Tetrahedron 66 (2010) 6912–6918

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Design and automated generation of artificial estrogen receptor as potential endocrine disruptor chemical binders

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ARTICLE INFO

Article history:
Received 21 March 2010
Received in revised form 14 June 2010
Accepted 15 June 2010
Available online 30 June 2010

Keywords:
Synthetic receptor
Endocrine disruptor
Peptide
Automated peptide synthesis
Library

ABSTRACT

We here report on our results concerning the systematic design and synthesis of a new type of artificial estrogen receptor candidates. Our interest herein originates from the known estrogenic activity of endocrine disrupting chemicals, environmental pollutants that have attracted increased attention due to their interference with the endocrine and reproductive system of wildlife and humans. An automated protocol for the generation of libraries of potential artificial receptors is described. The resulting structures could find application in novel SPE cartridges for EDC-preconcentration.

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1. Introduction

As human health is inextricably linked to the health of the environment, the last decades have witnessed an increasing attention to the role that certain environmental pollutants exert on the endocrine system of humans and wildlife. These environmental contaminants have the potential to interfere negatively with the normal functioning of the endocrine and reproductive system by mimicking or inhibiting endogenous hormone action, modulating the production of endogenous hormones, or altering hormone receptor populations. Due to the ability of these chemicals to interfere with the endocrine systems they have been termed Endocrine Disruptor Chemicals (EDCs).[1] EDCs encompass a variety of compounds, such as natural and synthetic hormones, plant constituents, pesticides, additives used in the plastic industry and in consumer products and other industrial by-products and pollutants. Many of these chemicals have been linked with developmental, reproductive, neural and immune problems in wildlife and laboratory animals. They may also adversely affect human health in similar ways resulting in declined fertility and increasing incidence or progression of some diseases including endometriosis and cancer.[2] Trace determination of EDCs in environmental samples is important because they are active even at concentrations below 1 ng/L. However, the analysis of EDCs in water is not straightforward due to the complexity of the environmental matrix and the low concentration at which EDCs are present.

A series of EDCs, such as diethylstilbestrol (DES), bisphenol-A (BPA), dioxin and dioxin-like compounds, polychlorinated biphenyls (PCBs) and pesticides (DDT), is known to exert their effect through binding within the hormone-binding domain of the Estrogen Receptor (ER-HBD). Current assessment of estrogenic activity of chemicals is based on a receptor binding assay with the human Estrogen Receptor.[3] Furthermore a chip-based nano ESI-MS method has been developed for investigation of the gas-phase stability of the human Estrogen Receptor α-hormone-binding domain (hERα-HBD) complexed with environmental contaminants.[4]

In this paper we report on the rational design and systematic synthesis of a library of potential artificial estrogen receptor.

2. Results and discussion

2.1. Rational design of an artificial receptor candidate

The ultimate purpose of this study is the development of a high-throughput system devoted to the preconcentration of EDCs present in waste water. The envisaged system is a solid-phase extraction (SPE) cartridge constituted by an artificial EDC-receptor anchored on a suitable polymer. Previously, simple hexapeptides were reported to show selective binding of estradiol over testosterone.[5] The amino acid constituents were selected among the binding pocket residues of the human steroid binding protein. However, in

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0040-4020/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2010.06.043

view of the apparent importance of the estrogen receptor in the toxicity profile of EDCs and in the above described methods for determination of EDC activity, our design of an artificial receptor is based on the known estrogenic activity of endocrine disruptors. The main feature of a synthetic receptor should thus consist in its ability to mimic the ligand-binding cavity of the Estrogen Receptor (ER-LBD).

The Estrogen Receptor is a steroid receptor belonging to the nuclear-receptor superfamily and regulating development and metabolism through control of gene expression.

Rational design of a suitable artificial receptor was based on the crystallographic structure of the ER-LBD. Careful analysis of the Protein Data Bank (PDB) revealed the presence of not only structural data for the ER-LBD with its natural ligand (17β-estradiol, E2), but also for complexes with two identified endocrine disruptors DES and BPA (Fig. 1). The deposited data were analyzed in detail to identify key interactions and similarities within the different complexes.

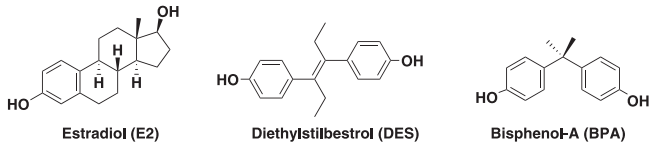


Figure 1. EDC's studied via X-ray diffraction of their corresponding ER-complexes.

As can be directly derived from the X-ray data, molecular recognition is achieved through a combination of specific hydrogen bonds and the complementarity of the binding cavity to the ligand's non-polar character.

2.2. Estrogen receptor α ligand-binding domain (ERα-LBD) complexed to estradiol

Structural data of PDB 1ERE were used for the analysis illustrated in Figure 2.[6] When the ER-LBD complexes E2, the phenolic hydroxyl of the A-ring engages in direct hydrogen bonding with the carboxylate of Glu 353, the guanidinium group of Arg 394, and a water molecule, while the 17-β hydroxyl shows a single hydrogen bond with His 524. The remainder of the molecule is involved in a pattern of hydrophobic contacts with its A, B- and D-ring. The A-ring, and the planar A/B-ring interface, interact with the side chains of Ala 350 and Leu 387 via its β face and Phe 404 and Leu 391 through its α face. The B-ring interacts with the side chain of Met 388. At the other end of the binding cavity the D-ring makes hydrophobic contacts with the side chain of His 524 and Leu 525.

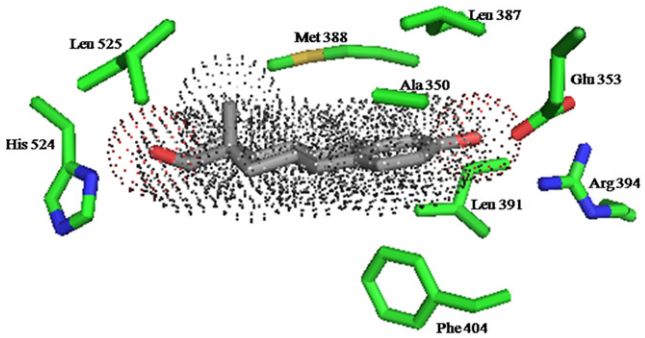


Figure 2. Detailed view of ERα-LBD complexed by estradiol (1ERE). For clarity, only amino acid side chains from contact residues are indicated.

2.3. Estrogen receptor α ligand-binding domain (ERα-LBD) complexed to diethylstilbestrol

Structural data of PDB 3ERD were used to study the interaction of DES with the ERα-LBD illustrated in Figure 3.[7] In general the interaction pattern resembles that of E2. As the phenolic rings of both DES and E2 are situated in the same position, the DES hydroxyl group of the A-ring forms similar hydrogen bonds with the side chain of Glu 353, Arg 394 and a water molecule. On the other hand, the hydroxyl group of the A'-ring forms hydrogen bond with His 524. Concerning the hydrophobic interactions, the A-ring interacts with the side chain of Leu 387, while the A'-ring interacts with Leu 525, Met 343 and Met 421. The remainder of DES is stabilized by the side chain of Phe 404, Ala 350 and Leu 386.

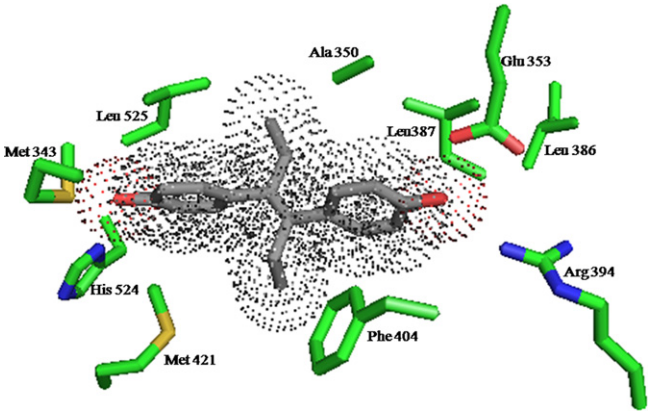


Figure 3. Detailed view of ERα-LBD complexed by diethylstilbestrol (3ERD). For clarity, only amino acid side chains from contact residues are indicated.

2.4. Estrogen-related receptor γ ligand-binding domain (ERRγ-LBD) complexed to bisphenol-A

In literature no crystallographic structures are available concerning complexation of bisphenol-A to the ER-LBD. Our investigation was therefore based on an X-ray structure of the estrogen-related receptor γ (ERRγ, PDB 2P7 G, see Fig. 4).[8] The ERR subfamily is closely related to the estrogen receptor and though sharing structural homology, these receptors do not bind estrogens.[8] The bisphenol-A in the ERRγ ligand-binding pocket is complexed mostly by hydrophobic residues, such as Phe 435, Met 306, Ala 272 and Leu 309 at the convex side of the molecule. The concave

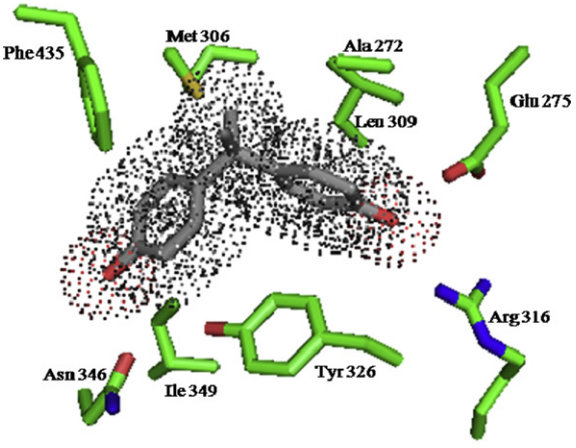


Figure 4. Detailed view of ERα-LBD complexed by Bisphenol-A (2P7 G). For clarity, only amino acid side chains from contact residues are indicated.

face of the ligand interacts with the side chain of Tyr 326 and Ile 349. The phenolic hydroxyl of the A-ring forms hydrogen bonds with the side chains of Glu 275 and Arg 316, while the hydroxyl group of the A'-ring presents a direct hydrogen bond to Asn 346.

This brief analysis of different LBD's complexed with E2, DES and BPA confirms that the ER-LBD is able to accommodate and recognise various compounds diverse in size, shape and chemical properties. Although the narrow arrangement of the binding cavity around the A-ring imposes the ligand to present an aromatic ring in that position, the remainder of the binding pocket can accept a number of different hydrophobic structures.[9]

Analysis of these X-ray structures has permitted gathering the data in Table 1. Table 1 summarizes all the amino acids involved in the different interactions and highlights, which of those can be considered crucial for the synthesis of an artificial estrogen receptor able to interact and thus show affinity towards EDCs (see Table 1).

Table 1
Crucial amino acids involved in interactions within the binding pocket. Bold residues have been identified as being common for all three complexes

Type of interactions	**E2** 1ERE[a]	**DES** 3ERD[a]	**BPA** 2P7G[a]
Hydrophilic	**Glu** 353	**Glu** 353	**Glu** 275
	Arg 394	**Arg** 394	**Arg** 316
	His 524	**His** 524	Asn 346
Hydrophobic	**Phe** 404	**Phe** 404	Tyr 326
	Ala 350	**Ala** 350	**Phe** 435
	Leu 387	**Leu** 387	**Leu** 309
	Leu 391	**Leu** 386	**Met** 306
	Leu 525	**Leu** 525	**Ala** 272
	His 524	**Met** 343	Ile 349
	Met 388	**Met** 421	

[a] PBD code of studied complex.

In summary the crucial amino acids involved in hydrophilic interactions are glutamic acid, arginine and histidine, while the hydrophobic interactions are supplied by phenyl alanine, alanine, methionine and leucine.

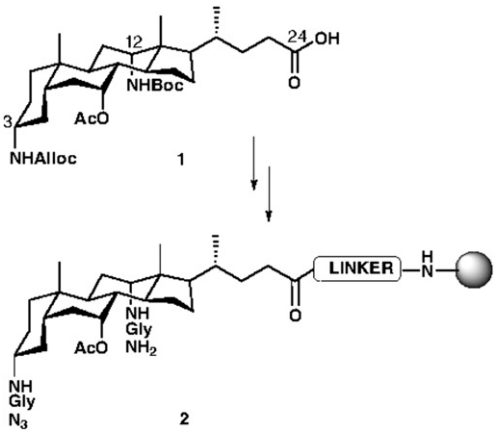
These seven amino acids can now be used in the preparation of simple linear peptides or more complicated structures based on flexible and rigid scaffolds.

2.5. Scaffold considerations: from a flexible to a rigid multipodal presentation of amino acids

As already mentioned, previous investigations were performed in the field of development of new materials for EDC-preconcentration. Vanni and co-workers[5] synthesized a series of linear peptides with affinity for E2. Furthermore, we have recently reported on the development of a library of EDCs-receptors based on a flexible tripodal scaffold.[10] However, no strong EDC affinity could be confirmed.

In the current study we focus on the combination of the crucial amino acids with the utilization of a rigid dipodal template as a new strategy for EDC-receptor synthesis. The chosen dipodal scaffold **1** (Scheme 1) is an orthogonally protected cholic acid derivative,[11] which allows the construction of two different parallel oriented peptide chains.

In earlier work this type of scaffold has been applied in a variety of designs[12] including the generation of cholate-derived translocases,[13] the construction of artificial carbohydrate receptors[14] and the synthesis of serine protease mimics.[15,16] The typical A-B cis connection with appendages originating from the C-3 and C-12 positions imposes a rigid and parallel orientation upon connection of peptide chains; this in contrast to the flexible nature of our previous tripodal design. Combining the more preorganized nature of the chains with the intrinsic hydrophobic cavity presented by the cholic acid should allow for the development of artificial receptors with more potent EDC binding properties.



Scheme 1. Dipodal steroid scaffold **1** and its derivative **2** suitable for automated synthesis.

2.6. Scaffold transformation: adaptation towards an automated library synthesis protocol

The dipodal steroid scaffold **1**, presents two amino functionalities orthogonally protected as Alloc (in position C-3) and Boc (in position C-12). Additionally, the carboxyl group in position C-24 provides an anchoring point to the solid support allowing for its application in solid-phase peptide synthesis.

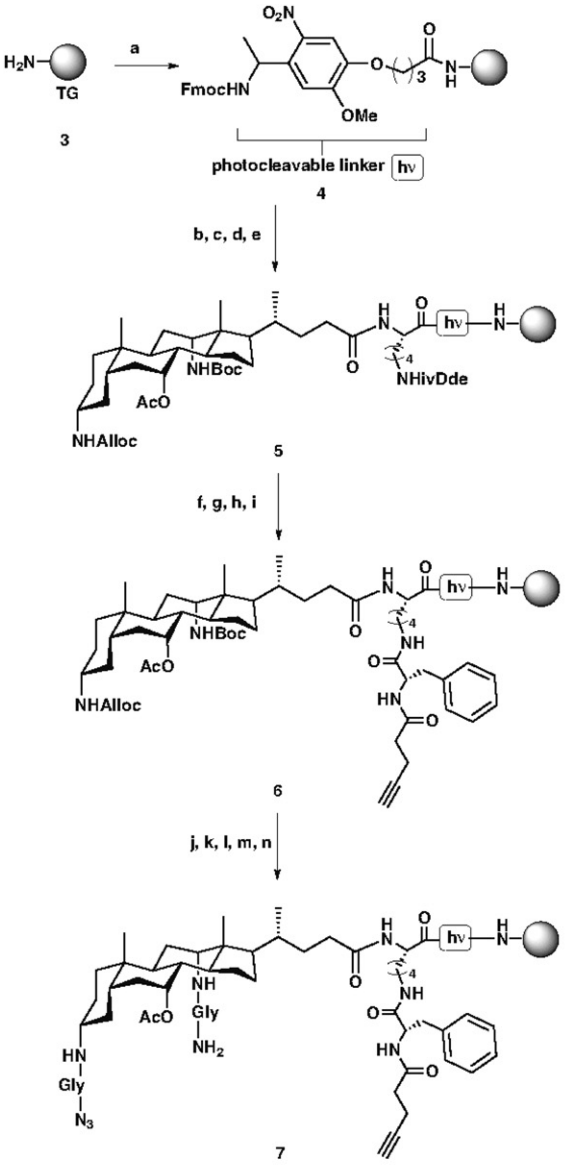
In view of later facilitation of screening it is our aim to generate parallel libraries of possible artificial receptor candidates. Although the cholic acid template has been used in a variety of designs, the reported and earlier developed synthetic methodology for construction of compound collections was centered around the manual generation of either single compound[17] or mix-split libraries.[15,16] As for generation of parallel libraries, using a standard automated peptide synthesis apparatus, the orthogonality scheme of **1** presents some serious drawbacks.

Indeed, the removal of the Boc protecting group requires acidic conditions, which can result in corrosion and thus damage of automated synthesis systems. Removal of the Alloc protecting group requires the utilization of an unstable catalyst and mostly the very volatile dichloromethane as a solvent, conditions that cannot be handled in the standard automated peptide synthesis systems. These incompatibilities inspired us to re-think the orthogonality scheme and chemically modify the scaffold to allow automated synthesis for parallel library generation.

When a synthesis is performed on solid support, cleavage of the product from the support is an important step both after each reaction and at the end. Intermediate resin detachment along the synthetic pathway allows controlling the reaction completeness, while the final cleavage should enable the quantitative release of the desired product in solution for easy screening.

A convenient handle for cleavage is the utilization of a photolabile linker (4-[4-1-(9-fluorenylmethoxycarbonyl-amino)ethyl]-2-methoxy-5-nitro-phenoxy)butanoic acid (Scheme 2), which can release the product in solution upon mere irradiation with UV-light.[18,19]

In first instance, the photolabile linker was immobilized on aminomethyl TentaGel™ resin (**3**) to generate compound **4**. Upon Fmoc deprotection, Fmoc-Lys(ivDde)OH was introduced and after Fmoc removal, dipodal steroid scaffold **1** was coupled (**5**). In all manual coupling reactions, the activating reagent 1*H*-benzotriazol-1-yloxy-tris(pyrrolidino)phosphonium hexa-fluorophosphate (PyBOP) and the tertiary base *N*,*N*′-diisopropylethylamine (DIPEA) were used. The orthogonality of the protecting groups ivDde, Alloc and Boc ensured



a) Fmoc-protected photocleavable linker, PyBOP, DIEA, DMF, RT 3 h; b) 20% piperidine/NMP; c) Fmoc-Lys(ivDde)-OH, PyBOP,DIEA, DMF, RT 2 h; d) 20% piperidine/NMP; e) scaffold, PyBOP,DIEA, NMP, RT 2 h; f) 10% $NH_2NH_2{\cdot}H_2O$/DMF, RT, 2 x 10 min; g) Fmoc-Phe-OH, PyBOP,DIEA, NMP, RT 2.5 h; h) 20% piperidine/NMP; i) 4-Pentynoic acid, PyBOP, DIEA, NMP, RT 3 h; j) $PhSiH_3$, $Pd(PPh_3)_4$, DCM (dry), RT 3 x 1h; k) N_3-Gly-OH, PyBOP, DIEA, NMP, RT 2 x 3 h; l) 20% TFA/DCM, RT 2 x 20 min; m) Fmoc-Gly-OH, PyBOP, DIEA, RT 2 x 3 h; n) 20% piperidine/NMP;

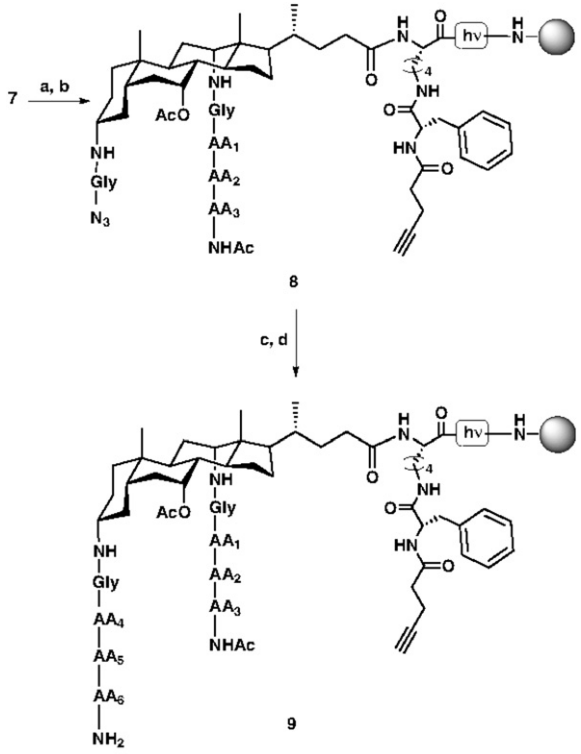
Scheme 2. Construction of starting material for library generation.

the removal of the ivDde group from **5** without perturbing the remaining protecting functionalities on the scaffold. The introduction of the extra Lys residue, allowed for introduction of a Phe unit in order to enhance the UV-activity of the molecule. In this way, later RP-HPLC analysis of intermediates is facilitated, allowing optimization through close monitoring of the critical Alloc deprotection step. Although the Alloc group has very frequently been applied in peptide chemistry, in our experience its deprotection requires careful monitoring and optimization in order to obtain reproducible results. Furthermore this side chain was employed for the provision of an alkyne moiety that serves the purpose of later immobilization via straightforward click-chemistry onto a suitable SPE support for screening and application of the artificial receptor.

Therefore, after Fmoc deprotection, an alkyne moiety was introduced by coupling 4-pentynoic acid (**6**). Directing the attention to the scaffold, Alloc deprotection was then tackled. Initial attempts involved the use of morpholine, Bu_3SnH and $Pd(PPh_3)_4$[20] leading to either incomplete deprotection or competitive allylation of free amino functionality. The Alloc protecting group was finally removed in good yield using $PhSiH_3$ as scavenger and $Pd(PPh_3)_4$ as catalyst. On the free amino group azido acetic acid was coupled. At this stage the product was subjected to acid treatment using a solution of 20% TFA in DCM, which removed the Boc protecting group. The primary amino group at C-12 was then coupled with Fmoc-Gly-OH, its further Fmoc deprotection provided the starting material (**7**) for a fully automated synthesis of the parallel library.

3. Library synthesis

In the current work six out of the identified seven amino acids were used, for similarity reasons we decided not to incorporate Ala. Six representative members were then arbitrarily selected for checking the automated parallel synthesis set-up (Scheme 3). In position C-12 an automated protocol was applied to generate a capped tripeptide chain. For automated synthesis PyBOP was replaced by *O*-benzotriazole-*N*,*N*,*N*′,*N*′,-tetramethyl-uronium-hexafluoro-phosphate (HBTU).

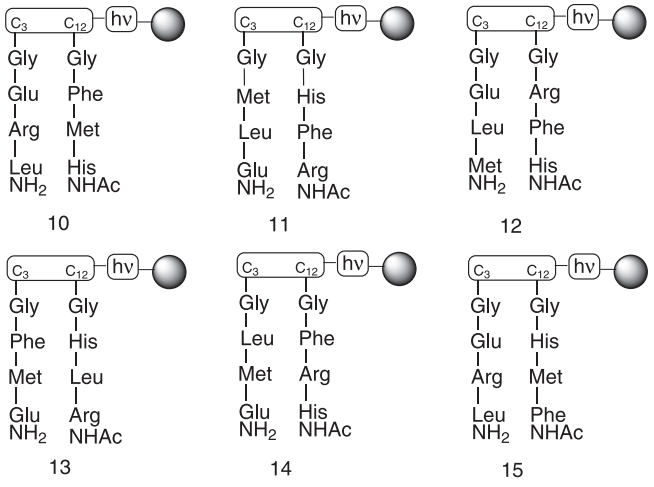


a) Automated synthesis; b) capping: Ac_2O/Py/NMP 1 : 3 : 5, RT 10 min; c) M_3P 0.75 M THF/H_2O, RT 2 x 2 h; d) Automated synthesis;

Scheme 3. Automated parallel synthesis of artificial estrogen receptor candidates.

Next, the azide was reduced revealing the primary amino group ready to react with the subsequent amino acids and generating the second tripeptide chain. Applying this fully automated synthetic protocol a first six-member library was prepared (Scheme 4).

After the complete synthesis all members were characterized by RP-HPLC-MS. We here include the resulting LC-MS for members **11** and **12** (Fig. 5, for other members see Supplementary data). In both spectra two peaks are noticeable, the main signal corresponding to



Scheme 4. Structures of the six members of the parallel library.

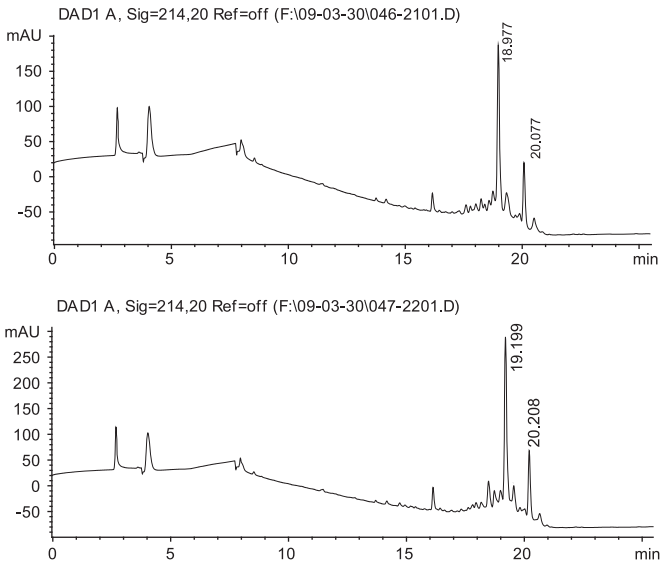


Figure 5. RP-HPLC of members **11** (above) and **12** (below). The major peaks represent the desired material.

the correct product while a smaller signal corresponding to a non-reduced azide intermediate is visible.

Although the generated products are not perfectly pure, they can be easily purified by RP-HPLC due to the difference in retention time between the peaks. These chromatograms confirm the good performance of the developed automated synthesis protocol, which can now be considered for further generation of large parallel combinatorial libraries.

4. Conclusions

Herein we have reported the rational design, based on the systematic analysis of available crystallographic data, and the synthesis of artificial biomimetic estrogen receptors. The described strategy allows the fully automated generation of a series of compounds with satisfying purity. In view of the above illustrated importance of the natural estrogen receptor in current techniques aimed at determination of EDC activity of emerging environmental pollutants, the currently developed compounds, once proven capable of binding EDCs, could find useful applications in the development of SPE cartridges for preconcentration of EDCs in waste water or alternative detection technologies.

5. Experimental section

5.1. General methods

Manual peptide synthesis on solid-phase was performed in a glass-vessel protected against light with aluminium foil and equipped with a sintered glass filter and a 3-way stopcock for easy filtration and washing. Automated synthesis was executed in plastic reaction vessels of 2 mL equipped with Teflon frits both purchased from Multisyntech Scientific. Photolysis of the product from the solid support was carried out with a 4 W Bioblock Scientific compact UV lamp set at 365 nm.

NMR spectra were recorded at 300 MHz for proton and at 75 MHz for carbon nuclei in chloroform-*d*. Chemical shifts are reported in units of parts per million (ppm), referenced relative to the residual ^{1}H or ^{13}C peaks of the used solvent as internal standard chloroform-*d*: ^{1}H 7.26 and ^{13}C 77.16. IR spectra were recorded on a Perkin–Elmers 1000 equipped with HATR and reported in wavenumber (cm^{-1}). Samples were prepared as a thin film on the sample-holder.

Mass spectra were recorded with an atmospheric pressure electrospray-ionization source (ESI) equipped with a LCQ ion trap (Hewlett–Packard 5988) and by MALDI-TOF mass spectrometry (Applied Biosystem Voyager-DE STR Biospectrometry Workstation) using 2,5-dihydroxybenzoic acid as matrix.

Accurate mass measurements were performed on a quadrupole/orthogonal-acceleration time-of-flight (Q/oaTOF) tandem mass spectrometer (qTof 2, Micromass, Manchester, UK) equipped with a standard electrospray ionization (ESI) interface. Cone voltage was set to 30 V, capillary voltage to 3000 V and resolution of the instrument was 8500. Samples were dissolved in a mixture of acetonitrile and water (1:1) and infused at 5 μL/min. The protonated ion for peptide YGGFL (m/z =556.2771), which was added to the sample (10 pmol/μL) was used as lock mass.

Purity and mass analysis were recorded on a HPLC Agilent 1100 Series using a Phenomenex Luna C18 (2) column (250×4.6 mm, 5 μL at 35 °C) and equipped with an ES-MSD type VL mass detector. The samples were analyzed using a flow rate of 1 mL/min with the following eluents: 5 mM NH_4OAc 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 applied, followed by 5 min of flushing with 100% B.

Unless otherwise stated, RP-HPLC analysis were performed on an Agilent 1100 Series instrument with a Phenomenex Luna C18(2) column (250×4.6 mm, 5 μm at 35 °C). A flow-rate of 1 ml/min and with the following solvent system: 0.1% TFA in H_2O A and MeCN (B). The column was flushed for 3 min with 100% A, then a gradient from 0% to 100% of B in 15 min was used, followed by 5 min of flushing with 100% B.

5.2. Materials

The chosen solid support was Tentagel-S-NH_2 (loading 0.27 mmol/g, 90 μm) from Irish BiotechGmbH. All the natural amino acids were purchased from NovaBiochem and Irish BiotechGmbH. DMF extra dry and dry DIEA were purchased from Aldrich. DMF and NMP peptide grade were purchased from Biosolve. All the chemicals were purchased and used without any further purification, except from dichloromethane, which was distilled from CaH_2.

5.3. Standard operating procedures

5.3.1. Swelling. Swelling the resin prior to a reaction guarantees a good penetration of the reagents between the beads and within the pores. Usually the resin is swollen in the solvent used in the reaction

for 10–15 min: the solvent (10 mL/g) is added, the resin is occasionally swirled after which the solvent is removed by filtration.

5.3.2. Washing. After each reaction the resin is thoroughly washed and dried to remove the excess of reagents and prepare the resin for the next synthetic step. The washing is carried out by repeating the application of solvent, stirring and filtration; the following sequence of solvents is usually applied: NMP or DMF (×3), MeOH (×3), DCM (×3) and Et_2O (×2).

5.3.3. Fmoc loading. Fmoc loading permits to quantify the amount of Fmoc protecting groups loaded on the resin via coupling of a Fmoc-containing molecule. It can thus be used to quantify coupling yields. A small sample of resin (~7 mg) is treated with a 20% piperidine/DMF solution and is occasionally swirled for a period of 20 min. The resin is let to settle and the solution is transferred to two UV-cuvettes. By measuring the absorbance at 300 nm of the piperidine–fulvene adduct, its concentration is determinated and thus the loading can be calculated.

5.3.4. Fmoc deprotection. The removal of the Fmoc protecting group allows to obtain a free amino group ready for the subsequent coupling.

For the Fmoc removal the resin is treated with a 20% piperidine/DMF solution (10 mL/g) for 1 min after which the suspension is filtered. This step is repeated for 5 min and 8 min. Finally the resin is washed following the above mentioned procedure.

5.3.5. NF31test[21]. The NF31 test is a colour test used to detect free amines, thus is a useful tool to monitor peptide coupling.

A small sample of resin (1 mg) is suspended in 50 μL of a 0.0002 M solution of NF31 in MeCN. The suspension is heated at 70 °C for 10 min, then the resin is washed with NMP or DMF (×3), MeOH (×3) and DCM (×3). If the beads turn red they containing free amino groups, if the beads are colourless no primary amino groups are present, thus the coupling can be considered complete.

5.3.6. Photolysis via UV irradiation. A small sample (1 mg) of resin is suspended in 100 μL of MeCN and subjected to irradiation of 365 nm light for 3 h.

5.4. Synthesis

5.4.1. Synthesis of azido acetic acid. According to literature,[22] in a dry flask NaN_3 (5.13 g, 2.1 equiv) was dissolved in DMSO (218 mL) and stirred at room temperature for 90 min. In a separate flask bromo acetic acid (5.21 g, 1 equiv) was dissolved in DMSO (2 mL). This solution was added dropwise to the NaN_3 solution, the overall reaction mixture was stirred at room temperature overnight. The solution was then diluted with water (174 mL) and acidified with HCl (6 N, 35 mL). The organic phase was dried on sodium sulfate and evaporated under reduced pressure to obtain a slightly orange oil (2.73 g, 73%). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ=11.2 ppm, δ=3.97 ppm; ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ=175.54 ppm, δ=50.00 ppm; IR ν=2111 (s) (cm^{-1}).

CAUTION The pure acid is insensitive to shock up to 175 °C and not explosively unstable up to 250 °C. In contrast the presence of iron or iron-salts leads to rapid exothermic decomposition of the acid at 25 °C, and explosion at 90 °C.[23]

In view of these safety issues we strongly recommend to avoid warming-up the waterbath during concentration under reduced pressure.

*5.4.2. Immobilization of photolinker on TentaGel™ resin (**4**).* To a suspension of resin (2 g, 0.27 mmol/g) were added Fmoc-protected photolabile linker (0.88 g, 0.1 M in DMF, 1.62 mmol), PyBOP (0.84 g, 1.62 mmol) and DIEA (0.56 mL, 3.24 mmol). The mixture was shaken at room temperature for 3 h, after which the resin was washed. The loading was calculated to be 0.17 mmol/g, which gave a coupling yield of 71.34%.

*5.4.3. Coupling of Fmoc-Lys(ivDde)-OH and dipodal scaffold (**5**).* Compound **4** (1.95 g, 0.17 mmol/g) was Fmoc deprotected. Then the resin was suspended in DMF (6.6 mL) and to it were added Fmoc-Lys(ivDde)-OH (0.95 g, 1.65 mmol), PyBOP (0.86 g, 1.65 mmol) and DIEA (0.57 mL, 3.29 mmol). The mixture was shaken at room temperature for 2 h, after which the resin was washed. The NF31 test remained colourless. A small sample was irradiated by UV-light for LC-MS analysis: t_R 17.81 min; exact mass calculated 573.32, found m/z 574.2 $[M+H]^+$.

Then the product (1.7 g, 0.17 mmol/g) underwent Fmoc deprotection. To a suspension of resin in NMP (6.6 mL) were added the dipodal scaffold (0.47 g, 0.74 mmol, 0.3 M), PyBOP (0.38 g, 0.74 mmol, 0.3 M) and DIEA (0.26 mL, 1.48 mmol, 2 N). The mixture was shaken at room temperature for 2 h, after which the resin was washed. The NF31 was performed and remained colourless. A small sample was cleaved from the resin via UV-irradiation for a LC-MS analysis: t_R 19.04 min, exact mass calculated: 965.65; found m/z 966.60 $[M+H]^+$.

*5.4.4. Synthesis of the external linker: coupling of Fmoc-Phe-OH and incorporation of alkyne moiety (**6**).* Product **5** (1.7 g, 0.17 mmol/g) was treated with a solution of 10% $NH_2NH_2 \cdot H_2O$/DMF (25 mL/g resin) for 10 min, after which the suspension was filtered. This treatment was repeated once, then the resin was washed. A small sample was cleaved by photolysis and analyzed by LC-MS and ESI-MS. LC-MS: t_R 14.69 min; exact mass calculated: 759.51, found m/z 760.4 $[M+H]^+$, ESI-MS: m/z 760.2 $[M+H]^+$ and 660.3 $[M-Boc]^+$. It's noteworthy mentioning that the compound at this stage is not UV-active.

To a suspension of resin in NMP were added Fmoc-Phe-OH (0.56 g, 1.44 mmol, 0.5 M), PyBOP (0.75 g, 1.44 mmol, 0.5 M) and DIEA (0.50 mL, 2.87 mmol, 2 N). The mixture was shaken at room temperature for 2.5 h, after which the resin was washed. The resin remained colourless after a NF31 test. A small sample of resin was subjected to photolysis under UV-irradiation for LC-MS analysis (gradient 75–100% MeCN in 15 min): t_R 9.23 min, exact mass calculated: 1128.65; found m/z 1129.6 $[M+H]^+$ and 1029.6 $[M-Boc]^+$.

The product (1.7 g, 0.17 mmol/g) was subjected to Fmoc deprotection. To a suspension of resin in NMP were added 4-pentynoic acid (0.14 g, 1.44 mmol, 0.5 M), PyBOP (0.75 g, 1.44 mmol, 0.5 M) and DIEA (0.50 mL, 2.87 mmol, 2 N). The mixture was shaken at room temperature for 3 h, after which the resin was washed. The NF31 colour test revealed colourless beads.

*5.4.5. Coupling of azido acetic acid and Fmoc-Gly-OH for the preparation of starting material for library generation (**7**).* Resin **6** (1.08 g, 0.17 mmol/g) was swollen and thoroughly washed with DCM (dry) under a stream of argon. The resin was suspended in DCM (dry, 0.3 mL/g resin) then the scavenger $PhSiH_3$ (0.52 mL, 4.22 mmol) and the catalyst $Pd(PPh_3)_4$ (19.5 mg, 0.02 mmol) were loaded. The mixture was shaken at room temperature for 1 h under anhydrous conditions, after which the resin was washed with DCM (dry) and the reaction was repeated twice. At the end of the Alloc removal the resin was washed following the standard procedure. A small sample was irradiate with UV-light for LC-MS analysis: t_R 13.09 min; exact mass calculated: 902.56 found, m/z 903.6 $[M+H]^+$ and 402.3 $[M-Boc+2]/2$.

To a suspension in NMP of the Alloc deprotected resin (1.08 g, 0.17 mmol/g) were added azido acetic acid (92 mg, 0.91 mmol, 0.5 M), PyBOP (0.47 g, 0.91 mmol, 0.5 M) and DIEA (0.32 mL, 1.82 mmol, 2 N). The reaction mixture was shaken at room

temperature for 3 h, after which the resin was washed. The reaction was repeated once. The photocleavage of a small sample permitted to obtain the product in solution ready for LC-MS analysis: t_R 15.99; exact mass calculated: 985.6; found m/z 986.6 $[M+H]^+$.

Then the product was subjected to Boc deprotection: the resin (1.08 g, 0.17 mmol/g) was treated with a solution of 20% TFA/DCM (20 mL/g resin) for 20 min, after which the solution was removed via filtration and the treatment repeated once. Finally the resin was washed with a solution of 10% TEA/DCM (×3) followed by the standard washing procedure. The deprotection was indicatively controlled by a NF31 colour test, which gave positive response. The product (1.08 g, 0.17 mmol/g) was suspended in NMP and the following reagents were added Fmoc-Gly-OH (0.27 g, 0.91 mmol, 0.5 M), PyBOP (0.47 g, 0.91 mmol, 0.5 M) and DIEA (0.312 mL, 1.82 mmol, 2 N). The reaction mixture was shaken at room temperature for 3 h, after which the resin was washed and the reaction repeated once. A small sample was photocleaved for LC-MS analysis: t_R 16.55 min; exact mass calculated 1164.64; found m/z 1165.6 $[M+H]^+$.

A small amount of resin (90 mg, 0.17 mmol/g) underwent Fmoc deprotection yielding product **7**.

The resin was split in six reaction vessels (2 mL) to perform the automated synthesis for the preparation of six members.

5.4.6. Automated synthesis. All reactions occurred at room temperature, without protecting atmosphere; the necessary solutions were prepared manually.

First, the synthesis of the six different members was programmed. The automated synthesis started with a 10 min swelling step followed by coupling of the first amino acid. During the automated coupling the correct amino acid is transferred in the corresponding plastic reactor, which is then filled with HBTU and DIPEA and swirled for 1 h; each coupling step is followed by Fmoc removal. After the coupling and Fmoc deprotection of the final amino acid of the first peptide chain, a capping step was carried out (**8**). Next, the azide moiety was reduced to amino group treating the resin with Me_3P (0.75 M THF/H_2O) and leaving it to react for 2 h, this treatment was repetes once. The supports were thoroughly washed with ^{i}PrOH and DMF, then swollen in DMF. The second peptide chain was synthesized as the first one but no capping was carried out to allow a better ionization of the product during mass analysis. This protocol allowed to obtain products from **10** to **15**.

Preparation of coupling reagents solutions: Fmoc-protected amino acid (5 equiv, 0.5 M in DMF except for Fmoc-Phe-OH dissolved in NMP), HBTU (5 equiv, 0.5 M in DMF) and DIEA (10 equiv, 2 M in NMP).

Preparation of the solutions for Fmoc deprotection (40% piperidine/NMP), capping (Ac_2O/Py/NMP 1: 3: 5), azide reduction Me_3P (5 equiv, 0.75 M THF/H_2O).

All library members (from **10** to **15**) were analyzed by RPHLC-MS and HRMS.

Here below the HRMS data are reported:

Member **10**: HRMS (ESI) for $C_{125}H_{171}N_{19}O_{20}S_2$: [M+2]/2 calculated 1162.1272, found 1162.1301.

Member **11**: HRMS (ESI) for $C_{125}H_{171}N_{19}O_{20}S_2$: [M+2]/2 calculated 1162.1272, found 1162.1224.

Member **12**: n.d.

Member **13**: HRMS (ESI) for $C_{125}H_{171}N_{19}O_{20}S_2$: [M+2]/2 calculated 1162.1272, found 1162.1238.

Member **14**: HRMS (ESI) for $C_{125}H_{171}N_{19}O_{20}S_2$: [M+2]/2 calculated 1162.1272, found 1162.1226.

Member **15**: HRMS (ESI) for $C_{125}H_{171}N_{19}O_{20}S_2$: [M+2]/2 calculated 1162.1272, found 1162.1239.

All the RPHPLC-MS chromatograms and spectra are included in the Supplementary data.

Acknowledgements

This research project has been supported by a Marie Curie Early Stage Research Training Fellowship of the European Community's Sixth Framework Programme under contract number (MEST-CT-2005-020643).

Supplementary data

Electronic Supplementary data (ESI) available: copies of RP-HPLC chromatograms and MS spectra for cleaved intermediates and final library members. Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tet.2010.06.043. These data include MOL files and InChIKeys of the most important compounds described in this article.

References and notes

1. Blair, R. M.; Fang, H.; Branham, W. S.; Hass, B. S.; Dial, S. L.; Moland, C. L.; Tong, W. D.; Shi, L. M.; Perkins, R.; Sheehan, D. M. *Toxicol. Sci.* **2000**, *54*, 138–153.
2. Preziosi, P. *Pure Appl. Chem.* **1998**, *70*, 1617–1631.
3. Thomas, P.; Smith, J. *Mar. Environ. Res.* **1993**, *35*, 147–151.
4. Bovet, C.; Wortmann, A.; Eiler, S.; Granger, F.; Ruff, M.; Gerrits, B.; Moras, D.; Zenobi, R. *Protein Sci.* **2007**, *16*, 938–946.
5. Tozzi, C.; Anfossi, L.; Giraudi, G.; Giovannoli, C.; Baggiani, C.; Vanni, A. *J. Chromatogr., A* **2002**, *966*, 71–79.
6. Brzozowski, A. M.; Pike, A. C. W.; Dauter, Z.; Hubbard, R. E.; Bonn, T.; Engstrom, O.; Ohman, L.; Greene, G. L.; Gustafsson, J. A.; Carlquist, M. *Nature* **1997**, *389*, 753–758.
7. Shiau, A. K.; Barstad, D.; Loria, P. M.; Cheng, L.; Kushner, P. J.; Agard, D. A.; Greene, G. L. *Cell* **1998**, *95*, 927–937.
8. Abad, M. C.; Askari, H.; O'Neill, J.; Klinger, A. L.; Milligan, C.; Lewandowski, F.; Springer, B.; Spurlino, J.; Rentzeperis, D. *J. Steroid Biochem. Mol. Biol.* **2008**, *108*, 44–54.
9. Anstead, G. M.; Carlson, K. E.; Katzenellenbogen, J. A. *Steroids* **1997**, *62*, 268–303.
10. Van der Plas, S. E.; Van Hoeck, E.; Lynen, F.; Sandra, P.; Madder, A. *Eur. J. Org. Chem.* **2009**, 1796–1805.
11. Barry, J. F.; Davis, A. P.; Perez-Payas, M. N.; Elsegood, M. R. J.; Jackson, R. F. W.; Gennari, C.; Piarulli, U.; Gude, M. *Tetrahedron Lett.* **1999**, *40*, 2849–2852.
12. Maitra, U. *Org. Biomol. Chem.* **2008**, *6*, 657–669.
13. Lambert, T. N.; Boon, J. M.; Smith, B. D.; Perez-Payan, M. N.; Davis, A. P. *J. Am. Chem. Soc.* **2002**, *124*, 5276–5277.
14. Davis, A. P.; Wareham, R. S. *Angew. Chem., Int. Ed.* **1999**, *38*, 2978–2996.
15. De Muynck, H.; Madder, A.; Farcy, N.; De Clercq, P. J.; Perez-Payan, M. N.; Ohberg, L. M.; Davis, A. P. *Angew. Chem., Int. Ed.* **2000**, *39*, 145–148.
16. Madder, A.; Li, L.; De Muynck, H.; Farcy, N.; Van Haver, D.; Fant, F.; Vanhoenacker, G.; Sandra, P.; Davis, A. P.; De Clercq, P. J. *J. Comb. Chem.* **2002**, *4*, 552–562.
17. Bode, C. A.; Bechet, T.; Prodhomme, E.; Gheysen, K.; Gregoir, P.; Martins, J. C.; Muller, C. P.; Madder, A. *Org. Biomol. Chem.* **2009**, *7*, 3391–3399.
18. Farcy, N.; De Muynck, H.; Madder, A.; Hosten, N.; De Clercq, P. J. *Org. Lett.* **2001**, *3*, 4299–4301.
19. Holmes, C. P. *J. Org. Chem.* **1997**, *62*, 2370–2380.
20. Verzele, D.; Goeman, J. L.; Madder, A. *Arkivoc* **2007**, *10*, 325–336.
21. Madder, A.; Farcy, N.; Hosten, N. G. C.; De Muynck, H.; De Clercq, P. J.; Barry, J.; Davis, A. P. *Eur. J. Org. Chem.* **1999**, 2787–2791.
22. Banaszynski, L. A.; Liu, C. W.; Wandless, T. J. *J. Am. Chem. Soc.* **2005**, *127*, 4715–4721.
23. *Handbook of Reactive Chemical Hazards*, 7th ed.; Urben, P. G, Ed.; Academic Press: Amsterdam, 2007.