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Towards a New SPE Material for EDCs: Fully Automated Synthesis of a Library of Tripodal Receptors Followed by Fast Screening by Affinity LC

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Dedicated to Professor Alain Krief

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A series of human estrogen receptor (hER) mimics were synthesised. On the basis of the knowledge on the structure of the hormone binding domain of the hER, three different peptide chains were constructed onto a tripodal scaffold. By using a fully automated solid-phase synthesis protocol, 120 members with known identity were synthesised in only a week. Affinity towards estrogenic compounds was checked by affinity LC. For this purpose, ethinylestradiol was "clicked" onto a modified-silica phase, and the obtained material was packed into an HPLC column. The results stemming from the affinity LC experiments were confirmed by clicking one library member to silica and by using this solid phase to extract different endocrine-disrupting chemicals (EDCs) from aqueous media.

Introduction

Worldwide concern has been growing on the increasing distribution of endocrine-disrupting chemicals (EDCs) over the last decade. The overall anxiety is caused by the effect of these substances on the endocrine system of wildlife and humans. For humans, the most disturbing effects, such as a decrease in sperm count in men and the early sexual maturation of women, are thought to originate from the binding of chemicals with the human estrogen receptor (hER).[1] There are numerous chemicals with estrogenic properties present in the environment. These include synthetic and natural hormones, industrial chemicals (insecticides, household detergents) and phyto-estrogens (plant derived). Structural inspection of this wide variety of chemicals shows that a common feature of these estrogenic compounds is the presence of an aromatic ring, which can be accommodated through a pincer-like arrangement of amino acids in the hormone binding cavity of the hER.[2] The establishment of a causal relationship between the presence of EDCs in the environment and their possible effects on human health is a challenging quest. Besides their low physiologically active concentrations, the complex environmental matrices in which they are present complicate identification and quantification of EDCs.[3] This problem can be tackled by a preconcentration step before the actual analysis. In general, solid-phase extraction (SPE) of aqueous samples is a widely applied technique to enrich pollutants.[4] With the current SPE cartridges, the extraction efficiency of the pollutants depends on their polarity. Polar compounds tend to stay in the water sample, while apolar compounds are retained on the solid phase, with poor selectivity of the currently applied SPE methods as a consequence. A solid-phase material that could selectively bind EDCs would greatly simplify the analysis.

Attempts to address this problem include the development of immunoaffinity based systems and synthetic alternatives such as molecular imprinted polymers (MIPs) and linear peptides. Polyclonal antibodies against estradiol, estrone and estriol were developed and coupled to Sepharose to make an immunoaffinity cartridge that is able to preconcentrate the three estrogens from women's urine.[5] Besides the disadvantage of facing a time-consuming, trial-and-error-based production process, the immunoaffinity systems are generally too specific and do not allow the preconcentration of a whole range of compounds as in the case of EDCs. Alternatively and more easily produced, molecular imprinted polymers have been developed by using estradiol and diethylstilbesterol as templates.[6] Though good properties have been obtained in organic solvents, trapping of analytes with MIPs remains difficult in aqueous media.[7]

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A remarkable contribution towards selective trapping of EDCs has been made by Tozzi et al. who used small linear peptides as recognition motifs for estrogenic chemicals.[8] By using the amino acids present in the hormone-binding domain of the human steroid binding protein as building blocks, a parallel library of dipeptides was generated. After incubation with a radioactively labelled estradiol ligand, the dipeptide with the highest affinity towards estradiol was selected and then used as a starting point for the generation of larger peptides. Though peptides with up to 8 amino acids were synthesised, it seemed that the tetrapeptide Arg–Ser–Ser–Val–OH showed the best binding properties, and a pre-concentration column was constructed with this solid-phase-bound peptide.

Inspired by these results, we decided to try to develop an alternative synthetic estrogen receptor for extracting EDCs from aqueous media. It was believed that an improved and a more-selective binding of EDCs could be obtained if the amino acids of the hormone-binding domain of the hER were selected as building blocks for the synthetic receptors. Moreover, instead of making linear peptides without a predefined structure, tripodal scaffold **1** was chosen as the backbone, while allowing the incorporation of 3 different peptide chains. Earlier molecular modelling results have indicated the potential of **1** to orient attached peptide chains in a parallel way.[9] Previous examples in the area of scaffolded peptides towards the selective binding of small organic molecules or ions[10] and as mimics of peptidases[11] show that by pre-organising functionalised groups good results can be obtained in terms of affinity and selectivity.

When using scaffolded peptides, generally, molecular diversity is obtained by synthesising split-and-mix libraries. Screening can then be performed by adding a labelled ligand and selecting the fluorescent or coloured beads.[10b,10c,12] By using such a combinatorial approach, it sometimes remains difficult to establish the identity of the active compound, a process that is often complicated by the low amount of product synthesised.[13] These problems can be bypassed if a parallel strategy is employed. Indeed, in that case, the identity of the different library members is known and sufficient amounts can be synthesised for further, more elaborate screening.[14] This paper presents the first fully automated synthesis of a library of tripodal structures and demonstrates that in a short period of time, a considerable amount of candidate receptors can be synthesised in a parallel way. Moreover, by using this automated parallel approach, suitable quantities of material are within reach to allow extensive analysis of affinity properties.

Results and Discussion

Synthesis

In order to mimic the hormone-binding domain of the hER, the amino acids responsible for ligand binding were selected on the basis of X-ray structures of the hER with estradiol.[15] The important binding interactions are schematically presented in Figure 1. From the different contacts with estradiol, Arg/Glu/His were selected in order to mimic the hydrogen bonding at the extremities of the steroid. Additionally, Phe/Leu/Met were chosen to provide an apolar environment for the hydrophobic skeleton of the ligand.

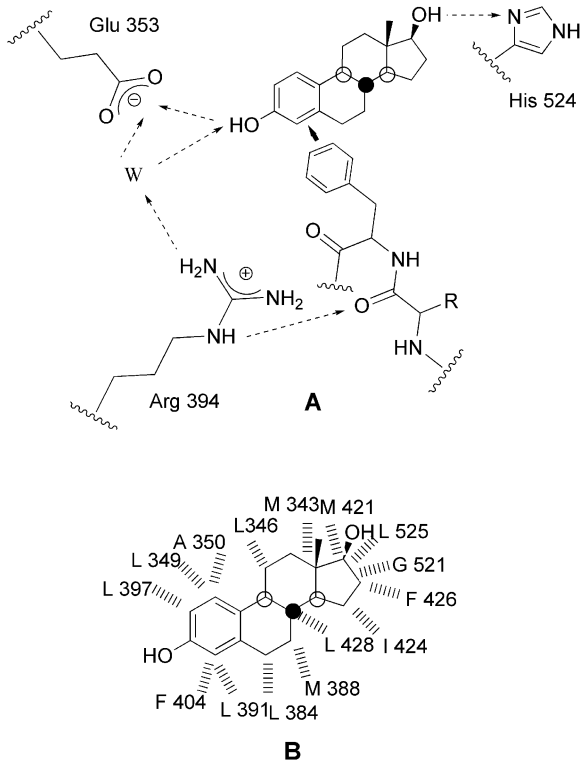
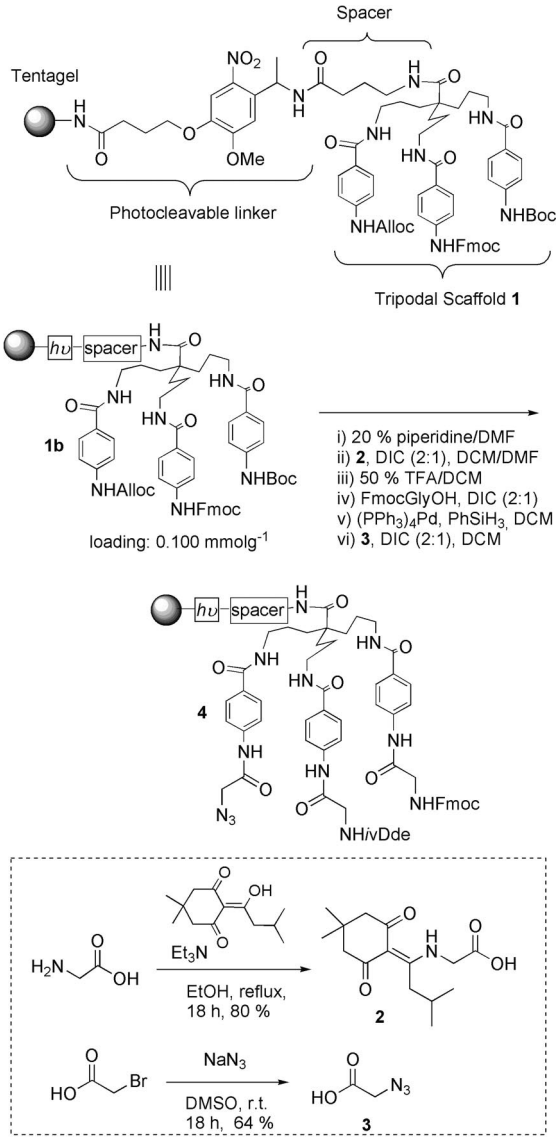


Figure 1. Schematic representation of (**A**) hydrogen-bonding network and (**B**) hydrophobic interactions between the amino acids (AAs) of the hormone-binding domain (HBD) of the hER and its natural ligand estradiol.

The orthogonally protected tripodal scaffold was synthesised and attached to a solid phase as described before.[16] A photocleavable linker[17] was first coupled with Tentagel, followed by the incorporation of a spacer, γ-aminobutyric acid (GABA). The tripodal scaffold was then attached by using a PyBOP/DIEA coupling protocol to yield construct **1b** (see Scheme 1). The weak nucleophilicity of the aromatic amines was then bypassed by incorporation of differently protected glycines through their symmetrical anhydrides.[18] The protecting groups of these glycines were chosen in such a way that their deprotection could be performed on an automated peptide synthesiser. Indeed, the removal of the acid labile Boc group and the Pd^0 labile Alloc were not compatible with the automated synthesiser present in our laboratory.[19] In first instance, the 2-nitrobenzenesulfonyl (*o*NBS) derivative of Gly was tested.[20] However, in our hands upon test coupling of an amino acid to one of the other scaffold "arms" with the use of PyBOP/DIEA, the *o*NBS sulfonamide linkage was partially acylated.

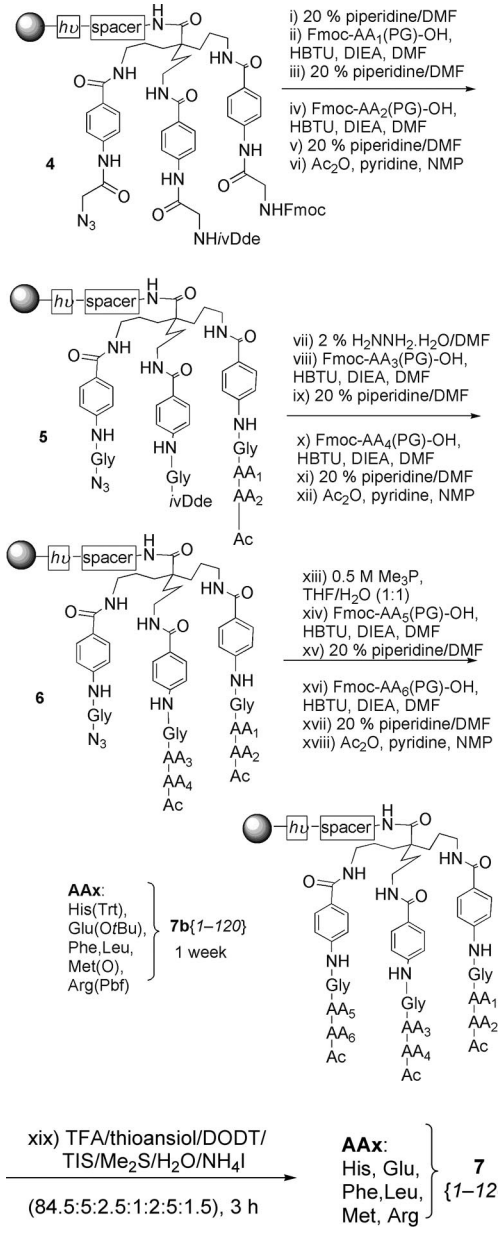
Therefore, the Boc and the Alloc group were replaced with 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (*iv*Dde)[21] and the azide[22] group. The former is smoothly cleaved by a 2% hydrazine solution in DMF, and the latter can be



Scheme 1. Bypassing the weak nucleophilicity of the aromatic amines by first incorporating glycine derivatives through their symmetrical anhydrides. FmocGlyOH, *iv*DdeGlyOH **2**, and azidoacetic acid (**3**) were attached.

reduced with Me_3P in THF/H_2O. As depicted in Scheme 1, the Fmoc group of **1** was deprotected and subsequently 2 equiv. **2**, synthesised from glycine and *iv*DdeOH, were activated in the presence of 1 equiv. DIC and added to the resin. After overnight reaction, the Boc group was deprotected and FmocGlyOH was coupled. Subsequently, the Alloc group was deprotected with Pd^0 and $PhSiH_3$,[23] and azidoacetic acid (**3**), prepared from bromoacetic acid,[24] was then coupled to give orthogonally protected **4**. Construct **4** was then used for the automated synthesis of a parallel library of 120 members. When 6 AAs are used for construction in 6 different positions, the size of the library can rise to 46656 members. Though molecular diversity can be generated with automated synthesis, it is practically impossible to synthesise a library of more then 46000 members. In order to constrain the size of the library, each member was designed to contain all 6 different, selected AAs. The size of the library was further substantially decreased by applying some simplifications. It was decided to omit the combinations in which one strand seems to be switched with another strand. A second method to restrict the size was to insert each AA only once into the tripodal structure. In this way, a small-sized library of 120 members was designed. Because the synthesiser was equipped with a reaction block that could contain 24 reaction vessels, the whole library was synthesised in 5 consecutive cycles.

Each cycle started by removing the Fmoc group, after which two AAs were inserted by using HBTU and DIEA (see Scheme 2). The first strand was terminated by capping



Scheme 2. Automated synthesis of 120 library members and subsequent deprotection of the side chain protecting groups including Met(O).

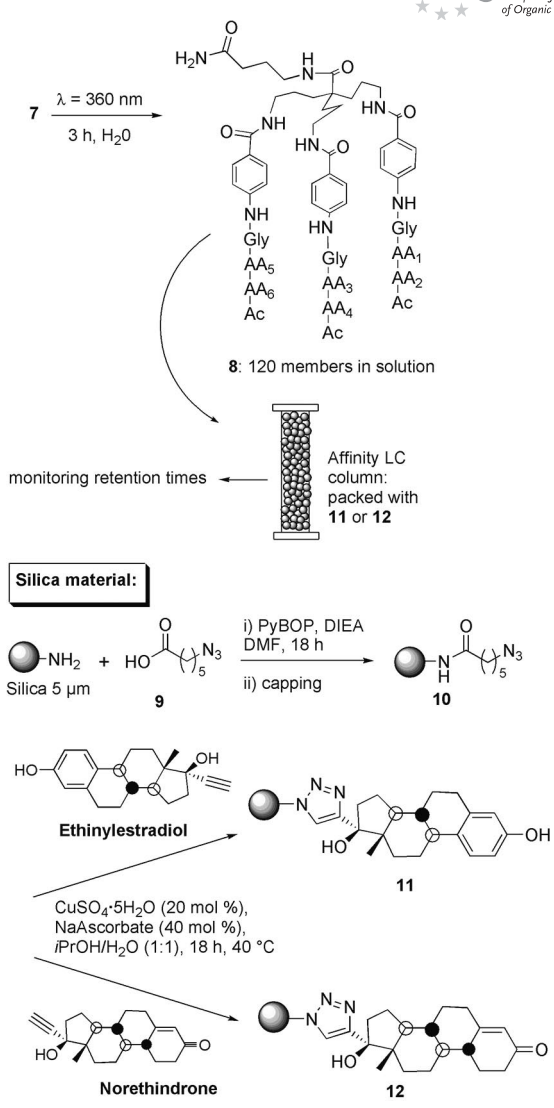
with Ac_2O. The second strand was accessed by removing the *iv*Dde group with a 2% hydrazine solution in DMF, which could be added to the different reactors with a robot arm. Again, two cycles of coupling and Fmoc deprotection were followed by a capping step. The last peptide chain was inserted after the azide was reduced. Reduction with Ph_3P resulted in the formation of the N–P ylide, but the sterically less-hindered Me_3P gave a smooth conversion after 2×10 min reaction time. For this reason, a 0.5 M solution of Me_3P in THF/H_2O (1:1) was added to the reactors through the robot arm. This deprotection step was followed by the coupling of the last two AAs, and the third strand was finally terminated by capping with Ac_2O. Each batch of 24 members could be synthesised in 20 h, and consequently, the whole library was synthesised in one week. The purity of the crude products was easily checked by irradiating a small sample of the resin with light (λ = 360 nm) for 3 h in EtOH.[17] Analysis of the resulting solution by HPLC showed the presence of two products. Further analysis with MALDI-TOF indicated that partial oxidation of the Met side chain had occurred during the synthesis, which explains the two different signals in the HPLC chromatogram. This problem was solved during the manual side chain deprotection step by adding Me_2S and NH_4I to the cleavage cocktail. After 3 h, not only were the different side chain protecting groups removed, but the sulfoxide was also reduced, to yield the desired library in good purity (see Supporting Information for analytical details of library members).

Screening

In order to screen the library for affinity towards estradiol, it was decided to develop an affinity LC based method (Scheme 3). By immobilising estradiol on silica and by using this material to pack an HPLC column, it should be possible to identify the most-active receptor by monitoring the retention times of the different library members. Indeed, if a receptor forms a strong complex with the silica-bound ligand, this receptor should be retained longer on the column than weakly binding receptors.

The affinity LC material was prepared by acylating aminopropylsilica (5 μm particle size) with azidohexanoic acid **9**, which was prepared in a similar fashion to azidoacetic acid **3**.[25] Ethinylestradiol was then attached to this material by using the Cu^I-mediated cycloaddition between an azide and an alkyne[26] to yield material **11**. This material was subsequently packed into a column (2.1×150 mm) that was then integrated into a LC-MS system. In this way, this technique has the additional advantage of confirming the identity of each member. Using an autosampler with the LC-MS system, the whole library can be screened in a few days.

Under isocratic conditions, with H_2O at pH 7.4, none of the members eluted from the column. In an effort to disrupt the hydrogen bonding, the pH was lowered to 3, but no improvement was observed. When H_2O was replaced with

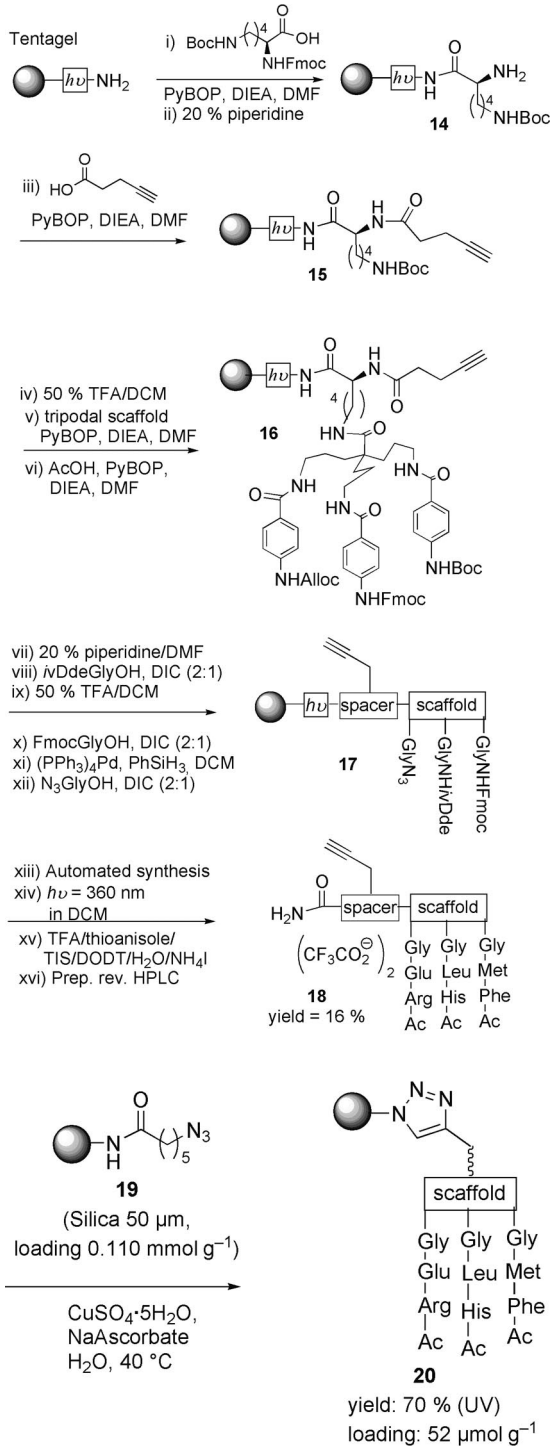


Scheme 3. Screening of the library in solution with an affinity-based method. Two new silica materials **11** and **12** were prepared by using "click" chemistry.

MeOH to target the hydrophobic interactions, the members finally eluted. However, the compounds all eluted in a similar time frame, which suggests that the different library members interact in a similar manner with the silica-bound estradiol. The selectivity was tested by designing a non-estrogenic norethindrone column packed with material **12**. From these experiments, it became clear that nonselective binding was observed, since all members interacted similarly with the silica-bound norethindrone. In a first attempt to evaluate the obtained results, a competition experiment was designed. The same LC-MS setup with the estradiol column **11** was envisaged, however, in the presence of increasing amounts of estradiol in the eluent. Normally, a shift in the peaks can be expected because the receptors should now also interact with the estradiol in solution. Unfortunately, the solubility of estradiol in MeOH was not sufficient to obtain the concentrations needed for the competition experiments.

In a second attempt to evaluate the obtained results and as a proof of principle, we decided to construct an SPE cartridge consisting of a solid support loaded with one library member. This cartridge could then be used to extract EDCs from an aqueous matrix, thus the efficiency of the synthesised receptor could be evaluated. Because Tentagel and other commercially available SPPS resins showed a too high nonselective background absorption of apolar compounds, including EDCs, silica was used to immobilise the library member. Direct synthesis on silica proved to be unsuccessful because cleavage of the side chain protecting groups of the AAs was not complete. This is probably due to the oxygen-rich backbone of the silica material, which can be protonated by TFA, which lowers the effective [H^+]. It was thus confirmed that silica is not a suitable support for the construction of these tripodal peptides, which is a fact that is not entirely unexpected. Hence, an alkyne-containing library member was synthesised on Tentagel, which allows further attachment to azido-functionalised silica through the Cu^I azide–alkyne cycloaddition. The synthesis started from Tentagel modified with the photocleavable linker to which FmocLys(Boc)OH was coupled (see Scheme 4). The α-N atom was first deprotected and capped with pentynoic acid. The side chain of Lys was subsequently used as a spacer arm to couple the tripodal scaffold with PyBOP and DIEA. After insertion of the 3 orthogonally protected glycines, construct **17** was used as starting point for the automated synthesis. The obtained protected peptide was then photocleaved from the resin, after which the side chain protecting groups were cleaved. The resulting crude material was purified by reversed-phase preparative HPLC to give library member **18** in a good purity with an overall yield of 16%.

Next, this compound was "clicked" to azido-functionalised silica **19** (50 μm particle size, loading 0.110 mmol g^{-1}) by using Cu^I as a catalyst. The reaction was monitored by UV, and in this way, the amount of reacted compound **18** could be determined. A loading of 52 μmol g^{-1} for material **20** was then used. This material was incubated with an aqueous solution of bisphenol-A (BPA), estradiol (E_2), ethinylestradiol (EE_2), estrone, diethylstilbesterol (DES) and testosterone (Tes). The concentrations of the analytes after incubation were determined by HPLC-UV. These values were correlated with the amount of EDC that was trapped on the material.



Scheme 4. Preparation of alkyne-modified library member **18** and subsequent attachment to silica.

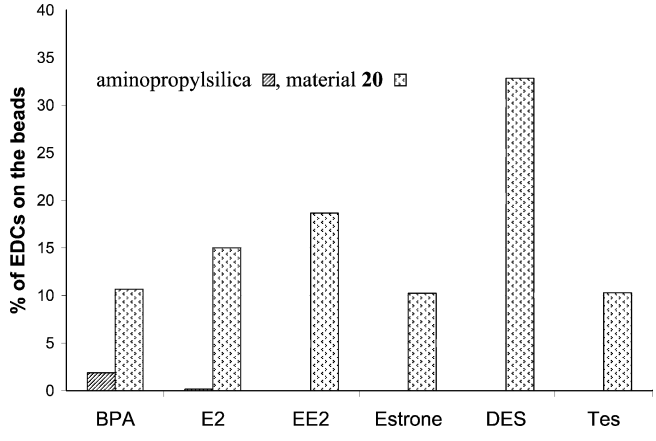


Figure 2. The percentage of EDC trapped on aminopropylsilica and material **20**. While the silica retains little or no EDCs, an increase is clearly obtained for material **20**. However, the silica-bound receptor is not able to differentiate between estrogenic compounds and testosterone.

From Figure 2, it is clear that the newly synthesised material **20** entraps the selected EDCs from an aqueous matrix and does so to a higher degree than the unmodified aminopropylsilica. As a further control, the earlier mentioned tetrapeptide Arg–Ser–Ser–Val–OH, developed by Tozzi and co-workers, was N-terminally treated with pentynoic acid and, after release, clicked onto azido-functionalised silica **19**. This material did not show any EDC retention in this experiment. These results confirm that there are interactions between the solid-phase-bound receptor **18** and the various ligands. However, as can be seen from the percentage of testosterone that is retained, no real selectivity has yet been obtained.

Conclusions

This paper presents the solid-phase preparation of a library of 120 members of tripodal-scaffolded peptides that are designed to mimic the hormone-binding domain of the hER. The originally designed scaffold was modified to allow the fully automated synthesis of a parallel library in a very short time frame. By using this parallel approach, the peptide sequences of the members were known (positional encoding), and, consequently, no time had to be invested in deciphering the identity of the compounds whilst a significant molecular diversity was still reached. The amount of available material allowed a fast solution screening to be performed by using a novel type of estradiol column. Besides the establishment of protocols for the efficient synthesis of new column materials with "click" chemistry, the fast screening of the whole library was possible by using this approach. Though strong interactions between the receptors and the silica-bound ligand were observed (relative to the interactions between the linear control peptide and the silica-bound ligand), no selectivity was obtained as witnessed after screening with a non estrogenic norethindrone column. These results were then evaluated and confirmed by "clicking" an alkyne-bearing library member on silica and by using this material as a solid-phase extraction cartridge. With the methodology for fast synthesis and screening now fully optimised, we currently focus on the design of new libraries with longer peptide chains in order to develop compounds with stronger and more selective EDC-extracting capacities.

Experimental Section

General Methods: All solid-phase reactions on 20 mg of resin or less were performed in polypropylene Chromabond columns of 1 mL with a polyethylene frit, closed at the bottom with a B7 septum from Aldrich. Solid-phase reactions on a bigger scale were performed in a peptide vessel protected against light with aluminium foil and comprising a sintered glass funnel and a 3-way stopcock for easy filtration and washing. All solution-phase reactions were conducted under an inert atmosphere of argon gas in oven dried glassware. The reactions were monitored by thin layer chromatography (TLC) by using SIL G-25 UV_{254} precoated silica gel plates (0.25 mm thickness). The TLC plates were visualised with an anisaldehyde (5% anisaldehyde in ethanol with 1% sulfuric acid) or a PMA (5% phosphomolybdic acid in ethanol) solution. Flash column chromatography was performed by using BIOSOLVE silica gel (0.063–0.200 mm particle size). NMR spectra were recorded at 500 MHz or 300 MHz for proton and at 125 MHz or 75 MHz for carbon nuclei in [D]chloroform, [D_6]DMSO, [D_4]MeOD or [D_6]-acetone. Chemical shifts are reported in units of parts per million (ppm), referenced relative to the residual ^1H or ^{13}C peaks of the used solvent as internal standards ([D]chloroform: ^1H 7.26 and ^{13}C 77.16; [D_6]DMSO: ^1H 2.50 and ^{13}C 39.52; [D_4]MeOD: ^1H 3.31 and ^{13}C 49.00; [D_6]acetone: ^1H 2.05 and ^{13}C 29.84 and 206.26). Infrared spectra (IR) were recorded on a Perkin–Elmer 1600 series FTIR spectrometer and reported in wavenumbers (cm^{-1}). Samples were prepared as a thin films (neat) on KBr plates. Low-resolution mass spectra were recorded with an atmospheric pressure electrospray-ionisation (ESI) Hewlett–Packard 5988 A mass spectrometer. HPLC analyses were performed on an Agilent 1100 Series instrument with a Phenomenex Luna C18(2) column (250 × 4.6 mm, 5μ at 35 °C) by using a flow rate of 1 mL/min and with the following solvent systems: 0.1% TFA in H_2O (A) and MeCN (B). Unless otherwise stated the column was flushed for 3 min with 100% A, then a gradient from 0 to 100% B over 15 min was used, followed by 5 min of flushing with 100% B. Photolyses were carried out with a 4 W Bioblock Scientific compact UV lamp set at 365 nm. Melting point ranges were determined with an Electrothermal 9100 melting point apparatus.

Materials: All amino acids and the solid support Tentagel-S-NH_2 were purchased from NovaBiochem. DMF extra dry was purchased from Aldrich. DMF peptide grade was purchased from Biosolve. All chemicals were purchased and used without any further purification, except tetrahydrofuran (THF), which was distilled from Na/benzophenone prior to use, and dichloromethane, which was distilled from CaH_2.

Standard Operating Procedures

Swelling: The resin is swollen by adding a solvent (10 mL/g) of choice (usually DMF or DCM) to the beads and shaking the suspension for 5 min. To remove the solvent, the resin is filtered at the end.

Washing: Unless stated otherwise, the resin is washed with DMF (3×), MeOH (3×) and DCM (3×). Finally, the resin can be dried by washing with diethyl ether (3×) or pentane (3×).

Fmoc Deprotection: A 20% piperidine/DMF solution is added to the resin (10 mL/g resin). The suspension is shaken for 1 min, after which the suspension is filtered. This step is repeated for 5 min and 10 min. Finally, the resin is washed with DMF (3×), MeOH (3×) and DCM (3×).

Boc Deprotection: The resin is treated with a 50% TFA/DCM solution for 5 min. After filtration, the same solution is applied, and the suspension is shaken for 25 min. Finally, the beads are washed with DCM (3×), MeOH (3×), 10% DIEA/DCM (3×) and DCM (3×).

Capping: The free amines are capped by adding a mixture of Ac_2O/pyridine/DCM (1:3:16) to the beads. After 1 h, the resin is filtered and washed.

Loading Determination: The resin is treated with a 20% piperidine/DMF solution for 30 min. After occasional swirling, the resin is left to settle. Subsequently, a small amount of the solution is transferred to two UV cuvettes (≈3 mL). By measuring the absorbance value at λ = 300 nm, the concentration of the piperidine/fulvene adduct can be determined. This concentration can be correlated to

the loading by taken into account the amount of resin used for the loading test.

Synthesis of *iv*DdeGlyOH (2): Et_3N (1.74 mL, 12.5 mmol, 1.5 equiv.) and *iv*DdeOH (2.36 mL, 10.8 mmol, 1.3 equiv.) were added to a suspension of glycine (623 mg, 8.30 mmol, 1 equiv.) in EtOH (16 mL). The suspension was heated to reflux overnight under inert atmosphere. After 18 h, the clear, yellow solution was concentrated under reduced pressure to give a yellow residue. After the residue was redissolved in a 1:1 dioxane/water mixture, a 4 N HCl solution was added dropwise until no more precipitation occurred. The white precipitate was dissolved in water/MeOH and was subsequently lyophilised to give a white powder (1.86 g, 80%). ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): δ = 9.99 (br. s, 1 H), 6.67 (br. s, 1 H), 5.87 (m, 1 H), 5.62 (s, 1 H), 5.27 (d, J = 17.0 Hz, 1 H), 5.18 (d, J = 10.1 Hz, 1 H), 4.56 (d, J = 4.7 Hz, 2 H), 3.96 (s, 2 H) ppm.

Synthesis of Azidoacetic Acid (3): NaN_3 (5.13 g, 78.9 mmol, 2 equiv.) was dissolved in DMSO (210 mL) and stirred for 90 min. Bromoacetic acid (5.17 g, 37.4 mmol, 1 equiv.) in DMSO (2 mL) was then added. After stirring for 18 h, the solution was diluted with H_2O (170 mL) and acidified with 6 N HCl (33 mL). The aqueous phase was then extracted three times with MTBE. The organic phases were pooled and extracted with a 0.1 N HCl solution saturated with NaCl. After drying and subsequent evaporation of the organic phase, an oil (2.40 g, 64%) was obtained. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 9.62 (br.s, 1 H), 3.97 (br.s, 1 H), ppm. IR: $\tilde{\nu}$ = 2111 (s) cm^{-1}.

Synthesis of Construct 4: Resin **1** (1.6 g, 0.100 mmol/g, 0.19 mmol, 1 equiv.) was Fmoc deprotected. During Fmoc deprotection, *iv*DdeGlyOH (646 mg, 2.3 mmol, 12 equiv.) was dissolved in DCM (4 mL), and the solution was cooled down until 0 °C. After adding DIC (170 μL, 1.1 mmol, 6 equiv.), the mixture was stirred for 30 min and subsequently added to the Fmoc-deprotected scaffold. To ensure complete solubility of the symmetrical anhydride, DMF (4 mL) was added. The resulting suspension was shaken for 18 h and after filtering the resin, the beads were thoroughly washed. A small sample was photocleaved in EtOH and analysed by LC, which showed the presence of a small amount of starting material. The coupling was thus repeated once, and no more starting material was observed. Next, the Boc group was deprotected. In the mean while, FmocGlyOH (683 mg, 2.3 mmol, 12 equiv.) was dissolved in DCM (4 mL) and cooled down until 0 °C. After adding DIC (170 μL, 1.1 mmol, 6 equiv.), the mixture was stirred for 30 min and subsequently added to the resin. To ensure complete solubility of the symmetrical anhydride, DMF (4 mL) was added. The resulting suspension was shaken for 18 h, and after filtering the resin, the beads were thoroughly washed. A small sample was photocleaved in EtOH and analysed by LC, which showed the presence of a small amount of starting material. The coupling was thus repeated once, and no more starting material was observed. Finally, the resin was suspended in DCM. First, the scavenger $PhSiH_3$ (585 μL, 4.75 mmol, 25 equiv.) was added to this suspension followed the Pd^0 catalyst (22 mg, 19 μmol, 10 mol-%). The resulting brown mixture was shaken for 1 h, filtered and washed three times with DCM. After repeating this procedure once, the Alloc group was fully removed as monitored by HPLC after photocleavage in EtOH. Next, azidoacetic acid (**3**, 230 mg, 2.3 mmol, 12 equiv.) was dissolved in DCM (8 mL) and cooled down until 0 °C. After adding DIC (170 μL, 1.1 mmol, 6 equiv.), the mixture was stirred for 30 min and subsequently added to the Alloc-deprotected resin. The resulting suspension was shaken for 18 h, and after filtering the resin, the beads were thoroughly washed. A small sample was photocleaved in EtOH and analysed by LC, which showed the presence of a small quantity of starting material. The coupling was thus repeated once, after which no more starting material was observed. LC-MS analysis of **4** after photocleavage in EtOH (1 mg of resin in 100 μL): calcd. for $[M + H]^+$ 1298.6; found 1299.2.

Automated Synthesis of Chemset 7b: All reactions were performed at ambient temperature and without a protective atmosphere. The resin was weighed into 2 mL syringes, depending on the amount of resin. In 24 plastic syringes of 2 mL, a small amount of resin **4** (1–3 mg, 0.09–0.27 μmol) was weighed. These reaction vessels were placed into the reaction block of the SYRO. After inserting the correct sequences in the software program and preparing the AA_x (0.5 M in DMF or NMP), HBTU (0.5 M in DMF), DIEA (2 M in NMP), piperidine (40% piperidine in DMF) and capping (Ac_2O/pyridine/NMP 1:3:10) solutions, the sequence was started. Firstly, the Fmoc group was deprotected. The first AA was coupled by addition of a solution of AA_x (60 μL) to the resin. Each syringe was then filled with the HBTU solution (60 μL) and the DIEA solution (30 μL). The suspension was vortexed for 40 min, after which it was drained and washed. This procedure was repeated once. The first strand was terminated by addition of the capping mixture (120 μL) and DMF (120 μL) to the resin. The *iv*Dde group was removed by addition of a 2% $H_2NNH_2{\cdot}H_2O$/DMF (180 μL) to the reaction vessel. After vortexing for 10 min, the resin was shortly washed with DMF, and the deprotection was repeated once. The subsequent AA_x were then coupled as already described. The second strand was terminated by capping the free amine with A_2O. Finally, the azide was reduced by adding 0.5 M Me_3P in THF/H_2O (180 μL) for 10 min to the reaction vessels. The reduction was repeated once, and then the resin was drained and washed thoroughly with DMF and *i*PrOH. The subsequent AA_x were then coupled as already described. The synthesis was terminated by capping the free amine. After the synthesis, the resins were washed with *i*PrOH (3×) and pentane (3×). In total, 120 members were synthesised by repeating this overall procedure over 5 synthesis cycles. MALDI-TOF analysis of **7b** after photocleavage in EtOH: calcd. for $[M + H]^+$ 2335.1; found 2094.3 $[M - Trt + H]^+$, 2115.5 $[M - Trt + Na]^+$, 2334.9 $[M + H]^+$, 2350.7 $[M + O + H]^+$, 2357.4 $[M + Na]^+$, 2373.4 $[M + O + Na]^+$ or $[M + K]^+$.

Side Chain Deprotection and Met(O) Reduction of Chemset 7b: The TFA cleavage mixture was prepared by mixing TIS (100 μL), DODT (250 μL), Me_2S (200 μL) and thioanisole (500 μL) with TFA (8.45 mL). For the reduction of Met(O), NH_4I (230 mg) was dissolved in H_2O (500 μL). Firstly, NH_4I_{aq} (10 μL) was added to each member, followed by the addition of the TFA cleavage mixture (190 μL). The suspension was left for 3 h, after which the beads were drained and washed thoroughly. ES analysis of **7** after photocleavage in H_2O: calcd. for $[M + H]^+$ = 1784.9; found 892.6 $[M + 2H]^{2+}$, 903.6 $[M + Na + H]^{2+}$.

Synthesis of Azidohexanoic Acid (9): Bromohexanoic acid (6 g, 31 mmol, 1 equiv.) was dissolved in DMF (20 mL). After adding NaN_3 (4 g, 62 mmol, 2 equiv.), the solution was heated to reflux for 18 h with an oil bath with a temperature of 85 °C. After cooling the solution, DMF was evaporated under vacuum to give an oil, which was redissolved in DCM. This organic phase was then extracted three times with a 0.1 N HCl solution. After drying the organic phase on $MgSO_4$ and evaporation, a light yellow oil was obtained (3.96 g, 81% yield). ^{1}H NMR + COSY (500 MHz, $CDCl_3$, 25 °C): δ = 10.37 (br. s, 1 H), 3.26 (t, J = 6.9 Hz, 2 H), 2.86 (t, J = 7.4 Hz, 2 H), 1.69–1.64 (m, 2 H), 1.62–1.58 (m, 2 H), 1.45–1.39 (m, 2 H) ppm. IR: $\tilde{\nu}$ = 2093 (s) cm^{-1}.

Attachment of 9 to Aminopropylsilica (5 μm): The silica material (2 g, max. loading 0.73 mmol g^{-1}) was suspended in DMF (10 mL)

and azidohexanoic acid (**9**, 691 mg, 4.4 mmol, 3 equiv.) was added to this suspension. After addition of PyBOP (2.3 g, 4.4 mmol, 3 equiv.) and DIEA (1.53 mL, 8.8 mmol, 6 equiv.), the suspension was shaken for 18 h. Subsequently, the solution was drained, and the remaining powder was washed thoroughly. After capping, the ninhydrine test gave a colourless result, which indicates that there was no more free amine left. IR: $\tilde{\nu}$ = 3414 (br. s), 2104 (s), 1642 (m), 1111 (br. s), 810 (s) cm^{-1}.

Clicking of Ethinylestradiol to Construct 10: Silica material **10** (6 g) was suspended in an *i*PrOH/H_2O (60 mL, ratio 1:1) solution. Ethinylestradiol (4.45 g, 15 mmol, 1 equiv.) was added to this suspension followed by $CuSO_4 \cdot 5H_2O$ (749 mg, 3 mmol, 20 mol-%) and sodium ascorbate (1.2 g, 6 mmol, 40 mol-%). The resulting orange solution was gently stirred in an oil bath with a temperature of 40 °C. The reaction mixture was left overnight, after which the suspension was filtered and the remaining silica material was washed with MeOH (3×), $EDTA_{aq}$ (3×), MeOH (3×) and diethyl ether (3×). All the azide functionalities had reacted, as shown by the disappearance of the azide absorption (≈2100 cm^{-1}) in the IR spectrum. Finally, the silica material **11** was dried overnight at an oven temperature of 60 °C. The column was packed by using the slurry packing method with a Haskel air driven pump (Burbobank, CA, USA).[27] The slurry solvent was THF/water (50:50) and the packing solvent used was deionised water, both were degassed. The derivatised silica (approx. 0.7 g) was slurried in slurry solvent (8 mL) in an ultrasonic bath, followed by packing at 450 bar. The columns were 15 cm in length and 2.1 mm in diameter. IR: $\tilde{\nu}$ = 3399 (br. s), 2936 (s), 1670 (s), 1050 (br. s), 800 (s) cm^{-1}.

"Clicking" of Norethindrone to Construct 10: Silica material **10** (1 g) was suspended in an *i*PrOH/H_2O (10 mL, 1:1) solution. Norethindrone (756 mg, 2.5 mmol, 1 equiv.) was added to this suspension, followed by $CuSO_4 \cdot 5H_2O$ (125 mg, 0.5 mmol, 20 mol-%) and sodium ascorbate (198 mg, 1.0 mmol, 40 mol-%). The resulting orange solution was gently stirred in an oil bath with a temperature of 40 °C. The reaction mixture was left overnight, after which the green suspension was filtered and the remaining silica material was washed with MeOH (3×), $EDTA_{aq}$ (3×), MeOH (3×) and ether (3×). Finally, the silica material **12** was dried overnight at an oven temperature of 60 °C. The column was packed in an analogous way as that described for **11**. IR: $\tilde{\nu}$ = 3430 (br. s), 2947 (m), 1647 (s), 1092 (br. s), 802 (s) cm^{-1}.

Synthesis of Construct 15: Resin **13** (1 g, 0.21 mmol, 1 equiv.) was weighed into a reaction vessel. After the addition of DMF (4.6 mL), FmocLys(Boc)OH (539 mg, 1.15 mmol, 5.5 equiv.) and PyBOP (598 mg, 1.15 mmol, 5.5 equiv.) were added to this suspension. Subsequently, DIEA (400 μL, 2.30 mmol, 11 equiv.) was added, and the suspension was shaken for 4 h and 30 min, after which the solution was drained and the beads were thoroughly washed. The ninhydrine test on a small sample was negative. The Fmoc group was then deprotected to give construct **14**. Next, pentynoic acid (68 mg, 0.69 mmol, 3.3 equiv.), PyBOP (359 mg, 0.69 mmol, 3.3 equiv.) and DIEA (241 μL, 1.38 mmol, 6.6 equiv.) were added to a suspension of **14** in DMF (4.6 mL). The suspension was shaken for 3 h and 30 min and drained, and the remaining beads were washed. The ninhydrine test was negative. ES analysis after photocleavage in EtOH: calcd. For $[M + H]^+$ 326.4; found 348.0 $[M + Na]^+$.

Attachment of the Tripodal Scaffold to Construct 15: Resin **15** (1.1 g, 0.23 mmol, 1 equiv.) was weighed in a reaction vessel and Boc deprotected. A solution of the tripodal scaffold (345 mg, 0.35 mmol, 1.5 equiv.) and PyBOP (180 mg, 0.35 mmol, 1.5 equiv.) in DMF (15 mL) was prepared. When the solution was homogeneous, it was transferred to the pre-swollen resin, and after the addition of DIEA (181 μL, 1.04 mmol), the suspension was shaken for 18 h. The resin was drained and washed thoroughly. The beads turned dark yellow with the TNBS test and red with NF31. The remaining free spacer amino groups were capped for 1 h with a 0.1 M solution of AcOH and PyBOP in the presence of DIEA (2 equiv.). The ninhydrin test was negative. The total loading was 0.101 mmol g^{-1}, which corresponded (maximal theoretical loading 0.173 mmol g^{-1}) to a yield of 58%.

Incorporation of the Three Differently Protected Glycines: For the incorporation of the glycine derivatives, the same protocol as that described for the synthesis of **4** was applied. The amount of resin **16** used was 1.1 g (110 μmol).

Preparation of Compound 18: Automated synthesis was performed in a manner similar to the protocol described for the preparation of library **7b**. Construct **17** (100 mg, 11 μmol) was subjected to this protocol. The protected peptide was cleaved from the resin by irradiating the beads with light at 360 nm. This was done under continuous vortexing of the beads in DCM (10 mL) for 18 h. The suspension was then drained, and the beads were washed with DCM (3×), EtOH (3×) and DCM (3×). The organic fractions were combined, and the solvents evaporated. This whole procedure was repeated five times, and the organic fractions were combined in the same flask. For side-chain deprotection, a mixture of TFA/TIS/DODT/thioanisole/Me_2S (89.5:1:2.5:5:2) was prepared. After adding NH_4I_{aq} (125 μL, 460 mg/mL) to the flask containing the protected peptide, the TFA mixture (2.5 mL) was added. The solution was stirred for 3 h, and then it was evaporated under a flow of Ar. The residue was subsequently dissolved in 10% AcOH/H_2O, and this aqueous fraction was extracted three times with MTBE ether. The water fraction was then lyophilised. The residue obtained after lyophilisation was redissolved in 10% AcOH/H_2O (1 mL). The desired compound was then purified by injecting 500 μL of this mixture on a preparative HPLC system (C_{18} column). A gradient from 0–40% MeCN in 40 min was used in combination with a 0.1% TFA_{aq} solution (from 100–60% in 40 min). After lyophilisation, **18** (3.5 mg, 16%) was obtained. MALDI-TOF analysis: calcd. for $[M - H]^-$ 1904.9; found 1905.1 $[M - H]^-$, 1928.7 $[M - H + Na]^-$, 1942.9 $[M - H + K]^-$.

Preparation of Azido-functionalised Silica 19: The silica particles (2.1 g) were put in a plastic falcon tube. DMF (6 mL) was added, followed by the addition of FmocGlyOH (119 mg, 0.4 mmol, 20 mol-%), AcOH (92 μL, 1.6 mmol, 80 mol-%), HBTU (759 mg, 2 mmol, 1 equiv.) and DIEA (700 μL, 4 mmol, 2 equiv.). After shaking for 90 min, the suspension was centrifuged and washed thoroughly. Determination of the loading gave a value of 0.110 mmol g^{-1}. Next, the Fmoc group was deprotected. The presence of free amine groups was confirmed by a positive TNBS test. The material (1 g, 0.066 mmol, 1 equiv.) was then suspended in DMF (4 mL). PyBOP (260 mg, 0.5 mmol, 7.6 equiv.), azidohexanoic acid (**9**, 79 mg, 0.5 mmol, 7.6 equiv.) and DIEA (174 μL, 1 mmol, 15.2 equiv.) were subsequently added to this suspension. After shaking for 4 h, the solution was drained, and the particles were thoroughly washed. Though no free amines were detected by the TNBS test, a capping step was still performed to ensure that there were no free amines. IR: $\tilde{\nu}$ = 3414 (br. s), 2104 (s), 1642 (m), 1111 (br. s), 810 (s) cm^{-1}.

"Clicking" of Tripodal Compound 18 to Azido-functionalised 19: Firstly, a UV-calibration curve was determined by preparing different concentrations (98, 9.8, 4.9, 2.5, 2.0 and 1.0 μM) of **18** in H_2O. By measuring the absorbance of the different concentrations, the following equation was determined: Abs = 63.082× $[\mathbf{18}]_{aq}$ –

0.0544 (R^2 = 0.9999). Next, $CuSO_4 \cdot 5H_2O$ (5.5 mg, 22 μmol) and sodium ascorbate (8.71 mg, 44.0 μmol) were dissolved in H_2O (20 mL). From this stock solution, 200 μL (16 mol-% $CuSO_4 \cdot 5H_2O$ and 32 mol-% sodium ascorbate) was pipetted in an eppendorf containing **18** (3.5 mg, 1.64 μmol, 8.2 mM 1 equiv.) and **19** (20 mg, 2.20 μmol). The resulting mixture was vortexed at 40 °C. After 48 h, 3 μL of the reaction mixture was transferred to a UV cuvette and 2997 μL H_2O was added. For the blank sample, 3 μL from the $CuSO_4 \cdot 5H_2O$ stock solution was diluted up to 3 mL in a UV cuvette. The absorbance from this sample was 0.121 mAu, which correlates to a concentration of 2.8 mM. From this value, a loading of ≈52 μmol/g was determined. The silica material **20** was isolated by centrifugation and subsequent decantation of the liquid. The material was washed thoroughly water (3×), $EDTA_{aq}$ (3×) and *i*PrOH (3×).

Screening by Affinity LC: LC-MS analyses were carried out on an Alliance 2690 LC system equipped with an online degasser and an autosampler (Waters Milford, MA, USA). The affinity LC analyses were carried out on the home-made estradiol or testosterone column (5 μm, 2.1 × 150 mm). The column was thermostatted at 37 °C. The mobile phase consisted of 10 mM NH_4Ac in water and methanol. The pH of the water was adjusted to 7.4 with NH_3. Different gradients were evaluated, and the best results were obtained using the following linear gradient. Firstly, the mobile phase consisted of 100% 10 mM NH_4Ac in water. Thereafter, the composition was changed to 100% MeOH in 10 min. Finally, this composition was maintained for 15 min. In the end, the mobile phase composition was returned to the initial conditions. The flow rate was 0.2 mL/min, and the injection volume was 10 μL, which corresponds to approximately 250 ppm.

SPE of an Aqueous Mixture of EDCs: The affinity of the aminopropyl silica and material **20** were evaluated by using the following procedure. Firstly, the materials were incubated with 900 μL of a 10 mM NH_4Ac/water solution, adjusted with NH_3 to obtain a pH of 7.4. The cartridge was then loaded with 900 μL of a 10 mM NH_4Ac/water solution, adjusted with NH_3 to obtain a pH of 7.4, spiked with the EDCs, which resulted in a concentration of 10 mg/L (ppm) for each EDC in the water. The flow rate was approximately 0.5 mL/min. The concentration of the EDCs in the water effluent was determined with HPLC-UV. After SPE extraction, the cartridges were rinsed with MeOH to remove the extracted EDCs from the resin so that the resins can be reused. The effluent obtained after the SPE procedure was separated on a Luna-C18 column (5 μm, 2.1 × 150 mm) (Phenomenex, USA). The column was thermostatted at 20 °C. The mobile phase consisted of acetonitrile and water. A linear gradient program was used from 40–60% acetonitrile in 15 min. Thereafter, the mobile phase was switched directly to 100% acetonitrile. This mobile phase composition was then maintained for 5 min. Finally, the mobile phase composition was returned to the initial conditions. The flow rate was 0.2 mL/min, and the injection volume was 10 μL. Ultraviolet detection was carried out by using a Waters 2487 dual λ absorbance detector (Waters Milford). Analyses were recorded at 230 nm.

Supporting Information (see footnote on the first page of this article): RPHPLC and MS data for cleaved compound **4** and detailed RPHPLC and MS data for 18 (15%) of the library members are presented.

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