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A stereoselective approach to peptidomimetic BACE1 inhibitors

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ABSTRACT

Aiming at identifying new scaffolds to generate beta-secretase (BACE1) inhibitors we developed peptidomimetics based on a 1,4-benzodiazepine core (**3a**–**d**), their seco-analogs (**4a**–**b**), and linear analogs (**5a**–**h**), by stereoselective approaches. We herein discuss the synthesis, molecular modeling and in vitro studies for the newly developed ligands. Compounds **5c** and **5h** behaved as BACE1 inhibitors on the isolated enzyme and in cellular studies. Particularly, for its low molecular weight, inhibitor **5h** is a prototypic hit to develop a series of BACE1 inhibitors more potent and active on whole-cells.

1. Introduction

Alzheimer's disease (AD) is a progressive neurological disease that leads to the irreversible loss of neurons. In the brain of AD patients there are relevant amounts of extracellular senile plaques, mainly constituted by amyloid β (Aβ) peptide aggregates and intracellular neurofibrillary tangles made of hyperphosphorylated *tau* protein [1,2]. It is widely documented that levels of soluble Aβ peptides are superior to amyloid deposits and correlate to cognitive decline in AD and synaptic dysfunction [3]. Aβ40 and Aβ42 are the two major isoforms of Aβ found in AD brains and are produced by the sequential proteolysis of the amyloid precursor protein (APP) by α-, β-, and γ-secretases [4]. β-Secretase (BACE1) catalyzes the rate-limiting step of the Aβ generation by cleaving the APP at the extracellular space [5]. The aspartic-protease BACE1 has been recognized as a primary drug target for drug development for AD [6,7]. Most of BACE1 inhibitors described to date do not fulfill the requirements for clinical development, and orally available highly efficient BACE1 inhibitors only recently have been identified [8]. Aiming at proposing new scaffolds for the development of inhibitors for overcoming the pharmacokinetic problems associated with peptide-like structures, and as a part of our research program [9], we developed a series of peptidomimetics as BACE1 inhibitors.

Inspired by piperazine-based biphenyl analogs (**1** [10], Fig. 1) and by peptidomimetics containing the hydroxyethylamine (HEA) function (e.g. **2** [11], Fig. 1), we developed 1,4-benzodiazepines (BDZ) and seco-analogs to inhibit BACE1. We initiated with BDZ-based compounds since the BDZ system is a privileged pharmacogenic structure endowed with a high degree of druggability [12,9]. Thus, the early structure activity relationship (SAR) studies [9] led to BDZs **3a**–**d** (Fig. 1 and Table 3) bearing at C_9 position protonatable and non-protonatable functions. To further explore the original scaffolds in the present manuscript we also discuss the

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; BACE1, β-Secretase; HEA, hydroxyethylamine; BDZ, 1,4-benzodiazepine.

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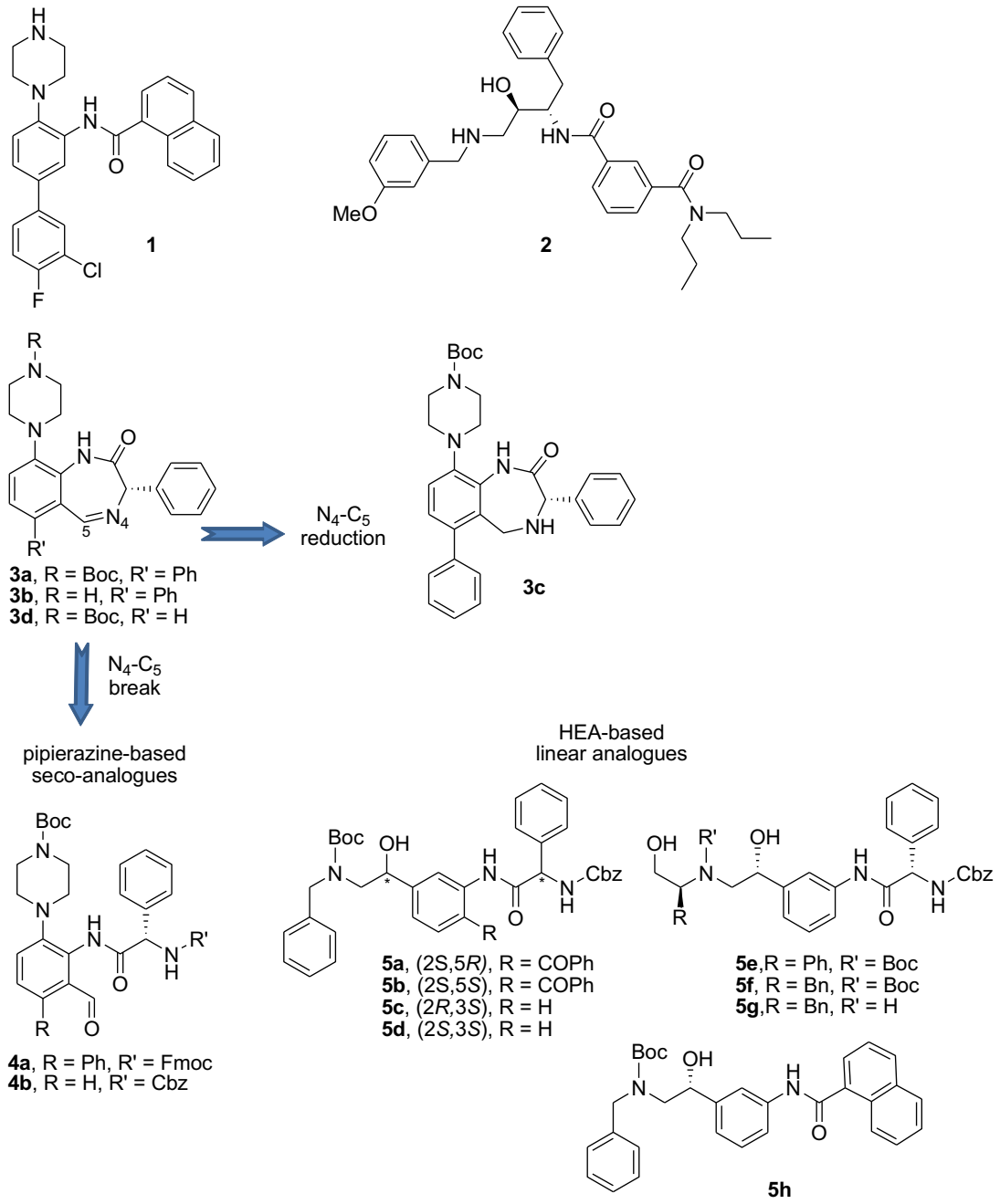


Fig. 1. Structure of reference compounds **1** and **2** and modifications performed for developing title compounds **3a**–**d**, **4a**, **b** and **5a**–**h** (See Tables 3 and 4 for the structures of the title compounds).

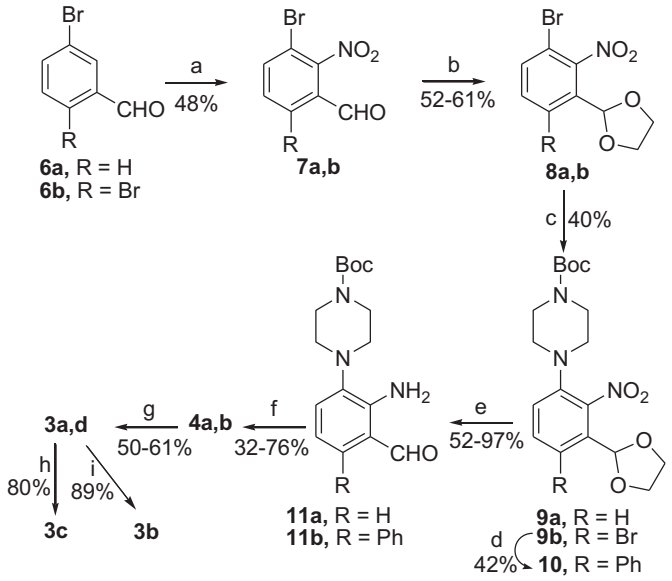
synthesis, biological studies and molecular modeling of seco-peptidomimetics and related analogs with the aim of identifying novel BACE1 inhibitors (**4a**,**b** and **5a**–**h**, Fig. 1 and Tables 3 and 4).

2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis of compounds *3a–d*, and seco-analogs *4a,b*

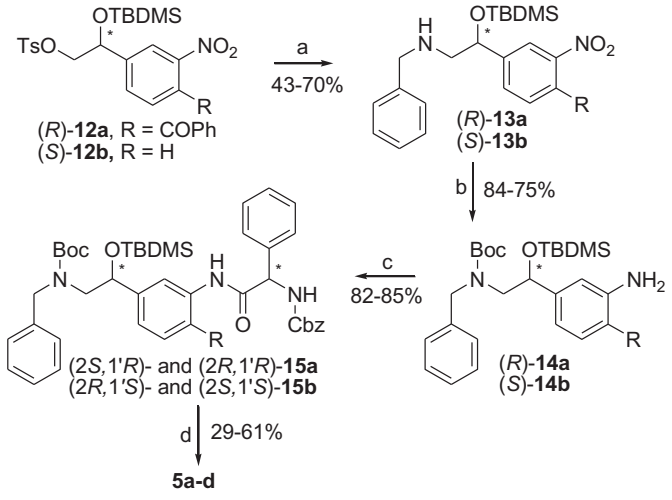
According to a recently discovered protocol to BDZs [13], compounds **3a**–**d** were synthesized as described in Scheme 1. Compounds **7a**,**b** were regioselectively obtained starting from the benzaldehydes **6a**,**b** after a classic aromatic nitration reaction. Protection of the carbonyl function of **7a**,**b** with ethylene glycol under microwave irradiation, afforded the acetals **8a**,**b**. Compounds **9a**,**b** were obtained by condensation of bromides **8a**,**b** with *N*-Boc-piperazine. Starting from **9b**, the phenyl substituent on the aromatic ring was introduced through a Suzuki cross-coupling reaction employing phenylboronic acid, which led to the biphenyl piperazine **10**. Reduction of the nitro group of **9a** and **10** furnished anilines **11a**,**b** in high yield where deprotection of the acetal functionality was also achieved, while leaving the Boc protection unaltered. Coupling of (*R*)-(−)-Fmoc/Cbz-phenylglycine with **11a**,**b** [13] afforded the anilides **4a**,**b**. Cleavage of the protecting groups resulted in the simultaneous cyclization (**3a**,**d**). *N*-Boc removal (**3a**) afforded the hydrochloride **3b** while reduction of the imine functionality of **3a** in glacial acetic acid and sodium cyanoborohydride gave the amine **3c** in good yield.



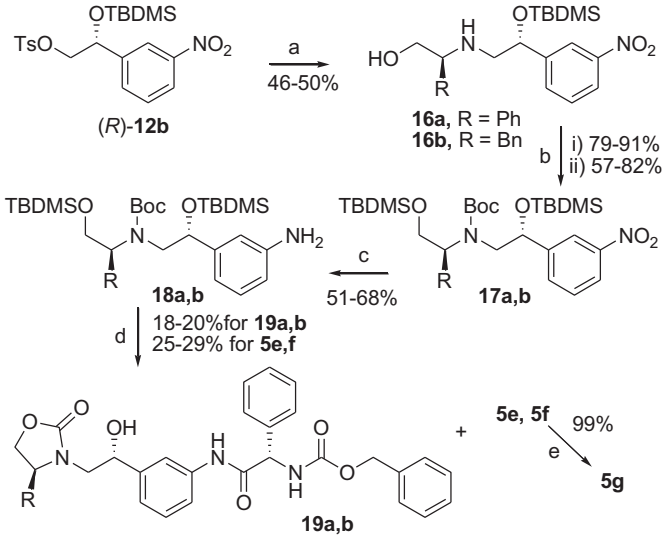
Scheme 1. Synthesis of BDZs **3a**–**d** and *seco*-analogues **4a**–**b**. Reagents and conditions: a) HNO_3, H_2SO_4, from 0 °C to rt, 1 h; b) ethylene glycol, PTSA, MW; c) Boc-piperazine, 120 °C, 48 h, d) from **9b**: $PhB(OH)_2$, $Pd(PPh_3)_4$, K_3PO_4, dioxane, 120 °C, 72 h; e) from **9a** and **10**: Fe, NH_4Cl, EtOH, refl, 3h; f) Fmoc- or Cbz-L-phenylGly, PPh_3, $(Cl_3C)_2CO$, DCM rt, from −10 °C to rt, 90 min; g) Et_2NH, DCM refl, 5 h (for **3a**); cyclohexene Pd/C, rt, refl, 4 h (for **3d**); h) from **3a**: $NaBH_3CN$, AcOH, rt, 10 min; i) from **3a**: AcCl, MeOH.

2.1.2. Synthesis of compounds *5a–h*

The synthesis of compounds **5a**–**d** was performed starting from **12a**,**b** (Scheme 2) which were prepared through the enantioselective synthetic approach described in the next paragraph (Scheme 5). Nucleophilic displacement of the tosyl-groups of the appropriate isomers (*R*)-**12a** and (*S*)-**12b** (Scheme 2) by benzylamine afforded intermediates **13a**,**b**. After Boc protection of the secondary amino-group, the reduction of the nitro group gave **14a**,**b**. Coupling of these anilines with L- or D-Cbz-protected phenylglycine gave **15a**,**b**. Desilylation of *tert*-butyldimethylsilylether afforded **5a**–**d**. The enantiomeric excess (e.e.) of compounds **5a**–**d** were calculated using Mosher method with 1-methoxy-1-trifluoromethyl-1-phenylacetic acid ester and was found >96% for all compounds (data not shown), thus demonstrating the preservation of the chiral integrity at all steps of the synthetic pathway.



Scheme 2. Synthesis of compounds **5a**–**d**. Reagents and conditions: a) benzylamine, DIPEA, DMSO, 75 °C, 20 h; b) i) $(Boc)_2O$, TEA, THF, rt, 12 h; ii) Fe, NH_4Cl, EtOH, reflux, 1 h; c) L- or D-Cbz-phenylGly, $(Cl_3C)_2CO$, PPh_3, DCM rt; d) TBAF, THF, 0 °C, 12 h.

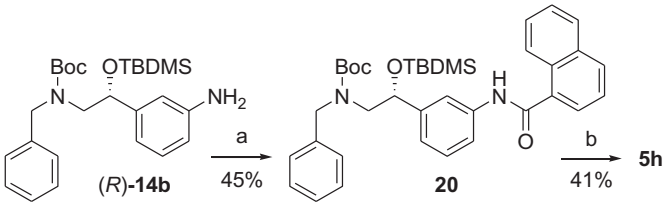


Scheme 3. Synthesis of compounds **5e**–**g**. Reagents and conditions: a) (*S*)-2-amino-2-phenylethanol (for **16a**), (*S*)-2-amino-3-phenylpropanol (for **16b**), DIPEA, DMSO, 75 °C, 20 h; b) i) $(Boc)_2O$, TEA, THF, rt, 12 h; ii) TBDMS, imidazole, DMF, 25 °C, 24 h; c) Fe, NH_4Cl, EtOH, reflux, 1 h; d) L-Cbz-phenylGly, $(Cl_3C)_2CO$, PPh_3, DCM rt; e) from **5f** AcCl, MeOH.

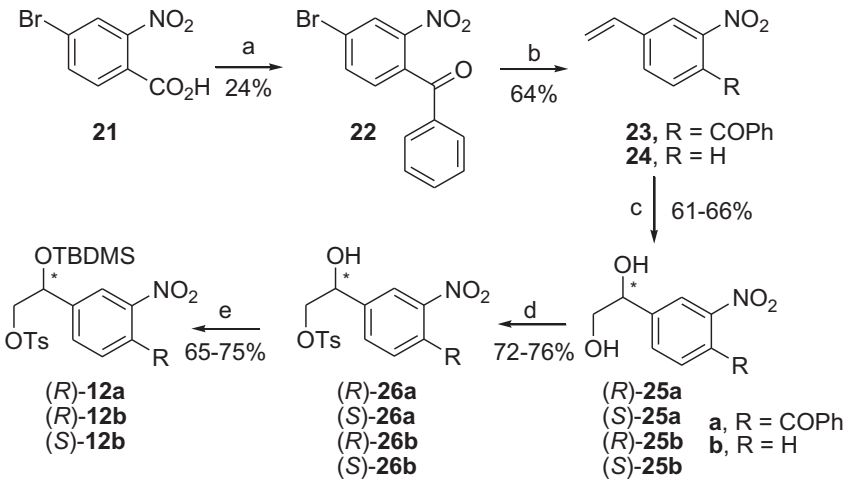
Compound (*R*)-**12b** was used as the starting material for the synthesis of compounds **5e**–**g** (Scheme 3). Accordingly, (*R*)-**12b** was heated at 75 °C in DMSO with two commercially available ethanolamines. After *N*-Boc protection of the resulting amines **16a**,**b**, the corresponding alcohols were protected as *tert*-butyldimethylsilylethers. Compounds **17a**,**b** thus obtained were submitted to a Fe/NH_4Cl-mediated reduction of the nitro group leading to anilines **18a**,**b**. Anilines **18a**,**b** were coupled with the in situ generated aminoacyl chloride with simultaneous cleavage of the *tert*-butyldimethylsilylether, thus resulting in compounds **5e**,**f**, along with the oxazolidinones **19a**,**b**. Compound **5f** was deprotected by exposure to hydrochloric acid to furnish compound **5g** as hydrochloride salt. Compound (*R*)-**14b** (obtained as described for (*S*)-**14b**) was acylated with 1-naphthoyl chloride (Scheme 4) affording intermediate **20**. Compound (*R*)-**5h** was obtained after deprotection of the *tert*-butyldimethylsilylether.

2.1.3. Enantioselective synthesis of compounds *12a,b*

For the generation of compounds **12a**,**b** we exploited an asymmetric dihydroxylation reaction starting from the appropriate vinylbenzenes. As described in Scheme 5, the vinyl-derivative **23** was prepared starting from 4-bromo-2-nitrobenzoic acid **21** which, after conversion in its corresponding acyl chloride, was submitted to a Friedel–Crafts reaction that afforded the benzophenone **22** in moderate yield. A Stille coupling reaction allowed conversion of **22** into the styryl-derivative **23**. Starting from **23** or the commercially available 3-nitrovinylbenzene **24**, application of the Sharpless asymmetric dihydroxylation protocol [14] provided diols **25a**,**b** in



Scheme 4. Synthesis of compound **5h**. Reagents and conditions: a) 1-naphthoyl chloride, DIPEA, DCM rt, 2 h; b) TBAF, THF 0 °C, 12 h.



Scheme 5. Synthesis of protected intermediates **12a**,**b**. Reagents and conditions: a) i) PCl_5 dry chlorobenzene, rt, 12 h; ii) benzene, $FeCl_3$, rt, 2 h; b) $Pd(PPh_3)_4$, tributyl(vinyl)tin, toluene, 125 °C, 3 h; c) AD beta mix for *R*-isomer, AD alpha mix for *S*-isomer, *t*-BuOH/H_2O, 0 °C, 8 h; d) TsCl, pyridine, DCM, rt, 4 h; e) TBDMSCl, imidazole, DCM, rt, 12 h.

their enantiomerically pure forms. Particularly, treatment of **23** and **24** with AD-mix-beta or AD-mix-alpha led to the formation of the desired (*S*)- or (*R*)-stereoisomers of **25a**,**b** respectively. Determination of the absolute configuration of compounds (*S*)- and (*R*)-**25b** was described in the literature [15], while the absolute configuration of the newly formed chiral centres of compound (*R*)-**25a** was determined as described in the following paragraph. Regioselective tosylation of **25a**,**b** provided compounds **26a**,**b**. Treatment of tosylates **26a**,**b** with TBDMSCl and imidazole furnished the key diol derivatives (*R*)-**12a** and (*S*)- and (*R*)-**12b** [16] opportunely protected at the secondary alcoholic functionality.

2.1.4. Determination of the absolute configuration of (+)-26a and (−)-26a

We used the ^{19}F for the determination of the e.e. of the Sharpless reaction [17]. In order to validate the methodology, we applied the same approach also to the known substrate **25b**. Tosyl alcohol (+)-**26a**, (−)-**26a** and *S*-(+)-**26b** *R*-(−)-**26b** were reacted with both *R*- and *S*-methoxy-1-trifluoromethyl-1-phenylacetic acid (MTPA) acyl chlorides in presence of a base and ^1H and ^{19}F spectra were recorded. After assignment of the proton signals, calculation of $\Delta\delta^{RS}$ of specific signals (indicated as L1 and L2, see Table 1) shifted by the anisotropic effect of the phenyl group was performed. For the known diol **25b**, the MTPA methods led to results that were in agreement with the configuration reported in the literature. Consequently, we decided to apply the methodology to the unknown alcohols (+)-**25a** and (−)-**25a** obtained from the reaction with αAD mix and βAD mix, respectively. The procedure was applied to the corresponding tosyl-derivative **26a** and the $\Delta\delta^{RS}$ of assigned signals are reported in Table 1. The positive or negative values of the selected signals were used to determine the absolute configuration of the unknown chiral centers (Tables 1 and 2). Our experiments showed that all L_1 or L_2 signals have the same positive or negative value, therefore we could assign the (*R*)-configuration to isomer (−)-**26a** and the (*S*)-configuration to isomer (+)-**26a** (Table 1). Analysis of $\Delta\delta^{RS}$ of ^{19}F signals led to coherent results (Table 1). The absolute configuration of the synthesized diols is summarized in Table 2.

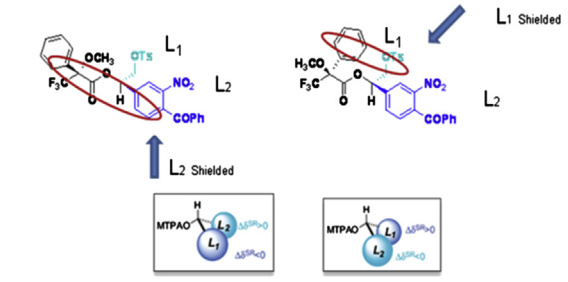
2.2. Binding assays, structure–activity relationships, and molecular modeling studies

The newly developed compounds (**3a**–**d**, **4a**,**b**, Table 3, and **5a**–**h**, Table 4) were submitted to an enzymatic assay based on the time resolved fluorescence resonance energy transfer (TR-FRET) [18] to evaluate their BACE1 inhibitory properties. The most active compounds were also evaluated in cell-based assays.

The optically pure (*S*)-3-phenylBDZ core structure was decorated at C_9 with functionalities bearing the piperazine system (**3a**–**d**). Starting from the biphenyl BDZ **3a**, a set of structural modifications to obtain compounds **3b**–**d** (Table 3) was performed: i) removal of the carbamoyl function from the piperazine N_4 (**3b**), ii) reduction (**3c**) of the N_4–C_5 imine bond iii) removal of the phenyl at C_6 position (**3d**). BDZ **3a** showed inhibition potency against BACE1 in the micromolar range, while all the analogs proved to be inactive. Results evidenced that geometry of the seven-membered ring together with the presence of the pendant phenyl ring are critical for activity (**3a** vs **3c** and **3a** vs **3d**). Removal of the carbamoyl function at piperazine N_4 was found detrimental for activity indicating that a protonatable function at that position is not required for activity. Due to the structural constrains for activity in the BDZ series we decided to investigate their advanced seco-intermediates which possess an "open" structure (N_4–C_5 seco-analogs **4a**,**b**) and lack natural aminoacidic substructures. As shown in Table 3, **4a**,**b** proved to be equally potent to **3a** in inhibiting BACE1. This finding provided inspiration for the investigation of a different class of compounds (**5a**–**h**, Fig. 1 and Table 4) characterized by a further modified structure, bearing an unnatural aminoacid (phenylglycine) and a stereodefined HEA substructure. Initially we synthesized the benzophenones **5a** and **5b**, and later we exploited the SARs with their flexible analogs **5c**–**h** through: i) the modification of the lateral chain containing the HEA moiety in terms of length and hindrance and/or stereochemistry, ii) the removal of the benzoyl group, iii) the modification of the lateral chain containing the phenylglycine moiety in terms of length and hindrance and/or stereochemistry. Design of **5h** was driven by the need of reducing molecular weight, molecular complexity, chiral centers and number of rotatable bonds.

As shown in Table 4, comparing **5a** and **5b** it is clear that there is not stereoselective interaction with the enzyme since *R*- and *S*-enantiomers showed comparable inhibition potencies. Removal of the benzoyl function of **5a**,**b** gave analogs **5c**,**d** 3 times more potent. Introduction of an extra HEA function generated the analog **5e** with an IC_{50} equal to 9.2 μM. Homologation of **5e** (benzylHEA in place of phenylHEA) led to a slightly more potent compound (**5f** vs **5e**). Removal of the Boc function from **5f** did not interfere with potency (**5g** vs **5f**). For minimizing the structure of these analogs we designed **5h**, characterized by a naphthoyl function, the absence of the urethane group (originally bear by the phenylglycine), and the

Table 1
Anisotropic effect of the chiral auxiliary MTPA exerted on the substituents (L_1 and L_2) of the analyzed isomers of **26a**.



$\Delta\delta(SR)L_1 = \delta L_1(S)-\delta L_1(R) < 0$ $\quad$ $\Delta\delta(SR)L_1 = \delta L_1(S)-\delta L_1(R) > 0$
$\Delta\delta(SR)L_2 = \delta L_2(S)-\delta L_2(R) > 0$ $\quad$ $\Delta\delta(SR)L_2 = \delta L_2(S)-\delta L_2(R) < 0$

Compound		^{1}H signals L_1/L_2 or ^{19}F signals	Chemical shifts (*R*)-MTPA ester	Chemical shifts (*S*)-MTPA ester	$\Delta\delta^{RS}$
(−)-**26a**	$L_1 > 0$, $L_2 < 0$ ⟹ **R**-configuration	A (L_1)	7.85	8.02	−0.17
		B (L_2)	7.69	7.70	−0.01
		C (L_1)	4.31	4.30	+0.01
		D (L_2)	2.44	2.40	+0.04
		^{19}F	−71.75	−71.60	−0.15
(+)-**26a**	$L_1 < 0$, $L_2 > 0$ ⟹ **S**-configuration	A (L_1)	8.02	7.80	+0.22
		B (L_2)	7.73	7.70	+0.03
		C (L_1)	4.29	4.31	−0.02
		D (L_2)	2.44	2.45	−0.01
		^{19}F	−71.60	−71.75	+0.15

absence of the extra alcoholic functionality in proximity of the HEA substructure. Furthermore, the peptidomimetic (*R*)-**5h** presents only one chiral center and lacks natural aminoacidic substructures. This compound showed an IC_{50} equal to 4.9 μM and it could be considered a hit to develop a novel class of BACE1 peptidomimetics.

To get an insight into the binding modes of our ligands into the BACE1 active site, ensemble-docking studies were performed using five X-ray structures representative for the enzyme flexibility [19]. Docking of our ligands in each of the BACE1 structures provided similar outcomes, with 2G94, 1W51 and 1FKN providing results more in line with the experimentally determined IC_{50}.

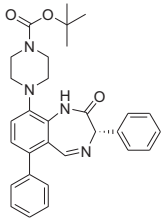
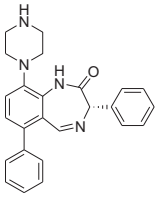
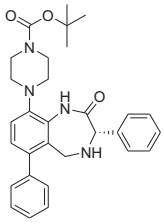
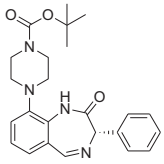
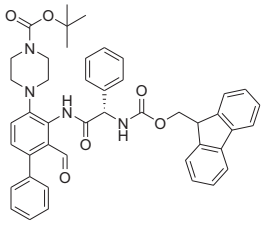
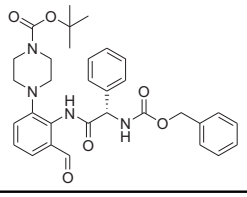
Analysis of **3a** docked structure into the BACE1 enzyme revealed that the BDZ scaffold lays under the flap region (Fig. 2). The *N*-Boc piperazine substituent present on the BDZ core H-bonds the T329 side chain but does not interact at all with the catalytic Asp residues (D32 and D228), which may account for the low inhibitory potency of the molecule. The phenyl at the C3 position (which is able to flip during docking calculations within Glide software) lays between the S2 and the S4 cavities, and the proximity of R235 would suggest the formation of a cation−π interaction. The phenyl ring at C6 is perfectly oriented into the S1 pocket establishing hydrophobic contacts with L30, F108 and I118 side chains and a T-shaped interaction with the W115 indole. The same phenyl ring at C6 seems to play an important role in binding, as suggested by the observed dramatic drop in affinity caused by its removal (**3d**). Also the withdrawal of the *N*-Boc group is not beneficial; in fact the protonated nitrogen of **3b** is not located in proximity of any of the catalytic Asp, and does not engage any fruitful interaction, but its proximity to K224 side chain may explain the drop of the affinity for **3b**. Further, for **3c** docking results are in line with the experimentally determined IC_{50} since the obtained binding pose shows that the protonated nitrogen at position 4 of the BDZ core forms a charge-reinforced H-bond with D228 side chain, which influences the binding mode of the molecule, thus preventing any other productive interaction.

Docking results for the seco-analog **4b** (Fig. 3) show that the benzyl-carbamate moiety contacts the Y198, I226 and T329 carbon atoms with the carbonyl oxygen H-bonding the protonated D32 carboxylate. On the other side, the benzaldehyde group lays into the S1 pocket establishing the same interacting pattern as described for **3a**. The long *N*-Boc piperazine substituent is stretched forward the

Table 2
Absolute configuration of diols **25a**,**b** and the evaluated enantiomeric excess (e.e.) of the Sharpless asymmetric dihydroxylation of compounds **23** and **24**.

Compd	e.e. (%)	Absolute configuration	AD mix used
(+)-**25a**	97	*S*	α
(−)-**25a**	97	*R*	β
(+)-**25b**	97	*S*	α
(−)-**25b**	98	*R*	β

Table 3
BACE1 inhibition activity of BDZs **3a**–**d** and *seco*-analogs **4a**,**b** as IC_{50} (μM).[a]

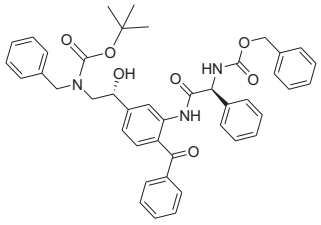
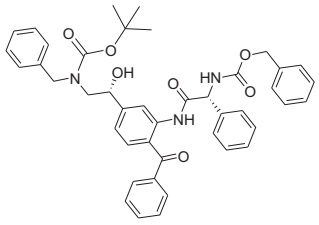
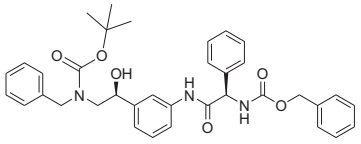
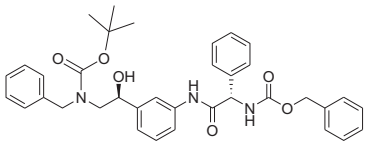
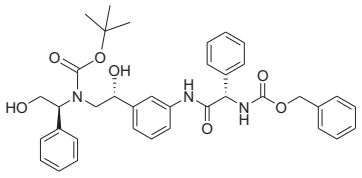
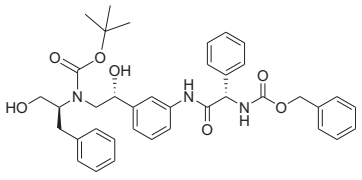
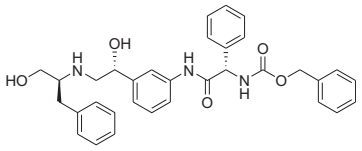
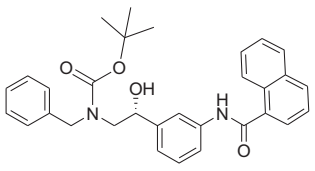
Compd	Compound structure	IC_{50} (μM)[b]
3a		12.8
3b		NA[c]
3c		NA[c]
3d		NA[c]
4a		14.8
4b		14.3

[a] Tests performed as described in SI.
[b] Each value is the mean of at least 3 experiments (SD are within 10%).
[c] NA not active.

S4 where no productive interactions are detected while the phenyl substituent on the chiral center engages hydrophobic interactions with the T72, T231 and V332 carbon atoms.

When **5c** was docked into BACE1 active site (2G94), the peptidomimetic backbone stretches along the cleft with the OH group H-bonding catalytic D32 and D228 carboxylates (Fig. 4 panel a). The aromatic ring of the benzyl-carbamate moiety engages a T-shaped interaction with W197 while the NH and CO H-bond with Y198 and R128, respectively. The neighboring phenyl ring forms a π–π

Table 4
BACE1 inhibition activity of linear derivatives **5a**–**h**, as IC_{50} (μM).[a]

Compd	Compound structure	IC_{50} (μM)[b]
5a		14.9
5b		18.1
5c		4.7
5d		5.2
5e		9.2
5f		6.4
5g		7.5
5h		4.9

[a] Tests performed as described in SI.
[b] Each value is the mean of at least 3 experiments (all SD are within 10%).

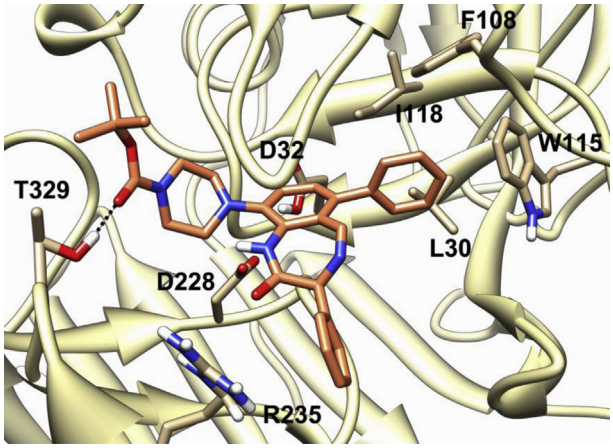


Fig. 2. Binding mode of **3a** into the BACE1 (PDB code: 2G94). Ligands carbon atoms are displayed in coral, key binding site residues as tan sticks. H-bonds are shown as black dotted lines.

interaction with Y71 and hydrophobic contacts with V69 side chain. Finally, the pendant phenyl ring, on the *N*-Boc side, fits the lipophilic cavity made up by L30, F108, W115 Y71 and I118 residues (S1 pocket), while the *N*-Boc group occupies the S3 cavity.

However, our docking calculations highlighted an alternative binding mode where the *N*-Boc and the pendant phenyl ring invert their positions. Both binding modes are in line with the lack of stereoselectivity of interaction of **5c** and **5d** as superimposable binding poses were obtained (Fig. 5 panel a). Binding data also suggested that the change of stereochemistry of the hydroxylated carbon atom of **5a** and **5b** (into their *S* isomers) does not substantially affect their binding mode. In fact, docking calculations on both compounds show that these isomers are still able to interact with the carboxylate groups of D228 and D32. However, the introduction of the phenone substituent makes the benzyl-carbamate moiety change its orientation thus losing the useful interactions as for **5c** (Fig. 5 panel b). This binding mode explains the lower activity of **5a** and **5b**. With regard to **5f**, a binding mode similar to that obtained for **5c** was detected. However the main difference relies on the positioning of the benzyl group which switches from S1 to S3 pocket due to the introduction of a hydroxymethylene group. Differently, in the case of **5e**, the semi-flexible docking program used for this study fails to furnish a reliable binding mode and this may be due to the rigid phenyl

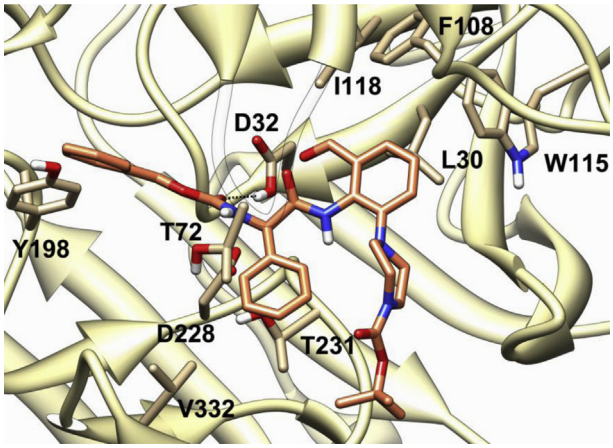


Fig. 3. Binding mode of **4b** into the BACE1 (PDB code: 2G94). Ligands carbon atoms are displayed in coral, key binding site residues as tan sticks. H-bonds are shown as black dotted lines and flap region cartoon are transparent for sick of clarity.

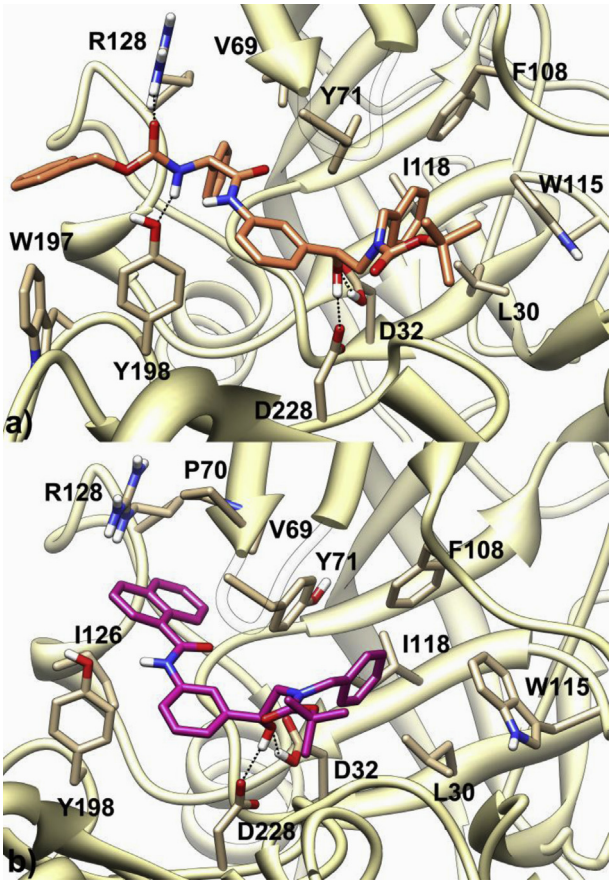


Fig. 4. Binding modes of **5c** (a) and **5h** (b) into the BACE1 (PDB code: 2G94). Ligands carbon atoms are displayed in coral and magenta, respectively, key binding site residues as tan sticks. H-bonds are shown as black dotted lines and the flap region is in transparent ribbon for sick of clarity.

substituent of **5e** which would require protein flexibility. Compared to **5c**, docking of **5g** showed that the HEA group can optimally interact with the catalytic dyad, the benzyl-carbamate moiety keeps its T-shaped interaction with the W197 side chain, while the nearby phenyl ring loses its position being mostly solvent exposed. The observed interactions (Fig. 5 panel c) are perfectly in line with the comparable IC_{50} values of **5c** and **5g**. The pose described for **5c** is similar to the one of **5h** (Fig. 4 panel b) in which the loss of the benzyl-carbamate moiety is partially compensated by the bulkier naphthalene group. In fact, hydrophobic contacts are detected with V69, P70, I126, Y198 and Y71 side chains, and the proximity of R128 would suggest the formation of a cation-π interaction. As a consequence, **5h** represents a new hit for developing small molecules as BACE1 inhibitors.

2.3. *In vitro cellular assays*

Since some BACE1 inhibitors active in isolated enzyme-assays fail when tested in cellular assays, as a further biological evaluation, we engaged the two most potent compounds of the series **5c** and **5h** in functional cell-based assays. At this purpose we employed two different assays [20]. One assay is performed on HEK293 cells expressing a specific APP construct containing a modified BACE1 cleavage sequence, NFEV and a K612V mutation thus preventing α-secretase processing. The other assay employs SHSY5Y cells expressing APP NFEV construct but contains the wild-type α-cleavage site. **5c** and **5h** were tested to determine their IC_{50} against the generation of sAPPβ (direct functional read out for BACE1

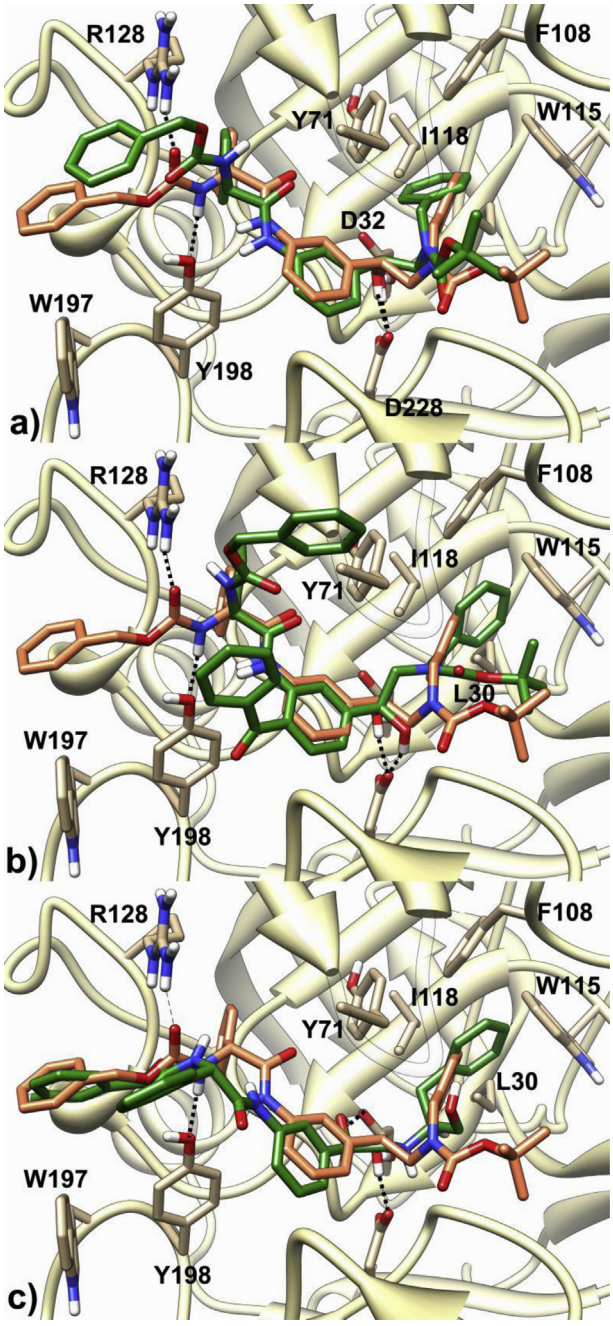


Fig. 5. Superimposition of docked structure of **5c** (coral sticks) with **5d** (a), **5a** (b) and **5g** (c) (forest green sticks) into the BACE1 (PDB code: 2G94). Flap region cartoon are transparent for sick of clarity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

activity) and confirmed as generation of AV40 and AV42 (AV40 and AV42 are the Aβ isoforms, derived from processing of the APP NFEV construct, and consequently bearing Glu and Val at the N-terminal portion). The determined IC_{50} values in HEK293 cells were >10 μM and >7 μM for **5c** and **5h** respectively. In the SHSY5Y cell line the efficacy values were confirmed for **5h** ($IC_{50} = 11$ μM) while for **5c** the determined IC_{50} was 10 times higher ($IC_{50} > 100$ μM).

3. Conclusion

In summary, we have herein described a stereoselective approach for the development of peptidomimetic BACE1 inhibitors. Binding studies on the isolated enzyme revealed a micromolar potency of inhibition of BACE1, and molecular modeling studies allowed rationalization of the observed SARs. In vitro cellular studies were performed for assessing cellular efficacy of the most active compounds, and we found out that cell-based activities were in line with the inhibition potencies determined by the TR-FRET assays. Taken together, these data indicate that, although weak, the identified compounds may be considered new peptidomimetic enzyme inhibitors active on whole-cells. Particularly, the peptidomimetic **5h** may be considered a hit to develop an improved series of BACE1 inhibitors.

4. Experimental

4.1. Chemistry

4.1.1. General procedures

Unless otherwise specified, materials were purchased from commercial suppliers and used without further purification. Reaction progress was monitored by TLC using silica gel 60 F254 (0.040–0.063 mm) with detection by UV. Silica gel 60 (0.040–0.063 mm) or aluminum oxide 90 (0.063–0.200 mm) were used for column chromatography. ^{1}H NMR, ^{13}C NMR, and ^{19}F NMR spectra were recorded on a Varian 300 MHz, spectrometer by using the residual signal of the deuterated solvent as internal standard. Splitting patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), quintet (p), and broad (br); the value of chemical shifts (δ) are given in ppm and coupling constants (J) in Hertz (Hz). Number of overlapping carbon signals are reported in brackets (equivalent carbon atoms are always counted once). ESI-MS spectra were performed by an Agilent 1100 Series LC/MSD spectrometer. Melting points were determined in Pyrex capillary tubes using an Electrothermal 8103 apparatus and are uncorrected. Optical rotation values were measured at room temperature using a Perkin–Elmer model 343 polarimeter (operating at $\lambda = 589$ nm, corresponding to the sodium D line) and at a Jasco P2000 polarimeter (operating at $\lambda = 436$ nm, corresponding to the mercury blue line). Yields refer to purified products and are not optimized. All moisture-sensitive reactions were performed under argon atmosphere using oven-dried glassware and anhydrous solvents. Elemental analyses were performed in a Perkin–Elmer 240C elemental analyzer, and the results were within ±0.4% of the theoretical values, unless otherwise noted.

4.1.2. 3-Bromo-2-nitrobenzaldehyde (*7a*)

A solution of nitric acid (0.6 mL), and concentrated sulfuric acid (8.1 mL), was stirred at 0 °C, then **6a** (3.0 g, 0.11 mmol) was added over a period of 20 min. The mixture was stirred at rt for 1 h and then poured into ice. The white solid formed was filtered and washed with water. The crude product was purified by flash chromatography (1:4, EtOAc/*n*-hexane) to obtain title compound as white solid (yield 48%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.60 (t, $J = 7.5$ Hz, 1H), 7.92 (d, $J = 2.4$ Hz, 1H), 7.94 (d, $J = 2.4$ Hz, 1H), 9.87 (s, 1H).

4.1.3. 3,6-Dibromo-2-nitrobenzaldehyde (*7b*)

Following the procedure described for **7a**, title compound was obtained as white solid (yield 48%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.70 (d, $J = 8.5$ Hz, 1H), 7.81 (d, $J = 8.5$ Hz, 1H), 10.21 (s, 1H).

4.1.4. 2-(3-Bromo-2-nitrophenyl)-1,3-dioxolane (*8a*)

A mixture of **7a** (45 mg, 0.20 mmol), ethylene glycol (163 μL, 2.93 mmol), and PTSA (29.30 mmol) was submitted to MW irradiation. The reaction mixture was cooled to rt and water (1 mL) was added. The aqueous phase was extracted with DCM (3 × 5 mL) and

the combined organic layers were dried over Na_2SO_4, filtered and evaporated. The crude product was purified by flash chromatography (1:4, EtOAc/*n*-hexane) to obtain title compound as a colorless oil (yield 52%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.99 (s, 4H), 5.98 (s, 1H), 7.36 (t, J = 8.1 Hz, 1H), 7.60 (d, J = 7.8 Hz, 1H), 7.64 (d, J = 8.1 Hz, 1H).

4.1.5. 2-(3,6-Dibromo-2-nitrophenyl)-1,3-dioxolane (**8b**)

Following the procedure described for **8a**, title compound was obtained as a white solid (yield 61%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.00 (m, 4H), 6.16 (s, 1H), 7.55 (m, 2H).

4.1.6. 2-[3-(4-tert-Butoxycarbonylpiperazin-1-yl)-2-nitrophenyl]-1,3-dioxolane (**9a**)

A mixture of **8a** (28 mg, 0.10 mmol) and 1-*tert*-butoxycarbonylpiperazine (95 mg, 0.50 mmol) was heated at 100 °C in a sealed tube for 48 h. After cooling, the mixture was partitioned between EtOAc (10 mL) and water (6 mL). The organic phase was separated, dried over Na_2SO_4, filtered and evaporated. The residue was purified by flash chromatography (1:4, EtOAc/*n*-hexane) to afford title compound as a yellow solid (yield 40%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (s, 9H), 2.88 (s, 4H), 3.49 (s, 4H), 3.99 (s, 4H), 5.92 (s, 1H), 7.25 (d, J = 7.2 Hz, 1H), 7.37–7.43 (m, 2H); MS (ESI) m/z: 418 $(M + K)^+$, 402 $(M + Na)^+$.

4.1.7. 2-[6-Bromo-3-(4-tert-butoxycarbonylpiperazin-1-yl)-2-nitrophenyl]-1,3-dioxolane (**9b**)

Following the procedure described for **9a**, title compound was obtained as a yellow solid (yield 40%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (s, 9H), 2.81 (m, 4H), 3.50 (m, 4H), 4.01 (m, 4H), 6.15 (s, 1H), 7.18 (d, J = 8.5 Hz, 1H), 7.65 (d, J = 8.8 Hz, 1H); MS (ESI) m/z: 480 $(M + H)^+$.

4.1.8. 2-[6-Phenyl-3-(4-tert-butoxycarbonylpiperazin-1-yl)-2-nitrophenyl]-1,3-dioxolane (**10**)

Phenylboronic acid (380 mg, 3.10 mmol) was dissolved in anhydrous 1,4-dioxane (20 mL). Then bromo-derivative **9b** (1.2 g, 2.6 mmol), tetrakis(triphenylphosphine)palladium(0) (3% mol), and K_3PO_4 (870 mg, 4.10 mmol), were added. The resulting suspension was refluxed for 72 h. Dioxane was evaporated and K_3PO_4 and the catalyst were removed by filtration through silica gel. The crude product was purified by flash chromatography (1:3, Et_2O/petroleum ether) to afford title compound as a colorless oil (yield 42%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.47 (s, 9H), 2.92 (m, 4H), 3.51 (m, 4H), 3.84 (m, 2H), 4.01 (m, 2H), 5.60 (s, 1H), 7.37 (m, 7H); MS (ESI) m/z: 477 $(M + Na)^+$.

4.1.9. [3-(4-tert-Butoxycarbonylpiperazin-1-yl)-2-amino] benzaldehyde (**11a**)

To a solution of **9a** (79 mg, 0.21 mmol) in ethanol (5 mL) and saturated solution of ammonium chloride (2 mL), iron powder (88 mg, 1.58 mmol) was added. The heterogeneous mixture was heated at reflux for 3 h, then poured into water (10 mL) and filtered through a small plug of Celite. The aqueous filtrate was extracted with DCM (3 × 50 mL) and the combined organic layers were washed with brine (5 mL), dried over Na_2SO_4, filtered and evaporated. The residue was purified by flash chromatography (1:5, EtOAc/*n*-hexane) to afford title compound as a yellow solid (yield 52%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.47 (s, 9H), 2.58–4.38 (m, 8H), 6.57 (br s, 2H), 6.69 (t, J = 9.0 Hz, 1H), 7.11 (d, J = 6.9 Hz, 1H), 7.27 (d, J = 6.3 Hz, 1H), 9.86 (s, 1H).

4.1.10. [6-Phenyl-3-(4-tert-butoxycarbonylpiperazin-1-yl)-2-amino]benzaldehyde (**11b**)

Following the procedure described for **11a**, title compound was obtained as a yellow solid (yield 97%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.49 (s, 9H), 2.81–2.97 (m, 6H), 4.11 (m, 2H), 6.57 (d, J = 7.0 Hz, 1H), 7.14 (d, J = 7.6 Hz, 1H), 7.32–7.40 (m, 5H), 9.83 (s, 1H); MS (ESI) m/z: 404 $(M + Na)^+$.

4.1.11. (2S)-[2-((9H-Fluoren-9-ylmethoxycarbonylamino)-2-phenylacetamido)-3-(4-tert-butoxycarbonylpiperazin-1-yl)-6-phenyl]benzaldehyde (**4a**)

To a stirred solution of L-Fmoc-phenylglycine (220 mg, 0.59 mmol) and triphenylphosphine (309 mg, 1.18 mmol) in dry DCM (10 mL), cooled to −10 °C, hexachloroacetone (45 μL, 0.29 mmol) was added dropwise. After 15 min, a solution of **11b** (150 mg, 0.39 mmol) in dry DCM (2 mL) was added. The reaction was stirred at −10 °C for 30 min, then 1 h at rt. The mixture was washed with 10% $NaHCO_3$ solution (10 mL) and the aqueous phase was extracted with DCM (3 × 5 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated. The residue was purified by chromatography (1:3, EtOAc/*n*-hexane) to afford title compound as a colorless solid (yield 76%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.48 (s, 9H), 2.75 (m, 4H), 3.27–3.38 (m, 4H), 4.21 (m, 1H), 4.36 (m, 2H), 5.42 (s, 1H), 6.19 (d, J = 5.2 Hz, 1H), 7.22–7.59 (m, 18H), 7.75 (m, 2H), 8.77 (s, 1H), 9.72 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.7, 43.8, 47.3, 51.7, 60.0, 67.4, 80.1, 120.2, 124.9, 125.3, 127.3, 127.9, 128.3 (2C), 128.5, 128.7 (2C), 129.0 (2C), 129.1, 129.5, 129.8, 130.1, 137.4, 137.7, 141.2, 141.5, 144.0, 154.8, 155.7, 168.4, 193.2; MS (ESI) m/z: 759 $(M + Na)^+$. Anal. ($C_{45}H_{44}N_4O_6$) C, H, N.

4.1.12. (2S)-[2-(Benzyloxycarbonylamino-2-phenylacetamido)-3-(4-tert-butoxycarbonylpiperazin-1-yl)]benzaldehyde (**4b**)

Starting from *Z*-L-phenylglycine, and following the same procedure reported for **4a**, title compound was obtained as a white solid (yield 32%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.49 (s, 9H), 2.59–2.65 (m, 4H), 3.22–3.27 (m, 4H), 5.10 (s, 2H), 5.41 (s, 1H), 6.03 (s, 1H), 7.27–7.64 (m, 13H), 8.34 (s, 1H), 9.84 (s, 1H); MS (ESI) m/z: 595 $(M + Na)^+$, 573 $(M + H)^+$. Anal. ($C_{32}H_{36}N_4O_6$) C, H, N.

4.1.13. (3S)-3,6-Diphenyl-9-(4-tert-butoxycarbonylpiperazin-1-yl)-1,3-dihydrobenzo[e][1,4]diazepin-2-one (**3a**)

To a solution of **4a** (288 mg, 0.39 mmol) in DCM (10 mL), DIEA (806 μL) was added. The reaction mixture was stirred at reflux for 5 h. The solvent was evaporated and the residue was purified by flash chromatography (1:3, EtOAc/*n*-hexane) to obtain title compounds as bright yellow solid (yield 61%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.50 (s, 9H), 2.77–3.13 (m, 4H), 3.62 (m, 4H), 4.87 (s, 1H), 7.31–7.50 (m, 10H), 7.62 (m, 2H), 8.34 (s, 1H), 8.70 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.4, 43.9, 51.9, 68.0, 80.1, 122.5, 123.1, 126.4, 127.1, 127.7, 128.1, 129.8, 130.1, 132.1, 135.9, 137.8, 138.5, 139.5, 142.6, 150.0, 168.1, 171.6; MS (ESI) m/z: 1015 $(2M + Na)^+$, 535 $(M + K)^+$, 519 $(M + Na)^+$, 497 $(M + H)^+$; $[\alpha]_D^{20}$ −16.5 (c = 0.2, MeOH). Anal. ($C_{30}H_{32}N_4O_3$) C, H, N.

4.1.14. (3S)-3,6-Diphenyl-9-(piperazin-1-yl)-1,3-dihydrobenzo[e][1,4]diazepin-2-one (**3b**)

To a solution of **4a** in dry MeOH was added a 0.1 N solution of HCl in MeOH, prepared by adding acetyl chloride (0.3 mmol) to dry MeOH (3 mL). The resulting mixture was evaporated under reduced pressure. The procedure was repeated until complete consumption of the starting material. The hydrochloride salt of title compound was obtained as bright yellow solid (yield 89%). ^{1}H NMR (300 MHz, CD_3OD) δ 3.21 (m, 2H), 3.51–3.61 (m, 6H), 5.87 (s, 1H), 7.49–7.89 (m, 11H), 7.91 (m, 1H), 8.77 (s, 1H); MS (ESI) m/z: 419 $(M + Na)^+$, 397 $(M + H)^+$. Anal. ($C_{25}H_{24}N_4O$) C, H, N (free base).

4.1.15. (3S)-3,6-Diphenyl-9-(4-tert-butoxycarbonylpiperazin-1-yl)-1,3,4,5-tetrahydrobenzo[e][1,4]diazepin-2-one (**3c**)

To a solution of **3a** (14 mg, 0.03 mmol), in glacial acetic acid (3 mL), sodium cyanoborohydride (3.5 mg, 0.05 mmol) was added

and the reaction was stirred at rt for 10 min. Water (3 mL) and a solution of Na_2CO_3 were added up to pH = 8. The aqueous phase was extracted with EtOAc (3 × 5 mL) and the combined organic layers were dried over Na_2SO_4, filtered and evaporated. The residue was purified by flash chromatography (1:2, EtOAc/*n*-hexane) to obtain title compound as a bright yellow solid (yield 80%). ^{1}H NMR (300 MHz, CD_3OD) δ 1.49 (s, 9H), 2.73–2.85 (m, 4H), 3.57 (m, 4H), 4.12 (m, 2H), 4.91 (s, 1H), 7.05 (d, J = 8.2 Hz, 1H), 7.14 (d, J = 8.2 Hz, 1H), 7.26–7.41 (m, 10H), 8.08 (s, 1H); MS (ESI) *m*/*z*: 1019 $(2M + Na)^+$, 521 $(M + Na)^+$, 499 $(M + H)^+$. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.49 (s, 9H), 2.73–2.85 (m, 4H), 3.58 (br s, 4H), 4.04 (m, 2H), 4.91 (s, 1H), 7.10 (m, 2H), 7.26–7.41 (m, 10H), 8.08 (s, 1H). Anal. ($C_{30}H_{34}N_4O_3$) C, H, N.

4.1.16. (3S)-3-Phenyl-9-(4-tert-butoxycarbonylpiperazin-1-yl)-1,3-dihydrobenzo[e][1,4]diazepin-2-one (**3d**)

To a suspension of **4b** (70 mg, 0.12 mmol) in EtOH (5 mL), cyclohexene (12 μL) and a catalytic amount of 10% palladium on carbon were added. The reaction was heated at reflux for 4 h, then cooled to rt and filtered. The solvent was removed under reduced pressure and the residue was purified by chromatography (1:3, EtOAc/*n*-hexane) to afford title compound as a brown amorphous solid (yield 50%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.49 (s, 9H), 2.73 (br s, 2H), 2.91–3.16 (m, 2H), 3.27–3.85 (m, 4H), 4.57 (s, 1H), 7.05–7.61 (m, 8H), 8.41 (s, 1H), 8.70 (d, J = 2.5 Hz, 1H); ^{13}C NMR (75 MHz, CDCl3) δ 28.6, 44.1, 52.1, 69.0, 80.4, 122.9, 124.5, 126.2, 126.5, 128.3, 128.6, 129.2, 132.2, 137.5, 142.8, 154.8, 162.4, 167.7; MS (ESI) *m*/*z*: 863 $(2M + Na)^+$, 459 $(M + K)^+$, 443 $(M + Na)^+$, 421 $(M + H)^+$. Anal. ($C_{24}H_{28}N_4O_3$) C, H, N.

4.1.17. 4-Bromo-2-nitrobenzophenone (**22**)

A solution of 4-bromo-2-nitrobenzoic acid **21** (1 g, 4.34 mmol) and phosphorous pentachloride (1 g, 4.77 mmol) in dry chlorobenzene (10 mL) was stirred at rt. After 12 h the solvent was removed to give 4-bromo-2-nitrobenzoyl chloride as yellow oil. The crude mixture was washed with *n*-hexane (2 × 10 mL) and used directly for the Friedel-Crafts reaction. A solution of 4-bromo-nitrobenzoyl chloride (2 mL, 23 mmol) in benzene was cooled to 0 °C and treated with anhydrous ferric chloride (0.77 g, 4.7 mmol) added over a period of 30 min. After stirring for 2 h at rt the reaction mass was added to 250 mL of ice and water. The benzene was removed by distillation as its water azeotrope and the reaction mass was extracted with 200 mL of EtOAc .The combined organic layers were washed with brine (20 mL), dried over Na_2SO_4, filtered and evaporated. The residue was purified by chromatography (1:10, EtOAc/*n*-hexane) affording title compound as an orange solid (yield 24%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.23–7.47 (m, 5H), 7.62 (d, J = 8.3 Hz, 1H), 7.88 (dd, J = 8.2, 1.7 Hz, 1H) 8.14 (d, J = 1.7 Hz, 1H).

4.1.18. 4-Vinyl-2-nitrobenzophenone (**23**)

To a solution of 4-bromo-2-nitrobenzophenone **22** (700 mg, 2.28 mmol) and tributylvinyltin (666 μL, 2.28 mmol) in 10 mL of dry toluene, tetrakis(triphenylphospine)palladium(0) (528 mg, 1.14 mmol), was added under argon, and the reaction was heated at reflux for 3 h. The residue was concentrated in vacuo and purified by chromatography (1:5, EtOAc/*n*-hexane) to afford title compound as a pale-yellow solid (yield 64%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.36 (d, J = 5.8 Hz, 1H), 5.70 (d, J = 5.7 Hz, 1H), 6.80 (m, 1H), 7.29–7.90 (m, 7H), 8.25 (s, 1H).

4.1.19. [4-((R)-1,2-Dihydroxyethyl)-2-nitrophenyl] phenylmethanone ((R)-**25a**)

A mixture of water (11.2 mL), *tert*-butyl alcohol (11.2 mL) and AD-mix-beta (3.1 g), was cooled to 0 °C whereupon some of dissolved salts precipitated and olefin **23** (557 mg, 2.2 mmol) was added. The heterogeneous slurry was stirred vigorously at 0 °C for 7 h then solid sodium sulfite (3.5 g) was added and the mixture was allowed to warm to rt and stirred for 30 min. DCM (10 mL) was added to the reaction and after the separation of the layers the aqueous phase was extracted with DCM (5 × 10 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated. The residue was purified by flash chromatography (1:5, EtOAc/*n*-hexane) to afford title compound as colorless oil (yield 61%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.26 (bs, 1H), 3.07 (bs, 1H), 3.70 (m, 1H), 3.95 (m, 1H), 5.00 (m, 1H), 7.46 (m, 3H), 7.60 (m, 1H), 7.77 (m, 3H), 8.27 (s, 1H); MS (ESI) *m*/*z*: 310 $(M + Na)^+$.

4.1.20. [4-((S)-1,2-Dihydroxyethyl)-2-nitrophenyl] phenylmethanone ((S)-**25a**)

Following the same procedure described for (*R*)-**25a** and using AD-mix-alpha, title compound was obtained as colorless oil (yield 70%). Spectroscopic data were identical to those obtained for (*R*)-**25a**.

4.1.21. (R)-1-(3-Nitrophenyl)ethane-1,2-diol ((R)-**25b**)

Starting from **24**, and following the same procedure reported for (*R*)-**25a**, title compound was obtained as a colorless oil (yield 61%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.67 (m, 1H), 3.86 (m, 1H), 4.96 (m, 1H), 7.55 (m, 1H), 7.73 (d, J = 7.0 Hz, 1H), 7.73 (d, J = 7.9 Hz, 1H), 8.20 (s, 1H); MS (ESI) *m*/*z*: 206 $(M + Na)^+$.

4.1.22. (S)-1-(3-Nitrophenyl)ethane-1,2-diol ((S)-**25b**)

Starting from **24** and AD-mix-alpha, and following the same procedure reported for (*R*)-**25a**, title compound was obtained as colorless oil (yield 70%). Spectroscopic data were identical to those obtained for (*R*)-**25b**.

4.1.23. Toluene-4-sulfonic acid (R)-2-(4-benzoyl-3-nitro-phenyl)-2-hydroxyethyl ester ((R)-**26a**)

A solution of diol (*R*)-**25a** (1.0 g, 3.49 mmol), and pyridine (1.4 mL, 17.4 mmol), in DCM (10 mL), was cooled to 0 °C and *p*-toluenesulfonyl chloride (1.7 g, 8.7 mol), was added in three portions over 1.5 h. The reaction mixture was stirred for 4 h at rt and a solution of sodium carbonate was added. The aqueous phase was extracted with DCM (5 × 10 mL), and the combined organic layers were dried over Na_2SO_4, filtered and evaporated. The residue was purified by flash chromatography (1:2, EtOAc/*n*-hexane) affording title compound as a colorless oil (yield 74%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.41 (s, 3H), 3.60 (bs, 1H), 4.11–4.25 (m, 2H), 5.14 (dd, J = 6.7, 4.0 Hz, 1H), 7.31 (m, 2H), 7.42 (m, 3H), 7.56 (m, 1H), 7.68–7.75 (m, 5H), 8.13 (d, J = 1.5 Hz, 1H); MS (ESI) *m*/*z*: 464 $(M + Na)^+$.

4.1.24. Toluene-4-sulfonic acid (S)-2-(4-benzoyl-3-nitro-phenyl)-2-hydroxyethyl ester ((S)-**26a**)

Starting from (*S*)-**25a** and following the same procedure described for (*R*)-**26a**, title compound was obtained as a colorless oil (yield 80%). Spectroscopic data were identical to those obtained for (*R*)-**26a**.

4.1.25. (R)-2-Hydroxy-2-(3-nitrophenyl)ethyl 4-methylbenzenesulfonate ((R)-**26b**)

Starting from (*R*)-**25b** and following the same procedure reported for (*R*)-**26a**, title compound was obtained as a colorless oil (yield 76%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.45 (s, 3H), 2.96 (bs, 1H), 4.11 (m, 1H), 4.21 (m, 1H), 5.11 (m, 1H), 7.33 (m, 2H), 7.53 (m, 1H), 7.72 (m, 3H), 8.17 (m, 2H); MS (ESI) *m*/*z*: 360 $(M + Na)^+$.

4.1.26. (S)-2-Hydroxy-2-(3-nitrophenyl)ethyl-4-methylbenzenesulfonate ((S)-**26b**)

Starting from (*S*)-**25b** and following the same procedure reported for (*R*)-**26a**, title compound was obtained as a colorless oil

(yield 72%). Spectroscopic data were identical to those obtained for (*R*)-**26b**.

*4.1.27. Toluene-4-sulfonic acid (R)-2-(4-benzoyl-3-nitrophenyl)-2-(tert-butyldimethylsilanyloxy)ethyl ester ((R)-**12a**)*

To a solution of (*R*)-**26a** (3.1 g, 7.0 mmol) in dry DCM (10 mL), *tert*-butyldimethylchlorosilane (2.6 g, 17.5 mmol), and imidazole (1.4 g, 21.0 mmol), were added and the mixture was stirred at rt for 12 h. A solution of sodium carbonate was added and the aqueous phase was extracted with DCM (5 × 10 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated. The residue was purified by flash chromatography (1:4, EtOAc/*n*-hexane) affording title compound as a colorless oil (yield 69%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.0 (s, 3H), 0.11 (s, 3H), 0.88 (s, 9H), 2.42 (s, 3H), 4.03–4.12 (m, 2H), 5.05 (m, 1H), 7.31 (m, 2H), 7.44 (m, 3H), 7.57 (m, 1H), 7.70 (m, 5H), 8.09 (s, 1H); MS (ESI) *m*/*z*: 594 $(M + K)^+$, 578 $(M + Na)^+$.

*4.1.28. Toluene-4-sulfonic acid (R)-2-(tert-butyldimethylsilanyloxy)-2-(3-nitrophenyl)ethyl ester ((R)-**12b**)*

Starting from (*R*)-**26b** and following the same procedure described for (*R*)-**12a**, title compound was obtained as a white solid (yield 65%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.06 (s, 3H), 0.07 (s, 3H), 0.86 (m, 9H) 2.40 (s, 3H), 4.01 (m, 2H), 4.98 (m, 1H), 7.26 (m, 2H), 7.48 (m, 1H), 7.64 (m, 3H), 8.09 (m, 2H); MS (ESI) *m*/*z*: 474 $(M + Na)^+$; $[\alpha]_{436}^{20} = -85.4$ ($c = 0.3$, $CHCl_3$).

*4.1.29. Toluene-4-sulfonic acid (S)-2-(tert-butyldimethylsilanyloxy)-2-(3-nitrophenyl)ethyl ester ((S)-**12b**)*

Starting from (*S*)-**26b** and following the same procedure described for (*R*)-**12a**, title compound was obtained as a colorless oil (yield 75%). Spectroscopic data were identical to those obtained for (*R*)-**12b**.

*4.1.30. 2-Benzoyl-5-((R)-1-(tert-butyldimethylsilyl)oxy)-2-(benzylamino)ethyl-1-nitrobenzene ((R)-**13a**)*

A mixture of (*R*)-**12a** (870 mg, 1.6 mmol), benzylamine (450 μL, 3.2 mmol), and DIPEA (280 μL, 1.6 mmol) in DMSO (10 mL) was heated at 75 °C for 20 h. The reaction mixture was diluted with EtOAc (20 mL) and water (20 mL) and stirred for further 15 min. Layers were allowed to separate and the organic phase was washed several times with NaCl saturated solution (5 × 20 mL), dried over Na_2SO_4, filtered and evaporated. The residue was purified by flash chromatography (1:2, EtOAc/*n*-hexane) to afford title compound as a colorless oil (yield 64%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.02 (s, 3H), 0.13 (s, 3H), 0.94 (s, 9H), 2.86 (m, 2H), 3.85 (m, 2H), 4.99 (m, 1H), 7.26–7.36 (m, 8H), 7.45 (t, $J = 7.6$ Hz, 1H), 7.62 (t, $J = 7.0$ Hz, 1H), 7.74 (m, 3H), 8.22 (s, 1H); MS (ESI) *m*/*z*: 491 $(M + H)^+$.

*4.1.31. (3-((R)-1-(tert-Butyldimethylsilyl)oxy)-2-(benzylamino) ethyl)nitrobenzene ((R)-**13b**)*

Starting from (*R*)-**12b**, title compound was obtained following the same procedure reported for (*R*)-**13a**. The residue was purified by flash chromatography (1:2, EtOAc/*n*-hexane) to afford pure compound as white oil (yield 44%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.11 (s, 3H), 0.07 (s, 3H), 0.90 (s, 9H), 1.71 (br s, 1H), 2.73–2.86 (m, 2H), 3.81 (m, 2H). 4.93 (m, 1H), 7.21–7.35 (m, 5H), 7.49 (t, $J = 7.9$ Hz, 1H), 7.65 (d, $J = 7.6$ Hz, 1H). 8.12 (dd, $J = 2.3$, 5.8 Hz, 1H), 8.22 (s, 1H); MS (ESI) *m*/*z*: 409 $(M + Na)^+$, 387 $(M + H)^+$; $[\alpha]_D^{20} = -45.8$ ($c = 0.2$, $CHCl_3$).

*4.1.32. (3-((S)-1-(tert-Butyldimethylsilyl)oxy)-2-(benzylamino) ethyl)nitrobenzene ((S)-**13b**)*

Starting from (*S*)-**12b**, title compound was obtained following the same procedure reported for (*R*)-**13a**. The residue was purified by flash chromatography (1:2, EtOAc/*n*-hexane) to afford pure compound as white oil (yield 43%). Spectroscopic data were identical to those obtained for (*R*)-**13b**; $[\alpha]_D^{20} = +45.73$ ($c = 0.1$, $CHCl_3$).

*4.1.33. (2-Benzoyl-5-(((R)-1-(tert-butyldimethylsilyl)oxy)-2-(benzyl(tert-butoxycarbonylamino))ethyl)phenyl)amine ((R)-**14a**)*

To a solution of (*R*)-**13a** (300 mg, 0.61 mmol) and TEA (250 μL, 1.8 mmol) in dry THF (5 mL) cooled to 0 °C, di-*tert*-butyl dicarbonate (200 mg, 0.92 mmol) was added. The mixture was allowed to warm to rt and stirred until complete consumption of the starting material. The solution was concentrated under vacuum and the residue was purified by chromatography (1:15, EtOAc/*n*-hexane) to give title compound as a colorless oil (yield 75%). ^{1}H NMR (300 MHz, $CDCl_3$) −0.02 (s, 3H), 0.15 (s, 3H), 0.97 (s, 9H), 1.52 (m, 9H), 3.06–3.13 (m, 1H), 3.44–3.62 (m, 1H), 4.37 (d, $J = 15.5$ Hz, 1H), 4.74 (d, $J = 15.7$ Hz, 1H), 5.15–5.30 (m, 1H), 7.19–7.35 (m, 7H), 7.42–7.46 (m, 2H), 7.57 (t, $J = 7.2$ Hz, 1H), 7.66–7.80 (m, 2H), 8.21 (m, 1H); MS (ESI) *m*/*z*: 629 $(M + K)^+$, 613 $(M + Na)^+$. To the above described nitro derivative (320 mg, 0.54 mmol) dissolved in a mixture of EtOH (15 mL) and saturated solution of ammonium chloride (13 mL), iron powder (240 mg, 4.3 mmol) was added. The mixture was heated at reflux for 1 h then cooled down to rt, poured into water (10 mL) and filtered through a small plug of Celite. The aqueous filtrate was extracted with DCM (3 × 10 mL) and the combined organic layers were washed with brine (10 mL), dried over Na_2SO_4, filtered and evaporated. The residue was purified by flash chromatography (1:3, EtOAc/*n*-hexane) to afford title compound as a yellow oil (yield 84%). ^{1}H NMR (300 MHz, $CDCl_3$) −0.03 (s, 3H), 0.10 (s, 3H), 0.95 (s, 9H), 1.51 (m, 9H), 2.96–3.14 (m, 1H), 3.32–3.56 (m, 1H), 4.32–4.41 (m, 1H), 4.59–4.74 (m, 1H), 4.91–5.09 (m, 1H), 6.16 (br s, 2H), 6.58 (dd, $J = 8.1$, 36.2 Hz, 1H), 6.73 (d, $J = 22.9$ Hz, 1H), 7.16–7.39 (m, 5H), 7.42–7.51 (m, 4H), 7.62 (d, $J = 7.1$ Hz, 1H); MS (ESI) *m*/*z*: 599 $(M + K)^+$, 583 $(M + Na)^+$.

*4.1.34. (3-(((R)-1-(tert-Butyldimethylsilyl)oxy)-2-(benzyl(tert-butoxycarbonylamino))ethyl)phenyl)amine ((R)-**14b**)*

To a solution of (*R*)-**13b** (320 mg, 0.83 mmol) and TEA (343 μL, 2.5 mmol) in dry THF (5 mL) cooled to 0 °C, di-*tert*-butyl dicarbonate (271 mg, 1.24 mmol) was added. The mixture was allowed to warm to rt and stirred until complete consumption of the starting material. The clear solution was concentrated under vacuum and the residue was purified by chromatography (1:10, EtOAc/*n*-hexane) to give title compound as a colorless oil (yield 79%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.10 (s, 3H), 0.09 (s, 3H), 0.93 (s, 9H), 1.51 (m, 9H), 2.97–3.52 (m, 2H), 4.29–4.68 (m, 2H), 5.05–5.22 (m, 1H), 7.13–7.32 (m, 5H), 7.47 (t, $J = 7.9$ Hz, 1H), 7.69 (d, $J = 7.6$ Hz, 1H). 8.13 (dd, $J = 2.3$, 5.8 Hz, 1H), 8.25 (s, 1H); MS (ESI) *m*/*z*: 509 $(M + Na)^+$; $[\alpha]_{436}^{20} = -77.4$ ($c = 0.2$, $CHCl_3$). Starting from the above described nitro derivative and following the same procedure reported for (*R*)-**14a**, title compound was obtained as a colorless oil (yield 69%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.09 (s, 3H), 0.06 (s, 3H), 0.91 (s, 9H), 1.49 (m, 9H), 2.92–3.11 (m, 1H), 3.23–3.50 (m, 1H), 3.62 (br s, 2H), 4.30–4.38 (m, 1H), 4.48–4.67 (m, 1H), 4.85–5.04 (m, 1H), 6.53–6.78 (m, 3H), 7.05–7.30 (m, 6H); MS (ESI) *m*/*z*: 495 $(M + K)^+$, 479 $(M + Na)^+$, 457 $(M + H)^+$, 357.

*4.1.35. (3-(((S)-1-(tert-Butyldimethylsilyl)oxy)-2-(benzyl(tert-butoxycarbonylamino))ethyl)phenyl)amine ((S)-**14b**)*

Following the same procedure described for (*R*)-**14b** the title compound was obtained as colorless oil. Spectroscopic data were identical to those obtained for (*R*)-**14b**.

*4.1.36. (2R)-2-(Benzyloxycarbonylamino)-N-(2-benzoyl-5-(((R)-1-(tert-butyldimethylsilyl)oxy)-2-(benzyl(tert-butoxycarbonylamino)) ethyl)phenyl)-2-phenylacetamide ((2R-1′R)-**15a**)*

Starting from D-Cbz-Phg-OH and (*R*)-**14a**, and following the same procedure reported for **4a**, title compound was obtained as an

amorphous solid (yield 82%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.05 (s, 3H), 0.10 (s, 3H), 0.95 (s, 9H), 1.49 (m, 9H), 3.01–3.08 (m, 1H), 3.39–3.59 (m, 1H), 4.30–4.38 (m, 1H), 4.69–4.79 (m, 1H), 5.05–5.17 (m, 3H), 5.41 (br s, 1H), 6.20 (br s, 1H), 7.05–7.67 (m, 22H), 8.67 (m, 1H), 11.47 (m, 1H); MS (ESI) *m*/*z*: 867 (M + K)$^+$; 850 (M + Na)$^+$.

4.1.37. (2S)-2-(Benzyloxycarbonylamino)-N-(2-benzoyl-5-(((R)-1-(tert-butyldimethylsilyl)oxy)-2-(benzyl(tert-butoxycarbonylamino)) ethyl)phenyl)-2-phenylacetamide ((2S-1′R)-**15a**)

Starting from L-Cbz-Phg-OH and (*R*)-**14a**, and following the same procedure reported for **4a**, title compound was obtained as an amorphous solid (yield 71%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.07 (s, 3H), 0.09 (s, 3H), 0.95 (s, 9H), 1.46 (m, 9H), 3.00–3.11 (m, 1H), 3.38–3.59 (m, 1H), 4.31–4.37 (m, 1H), 4.68–4.77 (m, 1H), 5.04–5.16 (m, 3H), 5.40 (br s, 1H), 6.18 (br s, 1H), 7.04–7.63 (m, 22H), 8.65 (m, 1H), 11.44 (m, 1H); MS (ESI) *m*/*z*: 867 (M + K)$^+$; 850 (M + Na)$^+$.

4.1.38. (2R)-2-(Benzyloxycarbonylamino)-N-(3-(((S)-1-(tert-butyldimethylsilyl)oxy)-2-(benzyl(tert-butoxycarbonylamino)) ethyl)phenyl)-2-phenylacetamide ((2R-1′S)-**15b**)

Starting from D-Cbz-Phg-OH and (*S*)-**14b** and following the same procedure reported for **4a**, title compound was obtained as an amorphous solid (yield 85%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.13 (s, 3H), 0.05 (s, 3H), 0.90 (s, 9H), 1.46 (m, 9H), 2.90–3.08 (m, 1H), 3.25–3.48 (m, 1H), 4.27–4.34 (m, 1H), 4.49–4.68 (m, 1H), 4.92–5.15 (m, 3H), 5.40 (br s, 1H), 6.22 (br s, 1H), 7.02–7.49 (m, 19H), 7.87–8.00 (m, 1H); MS (ESI) *m*/*z*: 746 (M + Na)$^+$.

4.1.39. (2S)-2-(Benzyloxycarbonylamino)-N-(3-(((S)-1-(tert-butyldimethylsilyl)oxy)-2-(benzyl(tert-butoxycarbonylamino)) ethyl)phenyl)-2-phenylacetamide ((2S-1′S)-**15b**)

Starting from L-Cbz-Phg-OH and (*S*)-**14b** and following the same procedure reported for **4a**, title compound was obtained as an amorphous solid (yield 32%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.12 (s, 3H), 0.05 (s, 3H), 0.91 (s, 9H), 1.46 (m, 9H), 2.90–3.09 (m, 1H), 3.25–3.48 (m, 1H), 4.28–4.35 (m, 1H), 4.49–4.68 (m, 1H), 4.92–5.14 (m, 3H), 5.44 (br s, 1H), 6.27 (br s, 1H), 7.02–7.45 (m, 19H), 7.83–7.93 (m, 1H); MS (ESI) *m*/*z*: 746 (M + Na)$^+$.

4.1.40. (2S)-2-(Benzyloxycarbonylamino)-N-(2-benzoyl-5-(((R)-1-hydroxy)-2-(benzyl(tert-butoxycarbonylamino))ethyl)phenyl)-2-phenylacetamide (**5a**)

To a solution of (2*S*-1′*R*)-**15a** (60 mg, 0.07 mmol) in dry THF (5 mL), 1 M solution of tetrabutylammonium fluoride in THF (100 μL, 0.09 mmol) was added and the reaction was stirred for 12 h. The solvent was removed under reduced pressure and the residue was taken up in DCM. The organic phase was washed with brine, dried over Na_2SO_4, filtered and evaporated. The residue was purified by flash chromatography (1:2, EtOAc/*n*-hexane) to afford title compound as an amorphous solid (yield 61%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.49 (s, 9H), 3.38–3.61 (m, 2H), 4.29–4.52 (m, 2H), 4.87–4.92 (m, 2H), 5.04–5.16 (m, 2H), 5.36 (br s, 1H), 6.15 (br d, 1H), 7.23–7.62 (m, 22H), 8.55 (br s, 1H), 11.42 (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 29.9, 53.0, 55.7, 60.7, 67.4, 74.4, 81.6, 118.7, 120.1, 122.3, 127.6, 127.7, 128.4 (2C), 128.5, 128.7, 128.9 (2C), 129.5 (2C), 130.0, 132.6, 134.4, 136.4, 137.6, 137.8, 138.8, 140.5, 149.9, 155.8, 158.6, 169.1, 199.5; MS (ESI) *m*/*z*: 736 (M + Na)$^+$; $[\alpha]_{436}^{20}$ = −49.2 (*c* = 0.9, $CHCl_3$). Anal. ($C_{43}H_{43}N_3O_7$), C, H, N.

4.1.41. (2R)-2-(Benzyloxycarbonylamino)-N-(2-benzoyl-5-(((R)-1-hydroxy)-2-(benzyl(tert-butoxycarbonylamino))ethyl)phenyl)-2-phenylacetamide (**5b**)

Starting from (2*R*-1′*R*)-**15a**, and following the same procedure reported for **5a** title compound was obtained as an amorphous solid (yield 57%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.49 (s, 9H), 3.39–3.64 (m, 2H), 4.30–4.47 (m, 2H), 4.79–4.91 (m, 2H), 5.05–5.16 (m, 2H), 5.38 (br s, 1H), 6.15 (br s, 1H), 7.22–7.60 (m, 22H), 8.56 (br s, 1H), 11.43 (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.6, 53.0, 55.6, 60.7, 67.4, 74.3, 81.5, 118.7, 120.1, 122.4, 127.6, 127.7, 128.3, 128.5 (2C), 128.7, 128.9 (2C), 129.4 (2C), 129.9, 132.5, 134.4, 136.4, 137.6, 137.9, 138.8, 140.4, 149.9, 155.8, 158.5, 169.1, 199.5; MS (ESI) *m*/*z*: 736 (M + Na)$^+$; $[\alpha]_{436}^{20}$ = −33.9 (*c* = 0.9, $CHCl_3$). Anal. ($C_{43}H_{43}N_3O_7$), C, H, N.

4.1.42. (2R)-2-(Benzyloxycarbonylamino)-N-(3-(((S)-1-hydroxy)-2-(benzyl(tert-butoxycarbonylamino))ethyl)phenyl)-2-phenylacetamide (**5c**)

Starting from (2*R*-1′*S*)-**15b**, and following the same procedure reported for **5a** title compound was obtained as an amorphous solid (yield 29%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.43 (s, 9H), 2.89 (br s, 1H), 3.18–3.43 (m, 2H), 4.44–4.75 (m, 2H), 4.96 (s, 1H), 5.10 (m, 2H), 5.51 (m, 1H), 6.23 (br s, 1H), 7.05 (m, 2H), 7.23–7.40 (m, 13H), 7.56–7.68 (m, 4H), 9.51 (s, 1H). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.46 (s, 9H), 3.23–3.47 (m, 2H), 4.17 (m, 1H), 4.42–4.78 (m, 2H), 4.81 (m, 1H), 5.09 (s, 2H), 5.41 (m, 1H), 6.20 (m, 1H), 6.97 (m, 2H), 7.15–7.61 (m, 17H), 8.02 (br d, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.6, 52.8, 55.8, 59.6, 67.5, 74.1, 81.3, 117.5, 119.4, 122.2, 127.5, 128.3, 128.4 (2C), 128.7, 128.8 (2C), 129.2, 129.4 (2C), 136.0, 137.6, 137.7, 138.1, 143.5, 156.2, 158.3, 168.4; MS (ESI) *m*/*z*: 632 [M + Na]$^+$; $[\alpha]_{436}^{20}$ = +24.6 (*c* = 0.7, $CHCl_3$). Anal. ($C_{36}H_{39}N_3O_6$) C, H, N.

4.1.43. (2S)-2-(Benzyloxycarbonylamino)-N-(3-(((S)-1-hydroxy)-2-(benzyl(tert-butoxycarbonylamino))ethyl)phenyl)-2-phenylacetamide (**5d**)

Starting from (2*S*-1′*S*)-**15b**, and following the same procedure reported for **5a**, title compound was obtained as an amorphous solid (yield 33%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.46 (s, 9H), 2.88 (br s, 1H), 3.19–3.45 (m, 2H), 4.44–4.80 (m, 2H), 4.96 (s, 1H), 5.10 (m, 2H), 5.53 (m, 1H), 6.25 (br s, 1H), 7.16 (m, 2H), 7.21–7.42 (m, 13H), 7.57–7.67 (m, 4H), 9.50 (s, 1H). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.45 (s, 9H), 3.24–3.47 (m, 2H), 4.10 (m, 1H), 4.42–4.78 (m, 2H), 4.81 (m, 1H), 5.09 (s, 2H), 5.41 (m, 1H), 6.20 (m, 1H), 6.97 (m, 2H), 7.15–7.61 (m, 17H), 8.02 (br d, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.6, 52.8, 55.8, 59.6, 67.5, 74.1, 81.3, 117.5, 119.4, 122.2, 127.5, 128.3, 128.4 (2C), 128.7, 128.8 (2C), 129.2, 129.4 (2C), 136.0, 137.6, 137.7, 138.1, 143.5, 156.2, 158.3, 168.4; MS (ESI) *m*/*z*: 632 (M + Na)$^+$; $[\alpha]_{436}^{20}$ = +46.0 (*c* = 0.1, $CHCl_3$). Anal. ($C_{36}H_{39}N_3O_6$), C, H, N.

4.1.44. (3-(((R)-1-(tert-Butyldimethylsilyl)oxy))-2-(((1S)-1-phenyl-(2-hydroxy)ethyl)(amino))ethyl)-1-nitrobenzene (**16a**)

A mixture of (*R*)-**12a** (174 mg, 0.38 mmol), (*S*)-2-amino-2-phenylethanol (105 mg, 0.771 mmol), DMSO (5 mL), and DIPEA (67 μL, 0.38 mmol), was heated at 75 °C for 20 h. To the reaction mixture was added to EtOAc (20 mL) and water (20 mL) and stirred for 15 min. Agitation was stopped, and the layers were allowed to separate. The organic layer was washed with water (20 mL) and concentrated. The residue was purified by flash chromatography (1:1, EtOAc/*n*-hexane) to afford title compound as colorless oil (yield 50%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.10 (s, 3H), 0.11 (s, 3H), 0.93 (s, 9H), 2.40 (br s, 2H), 2.59–2.77 (m, 2H), 3.49 (t, *J* = 9.6 Hz, 1H) 3.65–3.84 (m, 2H), 4.89–4.93 (m, 1H), 7.18–7.62 (m, 7H), 8.10 (d, *J* = 7.9 Hz, 1H), 8.16 (s, 1H); MS (ESI) *m*/*z*:439 (M + Na)$^+$; $[\alpha]_{436}^{20}$ = −55.4 (*c* = 0.2, $CHCl_3$).

4.1.45. (3-(((R)-1-(tert-Butyldimethylsilyl)oxy))-2-(((1S)-1-benzyl-(2-hydroxy)ethyl)(amino))ethyl)-1-nitrobenzene (**16b**)

Following the same procedure described for **16a** and using (*S*)-2-amino-3-phenylpropan-1-ol, title compound was obtained as colorless oil (yield 46%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.15 (s, 3H), 0.13 (s, 3H), 0.84 (s, 9H), 2.17 (br s, 2H), 2.73–2.88 (m, 5H), 3.30 (dd, *J* = 10.2, 4.9 Hz, 1H), 3.57 (dd, *J* = 10.6, 2.0 Hz, 1H), 4.81–4.84 (m, 1H) 7.14–7.31

(m, 5H), 7.47 (t, $J = 9.1$ Hz, 1H), 7.60 (d, $J = 7.3$ Hz, 1H), 8.11 (d, $J = 7.9$ Hz, 1H) 8.18 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 0.0, 0.2, 22.9, 30.6, 43.0, 60.4, 64.8, 67.3, 79.1, 125.9, 127.4, 131.4, 133.5, 134.0, 134.1, 136.9, 143.0, 150.5, 153.1; MS (ESI) m/z: 453 $(M + Na)^+$, 431 $(M + H)^+$.

*4.1.46. (3-(((R)-1-(tert-Butyldimethylsilyl)oxy))-2-(((1S)-1-phenyl-(2-(tert-butyldimethylsilyl)oxy)ethyl)(tert-butoxycarbonylamino)) ethyl)-1-nitrobenzene (**17a**)*

4.1.46.1. (3-(((R)-1-(tert-Butyldimethylsilyl)oxy))-2-(((1S)-1-phenyl-(2-hydroxy)ethyl)(tert-butoxycarbonylamino))ethyl)-1-nitrobenzene. To a solution of **16a** (80 mg, 0.19 mmol), TEA (80 μL, 0.57 mmol), in dry THF (5 mL), cooled at 0 °C, was added di-*tert*-butyl dicarbonate (63 mg, 0.28 mmol). The mixture was stirred at rt until consumption of the starting material as observed by TLC and the clear solution concentrated under vacuum to give a residue which was chromatographed (1:5, EtOAc/*n*-hexane) to give intermediate alcohol as a colorless oil (yield 91%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.10 (s, 3H), 0.1 (s, 3H), 0.92 (s, 9H), 1.39–1.51 (m, 9H), 1.78 (br s, 1H), 2.61–2.74 (m, 2H), 3.93–4.04 (m, 2H), 4.14 (dd, $J = 9.5$, 3.5 Hz, 1H), 4.85 (dd, $J = 6.0$, 4.1 Hz, 1H), 7.19–7.32 (m, 5H), 7.45 (m, 1H), 7.61 (d, $J = 7.6$ Hz, 1H), 8.08–8.16 (m, 2H); MS (ESI) m/z: 539 $(M + Na)^+$.

The above alcohol (100 mg, 0.19 mmol), was treated with dimethyl-*tert*-butylsilyl chloride (35 mg, 0.23 mmol) and imidazole (33 mg, 0.48 mmol) in DMF (350 μL) at 25 °C for 24 h. The reaction mixture was added to EtOAc (10 mL) and water (10 mL) and stirred for 15 min. Agitation was stopped, and the layers were allowed to separate. The organic layer was washed with water (20 mL) and concentrated. The residue was purified by flash chromatography (1:5, EtOAc/*n*-hexane) to afford title compound as white solid (yield 82%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.10–0.05 (m, 12H), 0.85 (m, 18H), 1.4 (s, 9H), 3.26–3.33 (m, 1H), 3.42–3.49 (m, 1H), 4.01–4.15 (m, 1H), 4.17–4.20 (m, 1H), 4.92–4.96 (m, 1H), 5.29 (br s, 1H), 7.11–7.16 (m, 5H), 7.32–7.36 (m, 1H), 7.53 (br s, 1H), 8.02–8.04 (m, 2H); MS (ESI) m/z: 653 $(M + Na)^+$; $[\alpha]_{436}^{20} = +27.5$ ($c = 0.3$, $CHCl_3$).

*4.1.47. (3-(((R)-1-(tert-Butyldimethylsilyl)oxy))-2-(((1S)-1-benzyl-(2-(tert-butyldimethylsilyl)oxy)ethyl)(tert-butoxycarbonylamino)) ethyl)-1-nitrobenzene (**17b**)*

Similarly to the procedure described for **17a**, the alcohol derivative (440 mg, 79%) was isolated starting from **16b**; (3-(((*R*)-1-(*tert*-butyldimethylsilyl)oxy))-2-(((1*S*)-1-benzyl-(2-hydroxy)ethyl)(*tert*-butoxycarbonylamino))ethyl)-1-nitrobenzene. ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.80–0.10 (m, 6H), 0.83–0.87 (m, 9H), 1.24–1.39 (m, 9H), 2.67–2.70 (d, $J = 7.0$ Hz, 1H), 2.78–2.94 (m, 1H), 3.11–3.19 (m, 1H), 3.31–3.36 (m, 1H), 3.66–3.82 (m, 1H), 3.98–4.14 (m, 1H), 4.39 (br s, 1H), 5.11 (br s, 1H), 5.50 (d, $J = 8.2$ Hz, 1H), 7.07–7.26 (m, 5H), 7.51–7.76 (m, 2H), 8.14 (d, $J = 8.2$ Hz, 1H), 8.29 (s, 1H); MS (ESI) m/z: 553 $(M + Na)^+$, 531 $(M + H)^+$.

The title compound was obtained starting from the above alcohol as a colorless oil (yield 57%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.10–0.08 (m, 12H), 0.88 (s, 18H), 0.92–1.27 (m, 9H), 2.51–2.55 (m, 1H), 2.82–2.91 (m; 1H), 3.15–3.29 (m, 2H), 3.67–3.79 (m, 1H), 3.89–4.01 (m, 1H), 4.83 (s, 1H), 5.23 (s, 1H), 7.06–7.43 (m, 5H), 7.44–7.49 (m, 1H), 7.70 (d, $J = 7.0$ Hz, 1H), 8.08 (d, $J = 7.9$ Hz, 1H), 8.24 (s, 1H); MS (ESI) m/z: 667 $(M + Na)^+$.

*4.1.48. N-(3-((((R)-1-(tert-Butyldimethylsilyl)oxy))-2-(((1S)-1-phenyl-(2-(tert-butyldimethylsilyl)oxy)ethyl)(tert-butoxycarbonylamino)) ethyl)phenyl)amine (**18a**)*

To a solution of **17a** (100 mg, 0.15 mmol) in EtOH (10 mL), and saturated solution of ammonium chloride (5 mL), was added iron powder (63 mg, 1.12 mmol). The heterogeneous mixture was heated at reflux for 3 h, poured into water (10 mL) and filtered through a bed of Celite which was subsequently washed with DCM (5 mL). The aqueous filtrate was extracted with DCM (3 × 5 mL) and the organic phases were combined, washed with brine (5 mL) and dried (Na_2SO_4). The solvent was removed and the residue was purified by flash chromatography (1:5, EtOAc/*n*-hexane) to obtain the title compound as yellow oil (yield 68%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.11–0.01 (s, 12H), 0.80 (s, 9H), 0.88 (s, 9H), 1.38 (br s, 9H), 3.27–3.31 (m, 3H), 3.40–3.42 (m, 1H), 4.06 (dd, $J = 10.2$, 4.6 Hz, 1H), 4.33–4.38 (m, 1H), 4.98 (br s, 2H), 6.58–6.67 (m, 3H), 6.98–7.05 (m, 1H), 7.20–7.29 (m, 5H); MS (ESI) m/z: 601 $(M + H)^+$.

*4.1.49. N-(3-((((R)-1-(tert-Butyldimethylsilyl)oxy))-2-(((1S)-1-benzyl-(2-(tert-butyldimethylsilyl)oxy)ethyl)(tert-butoxycarbonylamino)) ethyl)phenyl)amine (**18b**)*

Starting from **17b** and following the procedure described for **18a**, title compound was obtained as yellow oil (yield 51%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.12–0.08 (m, 12H), 0.86–0.94 (m, 18H), 1.35 (s, 9H), 2.47–2.69 (m, 1H), 2.87–3.24 (m, 2H), 3.66–3.74 (m, 2H), 3.94–4.10 (m, 1H), 4.75–4.79 (m, 1H), 5.11 (br s, 1H), 6.52–6.54 (m, 2H), 6.61 (d, $J = 7.3$ Hz, 1H), 6.69 (s, 1H), 6.77 (d, $J = 7.3$ Hz, 1H), 7.02–7.26 (m, 5H), 8.0 (s, 1H); MS (ESI) m/z: 637 $(M + Na)^+$.

*4.1.50. (2S)-2-(Benzyloxycarbonylamino)-N-(3-(((R)-1-hydroxy)-2-(((1S)-1-phenyl-2-hydroxyethyl)(tert-butoxycarbonylamino)) ethyl)phenyl)-2-phenylacetamide (**5e**)*

Following the same procedure described for **5a** title compound was obtained as an amorphous solid (yield 29%). ^{1}H NMR (300 MHz, DMSO) δ 1.29 (s, 9H), 2.69–2.82 (m, 1H), 2.92–3.05 (m, 1H), 3.92 (br s, 2H), 4.91 (d, $J = 6.7$ Hz, 1H), 5.01–5.06 (m, 3H), 5.24–5.29 (m, 2H), 5.40 (d, $J = 8.2$ Hz, 1H), 6.01 (d, $J = 13.4$ Hz, 1H), 6.96–6.99 (m, 1H), 7.19–7.35 (m, 12H), 7.48–7.54 (m, 5H), 8.07 (d, $J = 7.8$ Hz, 1H), 10.31 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.4, 50.8, 55.8, 62.0, 63.7, 67.5, 70.1, 81.0, 118.5, 119.4, 122.2, 127.0, 127.7 (2C), 128.1, 128.3 (2C), 128.7, 129.1, 129.4 (2C), 136.8, 137.6 (2C), 138.0, 143.3, 156.6, 157.5, 172.0; MS (ESI) m/z: 662 $(M + Na)^+$; $[\alpha]_{436}^{20} = -20.8$ ($c = 0.2$, $CHCl_3$). Anal. ($C_{37}H_{41}N_3O_7$), C, H, N.

The oxazolidyl-derivative **19a** was also recovered from the reaction mixture (yield 18%). ^{1}H NMR (300 MHz, CD_3OD) δ 2.76 (dd, $J = 14.1$, 6.1 Hz, 1H), 3.67 (dd, $J = 14.0$, 7.0 Hz, 1H), 4.02–4.05 (m, 1H), 4.50–4.53 (m, 2H), 4.76 (t, $J = 6.4$ Hz, 1H), 5.11 (s, 2H), 5.37 (s, 1H), 7.01 (d, $J = 7.6$ Hz, 1H), 7.13–7.15 (m, 2H), 7.25–7.37 (m, 12H), 7.49–7.51 (m, 4H); MS (ESI) m/z: 588 $(M + Na)^+$.

*4.1.51. (2S)-2-(Benzyloxycarbonylamino)-N-(3-(((R)-1-hydroxy)-2-(((1S)-1-benzyl-2-hydroxyethyl)(tert-butoxycarbonylamino))ethyl) phenyl)-2-phenylacetamide (**5f**)*

Following the same procedure described for **5a**, title compound was obtained as an amorphous solid (yield 25%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.26–1.35 (m, 9H), 2.59–2.74 (m, 2H), 3.04–3.21 (m, 2H), 3.24–3.37 (m, 3H), 3.62–3.76 (m, 2H), 4.38 (br s, 1H), 5.01 (br s, 1H), 5.11 (br s, 3H), 5.39 (s, 1H), 7.13–7.89 (m, 19H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 27.4, 35.6, 51.2, 55.6, 59.1, 62.2, 67.1, 70.5, 81.1, 118.7, 119.9, 122.0, 127.2, 127.5, 128.2 (2C), 128.3, 128.4 (2C), 128.8, 129.5 (2C), 136.6, 137.9 (2C), 138.1, 143.3, 156.8, 157.0, 173.1; MS (ESI) m/z: 676 $(M + Na)^+$. Anal. ($C_{38}H_{43}N_3O_7$), C, H, N.

The oxazolidyl-derivative **19b** was also recovered from the reaction mixture (yield 20%). ^{1}H NMR (300 MHz, CD_3OD) δ 2.63–2.71 (m, 1H), 2.99–3.10 (m, 1H), 3.20 (dd, $J = 14.0$, 5.5 Hz, 1H), 3.67–3.76 (m, 2H), 3.93–4.04 (m, 2H), 4.78 (t, $J = 6.7$ Hz, 1H), 5.10 (s, 2H), 5.38 (s, 1H), 7.06–7.35 (m, 16H), 7.42–7.50 (m, 2H), 7.61 (s, 1H); MS (ESI) m/z: 1180 $(2M + Na)^+$, 602 $(M + Na)^+$.

*4.1.52. (2S)-2-(Benzyloxycarbonylamino)-N-(3-(((R)-1-hydroxy)-2-((1S)-1-benzyl-2-hydroxyethyl)aminoethyl)phenyl)-2-phenylacetamide (**5g**)*

Following the procedure described for **3b** the title compound was obtained starting from **5f** to afford the hydrochloride salt as a

white solid (yield 99%). ^{1}H NMR (300 MHz, CD_3OD) δ 2.97–3.00 (m, 1H), 3.11–3.18 (m, 2H), 3.43–3.56 (m, 2H), 3.70–3.73 (m, 1H), 4.94 (d, J = 8.2 Hz, 1H), 5.11 (s, 2H), 5.36 (s, 1H), 7.15 (d, J = 7.3 Hz, 2H), 7.25–7.37 (m, 15H), 7.59–7.51 (m, 2H), 7.76 (s, 1H), 10.11 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 37.8, 51.9, 60.9, 64.3, 63.9, 67.1, 72.5, 119.1, 120.2, 122.1, 127.0, 127.2, 128.0128.2 (2C), 128.3, 128.9, 129.5 (2C), 129.8, 136.8, 137.1 (2C), 138.1, 143.6, 156.8, 170.2; MS (ESI) m/z: 576 $(M + Na)^+$, 554 $(M + H)^+$. Anal. ($C_{33}H_{35}N_3O_5$), C, H, N.

4.1.53. *N-(3-((((R)-1-(tert-Butyldimethylsilyl)oxy))-2-(benzyl(tert-butoxycarbonylamino))ethyl)phenyl)-1-naphthamide (**20**)*

To a stirred solution of (*R*)-**14a** (58 mg, 0.13 mmol), and DIPEA (35 μL, 0.19 mmol), in DCM (15 mL) was added 1-napthoyl chloride (26 mg, 0.13 mmol). The reaction mixture was stirred at rt for 2 h and then portioned between saturated aqueous Na_2CO_3 (10 mL) and DCM (10 mL). The organic phase was washed with 10% citric acid solution (20 mL), brine (20 mL) and dried (Na_2SO_4). The solvent was removed *in vacuum* and the residue was purified by chromatography (1:3, EtOAc/*n*-hexane) affording the title compound as an amorphous white solid (yield 45%). ^{1}H NMR (300 MHz, $CDCl_3$) δ −0.01 (3H, s), 0.14 (3H, s), 0.95 (s, 9H), 1.5 (m, 9H), 3.21 (m, 1H), 3.41 (m, 1H), 4.4 (d, J = 15 Hz, 1H), 4.62 (m, 1H), 5.13 (m, 1H), 7.17–7.41 (m, 8H), 7.57 (m, 2H), 7.82 (m, 2H), 8.02 (m, 2H),8.34 (s, 1H), 9.69 (s, 1H); MS (ESI) m/z: 633 $(M + Na)^+$, 511.

4.1.54. *N-(3-(((R)-1-Hydroxy)-2-(benzyl(tert-butoxycarbonylamino))ethyl)phenyl)-1-naphthamide (**5h**)*

To a solution of **20**, (0.10 mmol) in dry THF (5 mL), a 1 M solution of tetrabutylammonium fluoride in THF (0.13 mmol), was added. After stirring for 12 h, the solvent was removed and the residue was taken up in DCM. The organic phase was washed with brine, dried and concentrated. The residue was purified by means of flash chromatography (1:2, EtOAc/*n*-hexane) to give title compound as yellow amorphous solid (yield 41%). ^{1}H NMR (300 MHz, CD_3OD) δ 1.49 (s, 9H), 3.37–3.58 (m, 2H), 4.30–4.54 (m, 2H), 4.89 (s, 1H), 7.10 (d, J = 7.6 Hz, 1H), 7.20–7.38 (m, 6H), 7.47–7.60 (m, 4H), 7.75 (m, 2H), 7.88–7.98 (m, 2H), 8.35 (m, 1H). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.48 (s, 10H), 3.73–3.10 (m, 2H), 4.62–4.11 (m, 2H), 4.87 (s, 1H), 7.08 (d, J = 7.6 Hz, 1H), 7.39–7.12 (m, 6H), 7.62–7.39 (m, 4H), 7.70 (d, J = 6.9 Hz, 2H), 8.00–7.76 (m, 3H), 8.54–8.15 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.6, 52.8, 55.9, 74.2, 81.3, 117.5, 119.4, 122.2, 125.0, 125.3, 125.5, 126.8, 127.6 (2C), 128.6, 128.8 (2C), 129.5, 130.3, 131.3, 134.0, 134.6, 138.1, 138.4, 143.8, 158.4, 167.8; MS (ESI) m/z: 497 $[M + H]^+$; $[\alpha]_{436}^{20} = -51.2$ (c = 0.5, $CHCl_3$). Anal. ($C_{31}H_{32}N_2O_4$) C, H, N.

4.2. Molecular modeling

4.2.1. Docking simulations

The binding modes of compounds **3a**–**d**, **4a**–**b**, and **5a**–**h** were studied by means of docking experiments with the Glide 5.5 program [21]. Maestro 9.0.211 [22] was employed as the graphical user interface, and Figs. 2–5 were rendered by the Chimera software package [23].

4.2.2. Ligand and protein setup

The 3D structures of all the compounds were generated with the Maestro Build Panel. For the purpose of docking each molecule has been constructed in the protonation state suggested by the MarvinSketch 5.2.5.1 package (http://www.chemaxon.com) using a pH 7.0. The target BACE-1 structures (PDB codes: 1XN3, 1FKN, 1W51, 1TQF and 2G94) were prepared through the Protein Preparation Wizard of the graphical user interface Maestro 9.0.211 and the OPLS-2001 force field. Water molecules were removed. Hydrogen atoms were added (the side chain of D32 was taken as protonated) [24], and minimization was performed until the rmsd of all heavy atoms was within 0.3 Å of the crystallographically determined positions. The binding pocket was identified by placing a 20 Å cube centered on the mass center of the co-crystallized inhibitor. Molecular docking calculations were performed with the aid of Glide 5.5 in extraprecision (XP) mode, using Glidescore for ligand ranking [25,26]. For multiple ligand docking experiments, an output maximum of 5000 ligand poses per docking run with a limit of 100 poses for each ligand was adopted.

4.3. In vitro biological assays

4.3.1. BACE1 inhibition assay

The BACE1 inhibition fluorescence assays were conducted as previously reported [18] and the concentration–response curves for the tested compounds, with human recombinant BACE1 (Invitrogen, Carlsbad, CA) and BACE1 TR-FRET substrate [acetyl-C(W8044-Eu)-EVNLDAEFK-QSY7] (Perkin–Elmer Life Sciences, Turku, Finland) were determined. Briefly, enzymatic reactions were performed in 50 mM NaOAc and 0.005% Triton X-100 (pH 4.5), in a final volume of 30 mL with 200 nM of substrate and 6 nM of enzyme. Inhibitors were tested in solutions containing 3.3% of DMSO. Time resolved fluorescence was measured using the Analyst GT plate reader (Molecular Devices, Sunnyvale, CA) with a filter set allowing measurements at an excitation wavelength of 330 nm and an emission wavelength of 615 nm.

4.3.2. Cell based assay

The cell-based assays were performed at Merck RL and experimental details can be found in Ref. [20].

Briefly, HEK293 cells expressing a Merck RL proprietary APP construct containing a modified BACE cleavage sequence, NFEV and a K612V α-site sequence and SHSY5Y cells expressing APP NFEV and the wild-type α-cleavage site. Compounds were tested at a concentration of 100 μM with subsequent 3-fold dilution (10 points) to determine their 50% inhibitory capacity against the generation of sAPPβ NF (direct functional read out for BACE1 activity) as well as the EV40 and EV42 peptides.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.09.056.

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