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Bioorganic & Medicinal Chemistry Letters 17 (2007) 379–384
Bioorganic & Medicinal Chemistry Letters

Towards erythropoietin mimicking small molecules

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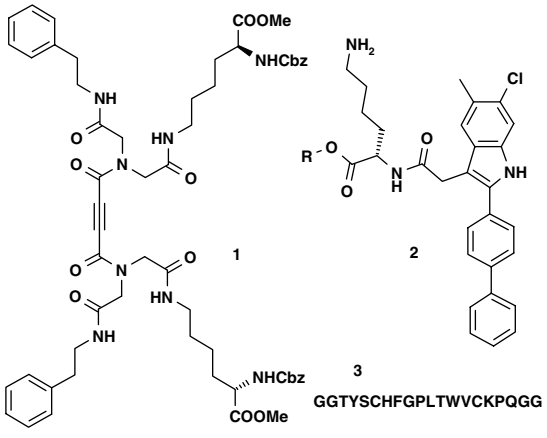
Received 27 July 2006; revised 17 October 2006; accepted 17 October 2006
Available online 20 October 2006

Abstract—Small molecules potentially mimicking the hormone erythropoietin have been discovered by screening of a library of rationally designed multicomponent reaction molecules in a functional cell-based assay.

The fortune of the biotechnology industry is significantly interrelated to the success of recombinant proteins involved in protein–protein interactions, for example, antibodies or hormones such as erythropoietin, insulin, or growth hormone. However due to the intrinsic disadvantages of proteins, for example, protease susceptibility, lack of oral bioavailability and rapid clearance, protein therapeutics often require frequent injections thereby placing a burden on patients. Strategies to overcome some of these disadvantages include transdermal delivery, inhalation, pegylation,[1] construction of dimeric proteins,[2] fusion to carriers that are believed to reduce clearance,[3] incorporation of D-amino acids or β-amino acids,[4] terminal capping and glycoengineering.[5] An alternative strategy is the development of small molecules which are devoid of these disadvantages. Indeed, several potent small molecule protein interaction antagonists have been recently discovered, inhibiting, for example, p53/mdm2, Bcl-2/Bax or XIAP/Caspase-3/-7.[6] Despite considerable effort, no small molecular weight EPO agonists capable of bringing receptors together in a functional manner have been developed or marketed so far. However recently small molecule thrombopoietin (TPO) mimetics, and a rather small peptidic CD40L mimic have been disclosed.[7–10]

Erythropoietin (EPO) is a 34 kDa glycopeptide hormone that acts primarily by inducing dimerisation of its unique cell surface receptor (Fig. 1).[30] Red blood cell production is thereby promoted by proliferation and differentiation of appropriate progenitor cells.[11] This biological activity is the basis for the use of recombinant EPO to treat anaemia stemming from, for example, cancer, kidney failure and AIDS. Moreover, EPO possesses biological effects independent of its erythropoietic activity. It appears to control the progress of myeloma in humans and animals,[12] and has been shown to be tissue protective in injury models of the central nervous system,[31] the eye[32] and the heart,[33] via interaction with an erythropoietin and common beta-subunit heteroreceptor.[34] Recently a few reports have described small peptides and even organic molecules capable of signalling by dimerisation of the EPO receptor, for example, **1**–**3**.[13–15]



Keywords: IMCR, Passerini; Ugi; Isocyanide; Erythropoietin; Design; Combinatorial chemistry.
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0960-894X/$ - see front matter © 2006 Published by Elsevier Ltd.
doi:10.1016/j.bmcl.2006.10.040

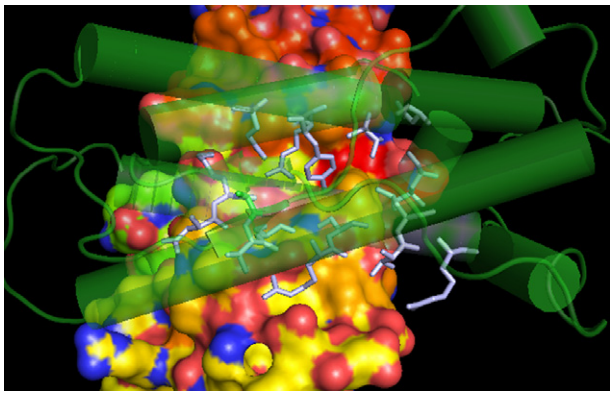


Figure 1. Above: Erythropoietin mimics. Below: Erythropoietin (green cylindrical helices) bound to its receptor leading to dimerisation (pdb ID: 1EER).[21] The interacting amino acid residues of erythropoietin are highlighted blue, the receptor is shown as a surface. The models herein are created using PyMol (www.pymol.com).

However, based on presently available compounds there is a need for more research in small molecular weight EPO agonists made from other scaffolds and exemplifying high potency, activity and oral bioavailability.[16] As part of our ongoing interest in small molecular weight protein interaction agonist and antagonists we describe here our preliminary results on the design, combinatorial synthesis and activity of novel EPO agonists.[17]

Boger et al. recently described small molecule EPO agonists stemming from the screening of a library of several hundred symmetric dicarboxylic acid derived compounds. The most potent disclosed compound, **1**, was found to be a partial agonist and selectively induced concentration-dependent proliferation of an EPO-dependent cell line. This work prompted us to design and synthesize combinatorial libraries based on isocyanide based multicomponent reactions (IMCRs).[18] Potentially, the application of MCR chemistry generates a chemical space that is complementary to classical peptide-coupling chemistry and is also more rapid and efficient because of its one-pot character.[19]

Based on the proposed mechanism of EPO-r dimerisation and the available structural information of the EPO/EPO-r complex[20,21] agonists are advantageously bi- or oligovalent.[22] In an active EPO mimicking peptide/EPO-r complex the two receptors are 39 Å apart in the EPO binding sites. The complex is almost symmetrical. Therefore, we felt a good starting point for small molecule EPO agonists should encompass two molecular binding units, connected by a distance-variable linker-yielding symmetrical molecules to capture the second EPO-r for dimerisation. The EPO/EPO-r affinity seems to be highly governed by a 'hot spot' of hydrophobic interaction. From phage display and alanine scan peptide libraries the crucial importance of hydrophobic aromatic amino acid residues is known, for example, Tyr, Trp and Phe (Fig. 2).[23]

We designed three different libraries using isocyanide-based MCR chemistry (Scheme 1).[25] Moreover, we

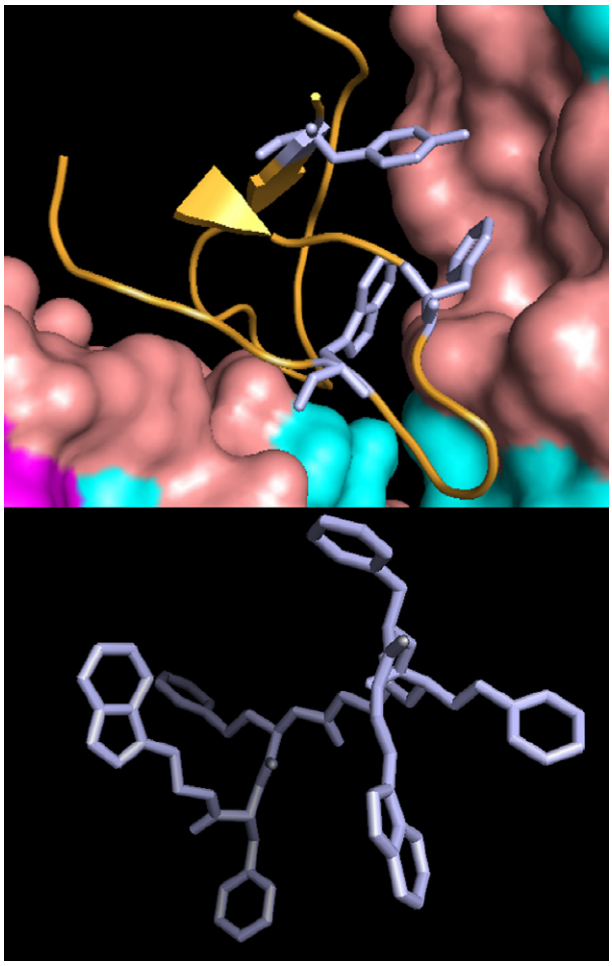
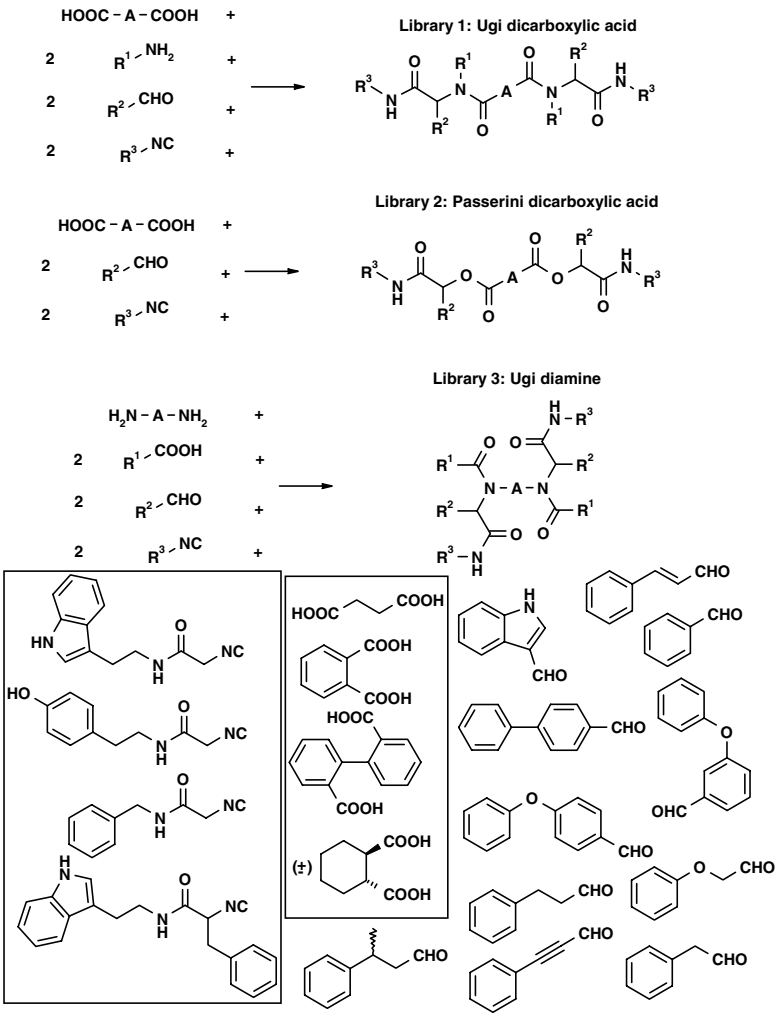


Figure 2. Above: The lipophilic 'hot spot' triade FTW of EBP1 (light blue) binding onto the surface of the EPO-r (pdb ID: 1EBP). Below: Energy minimized model of a library compound, showing the special arrangement of the three potential EPO-r binding elements two phenyls and one indole.

enriched our starting material set with aromatic moieties, for example, phenyl, 4-hydroxy phenyl and indole, corresponding to the aromatic amino acids found in the peptide agonist 'hot spot'. Thus we made extensive use of our recently described access to libraries of isocyano acetamides.[24]

For the synthesis of the three MCR libraries, we initially investigated the quality of the product formation of this liquid phase procedure.[25] Analysis of the crude mixture of a representative library by HPLC–MS revealed that 84 out of 88 compounds were formed. Seventy-six (86%) of these were formed in greater than 80% purity by HPLC–MS (Fig. 3). Screening of the starting materials revealed no activity. Thus in the light of the high quality of the reactions we decided to screen the crude reaction mixtures after evaporation of the solvent. Overall, we synthesized 88 compounds from each of the three scaffolds to explore the corresponding chemical scaffold space.

We chose to use a functional assay to discover EPO agonists, since it is known that binding and dimerization is



Scheme 1. Above: Three different shape-libraries based on IMCR yielding possibly EPO agonists. Below: Starting materials used in the construction of the Passerini-IMCR library.

not enough to yield receptor activation. However, it was found that there is a significant angle dependence of the resulting EPO/EPO-r complex.[26] The cytokine responsive cell-based assay used here is an alternative to similar cell-based assays used recently.[27]

The murine pre-B lymphoid cell line BaF3 is growth and survival dependent on IL-3 or IL-4. The BaF3-EPO-r cell line constitutively expresses a cloned human EPO receptor,[28] and its growth and survival can thus additionally be maintained with hEPO.[30] The BaF3 and BaF3-EPO-r cell lines were maintained in RPMI 1640 supplemented with 10% FBS (basal medium). Survival requirements were met with 1 nM murine IL-3 (mIL-3, R&D Systems) for BaF3 and 1 u/mL human erythropoietin (rhEPO, R&D Systems) for BaF3-EPO-r. Treated cells in 96-well trays were incubated for 24 h at 37 °C, after which 10 μL WST-1 reagent (Roche Biochemicals) was added, followed by 90 min additional incubation. Assays were concluded by lysing the cells with the addition of 15 μL of 10% SDS to each well, followed by A_{450} absorbance analysis in a plate reader (Wallac Victor[2]).

To detect EPO-r agonist activity, compounds (50 μM nominal concentrations) were added to wells containing 10^4 BaF3-EPOR cells in 100 μL basal medium. For EPO-r enhancement or antagonist activity, identical wells were additionally stimulated with 0.1 μ/ml rhEPO, the pre-determined EC_{50} dose, such that either stimulation or diminution of the EPO signal is detected. To control for non-specific enhancement or reduction of cell proliferation/survival, BaF3 cells were treated identically with compounds in the presence of 0.14 u/mL mIL-3, the pre-determined EC_{50} dose. This treatment also provided a control for interference with the assay read-out by light absorbance of test compounds at 450 nm.

Representative results of the screening of the crude Passerini library are shown in Figure 3. Apparently several compounds revealed functional EPO agonist behaviour. Compounds of interest were chosen for resynthesis according to the stimulation of EPO-responsive cells, as well as for potential selectivity, that is, little or no stimulation of non-EPO-responsive BaF3 cells. Resynthesized compounds were individually analysed at a

A

	1	2	3	4	5	6	7	8	9	10	11
A	11.1 13.7	20.9 29.7	7.7 30.1	10.2 11.9	0.8 3.4	16.1 15.9	-1.8 11.9	8.5 15.6	10.0 4.1	1.1 17.9	14.2 16.7
B	14.5 7.0	23.5 20.6	18.7 18.0	12.1 11.8	13.4 8.1	20.0 8.3	6.9 15.1	9.4 4.6	11.1 3.1	8.2 10.3	-1.6 5.8
C	13.0 10.9	24.8 23.6	14.2 28.9	9.7 3.8	10.6 12.9	29.6 12.7	-0.3 12.3	5.3 0.4	10.9 1.3	7.4 -1.2	9.1 9.9
D	5.9 7.1	6.1 22.7	7.5 30.7	8.7 2.7	4.3 0.2	20.0 18.3	5.8 19.3	-1.2 -4.8	7.5 0.6	6.1 18.2	3.5 0.8
E	12.1 13.0	10.0 21.2	9.3 17.8	8.5 0.6	8.0 14.7	34.0 17.4	6.5 7.4	23.9 11.2	18.5 -1.6	4.4 9.6	15.1 10.8
F	10.3 11.5	6.4 15.1	3.6 6.4	8.0 2.8	2.4 8.4	18.7 9.7	3.2 3.9	13.7 2.2	4.8 -3.0	0.4 7.3	6.0 7.2
G	9.8 8.6	7.0 12.1	6.9 7.1	8.3 -1.8	0.7 11.8	18.8 10.2	5.9 3.9	24.0 -4.0	5.7 -3.4	0.5 14.0	1.4 2.6
H	15.8 14.9	8.4 19.6	11.8 14.1	4.8 -0.5	9.5 17.4	25.9 16.2	4.3 9.0	24.4 1.5	3.8 -3.8	-0.8 13.9	10.1 10.6

B

	1	2	3	4	5	6	7	8	9	10	11
A	11.1 13.7	20.9 29.7	7.7 30.1	10.2 11.9	0.8 3.4	16.1 15.9	-1.8 11.9	8.5 15.6	10.0 4.1	1.1 17.9	14.2 16.7
B	14.5 7.0	23.5 20.6	18.7 18.0	12.1 11.8	13.4 8.1	20.0 8.3	6.9 15.1	9.4 4.6	11.1 3.1	8.2 10.3	-1.6 5.8
C	13.0 10.9	24.8 23.6	14.2 28.9	9.7 3.8	10.6 12.9	29.6 12.7	-0.3 12.3	5.3 0.4	10.9 1.3	7.4 -1.2	9.1 9.9
D	5.9 7.1	6.1 22.7	7.5 30.7	8.7 2.7	4.3 0.2	20.0 18.3	5.8 19.3	-1.2 -4.8	7.5 0.6	6.1 18.2	3.5 0.8
E	12.1 13.0	10.0 21.2	9.3 17.8	8.5 0.6	8.0 14.7	34.0 17.4	6.5 7.4	23.9 11.2	18.5 -1.6	4.4 9.6	15.1 10.8
F	10.3 11.5	6.4 15.1	3.6 6.4	8.0 2.8	2.4 8.4	18.7 9.7	3.2 3.9	13.7 2.2	4.8 -3.0	0.4 7.3	6.0 7.2
G	9.8 8.6	7.0 12.1	6.9 7.1	8.3 -1.8	0.7 11.8	18.8 10.2	5.9 3.9	24.0 -4.0	5.7 -3.4	0.5 14.0	1.4 2.6
H	15.8 14.9	8.4 19.6	11.8 14.1	4.8 -0.5	9.5 17.4	25.9 16.2	4.3 9.0	24.4 1.5	3.8 -3.8	-0.8 13.9	10.1 10.6

Figure 3. Differential cell-based screening of the crude Passerini shape-library in EPO and IL-3 dependent cell lines. Characteristic data in which triplicate compound samples were tested for effect on cell proliferation. Each square shows the mean values (normalized to percent maximum proliferation) of the effect on BaF3-EPO-r cells (upper value) and BaF3 cells (lower value). (A) Screening performed in the presence of EC_{50} concentration of EPO or IL-3, for upper and lower values, respectively. (B) Screening performed in the absence of added EPO or IL-3. Yellow fills denote those squares with the greatest differential between upper and lower values, that is, the maximum potential EPO-selective response.

concentration of 50 μM (Fig. 4). One such compound that retested positively, A5-24, appeared to stimulate BaF3-EPO-r cells with an EC_{50} of 25 nM while inhibiting BaF3 cells with an EC_{50} of 700 nM, suggesting a selective stimulation of the EPO receptor (Fig. 5).[29] Resynthesis of all apparently active compounds from the three libraries and SAR evaluation is currently ongoing.

In summary, we have synthesized three symmetrical libraries, based on IMCR chemistry and screened them for EPO agonists activity in an EPO-responsive cell-based assay. Thus we could identify several compounds with initial although very weak EPO agonistic behaviour.

Initiatives for future research will address selectivity of positive compounds towards other hormone receptors, a better understanding of the molecular mode-of-action of these compounds and the design of more potent and 'drug-like' small molecular weight EPO agonists.

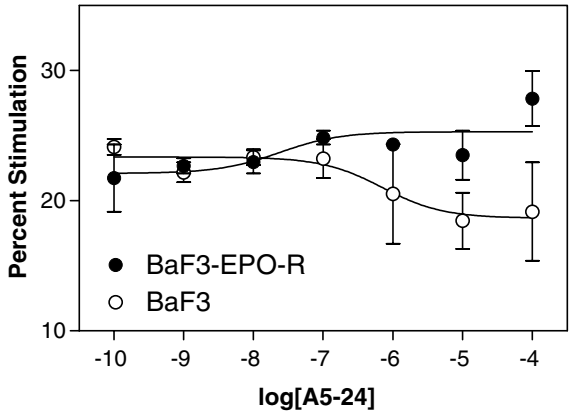


Figure 5. Activity of resynthesized compound in unstimulated cells. The compound A5-24 was tested in BaF3-EPO-r and in wild type BaF3 cell lines in the absence of added EPO or IL-3. In this preliminary test, proliferation data from duplicate wells were normalized based on full stimulation, respectively, by EPO or IL-3.

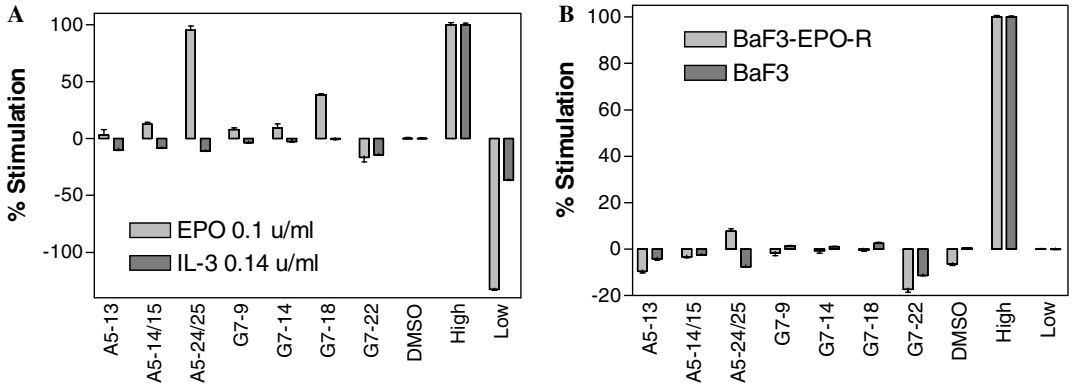


Figure 4. Proliferation analysis of individual resynthesized compounds at a concentration of 50 μM on BaF3-EPO-r and BaF3 cells. (A) Compounds were tested in the presence of EC_{50} concentrations of EPO or IL-3, in BaF3-EPOR and BaF3 cells, respectively, defined in this graph as 0% stimulation. (B) Compounds were tested without added EPO or IL-3. In this graph, Low is normalized to 0% stimulation. High, 10× EC_{50} concentration of EPO or IL-3, normalized to 100% stimulation; Low, no EPO or IL-3; DMSO, solvent only control.

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25. General procedure for the parallel, solution phase multicomponent synthesis of the Passerini library: as parallel reaction vessels deep-well 96 plates made of polypropylene were used. Stock solutions of the educts were prepared: 0.25 M dicarboxylic acids in THF, except *trans*-cylohexane dicarboxylicacid 0.125 (for solubility reasons); 0.5 M isocyanides in THF; 0.5 M or 0.25 M aldehydes in THF, respectively. Via multichannel pipettes each well was filled with each 50 μmol dicarboxylic acids (200 μL of the 0.25 M and 400 μM of the 0.125 M stock solution), 100 μmol aldehydes (200 μL of the 0.5 M and 400 μM of the 0.25 M stock solution) and 100 μmol isocyanides (200 μL of the 0.5 M stock solution). The 96-well plate was sealed with a nob mat and shaken for 2 days at 20 °C. The 96-well plate was opened and the THF allowed to evaporate for 24 h to concentrate the solution. The residual THF was evaporated in a GeneVac. Then 500 μL methanol per well was added. Several daughter plates were prepared for biological and analytical evaluation. The formation of the reaction products was confirmed and their purity was determined by HPLC–MS using a Hewlett-Packard LC 1100 driven electrospray MS instrument.
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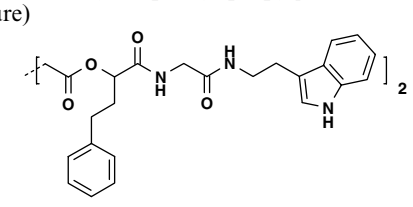
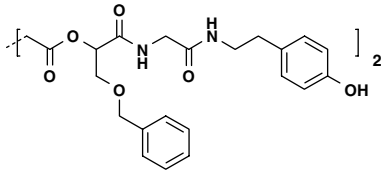
29. Succinic acid bis-[1-({[2-(1 H-indol-3-yl)-ethylcarbamoyl]-methyl}-carbamoyl)-3-phenyl-propyl] ester (diastereomeric mixture)



$C_{48}H_{52}N_6O_8$ = 840.98 Da
^{1}H NMR (CD_3OD, 400 MHz) = 1.10–1.18 (m, 4H, br), 1.61–1.79 (m, 4H, br), 1.82–1.95 (m, 4H, br), 2.10–2.18 (m, 4H, br), 2.29 (s, 4H), 2.40–2.52 (m, 4H, br), 2.67–2.86 (m, 4, br), 3.91–4.02 (m, 2H, br), 5.97–6.40 (m, 20H), 6.55 (s, 2H).
^{13}C NMR (CD_3OD, 100 MHz) = 24.1, 28.8, 30.9, 39.3, 40.2, 42.2, 73.8, 111.2, 111.8, 118.0, 118.4, 121.1, 122.3, 125.9, 127.5, 128.3, 140.9, 167.8, 169.9, 172.9.
R_t (HPLC–MS): 3.79; MH^+ = 841.7; M^+Na = 863.7.

Succinic acid bis-[2-benzyloxy-1-({[2-(4-hydroxy-phenyl)-ethylcarbamoyl]-methyl}-carbamoyl)-ethyl] ester



$C_{44}H_{50}N_4O_{12}$ = 826.90 Da

^{1}H NMR (CD_3OD) = 2.53–2.57 (tr, 4H, aryl-CH_2–CH_2), 2.76–2.85 (m, 4H, C-CH_2–CH_2), 3.06–3.11 (m, 1H, CH–CH_2–O), 3.19–3.24 (m, 1H, CH–CH_2–O), 3.33 (s, 2H, CO–CH_2–NH), 3.74–3.79 (m, 4H, OOC–CH_2–CH_2–COO–), 3.84–3.88 (m, 4H, OOC–CH_2–CH_2–COO–), 4.40–4.49 (q, 4H, aryl-CH_2–O), 5.22 (s, 2H, –NOC–CH–OCO–), 6.68–6.70 (d, 4H, phenyl–CH_o), 6.93–6.95 (d, 4H, phenyl–CH_m), 7.28 (s, 2× 5H, aryl-CH).

^{13}C NMR (CD_3OD) = 28.6, 34.4, 41.1, 42.3, 69.4, 73.2, 73.8, 115.1, 127.6, 128.3, 129.7, 137.8, 155.7, 169.6, 172.1.

R_t (HPLC–MS): 3.454; M^+Na = 849.7.

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