′ and ON153, 5′-ccggatctcgagttacccgcgaagcacgtgg-3′. The PCR product (ERβ-LBD residues 260–502) was digested with *Bam*HI and *Xho*I enzymes and subcloned into pHT401 to introduce a Strep-tag and His-tag at the N terminus of the ERβ-LBD resulting pHT504.

For expression and purification of Strep-His-ERα-LBD553 from pHT503[1] and Strep-His-ERβ-LBD502 from pHT504 were transformed into *E. coli* Rosetta 2 (DE3) pLacI cells (Novagen). A single colony was then grown in LB medium with 100 mg/L ampicilline and 34 mg/L chloramphenicol at 37 °C. At OD_{600} nm ~0.8, protein expression was induced with 0.1 mM IPTG for 16–18 hr at 16 °C. Bacteria were collected by centrifugation at 5000 rpm for 10 min. The cells were resuspended in washing buffer (50 mM sodium phosphate, 300 mM NaCl, 5% glycerol and 20 mM imidazole (pH 8.0)) containing 1 mM PMSF and disrupted by a sonicator (Branson) and centrifuged to collect the clear supernatant. The sample was loaded on a HisTrap HP 5 ml column (GE Healthcare) and the protein-bound column was washed with 100 ml washing buffer. Proteins were eluted by imidazole and fractions containing the Strep-His-ERα-LBD and Strep-His-ERβ-LBD proteins were desalted using on a PD-10 column (GE Healthcare) pre-equilibrated with storage buffer (25 mM NaCl, 20 mM Tris-HCl (pH 8.0), 10% glycerol, and 1 mM TCEP).

Miniprotein screening using phage display.

For construction of the phage libraries, DNA libraries were amplified by PCR using ON217, 5′-gccatgaattctggtggcgg-3′ and ON234, 5′- gtgcggccgcaagcttagtga-3′ with ON233, 5′-gccatgaattctggtggcggttgcaactgcaaagctccggaaaccrstnwwtgcnwwnwwnwwtgcnwwrstcactaagcttgcggccgcac-3′ (for library 1) and ON389B, 5′-gccatgaattctggtggcggttgcaactgcaaagctccggaaaccnwwnwwtgcnwwnwwnwwtgcnwwrstcactaagcttgcggccgcac-3′ (for library 2). The difference between libraries 1 and 2 are underlined. The DNA fragments were digested by *Eco*RI and *Hind*III and the resulting *Eco*RI-*Hind*III restriction fragment was cloned into T7Select *Eco*R I/*Hind*III Vector Arms (Novagen). The recombinant phage DNA was packaged by using a T7 packaging extract (Novagen). The resulting phage libraries contained 4.3×10^7 (library 1) and 8.6×10^7 (library 2) independent clones, as determined by plaque assays. The library was amplified once by infecting a mid-log-phase *E. coli* Origami^{TM} B 5615 culture (100 ml, OD600 ≈0.6) with the phage library at a multiplicity of infection of 0.001. The amplified library was then clarified by centrifugation and store in 8% glycerol at -70 °C.

For screenings, the phage libraries were cycled through five rounds of binding selection with ERα-LBD and ERβ-LBD. The ER proteins with strep-tag (0.05 mg/200µl) were immobilized on strep-tactin coated microplates (IBA) in TBS buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl and 10 µM beta-estradiol) through incubation for 1 h at room temperature. After coating, the plates were blocked with 1% ABS at room temperature for 30 min and then washed 3 times in TBS. Phage particles were incubated with the estrogen receptor-bound plates for 30 minutes at room temperature. The plates were washed 10 times using TBST buffer (TBS with 0.5% Tween 20). The bound phage particles were eluted with 1% SDS and immediately the titer was determined. The eluted sample was used to infect fresh *E. coli* Origami^{TM} B 5615. The resulting amplified phage was applied for a new round of selection. After 3-5 rounds of binding selection, two lines of experiment were followed. First, the selected libraries were used as templates to amplified DNA fragments using primers ON229 and ON230. These mixtures of selected DNA libraries were then cloned into pHT293 at *Xba*I and *Eco*RI. Plasmid pHT293 contains the backbone of plasmid pHT212[2] (Phan et al. 2010) and 6xHis-mCitrine with a part of the MCS sequence of the phage T7. Isolated colonies were used as templates to amplify using ON229, 5′-ggtgccacgcggatctgtgagcaagggcgaggagctg-3′ and ON230, 5′-

taagtaatatgaattaacccctcaagacccgtttag-3′. The resulting amplified fragments were subjected to DNA sequence analysis with primer ON127, 5′-ccgaaggctacgtccaggagc-3′. Second, individual phage particles from plaques were used as template to amplify the insert sequences using ON193, 5′-ccacggtggtcttcgcccag-3′ and T7Selectdown (Novagen). The amplified fragments were subjected to DNA sequence analysis with primer T7SelectUP (Novagen). The sequence results were analysed using ChromasPro and ClustalW2.

Phage display sequence results

Library 1	
CN**C**KAPETXX**C**XXX**C**XXH	X = D, E, F, H, I, K, L, N, Q, V, Y (charged / hydrophobic) X = A, G, S, T (small)
Hits	
CNCKAPETTLCKLLCDTH highly enriched → 17% (21/123) after three rounds of selection. **CNCKAPETALCFYLCETH** **CNCKAPETALCELLCFAH** **CNCKAPETGVCKHLCNGH** **CNCKAPETSLCDLICYSH** **CNCKAPETALCVLLCEGH** **CNCKAPETTLCFLLCEAH** **CNCKAPETALCVLLCEGH** **CNCKATETALCVEQCLGH** **CNCKATETTICYLLCDTH** **CNCKATETGICLLYCEGH** **CNCKATETALCDYLCFSH** **CNCKATETSVCEVLCEGH** **CNCKATETSLCLLFCKAH** **CNCKATETSLCELLCIGH** **CNCKATETTLCELLCIGH** **CNCKATETALCHFLCLAH**	Amino acid = polar Amino acid = basic Amino acid = acidic Amino acid = apolar

Library 2	
CN**C**KAPETXX**C**XXX**C**XXH	X = D, E, F, H, I, K, L, N, Q, V, Y (charged / hydrophobic) X = A, G, S, T (small)
Hits	
CNCKAPETLLCLLLCDGH highly enriched → 56% (70/126) after four rounds of selection **CNCKAPETLICLFLCNGH** **CNCKAPETILCHLLCVGH** **CNCKAPETLLCLFLCDGH**	Amino acid = polar Amino acid = basic Amino acid = acidic Amino acid = apolar

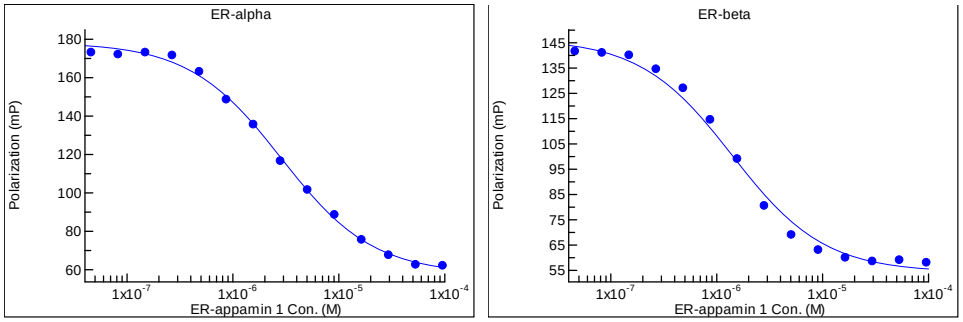
CNCKAPETLLCHLVCDGH CNCKAPETVLCDLLCVGH CNCKAPETLLCFILCDGH CNCKAPETLLCYYLCDSH CNCKAPETLQCEQLCDTH CNCKAPETLLCHLLCDSH CNCKAPETVVCHLLCDSH CNCKAPETILCHLLCVGH CNCKAPETVLCQLLCVTH CNCKAPETILCNLLCDAH CNCKAPETLLCLLLCYGH CNCKAPETVLCYYLCEAH CNCKAPETLLCYYLCDSH CNCKAPETFLCYFLCQGH CNCKAPETKICHFLCVAH CNCKAPETLLCHYLCLGH CNCKAPETIECLVHCHSH CNCKAPETLLCELLCFSH CNCKAPETVLCELLCFGH CNCKAPETELCQFLCFSH CNCKAPETLLCLFLCDGH CNCKAPETVLCLYLCLGH CNCKAPETVLCQYLCDGH CNCKAPETVLCHLLCVTH CNCKAPETILCHILCYGH CNCKAPETLLCVYLCEGH CNCKAPETLLCIFLCVGH CNCKAPETLLCNFDCLAH CNCKAPETLVCLILCDGH CNCKAPETFICLVLCLGH CNCKAPETKLCEILCFSH CNCKAPETLLCHFLCFSH CNCKAPETFLCKYLCVSH CNCKAPETFLCLYLCEGH CNCKAPETLLCHILCQSH CNCKAPETLLCFVFCYSH CNCKAPETVLCELLCHSH CNCKAPETILCLLLCLGH CNCKAPETVFCKLLCQGH CNCKAPETLFCYILCEGH CNCKAPETLFCLLLCYGH CNCKAPETVICLLLCDSH CNCKAPETLLCLLLCESH CNCKAPETLLCILLCEAH CNCKAPETVLCLLLCEAH CNCKAPETVLCYLVCDAH CNCKAPEPYYAYLLCEAH	

Immobilization based ER affinity screening of miniprotein hits.

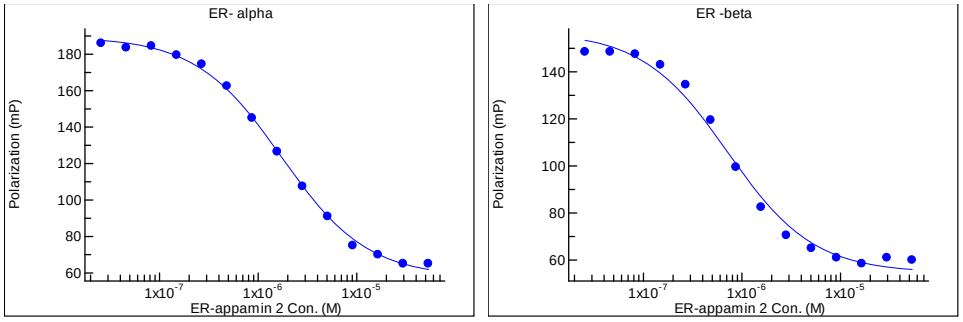
Strep-tag fusion proteins, ERα-LBD553 and ERβ-LBD502 were diluted to 10 μM in the binding buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 10 μM β-estradiol) containing 0.5% BSA. The above diluted proteins (200 μl) were incubated in Strep-Tactin coated microplates (IBA) at 4 °C for an hour. After incubation, the coated plates were washed 3 times using the binding buffer. The binding affinity of the fusion miniprotein-fluorescent proteins to the immobilized ER on the surface was determined by incubation of the wells with 200 μl of the m-Citrine-miniprotein fusion proteins in the binding buffer for 1 hour and subsequently washed 10 times with binding buffer. The wells were measured using a Safire2 plate reader (Tecan) with excitation at 480 ± 20 nm and emission at 530 ± 15 nm. Higher relative fluorescence signals were considered representative for higher binding affinity.

Competitive fluorescence polarization assay. The competitive fluorescence polarization assay was carried out analogously as described by Vaz *et al.* for the androgen receptor.[3] A reaction mixture containing a fluorescein-labeled coactivator (Src1Box2) peptide FL-CQLLTERHKILHRLLQEGSPSD (10 nM), the ERα-LBD553 or ERβ-LBD502 protein (1 μM) and 17β-estradiol (10 μM) was prepared in the assay buffer (20 mM Tris-HCl pH 8.0, 25 mM NaCl, 10% glycerol, 10μM beta-estradiol and 1 mM TCEP). Fluorescence polarization inhibition experiments were performed in 384-well plates (Optiplate-384 F, Perkin Elmer) by adding 10 μl of the reaction mixture to 40 μl of inhibitor peptides at increasing concentrations. Inhibitor peptide/miniprotein solutions were made by weighing the peptides and dissolving them in the assay buffer to yield stock solutions of 0.7 mM. After 2 h incubation at 4 °C, the fluorescence polarization of the labeled coactivator peptide was measured on a plate reader (Safire2, Tecan) at 25 °C with excitation at 470 nm and emission at 519 nm for fluorescein. The concentration of inhibitor peptide that resulted in a half-maximum decrease in the polarization value of the fluorescent coactivator peptide displayed from the purified ER was defined as IC_{50} and was determined from 3-4 independent experiments. Representative displacement curves are provided below.

ER-apamin 1



ER-apamin 2



SRC1Box2

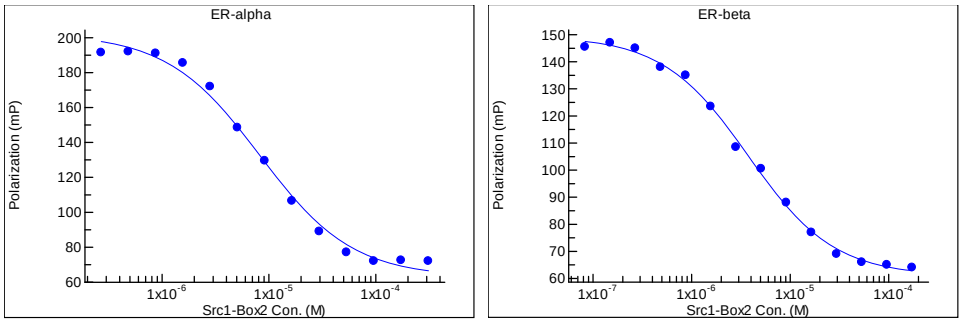


Figure S3.. Representative fluorescence polarization coactivator peptide displacement curves of ER-apamin 1, ER-apamin 2 and Src1Box2.

References

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[2] Phan,T.T., Nguyen,H.D. and Schumann,W. (2010) Establishment of a simple and rapid method to screen for strong promoters in *Bacillus subtilis*. *Protein Expr. Purif.* 71, 174-178.

[3] Vaz,B., Mocklinghoff,S., Folkertsma,S., Lusher,S., de,Vlieg .J. and Brunsveld,L. (2009) Computational design, synthesis, and evaluation of miniproteins as androgen receptor coactivator mimics. *Chem. Commun. (Camb.)* 5377-5379.