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ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 4810–4822
Bioorganic & Medicinal Chemistry

Design, synthesis, and biological evaluation of glycine-based molecular tongs as inhibitors of $A\beta_{1-40}$ aggregation in vitro

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Received 14 February 2008; revised 13 March 2008; accepted 20 March 2008
Available online 25 March 2008

Abstract—A series of N-terminus benzamides of glycine-based symmetric peptides, linked to *m*-xylylenediamine and 3,4′-oxydianiline spacers, were prepared and tested as inhibitors of β-amyloid peptide $A\beta_{1-40}$ aggregation in vitro. Compounds with good anti-aggregating activity were detected. Polyphenolic amides showed the highest anti-aggregating activity, with IC_{50} values in the micromolar range. Structure–activity relationships suggested that π–π stacking and hydrogen-bonding interactions play a key role in the inhibition of $A\beta_{1-40}$ self-assembly leading to amyloid fibrils.

1. Introduction

Cerebral amyloid deposits, formed by β-sheet fibrillar aggregates in selected brain areas, are the hallmarks of many neurodegenerative disorders, including Alzheimer's (AD), Huntingtons's disease, and Parkinson's disease.[1–3] The morphological and biochemical characterization of the deposits isolated from postmortem brain tissues of AD patients indicated β-amyloid peptides $A\beta_{1-40}$ and $A\beta_{1-42}$ as the main components of the amyloid plaques.[4,5] Compelling evidence suggests that Aβ oligomerization leading to (proto)fibrils may occur via distinct intermediates[6,7] and that low molecular weight oligomers, rather than fibrils, are the likely neuropathogenic species.[8–13] Any step in the formation and aggregation of Aβ may represent a target for potential therapeutic intervention. The most promising targets include: (i) proteolytic cleavage of the β-amyloid precursor protein (APP) by the β-site APP-cleaving enzyme (BACE) and by γ-secretase enzyme complex[14]; (ii) aggregation of Aβ into low oligomers and into insoluble amyloid fibrils[15]; and (iii) clearance of Aβ peptide from brain deposits.[16,17] The development of specific inhibitors that block oligomers and/or fibril formation is strongly hampered by lack of reliable three-dimensional structures of monomers and low oligomers that would have guided a structure-based inhibitor design.[18] Unfortunately, structural studies based on NMR methods in the solution and solid state addressed mature amyloid fibrils rather than oligomers and therefore resulted in limited use for a rational inhibitor design.[19–21] Nevertheless, starting from the well-known self-recognizing and -aggregating ability of Aβ, peptide inhibitors have been designed by mimicking sequences of the self-recognition motif, generally the 16–21 amino acid sequence.[22,23] Despite the good in vitro activity found for some of these compounds, their therapeutic usefulness is restricted due to their ability to self-aggregate and to the risk of being incorporated into amyloid fibrils.

The design of peptide inhibitors has been therefore modified and improved by combining the self-recognition motif with a β-sheet-disrupting element to produce molecules capable of binding specifically to Aβ while avoiding self-aggregation. Several strategies have been described to include β-sheet-disrupting elements: (i) use of amino acids with charged side chains at both ends of the Aβ recognition motif, presumably increasing solvent surface tension[24]; (ii) insertion of bulky groups (side chains) to sterically interfere with Aβ aggregation[25]; and (iii) incorporation of *N*-methyl (or *N*-alkyl)

Keywords: $A\beta_{1-40}$; β-Amyloid aggregation; Glycine-based molecular tongs; Polyphenolic amides inhibitors.
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doi:10.1016/j.bmc.2008.03.052

amino acids in the peptide sequence to impede β-sheet formation and/or stabilization, and/or aggregation.[26,27]

Successful approaches have recently been developed by using glycine-containing peptides, targeting different amino acid sequences, that is, the 25–35 and the GxMxG sequences in the C-terminus of Aβ.[28–31] These studies are authoritative examples of the rational design of peptide inhibitors; they are based on the specific role that glycine plays in acting as a flexible hinge to disrupt transient α-helical secondary structure and to facilitate flattened sheet-to-sheet packing due to the lack of side chains.[31,32] Actually, glycine-rich peptides are over-represented in highly fibrillogenic cores of several amyloidogenic proteins involved in human diseases, including the prion protein and α-synuclein.[33,34] This may also suggest an evolutionary role for this simple amino acid in controlling the intrinsic molecular aggregation propensity of peptides and proteins.[35]

It is well-known that stabilization of amyloid fibrils and globular proteins occurs through the synergistic action of hydrogen bonding and hydrophobic and ionic interactions, yet much remains to be explained about the respective boundaries of this synergism.[17,36,37] The high occurrence of aromatic amino acids in aggregation-prone fragments, observed in disease-related amyloidogenic proteins, reasonably supports the hypothesis that, besides hydrophobic interactions, non-bonded interactions (i.e., π–π stacking) between aromatic rings[38,39] might play a specific role in the self-assembly processes leading to amyloid fibril formation.[40] An opposite effect may be exerted by aromatic moieties of aggregation inhibitors leading to generalized anti-amyloidogenic activity.[41–43] In fact, (poly)aromatic compounds, including wine-related polyphenols, have been shown to be effective in inhibiting Aβ fibril formation and/or in disassembling preformed fibrils.[44–48]

Keeping in mind the previous observations, we designed and synthesized a series of glycine-based molecular tongs[39] bearing the general structure depicted in Figure 1, as potential inhibitors of the Aβ aggregation process. The physicochemical and structural properties of these compounds were systematically explored by varying the oligoglycine chain length, hydrophobicity, and the hydrogen bonding (HB) and π–π stacking abilities. Our design enabled us to obtain a progressive decrease in hydrophobicity along with a parallel increase in the number of potential HBs. Moreover, taking into account the remarkable anti-aggregating activity of several aromatic compounds, especially some natural polyphenols, we modified the glycine N-terminus via a simple amide linkage with benzoic and substituted benzoic acids. The π–π stacking potential of these β-sheet-disrupting aromatic elements was assessed by varying the substituent electronic properties.[48,49] The symmetric 'tong structure' of the compounds in Figure 1 was selected hypothesizing an interaction between one or both molecular arms with putative peptide sequences of Aβ involved in oligomerization.[50]

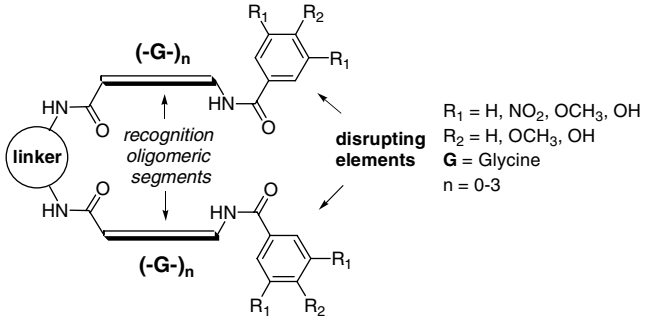


Figure 1. General structure of the targeted compounds. G is a glycine residue.

Our series of molecules bears two different linkers: *m*-xylylenediamine and 3,4′-oxydianiline, the latter having been preferred to the carcinogenic 4,4′-isomer. These two homo-bivalent scaffolds differ strongly in both their physicochemical properties and putative linking topologies which might allow the exploration of a diverse conformational domain through the two peptidic arms.

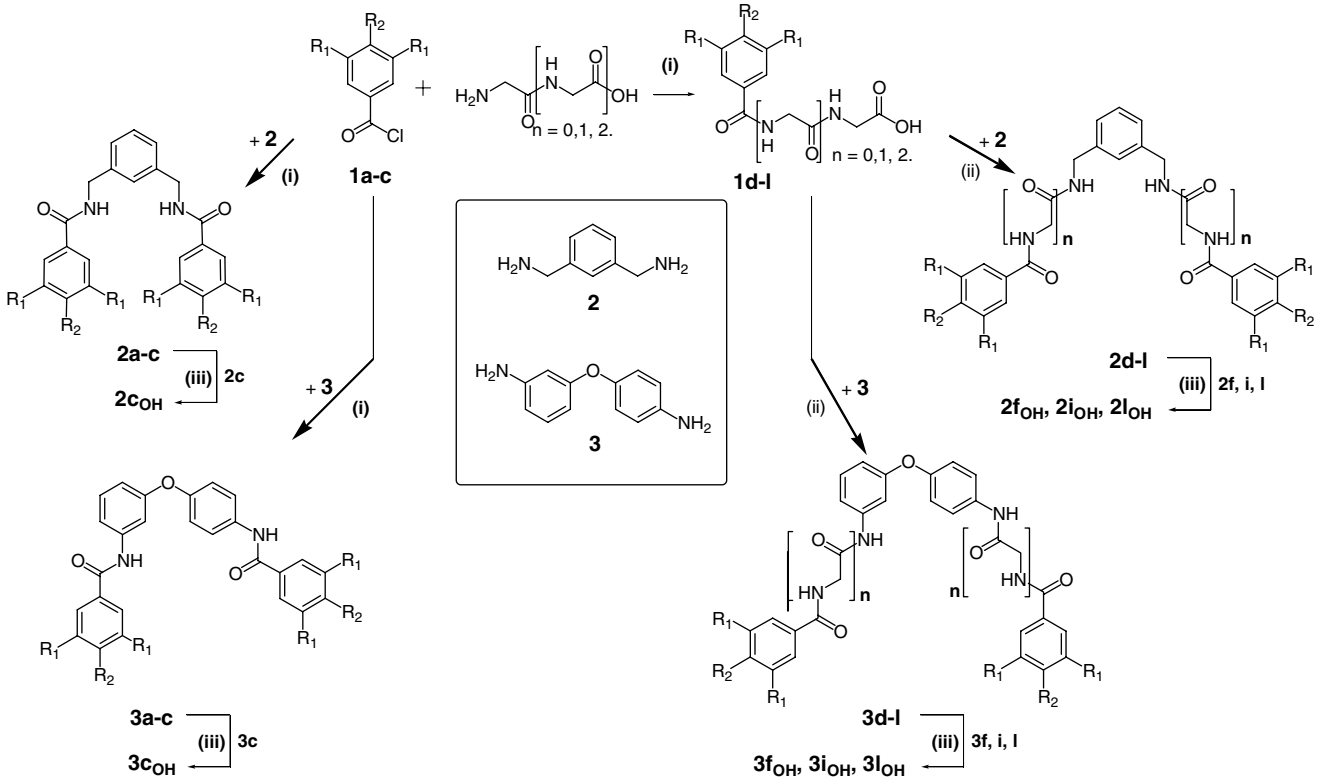
2. Results and discussion

2.1. Chemistry

The synthetic pathway leading to compounds with the general structure depicted in Figure 1 is reported in Scheme 1. The known *N*-aroylglycine-, di-glycine-, and tri-glycine-amides (**1e–l**, Table 1) were prepared according to standard methods.[51] Their physicochemical and spectroscopic data were in full agreement with the literature data.[52–56] *N*-aroyl derivatives **2a–c**, $\mathbf{2c_{OH}}$ (Table 2) of *m*-xylylenediamine were prepared according to procedures analogous to those yielding **1d–l**.[57] The novel synthesized *N*-aroyl derivatives **3a–c** of 3,4′-oxydianiline (Table 2) were synthesized according to the reaction pathways illustrated in Scheme 1. Coupling reactions between diamino linkers **2** and **3** and peptide derivatives **1d–l**, using PyBOP, produced compounds **2d–l** and **3d–l** in moderate-to-good yields. The demethylation reaction of the trimethoxy congeners **2c** and **3c**, **f**, **i**, **l**, was carried out with BBr_3 to achieve the corresponding polyphenol derivatives $\mathbf{2c_{OH}}$, $\mathbf{3c_{OH}}$, $\mathbf{3f_{OH}}$, $\mathbf{3i_{OH}}$, and $\mathbf{3l_{OH}}$ (Scheme 1).

2.2. Biological assays

Traditional methods to screen for inhibitors of Aβ aggregation present several limitations which result from subtle and unforseeable experimental alterations affecting the slow and complex kinetics of fibril formation.[58] Most of the reported experimental procedures are negatively impacted, indeed, by the long incubation time required by Aβ peptides to form fibrils, rendering their use particularly laborious for testing small/medium libraries of potential Aβ aggregation inhibitors. To overcome these difficulties, we recently developed a faster spectrofluorimetric method for medium-throughput screening that enabled to test both small-/medium-sized molecular libraries designed and prepared in our



Scheme 1. Reagents and conditions: (i) aq NaOH, THF, rt, 4 h; (ii) PyBOP, DIPEA/CH_2Cl_2, rt, overnight; (iii) BBr_3, CH_2Cl_2, rt, 20 h. Compounds **1a**–**l**, **2a**–**l**, and **3a**–**l** are listed in Tables 1 and 2, respectively.

Table 1. Starting aroyl chlorides **1a**–**c** and corresponding benzamide derivatives of glycine (hippuric acids, $n = 1$), glycylglycine ($n = 2$) and glycylglycylglycine ($n = 3$) (see Scheme 1)

Compound	*n*	R_1	R_2
1a	—	H	H
1b	—	NO_2	H
1c	—	OCH_3	OCH_3
1d	1	H	H
1e[52]	1	NO_2	H
1f[53]	1	OCH_3	OCH_3
1g[54]	2	H	H
1h[52]	2	NO_2	H
1i[55]	2	OCH_3	OCH_3
1j[56]	3	H	H
1k[52]	3	NO_2	H
1l[55]	3	OCH_3	OCH_3

laboratories, or available from commercial sources, in much less time.[59]

Considering the data in the literature that indicate a net enhancing effect of fluorinated alcohols on Aβ aggregation,[60,61] we evaluated $A\beta_{1-40}$ aggregation in phosphate-buffered saline solution containing 2% (v/v) 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). Under these experimental conditions, maximum fluorescence emission of thioflavin T (ThT) was attained within 24 h compared to the several days required by the traditional spectrofluorimetric test.[58] A quasi-saturated effect with 2% HFIP was observed at nearly 2 h, with loss of the typical initial lag phase (Fig. 2). Moreover, we proved that the addition of increasing volumes of DMSO (up to 10%; later 5% was used for testing) did not significantly affect the aggregation process and this enabled the testing of low water-soluble compounds even at high concentrations, up to 200 μM.

Because slight variations in the experimental protocols may lead to dramatically different results in the inhibition assay, we assessed the reliability of our method by screening a number of known Aβ anti-aggregating molecules. Very satisfactorily, our inhibition measures (data not shown) on well-known anti-aggregating agents, such as rolitetracycline, tetracycline, rifampicin, and doxorubicin were comparable to those reported in the literature.[62,63] In particular for quercetin we measured an IC_{50} equal to 0.8 μM that compared well with the literature data (IC_{50} = 0,25 μM and 0.75 μM in Refs. 64 and 44, respectively). Although comparison of IC_{50}s for A-β_{1-40} aggregation studies is a common practice in the literature, the dependence of this parameter from the ratio inhibitor/peptide should be kept in mind. However we used a ratio close to the ones commonly reported in the literature and the closeness of our IC_{50} values with the ones reported in the literature may be taken as a good proof of the validity of our methodological approach.

To further validate our method, scanning electron microscopy (SEM) was carried out on sample where Aβ peptide was incubated by a traditional method, that is, in the absence of HFIP, without and with the inhibitor. Fibrils formation took place only in the former case

Table 2. Inhibition data of $A\beta_{1-40}$ aggregation of *m*-xylylenediamine (**2x**) and 3,4′-oxydianiline (**3x**) derivatives

Compound	*n*	R_1	R_2	Inhibition at 100 μM, (% ±SEM)	IC_{50}[a] (μM)
2a	0	H	H	3 ± 2	n.d.
3a	0	H	H	−16 ± 4	n.d.
2b	0	NO_2	H	4 ± 3	n.d.
3b	0	NO_2	H	−20 ± 6	n.d.
2c	0	OCH_3	OCH_3	19 ± 3	n.d.
3c	0	OCH_3	OCH_3	−12 ± 4	n.d.
$2c_{OH}$	0	OH	OH	61 ± 3	n.d.
$3c_{OH}$	0	OH	OH	70 ± 2	12
2d	1	H	H	9 ± 7	n.d.
3d	1	H	H	7 ± 5	n.d.
2e	1	NO_2	H	33 ± 6	n.d.
3e	1	NO_2	H	44 ± 2	n.d.
2f	1	OCH_3	OCH_3	28 ± 4	n.d.
3f	1	OCH_3	OCH_3	9 ± 2	n.d.
$2f_{OH}$	1	OH	OH	39 ± 4	n.d.
$3f_{OH}$	1	OH	OH	79 ± 2	1.5
2g	2	H	H	10 ± 4	n.d.
3g	2	H	H	−3 ± 2	n.d.
2h	2	NO_2	H	23 ± 3	n.d.
3h	2	NO_2	H	30 ± 3	n.d.
2i	2	OCH_3	OCH_3	31± 4	n.d.
3i	2	OCH_3	OCH_3	40 ± 4	n.d.
$2i_{OH}$	2	OH	OH	5 ± 4	n.d.
$3i_{OH}$	2	OH	OH	80 ± 2	28
2j	3	H	H	19 ± 2	n.d.
3j	3	H	H	−3 ± 2	n.d.
2k	3	NO_2	H	13 ± 2	n.d.
3k	3	NO_2	H	4 ± 2	n.d.
2l	3	OCH_3	OCH_3	15 ± 2	n.d.
3l	3	OCH_3	OCH_3	20 ± 2	n.d.
$2l_{OH}$	3	OH	OH	4 ± 3	n.d.
$3l_{OH}$	3	OH	OH	24 ± 7	n.d.

[a] SEMs (standard error of mean) of IC_{50} are within ±15% of the reported values; n.d., not determined.

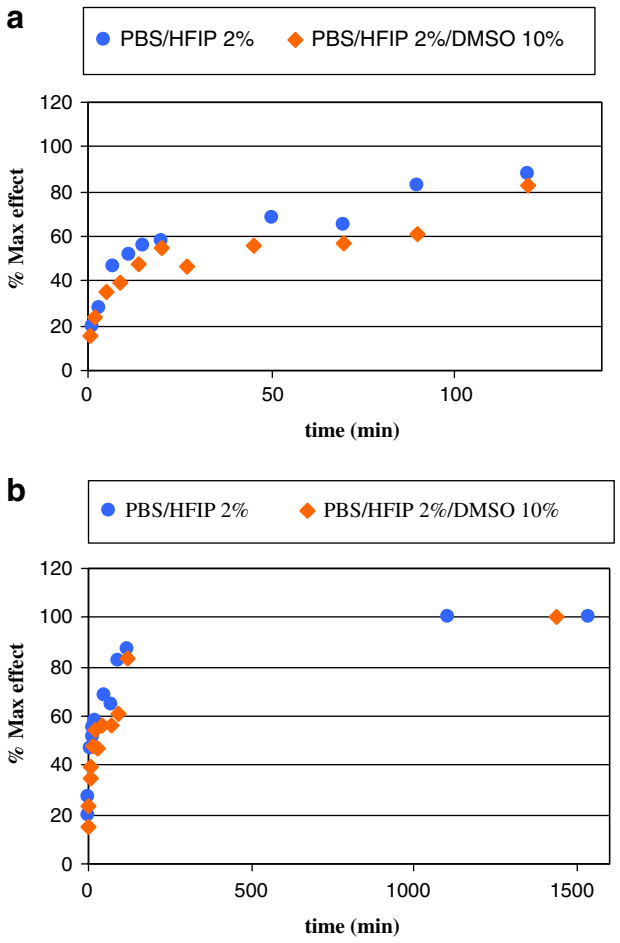


Figure 2. Aggregation rates of $A\beta_{1-40}$ in PBS/HFIP 2%, with and without DMSO 10%, within (a) 2 h and (b) 24 h.

(compare Fig. 3a with b and c). Due to the complete evaporation of solvent under high vacuum conditions required by SEM analysis, deposition of peptide, together with buffer salts and inhibitor compound, occurred as amorphous aggregates visible in Figure 3b and c. We are aware that the use of fluorinated solvents might imply a different aggregation kinetic pathway[61] but our first comparative controls suggested that our method might be favorably used for a first and rapid screening of Aβ anti-aggregating properties of synthetic and natural compounds.

2.3. Structure–activity relationships (SARs) of $A\beta_{1-40}$ aggregation inhibitors

The anti-aggregating activity of the examined compounds is listed in Table 2 as the percentage of aggregation inhibition at 100 μM or, for the most active inhibitors, as IC_{50}. The same data were also summarized in a graph (Fig. 4a and b) as a function of glycine arm length. Dose–response curve of most active compound **$3f_{OH}$** is depicted in Figure 5. Notably, the simplest benzamides **2a**–**c** of the *m*-xylylenediamine seemed to have a low, but significant, pro-aggregating activities. No sound explanation may be provided for this unexpected activity.

To facilitate the discussion of SARs, inhibition data were arbitrarily ranked into three groups: low (up to 30%), medium (from 30% to 60%), and high (from 60% to 90%) inhibitory activities. As a general trend, the 3,4′-oxydianiline derivatives **3a**–**l** were more active than the corresponding *m*-xylylenediamine derivatives **2a**–**l**, thus corroborating the notion that linker size and flexibility should play a role in positioning putative assembly-preventing structural elements close to those in $A\beta_{1-40}$ that promote oligomerization. In addition, some common SARs for both series can be inferred: (1) the 'tongs' bearing one or two glycine branches were generally more active than the tri-glycine derivatives, particularly in the 3,4′-oxydianiline series (e.g., **3j**, **3k**, **3l**, and **$3l_{OH}$** vs **3g**, **3h**, **3i**, and **$3i_{OH}$**, respectively); and (2) unsubstituted benzamido derivatives exhibited lower activity than the substituted benzamido congeners, pointing out that electron-donating and -withdrawing properties of aromatic substituents play a role in peptide-inhibitor binding.

The inhibitory activity of polyphenol derivatives in the *m*-xylylenediamine series (**$2c_{OH}$**, **$2f_{OH}$**, **$2i_{OH}$**, and **$2l_{OH}$**) decreased as the number of glycine units in the peptide arms increased. In fact, only compound **$2c_{OH}$**, lacking the glycine spacer, displayed a good inhibitory activity.

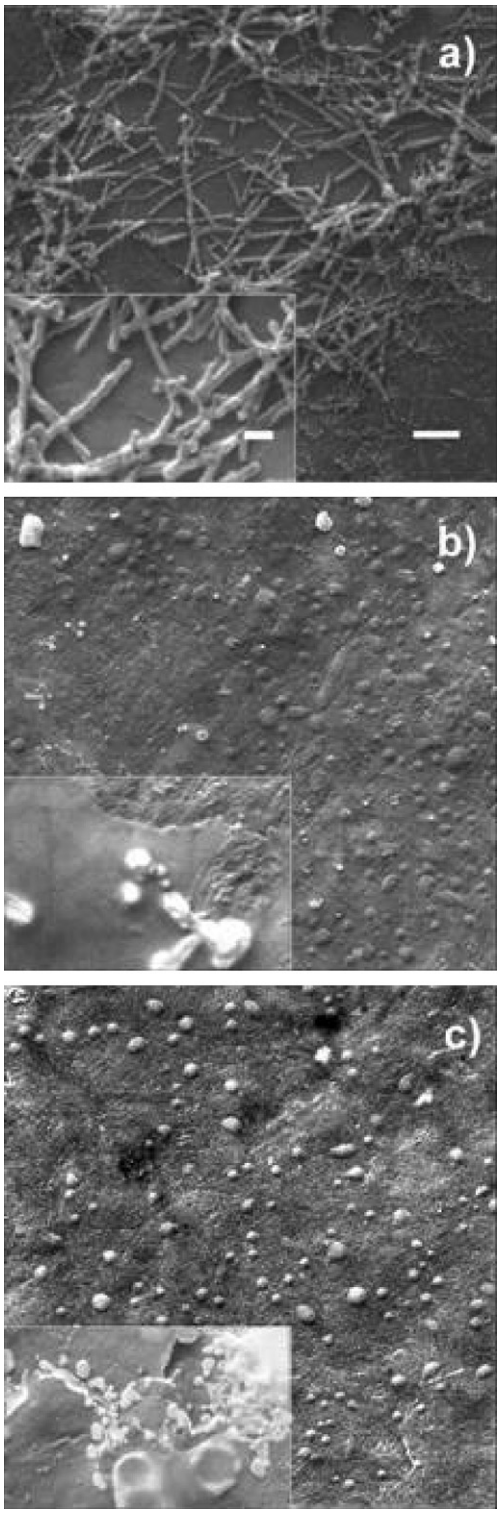


Figure 3. Scanning electron microscopy images, by secondary electrons, of $A\beta_{1-40}$ (30 μM) after incubation. (a) Amyloid fibrils formed after 3 weeks of incubation in PBS, 5% DMSO at 25 °C. (b) and (c) The same $A\beta_{1-40}$ samples obtained after co-incubation with 100 μM of quercetin and **3f$_{OH}$**, respectively. Magnifications are reported as insets. Images and insets are shown with a white calibration bar of 1 μm and 200 nm, respectively.

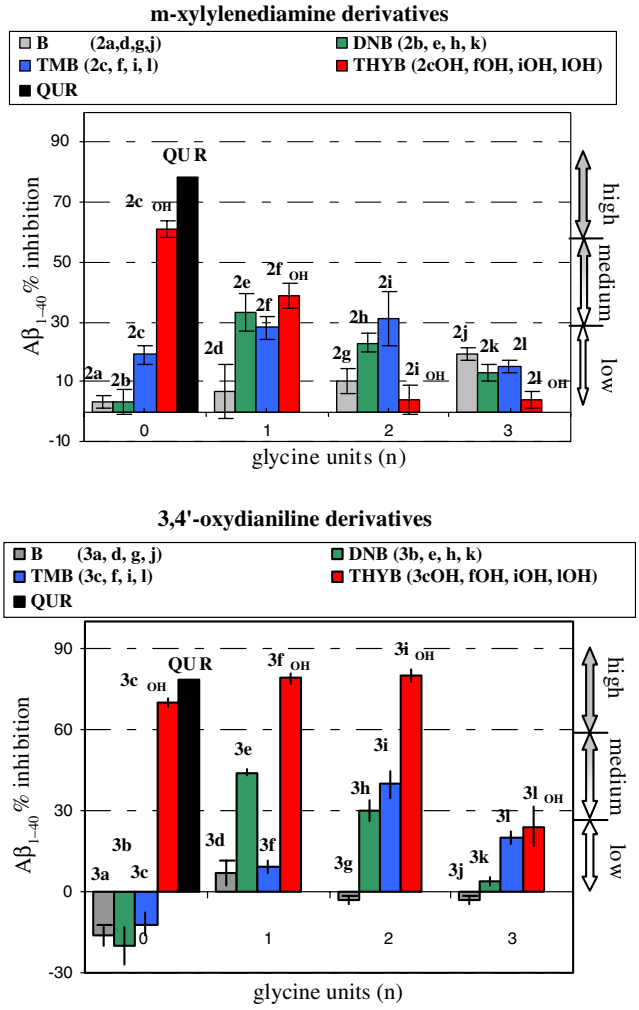


Figure 4. Histograms of $A\beta_{1-40}$ aggregation from inhibition data in Table 2. B = bis-*N*,*N*′-benzamido derivatives; DNB = bis-*N*,*N*′-3,5-dinitrobenzamido derivatives; TMB = bis-*N*,*N*′-3,4,5-trimethoxy-benzamido derivatives; THYB = bis-*N*,*N*′-3,4,5-trihydroxy-benzamido derivatives; QUR = quercetin.

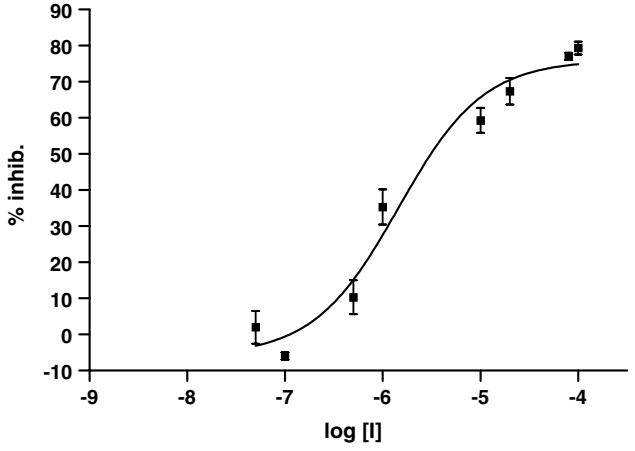


Figure 5. Representative inhibition plot for compound **3f$_{OH}$**.

The activities of the corresponding compounds of the 3,4′-oxydianiline series (**3c$_{OH}$**, **3f$_{OH}$**, and **3i$_{OH}$**) were significantly higher (Table 2, Fig. 4). The most active compound **3f$_{OH}$** exhibited an IC_{50} very close to that of quercetin, a well-known natural polyphenol that in vitro acts as a strong assembly inhibitor of various

amyloidogenic proteins.[64,65] However, derivative **3l$_{OH}$**, bearing two equal tri-glycine arms, showed a dramatic reduction of activity, suggesting possible intra-molecular folding of the two peptide chains, most likely caused by the formation of inter-chain (-arm) HBs involving the second and third glycine residues. Conformational analysis in *solvent continuum* (apolar) (data not shown) seems to support such a hypothesis.

To test the validity of our design, we prepared and assayed some truncated and nearly isolipophilic analogues of selected compounds from the two series, as shown in Scheme 2. The inhibitory activities of compounds **5**, **7**, **8**, and **9** are reported in Table 3 along with similar data referring to the corresponding 3,4′-oxydianiline and, when appropriate, *m*-xylylenediamine derivatives. Compound **8**, a truncated analogue of **3h**, retaining only one 3,5-dinitrobenzamido di-glycine branch in position 4′ showed a much lower inhibitory activity than **3h**. These findings proved the advantage of the 'tong structure' for efficient binding to Aβ_{1-40} or its low oligomers.

Replacement of the di-glycine segment of **3i** with the more flexible 5-aminovaleric acid in compound **5** resulted in a dramatic fall of activity. A similar decrease of activity was observed in compound **9**, where the 3,4′-oxydianiline spacer was replaced by 3,3′-diaminodipropylamine.

Interestingly, bis-cyclohexylamide derivative **7**, devoid of the aromaticity and the quadrupole moment associated with an aromatic ring in the amidic peptide tail, regained weak but detectable activity while the aromatic homologue **3g** remained inactive. These results warrant further studies to find out whether aromaticity and hydrophobicity play synergistic or distinctive roles both in promoting β-sheet formation and amyloid fibrils stabilization.

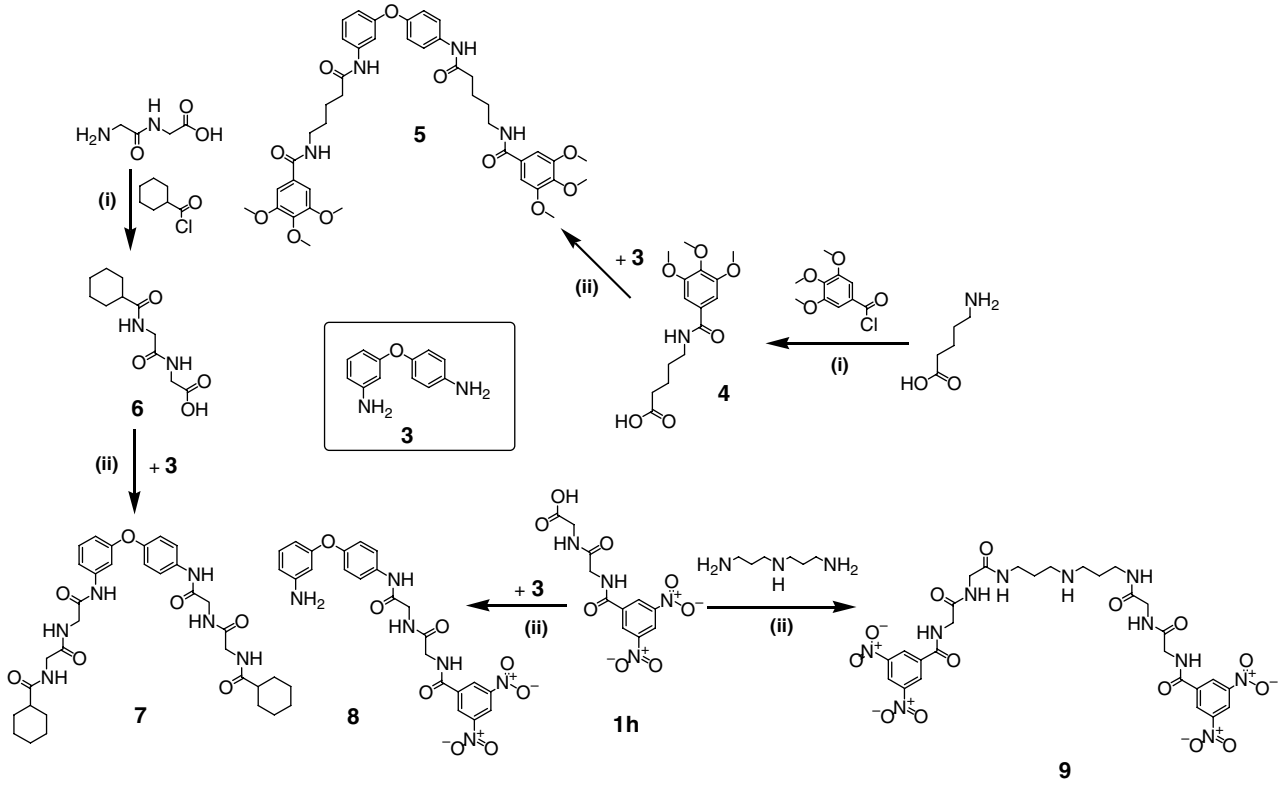
Table 3. Comparison of Aβ_{1-40} aggregation inhibition data of the listed compounds[a]

Compound	% Inhibition at 100 μM		Compound
5	~0	*40 ± 5*	**3i**
7	17 ± 5	*~0*	**3g**
8	~0	*30 ± 3*	**3h**
9	9 ± 6	*30 ± 3*	**3h**
		23 ± 3	**2h**
1h	36 ± 4	*23 ± 3*	**2h**
		30 ± 3	**3h**
1i	11 ± 2	*31 ± 9*	**2i**
		40 ± 5	**3i**

[a] Inhibition data in italics refer to compounds reported in the far-right column.

3. Conclusions and prospects

A certain number of the compounds analyzed proved to be effective inhibitors of Aβ_{1-40} aggregation in vitro. The 3,4,5-trihydroxy-benzamido derivatives **3c$_{OH}$**,**3f$_{OH}$**, and **3i$_{OH}$** displayed IC_{50} values in the micromolar range.



Scheme 2. Reagents and conditions: (i) aq NaOH, THF, rt, 4 h; (ii) PyBOP, DIPEA/CH_2Cl_2, rt, overnight.

The most active inhibitor was **3f$_{OH}$** with a potency similar to that of quercetin.

Potential aromatic π–π stacking interactions modulating Aβ_{1-40} ligand binding were hypothesized on the basis of the significant increase of inhibitory activity observed in the substituted benzamides compared to the unsubstituted congeners. Our data suggest that different π–π interactions of the aromatic amino acid of Aβ_{1-40} with the opposite quadrupole moments of aromatics (3,5-dinitrophenyl or 3,4,5-trimethoxyphenyl derivatives) might occur, generating attractive forces between interacting aromatic rings. However, different forces, such as hydrophobic interactions, might explain the aggregation inhibitory activity observed for di-cyclohexylamido derivative **7**, which is unable to establish π–π stacking interactions.

The length of the peptide arms modulated the anti-aggregating activity that reached the highest values when one or two glycine residues were present. Finally, the advantage of the 'tong structure' was confirmed by the inactivity of mono-branched analogue **8**.

In addition, our study showed that for the detection of amyloid fibrils, SEM appeared a valid and cheaper tool, alternative to the more advanced transmission electron microscopy (TEM). Further studies, currently in progress in our laboratories might lead to the discovery of more potent Aβ_{1-40} aggregation inhibitors and to a deeper understanding of the main molecular determinants of Aβ_{1-40} inhibitor binding.

4. Experimental

4.1. Chemistry

Starting materials, reagents, and analytical grade solvents were from commercial sources. Chromatographic separations were performed on silica gel (15–40 mesh, Merck) by flash methodology. Reaction progress was monitored using analytical thin layer chromatography (TLC) on precoated silica gel Kiesegel 60 F254 plates and the spots were detected under UV light (254 nm). Melting points were determined by the open capillary method on a Stuart-Scientific SMP3 electrothermal apparatus and are uncorrected. ^{1}H NMR spectra were recorded in the indicated deuterated solvents at 300 MHz on a Varian Mercury 300 instrument. Chemical shifts are expressed in δ (ppm) and coupling constants J in Hertz (Hz). The following abbreviations were used: br (broad), s (singlet), d (doublet), t (triplet), m (multiplet); signals due to OH and NH protons were located by deuterium exchange with D_2O. Electron spray ionization mass spectroscopy (ESI-MS) were recorded in a quadrupolar Agilent 1100 series LC-MSD Trap System VL apparatus. Elemental analyses were performed on a Carlo Erba C, H, N analyzer. Fully purified products had satisfactory (within 0.4% of theoretical values) C, H, N analyses. Parallel reactions were generally performed using Büchi Syncore® instrument and carried out under an argon atmosphere. The HPLC analyses were performed on a Agilent Zorbax C3 (4.6. × 250 mm, 5 μm) column or on a Waters XTerra RP8 (3.0. × 150 mm, 5 μm) column with a Multisolvent Delivery System Waters 600 instrument. As detector, UV Waters 486 was used.

Intermediates **1e–l** were prepared according to the reported procedures (see references in Table 1).

4.2. General procedure for the preparation of benzamides 2a–c and 3a–c

m-Xylylenediamine or 3,4′-oxydianiline (1.00 mmol), (±)-propylene oxide (0.42 mL, 6.0 mmol), and the appropriate benzoyl chloride (3.0 mmol) were dissolved in THF (15 mL) at 0 °C. After stirring at room temperature overnight the solvent was removed by evaporation to give a solid that was crystallized from the solvents reported below.

4.2.1. *N*-({3-[(Phenylformamido)methyl]phenyl}methyl)benzamide (2a). 67% Yield; mp 174–176 °C from EtOH. ^{1}H NMR (DMSO-d_6, δ): 9.03 (t, J = 6.0 Hz, 2H), 7.85–7.83 (m, 4H), 7.53–7.40 (m, 6H), 7.29–7.16 (m, 4H), 4.44 (d, J = 6.0 Hz, 4H). ESI-MS: $[M+H]^+$ = 345 *m*/*z*. Anal. calcd for $C_{22}H_{20}N_2O_2$: C, 76.72; H, 5.85; N, 8.13. Found: C, 76.94; H, 5.59; N, 8.33.

4.2.2. *N*-[(3-{[(3,5-Dinitrophenyl)formamido]methyl}-phenyl)methyl]-3,5-dinitrobenzamide (2b). 70% Yield; mp 236–238 °C from EtOH. ^{1}H NMR (DMSO-d_6 δ): 9.77 (t, J = 5.6 Hz, 2H), 9.05 (m, 4H), 8.93 (m, 2H), 7.34–7.24 (m, 4H), 4.53 (d, J = 5.6 Hz, 4H). ESI-MS: $[M-H]^-$ = 523 *m*/*z*. Anal. calcd for $C_{22}H_{16}N_6O_{10}$: C, 50.39; H, 3.08; N, 16.03. Found: C, 50.42; H, 3.37; N, 16.11.

4.2.3. 3,4,5-Trimethoxy-*N*-[(3-{[(3,4,5-trimethoxy-phenyl)-formamido]methyl}phenyl)-methyl]benzamide (2c). 58% Yield; mp 230–232 °C from EtOH. ^{1}H NMR (DMSO-d_6, δ): 8.98 (t, J = 5.8 Hz, 2H), 7.28–7.25 (m, 2H), 7.20–7.17 (m, 6H), 4.45 (d, J = 5.8 Hz, 4H), 3.77 (s, 12H), 3.68 (s, 6H). ESI-MS: $[M-H]^-$ = 523 *m*/*z*. Anal. calcd for $C_{28}H_{32}N_2O_8$: C, 64.11; H, 6.15; N, 5.34. Found: C, 64.27; H, 5.85; N, 5.13.

4.2.4. *N*-[4-(3-Benzamidophenoxy)phenyl]benzamide (3a). 70% Yield; mp 203–205 °C from THF/EtOH. ^{1}H NMR (DMSO-d_6, δ): 10.28 (s, 2H), 7.95–7.89 (m, 4H), 7.80 (d, J = 8.8 Hz, 2H), 7.58–7.49 (m, 8H), 7.35–7.30 (m, 1H), 7.06 (d, J = 8.8 Hz, 2H), 6.76–6.73 (m, 1H). ESI-MS: $[M-H]^-$ = 407 *m*/*z*. Anal. calcd for $C_{26}H_{20}N_2O_3$: C, 76.45; H, 4.94; N, 6.86. Found: C, 76.72; H, 4.85; N, 7.13.

4.2.5. *N*-(4-{3-[(3,5-Dinitrobenzene)amido]phenoxy}-phenyl)-3,5-dinitrobenzamide (3b). 68% Yield; mp 95–97 °C from EtOH. ^{1}H NMR (DMSO-d_6, δ): 10.89 (s, 1H), 10.87 (s, 1H), 9.16–9.11 (m, 4H), 8.99–8.96 (m, 2H), 7.82 (d, J = 9.1 Hz, 2H), 7.58–7.37 (m, 3H), 7.13 (d, J = 9.1 Hz, 2H), 6.86–6.83 (m, 1H). ESI-MS: $[M-H]^-$ = 587 *m*/*z*. Anal. calcd for $C_{26}H_{16}N_6O_{11}$: C, 53.07; H, 2.74; N, 14.28. Found: C, 53.37; H, 2.96; N, 14.41.

4.2.6. 3,4,5-Trimethoxy-*N*-(3-{4-[(3,4,5-trimethoxybenzoyl)-amino]phenoxy}phenyl)-benzamide (3c). 64% Yield; mp 168–170 °C from EtOH. ^{1}H NMR (DMSO-d_6, δ): 10.24 (s, 1H), 10.23 (s, 1H), 7.68 (d, J = 9.0 Hz, 2H), 7.40–7.30 (m, 3H), 7.21–7.18 (m, 4H), 7.04 (d, J = 9.0 Hz, 2H), 6.76–6.73 (m, 1H), 3.81 (s, 6H), 3.79 (s, 6H), 3.68 (s, 3H), 3.67 (s, 3H). ESI-MS: $[M-H]^-$ = 587 m/z. Anal. calcd for $C_{32}H_{32}N_2O_9$: C, 65.30; H, 5.48; N, 4.76. Found: C, 65.23; H, 5.11; N, 4.33.

4.3. General procedure for the preparation of 2d–l and 3d–l

m-Xylylenediamine **2** (or 3-4′-oxydianiline **3**) (1.00 mmol), *N*-aroylamide **1d–l** (1.5–6.00 mmol), DIPEA (0.52 mL, 3.00 mmol), and PyBOP (1.56 g, 3.00 mmol) were dissolved in THF (25 ml) under Ar at 0 °C. After stirring at room temperature overnight, the solid precipitate was filtered and purified by crystallization.

4.3.1. 2-(Phenylformamido)-*N*-[(3-{[2-(phenylformamido)-acetamido]methyl}phenyl)methyl]-acetamide (2d). 67% Yield; mp 211–213 °C from EtOH. ^{1}H NMR (DMSO-d_6, δ): 8.78 (t, J = 5.8 Hz, 2H), 8.43 (t, J = 6.1 Hz, 2H), 7.89–7.83 (m, 4H), 7.55–7.43 (m, 6H), 7.27–7.22 (m, 1H), 7.15–7.11 (m, 3H), 4.26 (d, J = 5.8 Hz, 4H), 3.90 (d, J = 6.1 Hz, 4H). ESI-MS: $[M-H]^-$ = 457 m/z. Anal. calcd for $C_{26}H_{26}N_4O_4$: C, 68.11; H, 5.72; N,12.22. Found: C, 68.26; H, 6.01; N, 12.47.

4.3.2. 2-[(3,5-Dinitrophenyl)formamido]-*N*-{[3-({2-[(3,5-dinitrophenyl)formamido]acetamido}-methyl)phenyl]-methyl}-acetamide (2e). 64% Yield; mp 211–213 °C from EtOH. ^{1}H NMR (DMSO-d_6, δ): 9.57 (t, J = 5.8 Hz, 2H), 9.09–9.06 (m, 4H), 8.96–8.94 (m, 2H), 8.57 (t, J = 5.8 Hz, 2H), 7.38–7.24 (m, 1H), 7.16–7.12 (m, 3H), 4.29 (d, J = 5.8 Hz, 4H), 3.98 (d, J = 5.8 Hz, 4H). ESI-MS: $[M-H]^-$ = 637 m/z. Anal. calcd for $C_{26}H_{22}N_8O_{12}$: C, 48.91; H, 3.47; N,17.55. Found: C, 48.83; H, 3.61; N, 17.24.

4.3.3. 2-[(3,4,5-Trimethoxyphenyl)formamido]-*N*-{[3-({2-[(3,4,5-trimethoxyphenyl)formamido]acetamido}-methyl)-phenyl]methyl}acetamide (2f). 65% Yield; mp 242–244 °C from EtOH. ^{1}H NMR (DMSO-d_6, δ): 8.77 (t, J = 5.8 Hz, 2H), 8.43 (t, J = 5.9 Hz, 2H), 7.27–7.11 (m, 8H), 4.27 (d, J = 5.9 Hz, 4H), 3.90 (d, J = 5.8 Hz, 4H), 3.80 (m, 12H), 3.68 (s, 6H). ESI-MS: $[M+Na]^+$ = 661 m/z. Anal. calcd for $C_{32}H_{38}N_4O_{10}$: C, 60.18; H, 6.00; N, 8.77. Found: C, 60.55; H, 6.20; N, 9.13.

4.3.4. 2-(Phenylformamido)-*N*-[({[3-({2-[2-(phenylformamido)acetamido]acetamido}methyl)phenyl]methyl}carbamoyl)methyl]acetamide (2g). 68% Yield; 198–200 °C from EtOH. ^{1}H NMR (DMSO-d_6, δ): 8.86 (t, J = 5.8 Hz, 2H), 8.32–8.28 (m, 4H), 7.86–7.83 (m, 4H), 7.56–7.42 (m, 6H), 7.24–7.19 (m, 1H), 7.15–7.11 (m, 3H), 4.26 (d, J = 5.8 Hz, 4H), 3.91 (d, J = 5.8 Hz, 4H), 3.76 (d, J = 5.8 Hz, 4H). ESI-MS: $[M-H]^-$ = 571.0 m/z. Anal. calcd for $C_{30}H_{32}N_6O_6$: C, 62.93; H, 5.63; N, 14.68. Found: C, 63.12; H, 5.81; N, 14.88.

4.3.5. 2-[(3,5-Dinitrophenyl)formamido]-*N*-[({[3-({2-[2-(phenylformamido)acetamido]acetamido}-methyl)phenyl]-methyl}carbamoyl)methyl]acetamide (2h). 67% Yield; mp 139–141 °C from AcOH. ^{1}H NMR (DMSO-d_6, δ): 9.59 (t, J = 5.8 Hz, 2H), 9.06–9.02 (m, 4H), 8.97–8.93 (m, 2H), 8.40 (t, J = 5.8 Hz, 2H), 8.29 (t, J = 6.0 Hz, 2H), 7.23–7.08 (m, 4H), 4.24 (d, J = 6.0 Hz, 4H), 3.98 (d, J = 5.8 Hz, 4H), 3.76 (d, J = 5.8 Hz, 4H). ESI-MS: $[M-H]^-$ = 751 m/z. Anal. calcd for $C_{30}H_{28}N_{10}O_{14}$: C, 47.88; H, 3.75; N, 18.61. Found: C, 47.49; H, 3.84; N, 18.77.

4.3.6. 2-[(3,4,5-Trimethoxyphenyl)formamido]-*N*-[({[3-({2-[2-(phenylformamido)acetamido]acetamido}-methyl)phenyl]-methyl}carbamoyl)methyl]acetamide (2i). 74% Yield; mp 137–139 °C from EtOH. ^{1}H NMR (DMSO-d_6, δ): 8.86–8.82 (m, 2H), 8.34–8.32 (m, 2H), 8.29–8.27 (m, 2H), 7.19 (s, 4H), 7.12–7.09 (m, 4H), 4.27–4.23 (m, 4H), 3.91–3.87 (m, 4H), 3.77 (s, 12H), 3.77–3.73 (m, 4H), 3.68 (s, 6H). ESI-MS: $[M-H]^-$ = 751 m/z. Anal. calcd for $C_{36}H_{44}N_6O_{12}$: C, 57.44; H, 5.89; N, 11.16. Found: C, 57.85; H, 5.65; N, 11.49.

4.3.7. 2-(Phenylformamido)-*N*-[({3-[(2-{2-[2-[(phenylformamido)acetamido]acetamido}acetamido)methyl]phenyl]-methyl}carbamoyl)methyl]acetamide (2j). 64% Yield; mp 240–242 °C (dec) from EtOH. ^{1}H NMR (DMSO-d_6, δ): 8.80 (t, J = 5.8 Hz, 2H), 8.29–8.16 (m, 6H), 7.87–7.85 (m, 4H), 7.55–7.43 (m, 6H), 7.22–7.17 (m, 1H), 7.08–7.0.6 (m, 3H), 4.22 (d, J = 5.8 Hz), 3.89 (d, J = 5.8 Hz, 4H), 3.76–3.73 (m, 8H). ESI-MS: $[M-H]^-$ = 751 m/z. Anal. calcd for $C_{34}H_{38}N_8O_8$: C, 59.47; H, 5.58; N, 16.32. Found: C, 59.21; H, 5.89; N, 16.38.

4.3.8. 2-[(3,5-Dinitrophenyl)formamido]-*N*-[({3-[(2-{2-[2-[(phenylformamido)acetamido]-acetamido}-acetamido)-methyl]phenyl]methyl}carbamoyl)methyl]-acetamide (2k). 65% Yield; mp 208–210 °C from EtOH. ^{1}H NMR (DMSO-d_6, δ): 9.57 (t, J = 5.8 Hz, 2H), 9.06–9.05 (m, 4H), 8.96–8.95 (m, 2H), 8.38 (t, J = 5.4 Hz, 2H), 8.30 (t, J = 5.8 Hz, 2H), 8.18 (t, J = 5.8 Hz, 2H), 7.20–7.18 (m, 1H), 7.09–7.06 (m, 3H), 4.22 (d, J = 5.8 Hz,4H), 3.98 (d, J = 5.8 Hz, 4H), 3.78–3.73 (m, 8H). ESI-MS: $[M-H]^-$ = 865 m/z. Anal. calcd for $C_{34}H_{34}N_{12}O_{16}$: C, 47.12; H, 3.95; N, 19.39. Found: C, 47.38; H, 4.14; N, 19.46.

4.3.9. 2-[(3,4,5-Trimethoxyphenyl)formamido]-*N*-[({3-[(2-{2-[2-[(phenylformamido)acetamido]-acetamido}acetamido)methyl]phenyl]methyl}-carbamoyl)methyl]-acetamide (2l). 64% Yield; mp 192–194 °C from MeOH. ^{1}H NMR (DMSO-d_6, δ): 8.83 (t, J = 5.5 Hz, 2H), 8.30–8.19 (m, 6H), 7.21–7.16 (m, 5H), 7.08–7.05 (m, 3H), 4.21 (d, J = 5.5 Hz, 4H), 3.89 (d, J = 5.5 Hz, 4H), 3.79 (s, 12H), 3.76–3.73 (m, 8H), 3.68 (s, 6H). ESI-MS: $[M-H]^-$ = 865 m/z. Anal. calcd for $C_{40}H_{50}N_8O_{14}$: C, 55.42; H, 5.81; N, 12.93. Found: C, 55.80; H, 5.92; N, 13.01.

4.3.10. 2-(Phenylformamido)-*N*-(4-{3-[2-(phenylformamido)acetamido]phenoxy}phenyl)-acetamide (3d). 64% Yield; mp 206–208 °C from MeOH. ^{1}H NMR (DMSO-d_6, δ): 10.11 (s, 1H), 10.09 (s, 1H), 8.84 (t,

J = 5.8 Hz, 1H), 8.80 (t, J = 5.8 Hz, 1H), 7.90–7.85 (m, 4H), 7.61 (d, J = 8.9 Hz, 2H), 7.55–7.44 (m, 6H), 7.28–7.24 (m, 3H), 7.00 (d, J = 8.9 Hz, 2H), 6.68–6.65 (m, 1H), 4.04 (d, J = 5.8 Hz, 2H), 4.00 (d, J = 5.8 Hz, 2H). ESI-MS: $[M-H]^-$ = 521 *m*/*z*. Anal. calcd for $C_{30}H_{26}N_4O_5$: C, 68.95; H, 5.02; N, 10.72. Found: C, 69.09; H, 4.82; N, 10.38.

4.3.11. 2-[(3,5-Dinitrophenyl)formamido]-*N*-[4-(3-{2-[(3,5-dinitrophenyl)formamido]acetamido}phenoxy)-phenyl]acetamide (3e). 70% Yield; mp 131–133 °C (dec) from EtOH. ^{1}H NMR (DMSO-d_6, δ): 10.19 (s, 1 H), 10.16 (s, 1H), 9.65 (t, J = 5.8 Hz, 1H), 9.61 (t, J = 5.8 Hz, 1H), 9.08–9.06 (m, 4H), 8.97–8.95 (m, 2H), 7.60 (d, J = 8.8 Hz, 2H), 7.28–7.26 (m, 3H), 7.01 (d, J = 8.8 Hz, 2H), 6.70–6.66 (m, 1H), 4.13–4.09 (m, 4H). ESI-MS: $[M-H]^-$ = 701 *m*/*z*. Anal. calcd for $C_{30}H_{22}N_8O_{13}$: C, 51.29; H, 3.16; N, 15.95. Found: C, 51.46; H, 3.29; N, 15.69.

4.3.12. 2-[(3,4,5-Trimethoxyphenyl)formamido]-*N*-[4-(3-{2-[(3,4,5-trimethoxyphenyl)formamido]acetamido}-phenoxy)-phenyl]acetamide (3f). 63% Yield; mp 116–118 °C from EtOH/Et_2O. ^{1}H NMR (DMSO-d_6, δ): 10.16 (s, 1H), 10.13 (s, 1H), 8.87 (t, J = 5.8 Hz, 1H), 8.82 (t, J = 5.8 Hz, 1H), 7.92 (d, J = 8.8 Hz, 2H), 7.70–7.60 (m, 3H), 7.23 (s, 2H), 7.21 (s, 2H), 6.99 (d, J = 8.8 Hz, 2H), 6.68–6.58 (m, 1H), 4.03 (d, J = 5.8 Hz, 2H), 4.00 (d, J = 5.8 Hz, 2H), 3.81 (s, 6H), 3.80 (s, 6H), 3.69 (s, 3H), 3.68 (s, 3H). ESI-MS: $[M-H]^-$ = 701 *m*/*z*. Anal. calcd for $C_{36}H_{38}N_4O_{11}$: C, 61.53; H, 5.45; N, 7.97. Found: C, 61.64; H, 5.62; N, 8.13.

4.3.13. *N*-{[(3-{4-[*N*-(Carbamoylmethyl)-2-(phenylformamido)acetamide]phenoxy}phenyl)carbamoyl]methyl}-2-(phenylformamido)acetamide (3g). 65% Yield; mp 243–245 °C from EtOH. ^{1}H NMR (DMSO-d_6, δ): 9.84 (s, 1H), 9.82 (s, 1H), 8.91 (t, J = 5.8 Hz, 1H), 8.88 (t, J = 5.8 Hz, 1H), 8.36 (t, J = 5.8 Hz, 1H), 8.31 (t, J = 5.8 Hz, 1H), 7.91–7.85 (m, 4H), 7.65 (d, J = 9.1 Hz, 2H), 7.56–7.42 (m, 6H), 7.34–7.24 (m, 3H), 7.00 (d, J = 9.1 Hz, 2H), 6.69–6.65 (m, 1H), 3.93–3.75 (m, 8H). ESI-MS $[M-H]^-$ = 635 *m*/*z*. Anal. calcd for $C_{34}H_{32}N_6O_7$: C, 64.14; H, 5.07; N, 13.20. Found: C, 64.36; H, 5.19; N, 13.33.

4.3.14. *N*-{[(3-{4-[*N*-(Carbamoylmethyl)-2-[(3,5-dinitrophenyl)formamido]acetamide]phenoxy}phenyl)carbamoyl]-methyl}-2-[(3,5-dinitrophenyl)formamido]-acetamide (3h). 66% Yield; mp 106–108 °C (dec) from EtOH. ^{1}H NMR (DMSO-d_6, δ): 9.95 (s, 1H), 9.92 (s, 1H), 9.68–9,65 (m, 1H), 9.63–9.61 (m, 1H), 9.08–9.06 (m, 4H), 8.96–8.95 (m, 2H), 8.50 (t, J = 5.7 Hz, 1H), 8.43 (t, J = 5.7 Hz, 1H), 7.62 (d, J = 9.0 Hz, 2H), 7.27–7,23 (m, 3H), 7.00 (d, J = 9.0 Hz, 2H), 6.68–6.65 (m, 1H), 4.02–3.85 (m, 8H). ESI-MS: $[M-H]^-$ = 815 *m*/*z*. Anal. calcd for $C_{34}H_{28}N_{10}O_{15}$: C, 50.01; H, 3.46; N, 17.15. Found: C, 49.68; H, 3.73; N, 17.36.

4.3.15. *N*-{[(3-{4-[*N*-(Carbamoylmethyl)-2-[(3,4,5-trimethoxyphenyl)formamido]acetamide]phenoxy}phenyl)carbamoyl]methyl}-2-[(3,5-trimethoxyphenyl)-formamido]-acetamide (3i). 69% Yield; mp 154–156 °C (dec) from EtOH. ^{1}H NMR (DMSO-d_6, δ): 9.84 (s, 1H), 9.80 (s, 1H), 8.92 (t, J = 5.2 Hz, 1H), 8.87 (t, J = 5.5 Hz, 1H), 8.37 (t, J = 5.8 Hz, 1H), 8.30 (t, J = 5.8 Hz, 1H), 7.66 (d, J = 9.1 Hz, 2H), 7.34–7.31 (m, 3 H), 7.23 (s, 2H), 7.22 (s, 2H), 6.99 (d, J = 9.1 Hz, 2H), 6.67–6.63 (m, 1H), 3.92–3.84 (m, 8H), 3.79 (s, 12H), 3.68 (s, 6H). ESI-MS: $[M-H]^-$= 815 *m*/*z*. Anal. calcd for $C_{40}H_{44}N_6O_{13}$: C, 58.82; H, 5.43; N, 10.29. Found: C, 58.98; H, 5.23; N, 10.47.

4.3.16. *N*-{[(3-{4-[(*N*-(Carbamoylmethyl)-2-(phenylformamido)acetamido)acetamide]phenoxy}phenyl)carbamoyl]-methyl}-2-(phenylformamido)-acetamide (3j). 68% Yield; mp 203–205 °C (dec) from DMSO/H_2O. ^{1}H NMR (DMSO-d_6, δ): 9.86 (s, 1H), 9.75 (s, 1H), 8.82–8.80 (m, 2H), 8.38 (t, J = 5.2 Hz, 1H), 8.31 (t, J = 5.8 Hz, 1H), 8.29 (t, J = 6.3 Hz, 1H), 8.20 (t, J = 5.5 Hz, 1H), 7.87–7.86 (m, 2H), 7.85–7.84 (m, 2H), 7.61 (d, J = 8.7 Hz, 2H), 7.54–7.40 (m, 6H), 7.27–7.19 (m, 3H), 6.91 (d, J = 8.7 Hz, 2H), 6.64–6.61 (m, 1H), 3.94–3.75 (m, 12H). ESI-MS: $[M-H]^-$ = 749 *m*/*z*. Anal. calcd for $C_{38}H_{38}N_8O_9$: C, 60.79; H, 5.10; N, 14.93. Found: C, 60.85; H, 5.48; N, 14.72.

4.3.17. *N*-{[(3-{4-[(*N*-(Carbamoylmethyl)-2-[(3,5-dinitrophenyl)formamido]acetamido)acetamide]phenoxy}phenyl)-carbamoyl]methyl}-2-[(3,5-dinitrophenyl)formamido]-acetamide (3k). 65% Yield; mp 208–210 °C (dec) from MeOH. ^{1}H NMR (DMSO-d_6, δ): 9.85 (s, 1H), 9.80 (s, 1H), 9.59–9.57 (m, 2H), 9.05–8.94 (m, 6H), 8.49–8.45 (m, 2H), 8.26–8.18 (m, 2H), 7.58 (d, J = 8.6 Hz, 2H), 7.24–7.17 (m, 3H), 6.91 (d, J = 8.6 Hz, 2H), 6.60–6.58 (m, 1H), 4.00–3.77 (m, 12H). ESI-MS: $[M-H]^-$ = 929 *m*/*z*. Anal. calcd for $C_{38}H_{34}N_{12}O_{17}$: C, 49.04; H, 3.68; N, 18.06. Found: C, 49.39; H, 3.77; N, 18.13.

4.3.18. *N*-{[(3-{4-[(*N*-(Carbamoylmethyl)-2-[(3,4,5-trimethoxyphenyl)formamido]acetamido)acetamide]phenoxy}-phenyl)carbamoyl]methyl}-2-[(3,4,5-trimethoxyphenyl)-formamido]-acetamide (3l). 61% Yield; mp 144–146 °C from EtOH. ^{1}H NMR (DMSO-d_6, δ): 9.81 (s, 1H), 9.73 (s, 1H), 8.83 (t, J = 5.5 Hz, 1H), 8.81 (t, J = 5.5 Hz, 1H), 8.38 (t, J = 5.5 Hz, 1H), 8.32 (t, J = 5.5 Hz, 1H) 8.27 (t, J = 5.5 Hz, 1H), 8.19 (t, J = 5.5 Hz, 1H), 7.58 (d, J = 8.9 Hz, 2H), 7.27–7.17 (m, 7H), 6.91 (d, J = 8.7 Hz, 2H), 6.62–6.59 (m, 1H), 3.93–3.84 (m, 8H), 3.78–3.74 (m, 16H), 3.67–3.66 (m, 6H). ESI-MS: $[M-H]^-$ = 929 *m*/*z*. Anal. calcd for $C_{44}H_{50}N_8O_{15}$: C, 56.77; H, 5.41; N, 12.04. Found: C, 56.94; H, 5.71; N, 12.16.

4.4. General procedure for the preparation of polyphenols derivatives 2(3)c_{OH}, i_{OH}, f_{OH}, l_{OH}

Appropriate 3,5-trimethoxyphenyl derivative (**2i**, **f**, **l** or **3c**, **i**, **f**, **l**) (0.90 mmol) was suspended in CH_2Cl_2 (30 mL) and cooled at 0 °C. BBr_3 (1 N in CH_2Cl_2, 9.00 mL, 9.00 mmol) was added and the mixture was stirred at room temperature for 20 h. After quenching with 50 mL of H_2O, the aqueous phase was extracted with CH_2Cl_2 and then with AcOEt. The organic solvents were combined, dried over Na_2SO_4 and evaporated to dryness. The residue was triturated with Et_2O, yielding

the desired polyphenol that was crystallized from the solvent indicated below. (Compound **2c$_{OH}$** was prepared according to the literature procedure).[56]

4.4.1. 2-[(3,4,5-Trihydroxyphenyl)formamido]-*N*-{[3-({2-[(3,4,5-trihydroxyphenyl)formamido]acetamido}-methyl)-phenyl]methyl}acetamide (2f$_{OH}$). 51% Yield; mp 139–141 °C from H_2O. ^1H NMR (DMSO-d_6, δ): 8.35–8.31 (m, 4H), 7.26–7.10 (m, 5H), 6.85–6.71 (m, 3H), 6.00–3.97 (m, 6H), 4.25 (d, J = 5.8 Hz, 4H), 3.81 (d, J = 5.5 Hz, 4H). ESI-MS $[M-H]^-$ = 553 *m*/*z*. Anal. calcd for $C_{26}H_{26}N_4O_{10}$: C,56.32; H, 4.73; N, 10.10. Found: C, 56.72; H, 4.44; N, 9.97.

4.4.2. 2-[(3,4,5-Trihydroxyphenyl)formamido]-*N*-[({[3-({2-[2-(phenylformamido)acetamido]acetamido}-methyl)-phenyl]methyl}carbamoyl)methyl]acetamide (2i$_{OH}$). 55% Yield; mp 157–159 °C (dec) from H_2O. ^1H NMR (DMSO-d_6, δ): 8.41–8.22 (m, 6H), 7.24–7.07 (m, 5H), 6.85–6.70 (m, 3H), 6.15–4.00 (s br, 6H), 4.21–4.17 (m, 4H), 3.85–3.74 (m, 4H), 3.67–3.63 (m, 4H). ESI-MS: $[M-H]^-$ = 691 *m*/*z*. Anal. calcd for $C_{30}H_{32}N_6O_{12}$: C, 53.89; H, 4.82; N, 12.57. Found: C, 53.78; H, 4.63; N, 12.46.

4.4.3. 2-[(3,4,5-Trihydroxyphenyl)formamido]-*N*-[({3-[(2-{2-[2-[(phenylformamido)acetamido]-acetamido}acetamido)methyl]phenyl]methyl}-carbamoyl)methyl]-acetamide (2l$_{OH}$). 48% Yield; mp 149–151 °C (dec) from H_2O. ^1H NMR (DMSO-d_6, δ): 8.40–8.20 (m, 8H), 7.26–7.01 (m, 5H), 6.90–6.71 (m, 3H), 6.00–3.91 (m, 6H), 4.18 (d, J = 5.8 Hz, 4H), 3.79 (d, J = 5.8 Hz, 4H), 3.66–3.50 (m, 8H). ESI-MS $[M-H]^-$ = 781 *m*/*z*. Anal. calcd for $C_{34}H_{38}N_8O_{14}$: C, 52.17; H, 4.89; N, 14.32. Found: C, 52.55; H, 4.73; N, 14.51.

4.4.4. 3,4,5-Trihydroxy-*N*-(4-{3-[(3,4,5-trihydroxybenzene)-amido]phenoxy}phenyl)benzamide (3c$_{OH}$). 47% Yield; mp 150–152 °C from H_2O. ^1H NMR (CD_3OD, δ): 7.62 (d, J = 8.9 Hz, 2H), 7.44–7.43 (m, 1H), 7.37–7.26 (m, 2H), 7.02 (d, J = 8.9 Hz, 2H), 6.96–6.93 (m, 4H), 6.75–6.73 (m, 1H). ESI-MS $[M-H]^-$ = 509 *m*/*z*. Anal. calcd for $C_{26}H_{20}N_2O_9$: C, 61.91; H, 4.00; N, 5.55. Found: C, 62.06; H, 4.12; N, 5.21.

4.4.5. 2-[(3,4,5-Trihydroxyphenyl)formamido]-*N*-[4-(3-{2-[(3,5-dinitrophenyl)formamido]acetamido}-phenoxy)-phenyl]-acetamide (3f$_{OH}$). 44% Yield; mp 164–166 °C from H_2O. ^1H NMR (CD_3OD, δ): 7.56 (d, J = 8.9 Hz, 2H), 7.32–7.30 (m, 1H), 7.26–7.25 (m, 2H), 6.97 (d, J = 8.9 Hz, 2H), 6.92–6.90 (s, 4H), 6.72–6.68 (m, 1H), 4.13 (s, 2H), 4.09 (s, 2H). ESI-MS $[M-H]^-$ = 617 *m*/*z*. Anal. calcd for $C_{30}H_{26}N_4O_{11}$: C, 58.25; H, 4.24; N, 9.06. Found: C, 58.45; H, 4.16; N, 9.21.

4.4.6. *N*-{[(3-{4-[*N*-(Carbamoylmethyl)-2-[(3,4,5-trihydroxyphenyl)formamido]acetamide]phenoxy}phenyl)carbamoyl]methyl}-2-[(3,5-trihydroxyphenyl)-formamido]-acetamide (3i$_{OH}$). 49% Yield; mp 186–188 °C from H_2O. ^1H NMR (DMSO-d_6, δ): 9.84 (s, 1H), 9.74 (s, 1H), 9.07 (s, 2H), 9.05 (s, 2H), 8.70 (s, 1H), 8.69 (s, 1H), 8.49 (t, J = 5.2 Hz, 1H), 8.41 (t, J = 5.6 Hz, 1H), 8.27 (t, J = 5.8 Hz, 1H), 8.19 (t, J = 5.6 Hz, 1H), 7.67 (d, J = 9.1 Hz, 2H), 7.32–7.24 (m, 3H), 7.01 (d, J = 9.1 Hz, 2H), 6.88 (s, 2H), 6.85 (s, 2H), 6.69–6.65 (m, 1H), 3.86–3.77 (m, 8H). ESI-MS $[M-H]^-$ = 731 *m*/*z*. Anal. calcd for $C_{34}H_{32}N_6O_{13}$: C, 55.74; H, 4.40; N, 11.47. Found: C, 55.65; H, 4.15; N, 11.26.

4.4.7. *N*-{[(3-{4-[(*N*-(Carbamoylmethyl)-2-[(3,4,5-trihydroxyphenyl)formamido]acetamido)acetamide]phenoxy}-phenyl)carbamoyl]methyl}-2-[(3,4,5-trihydroxyphenyl)form-amido]-acetamide (3l$_{OH}$). 53% Yield; mp 190–192 °C (dec) from H_2O. ^1H NMR (DMSO-d_6, δ): 9.86 (s, 1H), 9.80 (s, 1H), 9.05–8.95 (m, 4H), 8.80–8.60 (m, 2H), 8.38–8.17 (m, 6H); 7.59 (d, J = 9.1 Hz, 2H), 7.27–7.19 (m, 3H), 6.96 (d, J = 9.1 Hz, 2H), 6.84–6.83 (m, 4H), 6.64–6.62 (m, 1H), 3.88–3.72 (m, 12H). ESI-MS $[M-H]^-$ = 845 *m*/*z*. Anal. calcd for $C_{38}H_{38}N_8O_{15}$: C, 53.90; H, 4.52; N, 13.23. Found: C, 53.80; H, 4.77; N, 13.06.

4.5. 5-[(3,4,5-Trimethoxyphenyl)formamido]-pentanoic acid (4)

3,4,5-Trimethoxybenzoyl chloride **1c** (0.46 g, 2.00 mmol) dissolved in 10 mL of dioxane was added by a dropping funnel to 5-aminovaleric acid (0.23 g, 2.00 mmol) dissolved in 10 mL of aqueous solution of NaOH (1 N) at 0 °C. The mixture was stirred for 1 h and then acidified with an aqueous solution of HCl 1 M. The reaction mixture was evaporated yielding a solid residue that was purified by crystallization. 57% Yield; mp 138–140 °C (dec) from EtOH/Et_2O. ^1H NMR (DMSO-d_6, δ): 12.00 (s br, 1H), 8.39 (t, J = 5.5 Hz; 1H), 7.21–7.14 (m, 2H), 3.79 (s, 6H), 3.67 (s, 3H), 3.23–3.21 (m, 2H), 2.25–2.20 (m, 2H), 1.53–1.48 (m, 4H). ESI-MS: $[M-H]^-$ = 310 *m*/*z*. Anal. calcd for $C_{15}H_{21}NO_6$: C, 57.87; H, 6.80; N, 4.50. Found: C, 57.64; H, 6.72; N, 4.21.

4.6. 5-[(3,4,5-Trimethoxyphenyl)formamido]-*N*-[4-(3-{5-[(3,4,5-trimethoxyphenyl)formamido]-pentanamido}-phenoxy)-phenyl]pentanamide (5)

Title compound was obtained by reacting 3,4′-oxydianiline **3** (0.22 g, 1.12 mmol) and **4** (0.70 g, 2.24 mmol) according to the procedure described for the preparation of compound **3d**.

55% Yield; mp 210–212 °C (dec) from EtOH. ^1H NMR (DMSO-d_6, δ): 9.90 (s, 2H), 8.43–8.37 (m, 2H), 7.59 (d, J = 9.0 Hz, 2H), 7.29–7.14 (m, 7H), 6.96 (d, J = 9.0 Hz, 2H); 6.63–6.59 (m, 1H), 3.80 (s, 12H), 3.67 (s, 6H), 3.30–3.23 (m, 4H), 2.34–2.26 (m, 4H), 1.63–1.51 (m, 8H). ESI-MS: $[M+Na]^+$ = 809 *m*/*z*. Anal. calcd for $C_{42}H_{50}N_4O_{11}$: C, 64.11; H, 6.40; N, 7.12. Found: C, 63.97; H, 6.32; N, 7.03.

4.7. [2-(Cyclohexanecarbonyl-amino)-acetylamino]-acetic acid (6)

Glycylglycyne (0.50 g, 3.78 mmol), (±) propylene oxide (0.79 mL, 11.35 mmol), and cyclohexanecarbonyl chloride (0.51 mL, 3.78 mmol) were dissolved in THF (25 mL) at 0 °C and the mixture was stirred at room temperature for 15 h. The reaction mixture was filtered,

and the solvent evaporated yielding a residue that was purified by crystallization affording **6**. 56% Yield; mp 194–196 °C from EtOH. ^{1}H NMR (DMSO-d_6, δ): 12.50 (s br, 1H), 8.02 (t, J = 5.7 Hz, 1H), 7.93 (t, J = 5.7 Hz, 1H), 3.73 (d, J = 5.7 Hz, 2H), 3.66 (d, J = 5.7 Hz, 2H), 2.18–2.06 (m, 1H), 1.69–1.57 (m, 5H), 1.34–1.14 (m, 5H). ESI-MS: $[M+Na]^+$ = 265 *m*/*z*. Anal. calcd for $C_{11}H_{18}N_2O_4$: C, 54.53; H, 7.49; N, 11.46. Found: C, 54.40; H, 7.28; N, 11.34.

4.8. *N*-{[(3-{4-[*N*-(Carbamoylmethyl)-2-(cyclohexylformamido)acetamide]phenoxy}phenyl)carbamoyl]methyl}-2-(cyclohexylformamido)acetamide (7)

Title compound was obtained from 3,4′-oxydianiline (0.40 g, 2.00 mmol) and **6** (1.00 g, 4.00 mmol) by a procedure analogous to that used for the preparation of compounds **3d**–**l**. 58% Yield; mp 211–213 °C from EtOH. ^{1}H NMR (DMSO-d_6, δ): 9.80 (s, 1H), 9.79 (s, 1H), 8.18–8.04 (m, 4H), 7.63 (d, J = 9.2 Hz, 2H), 7.34–7.23 (m, 3H), 6.98 (d, J = 8.8 Hz, 2H), 6.68–6.64 (m, 1H), 3.86–3.81 (m, 4H), 3.73–3.65 (m, 4H), 2.21–2.09 (m, 2H), 1.73–1.56 (m, 10H), 1.35–1.12 (m, 10H). ESI-MS: $[M+Na]^+$ = 671 *m*/*z*. Anal. calcd for $C_{34}H_{44}N_6O_7$: C, 62.95; H, 6.84; N, 12.95. Found: C, 63.10; H, 6.66; N, 12.70.

4.9. *N*-({[4-(3-Aminophenoxy)phenyl]carbamoyl}-methyl)-2-[(3,5-dinitrophenyl)formamido]acetamide (8)

3,4′-Oxydianiline (0.15 g, 0.77 mmol), **1h** (0.25 g, 0.77 mmol), DIPEA (0.13 mL, 0.77 mmol), and PyBOP (0.40 g, 0.77 mmol) were dissolved in THF (25 mL) at 0 °C and the mixture was stirred at room temperature for 15 h. The reaction mixture was evaporated and the residue was crystallized, obtaining **8**. 56% Yield; mp 168–170 °C from H_2O. ^{1}H NMR (DMSO-d_6, δ): 9.88 (s, 1H), 9.66 (t, J = 5.8 Hz, 1H), 9.09–9.08 (m, 2H), 8.96–8.95 (m, 1H), 8.50 (t, J = 5.8 Hz, 1H), 8.20 (s, 2H), 7.58 (d, J = 9.1 Hz, 2H), 6.95 (d, J = 9.1 Hz, 2H), 6.94–6.92 (m, 1H), 6.28–6.07 (m, 3H), 4.01 (d, J = 5.8 Hz, 2H), 3.90 (d, J = 5.8 Hz, 2H). ESI-MS: $[M-H]^-$ = 507 *m*/*z*. Anal. calcd for $C_{23}H_{20}N_6O_8$: C, 54.33; H, 3.96; N, 16.53. Found: C, 54.02; H, 3.78; N, 16.21.

4.10. 2-{[(3,5-Dinitrophenyl)formamido]acetamido}-*N*-{3-[(3-{2-[(3,5-dinitrophenyl)formamido]acetamido}-propyl)-amino]propyl}acetamide (9)

1h (0.50 g, 1.53 mmol), DIPEA (0.27 mL, 1.53 mmol), PyBOP (0.80 g, 1.53 mmol), and bis(3-aminopropyl)amine (0.11 mL, 0.77 mmol) were dissolved in THF (25 mL) at 0 °C and the mixture was stirred at rt for 15 h. The reaction mixture was filtered and the solvent evaporated to dryness yielding a residue that was purified by crystallization. 51% Yield; mp 138–140 °C from EtOH. ^{1}H NMR (DMSO-d_6, δ): 9.65–9.63 (m, 2H), 9.05–9.04 (m, 4H), 8.95–8.94 (m, 2H), 8.43–8.42 (m, 3H), 7.95–7.94 (m, 2H), 3.98 (d, J = 5.2 Hz, 4H), 3.68 (d, J = 5.5 Hz, 4H), 3.14–3.12 (m, 4H), 2.84–2.79 (m, 4H), 1.71–1.69 (m, 4H). ESI-MS: $[M+Na]^+$ = 748 *m*/*z*. Anal. calcd for $C_{28}H_{33}N_{11}O_{14}$: C, 44.98; H, 4.45; N, 20.61. Found: C, 45.11; H, 4.67; N, 20.81.

4.11. Biology

For the measurement of amyloid peptide aggregation, the classic spectrofluorimetric method of LeVine,[65] based on fluorescence emission of thioflavin T (ThT) was followed. The solution of ThT (25 μM) was prepared in phosphate buffer 0.025 M, pH 6.0, filtered through 0.45 μM teflon filters and stored at 4 °C. Samples for aggregation tests were prepared in phosphate-buffered saline (PBS; 0.01 M, NaCl 0.1 M, pH 7.4) containing 2% HFIP and 5% DMSO. In order to obtain batches of $A\beta_{1-40}$ free from preaggregates, commercial peptide (purity >95%; Anaspec, USA) was dissolved in HFIP, lyophilized and stored at −20 °C as described by Liu.[66] Inhibitors were first tested at 100 or 50 μM concentrations (30 μM peptide concentration), with incubation in triplicate at 25 °C for 2 h. IC_{50}, were determined by testing in triplicate 5–7 concentrations of inhibitors in three independent experiments. Fluorimetric measures were performed in a 700 μL cuvette with a Perkin-Elmer LS55 spectrofluorimeter, using FLWinlab program. Parameters were set as follows: excitation at 440 nm (slit 5 nm); emission at 485 nm (slit 10 nm); integration time 2 s. Biological activity was determined as percent of inhibitory activity V_i for each compound according to the formula

$$V_i = 100 - \{[(F_i - F_b)/F_0] \times 100\},$$

where F_i is the fluorescence value of the sample, F_b its blank value, and F_0 the fluorescence value for free aggregation of a sample of $A\beta_{1-40}$ incubated in the absence of inhibitors. For the most active inhibitors IC_{50}s and the corresponding statistics were calculated with GraphPad Prism 4.0 software.

For electron microscopy assays, samples of $A\beta_{1-40}$ 30 μM (with or without test compounds) in PBS/5% DMSO were incubated for 3 weeks at 25 °C before SEM (scanning electron microscopy) analysis.

4.12. Scanning electron microscopy (SEM)

Samples were examined using a Zeiss EVO 50XVP Scanning Electron Microscope equipped with a tungsten filament emitter, allowing a resolution of 3.5 nm (i.e., a magnification of 300,000×). Operating conditions of the SEM were: accelerating potential 30 kV and probe current 200 pA. The samples were prepared as follows: a little drop (10 μL) of sample was placed on a carbon stub. Sedimentation of fibrils on the carbon film occurred during 15 min, then the excess of incubation solution was drained off by means of a filter paper and the specimen was transferred to the electron microscope for examination. Images of the specimens illustrated in Figure 3a–c were digitally collected using a secondary electron detector.

Acknowledgments

This work was supported by a grant from MIUR (Rome, Italy, FIRB project RBAU01LSCE). MC grate-

fully acknowledge the valuable guide of Prof. Fabrizio Chiti, University of Florence (Italy) in the development of the spectrofluorimetric method to assess the $A\beta_{1-40}$ aggregation in the presence of fluorinated solvents.

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