ChemPubSoc Europe

DOI: 10.1002/cmdc.201300078

Alzheimer's Disease: Identification and Development of β-Secretase (BACE-1) Binding Fragments and Inhibitors by Dynamic Ligation Screening (DLS)

María Isabel Fernández-Bachiller,[a] André Horatscheck,[a] Michael Lisurek,[a] and Jörg Rademann*[a, b]

The application of dynamic ligation screening (DLS), a methodology for fragment-based drug discovery (FBDD), to the aspartic protease β-secretase (BACE-1) is reported. For this purpose, three new fluorescence resonance energy transfer (FRET) substrates were designed and synthesized. Their kinetic parameters (V_{max}, K_M, and k_{cat}) were determined and compared with a commercial substrate. Secondly, a peptide aldehyde was designed as a chemically reactive inhibitor (CRI) based on the Swedish mutation substrate sequence. Incubation of this CRI with the protease, a FRET substrate, and one amine per well taken from an amine library, which was assembled by a maximum common substructure (MCS) approach, revealed the fragment 3-(3-aminophenyl)-2*H*-chromen-2-one (**1**) to be a competitive BACE-1 inhibitor that enhanced the activity of the CRI. Irreversibly formed fragment combination products of **1** with the initial peptide sequence were active and confirmed the targeting of the active site through the ethane-1,2-diamine isostere. Finally, structure-assisted combination of fragment **1** with secondary fragments that target the S1 site in hit optimization yielded novel, entirely fragment-based BACE-1 inhibitors with up to 30-fold improved binding affinity. Interactions with the protein were explained by molecular modeling studies, which indicate that the new fragment combinations interact with the catalytic aspartic acid dyad, as well as with the adjacent binding sites required for potency.

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder of the central nervous system, which results in severe cognitive deficits along with psychiatric complications and represents the most common form of dementia. Today, more than 37 million individuals in the world suffer from this condition; the number is expected to continue growing dramatically and it is predicted to reach 80 million by 2040, with AD being recognized as a major global social and financial burden.[1,2] Currently, there is no treatment available for AD and medical therapies are usually limited to acetylcholinesterase (AChE) inhibitors and *N*-methyl-D-aspartate (NMDA) antagonists. In this context, five drugs are currently on the market, the cholinesterase inhibitors donepezil, rivastigmine, galantamine, and tacrine, supplemented by the NMDA receptor modulator memantine. All of these clinically admitted inhibitors, however, were only approved for the symptomatic treatment of AD and are unable to halt or reverse the disease progression.

The major pathological hallmark of AD is the deposition and aggregation of amyloid-β peptide (Aβ40,42) in the brain tissue, which produces the extracellular formation of senile plaques.[3] These Aβ peptides consist in fragments of 39–43 amino acid residues and are produced under pathological conditions by the sequential action of two proteases, named β- and γ-secretase, on amyloid precursor protein (APP).[4] The β-secretase cleavage of APP is the first step in the generation of Aβ peptides. In this context, β-secretase, also called β-amyloid-converting enzyme 1 (BACE-1), has been identified as the rate-limiting step in the production of Aβ peptides and the main approach for avoiding Aβ formation involves targeting β-secretase.[5,6] In addition, γ-secretase inhibitors have been found to interfere with the Notch signaling pathway and were thus disqualified from drug use.[7] For these reasons, the inhibition of BACE-1 is considered to be one of the most prominent targets for intervention in AD.[8,9] Over the past decade, many BACE-1 inhibitors have been reported and these inhibitors are divided into two classes, peptidomimetic and non-peptidomimetic inhibitors.[10] Although the first class of inhibitors showed the highest potency against BACE-1, their relatively large molecular size, low metabolic stability, and poor bioavailability render their development into therapeutic drug candidates difficult.[11,12] On the other hand, a great deal of effort has been put into the discovery of nonpeptidic organic compounds with better pharmacological properties as drugs leads.[13–15] Some of these compounds have been identified as potent BACE-1 inhibitors with balanced adsorption, distribution, metabolism, and

[a] *Dr. M. I. Fernández-Bachiller, Dr. A. Horatscheck, Dr. M. Lisurek, Prof. Dr. J. Rademann*
Medicinal Chemistry Department
Leibniz Institut für Molekulare Pharmakologie (FMP) Campus Berlin-Buch
Robert-Rössle Str. 10, 13125 Berlin (Germany)

[b] *Prof. Dr. J. Rademann*
Free University Berlin, Medicinal Chemistry, Institute of Pharmacy
Königin-Luise Str. 2+4, 14195 Berlin (Germany)
E-mail: joerg.rademann@fu-berlin.de

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201300078.

excretion toxicity (ADMET) properties, which will make the chemicals more favorable for further development into leads and drugs.[16]

Generally, these nonpeptidic inhibitors have emerged by the implementation of high-throughput screening (HTS) programs and, more recently, due to the application of fragment-based drug discovery (FBDD) methodologies.[17–20] In the latter case, different detection methods have been used, such as X-ray analysis, nuclear magnetic resonance (NMR) spectroscopy, and surface plasmon resonance (SPR) spectroscopy. These biophysical methods directly provide thermodynamic and, at least partially, structure information on the fragment–protein interaction. However, the low throughput and the high time and protein consumption are limiting factors for the application of each technique for high-throughput screening (HTS). Moreover, these biophysical methods have been demonstrated to provide low-affinity binders of proteins; however, they do not provide information about the linking of primary fragment hits to transform low-affinity fragments into high-affinity fragment combinations. Therefore, exhaustive synthetic efforts are required for the chemical optimization of primary fragment hits.[21–25]

More recently, dynamic ligation screening (DLS) has been introduced as a method in FBDD that combines dynamic, target-assisted formation of fragment combinations as inhibitory species with detection through a biochemical assay.[26,27] For example, the enzymatic turnover of a fluorogenic substrate has been used to amplify the active fragment combination, which allowed sensitive detection of low-affinity fragments with a drastic decrease in the amount of protein required.[26] In addition, DLS has enabled the implementation of this methodology in an HTS format (Figure 1). In the initial setup, the method was established for cysteine proteases. Peptide aldehydes **V** were used as chemically reactive inhibitors (CRIs). The CRIs served as directing probes; they were able to bind to the protease's active cleft through backbone and side-chain interactions while the aldehyde electrophile was attacked by the thiolate nucleophile of the active site cysteine residue to yield a hemithioacetal intermediate in equilibrium.[28] The reversibility of the hemithioacetal formation finally permitted attack of a nucleophilic fragment (for example, an amine, **VI**) at the CRI aldehyde functionality in presence of the protein to yield, reversibly, a dynamic ligation product **VIII**, which competed with a fluorogenic substrate for binding to the protein surface. For an aspartic protease like BACE-1, the assay has to be modified at several points. Peptide aldehyde inhibitors cannot react with an aspartic protease under the formation of a hemithioacetal. Instead, the aldehyde hydrate has to be formed and binds to both aspartic acid residues of the active site (**VII**) through hydrogen bonding.[29] Dynamic ligation of the peptide aldehyde with an amine nucleophile can nevertheless form an imine product reversibly, which can possibly be hydrated into the respective hemiaminal in equilibrium. Both products should be able to interact with the active site and with other binding pockets close to the active site like the imine bound by the cysteine proteases. In addition, the substrate must be modified for the BACE-1 assay. Whereas 7-aminomethylcoumarin peptides served as fluorogenic substrates for cysteine proteases, BACE-1 requires fluorescence resonance energy transfer (FRET) substrates, in which the fluorescence of a donor fluorophore is suppressed by resonance energy transfer to a quencher dye. After the substrate has been cleaved by the protein, the donor fluorophore and the quencher are separated and the fluorescence of the fluorogenic part is restored (**IX**). Thus, to extend the DLS methodology from cysteine proteases to an aspartic protease such as β-secretase (BACE-1) and apply it for the identification of new chemical entities (NCEs) or fragments as BACE-1 inhibitors, the following steps have to be taken: a) the design, synthesis, and kinetic parameter determination for new FRET BACE-1 substrates **I**–**III**, b) the design and synthesis of peptide aldehyde **V** as the chemically reactive inhibitor, c) assay optimization, d) assay validation, e) hit identification, f) hit validation with a secondary assay, and finally, g) hit-to-lead optimization.

Results and Discussion

BACE-1 FRET substrates I–III: design, synthesis, and determination of kinetic parameters

To facilitate the research on specific BACE-1 inhibitors and to facilitate studies of the BACE-1 enzymology, it is necessary to have a sensitive and continuous assay for enzyme activity. For these reasons, fluorogenic peptide substrates that contain suitable fluorophore and quencher groups in the same molecule should be developed. In this context, the FRET substrates **I**–**III** were synthesized. Their kinetic parameters were determined and compared with the commercial substrate **IV** with a similar structure. The new FRET sub-

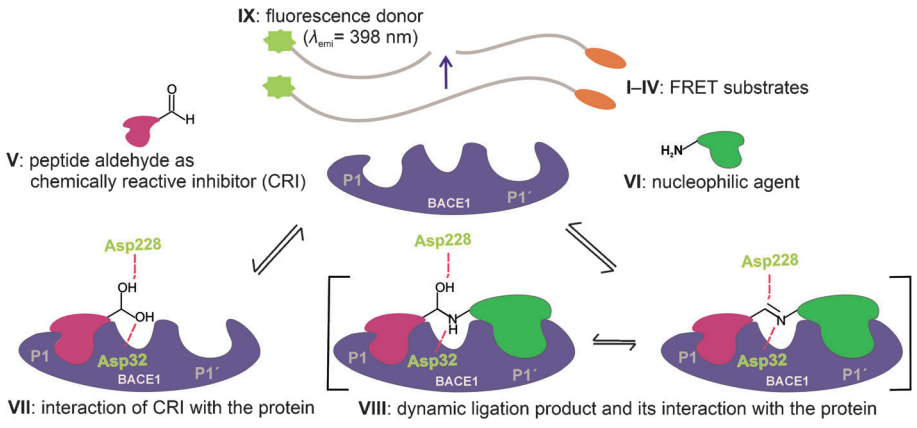


Figure 1. The concept of dynamic ligation screening (DLS) applied to the identification of BACE-1 inhibitors.

strates were designed based on a peptide containing ten amino acid residues of the putative substrate belonging to the Swedish mutant (SEVN**LD**AEFR; Michaelis–Menten constant (K_M) = 9 μM, catalytic rate (k_{cat}) = 0.002 s^{-1})[30] and were modified by the introduction of a fluorophore (7-methoxy-coumarin-4-yl-acetyl (MOCAc) or 7-amino-4-methylcoumarin-2-yl-acetyl (AMCA)) and a quencher like 2,4-dinitrophenyl (DNP), 2,4-dinitrobenzoyl (DNB), or 2-(2,4-dinitrophenylamino)acetyl (DNPA) at the N- and C-terminal positions, respectively (Table 1). The

Table 1. Kinetic parameters for the hydrolysis of the synthesized BACE-1 fluorescence substrates **I**–**III** mediated by the BACE-1 full protein and in comparison with commercial substrate **IV**.[a,b]

Substrate	V_{max} [μM s^{-1}] × 10^{-2}	K_M [μM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} M^{-1}]
I[c]	0.142 ± 0.016	4.97 ± 1.01	0.08	15687
I[d]	0.092 ± 0.003	6.71 ± 1.51	0.05	7705
II[c]	0.398 ± 0.006	40.2 ± 3.05	0.26	6388
III[c,e]	0.053 ± 0.005	128 ± 12.0	0.215	28
IV[c]	0.053 ± 0.005	9.27 ± 1.56	0.03	3405
IV[d]	0.038 ± 0.002	12.8 ± 2.53	0.02	2078

[a] The data points are an average of three independent experiments. Substrate **I**: (MOCAc)SEVN**LD**AEFK(DNPA)RR; **II**: (MOCAc)SEVN**LD**AEFK-(DNB)RR; **III**: (DNPA)SEVN**LD**AEFRRG(AMCA); **IV**: (MOCAc)SEVN**LD**AEFRK-(DNP)RR. The P1 and P1′ residues are shown in bold. [b] Unless otherwise stated, the enzyme reaction was carried out in 100 mM sodium acetate (pH 4.0) containing 0.01% Triton X-100, in the presence of 5–100 μM substrate and 18.6 nM enzyme (25 ng) with 5 or 10% DMSO at room temperature. [c] 5% DMSO. [d] 10% DMSO. [e] The enzyme reaction was carried out in 100 mM sodium acetate (pH 4.0) containing 0.01% Triton X-100, in the presence of 5–500 μM substrate and 148.8 nM enzyme (200 ng) with 5% DMSO at room temperature.

fluorescence signals in the uncleaved substrates were quenched by resonance energy transfer between the fluorophore and the quencher groups. After cleavage of the peptide chain, the quenching efficiency decreased, which resulted in an increase of the fluorescence signal at 398 nm. To increase the solubility of the FRET substrates in the buffer assay, the peptides were modified by the introduction of additional arginine residues into the sequence. The synthesis of FRET substrates was conducted by using 2-chloro-2-trityl resin (substrates **I** and **II**) or Rink amide (substrate **III**) as solid supports (see schemes S1 and S2 in the Supporting Information and the Experimental Section) for 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis (SPPS). The products were obtained in good yields (60%). Knowledge of the reaction kinetics and the K_M value of the substrate is a mandatory requirement for defining the assay conditions of the enzymatic reaction. Next, the FRET substrates **I**–**III** were characterized with respect to their kinetic properties by measuring the initial velocities at various substrate concentrations (figures S6–S10 in the Supporting Information). For this purpose, the BACE-1_{1-501} (BACE-1, His*Tag, human, recombinant, NSO cells, Calbiochem, PF125) full protein was selected. The effects of enzyme concentration and incubation time, as well as the influence of dimethyl sulfoxide (DMSO), on the rate of substrate cleavage were investigated. Thus, various amounts of the protein (25–100 ng; 8.6–74.4 nM) were incubated for 60 min at 25 °C with 25 μM substrate **I** and the development of the product was monitored through continuous measurements every minute. After these experiments, 25 ng (18.6 nM, final concentration) of the protein were used for the end-point estimation for the next experiments. The experimental conditions such as pH value and detergent were adapted from published data.[31,32]

With these results in mind, substrates **I**–**III** were digested in vitro with BACE-1 and their kinetic parameters were determined and compared with those of the commercial substrate **IV** with a similar amino acid sequence but with a different fluorophore and quencher pair (Table 1). The initial velocity (V) for each substrate was calculated from the linear part of the fluorescence curve recorded over the time between 1–20 min. The K_M and maximal velocity (V_{max}) values were determined by using the Michaelis–Menten representation. The k_{cat} value was calculated from the equation $k_{cat} = V_{max}/[\text{BACE-1}]_{total}$, in which $[\text{BACE-1}]_{total}$ is the total enzyme concentration in μM. The kinetic parameters for the cleavage of these fluorogenic substrates are represented in Table 1. Relative to the commercial substrate **IV**, our best substrate **I** displayed a twofold increase in the substrate affinity (K_M) and an almost fivefold increase in the rate of the enzymatic reaction (k_{cat}/K_M), which determines the sensitivity of the substrate in the enzyme assay.

This effect was achieved by replacement of 2,4-dinitrophenyl (DNP) with 2-(2,4-dinitrophenylamino)acetyl (DNPA) as a quencher in the C-terminal substrate position. For comparison, the kinetic parameters for our first FRET substrate **III**, which employed the synthetically favorably DNPA/AMCA pair in the reversed positioning, were decreased by a factor of 26 in terms of substrate affinity and by 560-fold with respect to the rate of the enzymatic reaction. Likewise, the substitution of a 2-(2,4-dinitrophenylamino)acetyl moiety by a dinitrobenzoyl group increased the K_M value eightfold and reduced the k_{cat}/K_M value 2.5-fold (compare substrates **I** and **II**). The kinetic parameters were also modified by the DMSO content in the assay. The K_M values of substrates **I** and **IV** against BACE-1 were increased 1.3-fold when the DMSO concentration was increased (Table 1). The highest affinity (lowest K_M value) and enzymatic activity (highest k_{cat}/K_M value) of substrate **I** proved to be the most suitable for a sensitive, rapid, and specific substrate-based BACE-1 assay. All of the FRET substrates **I**–**IV** were digested in vitro with BACE-1 and the generated peptide fragments were analyzed and identified by LC–MS (electrospray ionization), with the conclusion that they have a single cleavage site between the leucine and aspartate residues (**L↓D**; figure S11 in the Supporting Information).

Assay validation

The sensitivity of the biochemical assay was validated by reproducing the IC_{50} value of the peptide inhibitor H-Glu-Val-Asn-[(2*R*,4*S*,5*S*)-5-amino-4-hydroxy-2,7-dimethyloctanoyl]-Ala-Glu-Phe-OH, an OM99-2 derivative. This statine inhibitor has a peptide sequence similar to a known enzyme substrate, but the scissile bond is replaced by the statine isostere to mimic

the tetrahedral reaction intermediate of aspartic proteases. The compound has been reported to be a potent inhibitor of human brain BACE-1, with inhibition constant (K_i) values of 1.2–9.8 nM.[33,34] Thus, the BACE-1 full protein was treated with different inhibitor concentrations in the range of 1–250 nM and the product progress curves in the presence of this product afforded a K_i value of 5.47 nM, which is in good agreement with the published data.

Peptide aldehyde V as a chemically reactive inhibitor of BACE-1: design, synthesis, and biological evaluation

Peptide aldehydes have been described frequently as inhibitors of serine and cysteine proteases and have been studied intensively as biochemical tools and potential peptide drugs.[35,36] In general, peptide aldehydes inhibit cysteine proteases through the reversible formation of covalent, tetrahedral intermediates such as hemiacetals (serine proteases) or hemithioacetals (cysteine proteases), both of which mimic the transition states of the cleaved substrates.[37] Peptide aldehydes have also been suggested as inhibitors of aspartic proteases because they are potential transition state analogues by themselves.[29a] For these cases, it was assumed that the hydrated form of the aldehyde accounts for its activity.[38] To our knowledge, for the aspartic protease BACE-1, peptide aldehydes have not yet been described as inhibitors. Thus, for our purposes, we have investigated a peptide aldehyde as a chemically reactive inhibitor (CRI) of BACE-1 and as a directing probe for dynamic ligation screening. Based on the reported cleavage site of BACE-1 in the Swedish mutant substrate SEVN**LD**AEFR, the N-acetylated pentapeptide aldehyde *N*-acetyl-Ser-Glu-Val-Asn-Leu-H (**V**) was synthesized as a potential inhibitor. By using the preloaded aldehyde resin H-Leu-HNovaSyn TGResin as a solid support,[39] product **V** was obtained in 38% overall yield and with good purity by Fmoc solid-phase peptide synthesis. The biological activity of this peptide aldehyde against BACE-1 protease was determined and an IC_{50} value of (98 ± 4) μM was measured (Table 2). This result was in agreement with our initial hypothesis that peptide aldehydes can be inhibitors of aspartic proteases such as β-secretase. The inhibitory activity can be attributed to the formation of a tetrahedral, hydrated form of the peptide aldehyde, which may act as a transition state analogue recognized by the two active site Asp residues (Figure 1, **VII**).

Fragment screening: hit identification and subsequent SAR development by using the protein structure input

Next, a dynamic ligation assay was implemented by using substrate **I** and peptide aldehyde **V**. The directing probe **V** was employed at a concentration of 75 μM, which resulted in partial inhibition of the protease (63% of initial activity) and left room for the detection of additional inhibitory effects exerted by interacting nucleophilic fragments. For screening, a collection of 450 amine nucleophiles was assembled by employing a maximum common substructure (MCS) concept described recently.[40] In this concept, a structural analysis of the World Drug Index (WDI) revealed 561 cyclic substructures that were classified as privileged bioactive scaffolds. The small fragment library was assembled in order to represent the substructure composition of the WDI, together with an application of filters for reactivity and for physicochemical properties, such as the number of hydrogen bonding donors ≤ 3, the clog*P* value, and molecular weights ≤ 300 Da. For the assay, 18.6 nM BACE-1 protease was incubated with peptide aldehyde **V** (75 μM, final concentration) and a 2.6-fold excess of one nucleophilic fragment (200 μM, final concentration) per well in a 384-well microtiter plate for one hour at room temperature. FRET substrate **I** (25 μM) was then added and the rate of the enzymatic reaction was recorded for each fragment–enzyme combination. Rate differences in the turnover of the substrate were observed and quantified to identify the active-fragment hits. Under these experimental conditions, the fragment 3-(3-aminophenyl)coumarin (APC; **1**) was identified as an inhibitory fragment due to its ability to decrease the enzymatic activity in the presence of the peptide aldehyde. To our surprise, several coumarin-containing structures have been discovered as novel β-secretase inhibitors over the last few years; however, there has been no indication of the binding site of these molecules yet.[41–45] Thus, fragment **1** was validated as a potential starting point for further ligand development. For this purpose, the binding mode and K_i value were first determined by conducting concentration-dependent inhibition assays. The initial velocities of the enzymatic reaction at five different concentrations of fragment **1** (25–250 μM) and increasing substrate concentrations (1–25 μM) were represented in a Lineweaver–Burk plot (Figure 2a). Values of the reciprocal velocity ($1/V$) for each inhibitor concentration were linear with respect to the reciprocal substrate concentration ($1/[S]$) and all of the lines intercepted the *Y* axis in roughly the same point and the *X* axis at different points ($-1/K_M(1+I/K_i)$), which indicated that this compound displayed a competitive inhibition mode. In a second graph,

Table 2. IC_{50} determination for some modified peptide–coumarin derivatives against BACE-1.

Compound	IC_{50} [μM][a]	K_i [μM][b]
1	157 ± 6.5	146 ± 5[c]
2	72 ± 3	15.2 ± 0.6
5	> 250	> 250
7	96 ± 3	20.3 ± 0.6
8	> 250	> 250
V (CRI)	98 ± 4	20.7 ± 0.8

[a] For IC_{50} determination, full-length protein BACE-1 (His*Tag, human, recombinant NSO cells, PF 125, [E] = 18.6 nM, 25 ng), (MOCAc)SEVNLDAEFK-(DNPA)RR, FRET substrate **I** ([S] = 25 μM), and 100 mM sodium acetate (pH 4) were used. The IC_{50} values are the mean ($n = 3$) ± SD and were determined by the GraphPad Prism program by using log [Inhibitor] versus normalized response−variable slope; $\lambda_{abs} = 328$ nm, $\lambda_{emi} = 398$ nm. The assay was carried out with 10% DMSO (final concentration). [b] Unless noted, the K_i values for these inhibitors were calculated by using the equation of Cheng and Prusoff ($K_i = IC_{50}/(1+[S]/K_M)$), taking into account a competitive binding mode.[59] [S] = 25 μM and $K_M = (6.71 \pm 1.51)$ μM for substrate **I** (see Table 1). [c] This K_i value was calculated from the Lineweaver–Burk representation by using five different inhibitor concentrations (25–250 μM) and increasing substrate concentrations (1–25 μM).

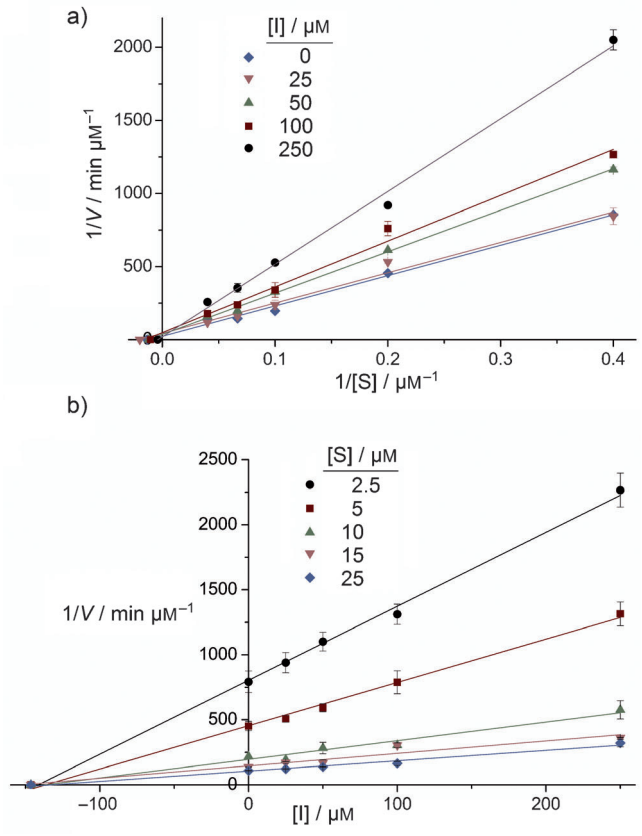


Figure 2. a) Kinetics of β-site amyloid precursor protein cleaving enzyme 1 inhibition with the 3-(3-aminophenyl)-2*H*-chromen-2-one (**1**) inhibitor: Lineweaver–Burk plot. The experiment was carried out with increasing substrate concentrations ([S]; 1–25 μM) (◆) and **1** at concentrations of 25 (▼), 50 (▲), 100 (■), and 250 μM (●) with a final enzyme concentration of 37.2 nM (50 ng; $V_{max} = 0.019 \times 10^{-2}$ μM s^{-1} and $K_M = 14.3$ μM). The data obtained with this FRET assay confirm the competitive binding mode. *V*: velocity. b) Determination of the K_i value (146 μM) for the BACE-1 inhibitor (**1**): Dixon-plot. The experiment was carried out with increasing inhibitor concentrations ([I]; 25–250 μM) and substrate at concentrations of 2.5 (●), 5 (■), 10 (▲) 15 (▼), and 25 μM (◆). Data are the mean ± of two experiments.

the Dixon plot, the reciprocal initial velocity was represented as a linear function of the inhibitor concentration (Figure 2 b). The intersection point of all of these lines with the *X* axis yielded a K_i value for this compound of 146 μM.

In addition, analysis of the inhibition mechanism was also conducted by using nonlinear least-squares fitting (NLSF) with the Solver macro in the Excel program.[46] Thus, experimental data in *x*/*y* form and data calculated from a regression equation are inputted and plotted in a Microsoft Excel worksheet and the addition of squares residual (SSR) is computed and minimized by using the Solver add-in to obtain the parameter-values set that best describes the experimental data. Thus, with consideration of the five different inhibitor concentrations (25–250 μM) and substrate concentrations (1–25 μM) and the Michaelis–Menten constant for substrate **I** (kinetic parameters of $V_{max} = 0.019 \times 10^{-2}$ μM s^{-1} and $K_M = 14.3$ μM for a final enzyme concentration of 37.2 nM (50 ng)), the best fit was obtained for a competitive binding mode, with SSR = 7.967 × 10^{-6} μM. As a third control experiment, the inhibitory capacity of **1** was confirmed by liquid chromatography/electrospray-ionization time-of-flight mass spectrometry (LC-ESI-TOF-MS). FRET substrate **I** was cleaved by BACE-1 to generate the two cleavage products **CP1** and **CP2** (figure S11 in the Supporting Information). The extracted ion chromatogram (EIC) peak areas for each cleavage products were normalized to 100% enzyme activity (see figure S13 and table S1 in the Supporting Information). In the presence of increasing concentrations of inhibitor **1** (0–250 μM), the peak areas for each cleavage product were decreased in correspondence with the enzyme activity. At 50 μM inhibitor **1**, the enzyme activity was decreased by 20% (for **CP1**) or by 17% (for **CP2**), whereas at 250 μM **1**, the enzyme activity was completely deleted. Analysis of peak areas for different concentrations yielded a mean IC_{50} value of 135 μM for fragment **1**.

In the next step, the concentration-dependent effects of fragment **1** were validated in the dynamic ligation assay (Figure 3). For this purpose, different concentrations of fragment **1** (0–150 μM) were added to buffer solutions containing the enzyme, substrate, and peptide aldehyde (PA; 0 or 75 μM) and the rates of the enzymatic reactions were determined. Enzymatic activity was decreased to 63% in the presence of PA alone (75 μM) and to a similar degree by the inhibitory fragment APC alone at the same concentration (60%, 75 μM). However, if equal concentrations of PA and APC (75 μM each) were investigated in the enzyme reaction, an additive effect was recorded, with 48% enzyme activity, not the cooperative (over-additive) effect reported earlier for other dynamic ligations assays. In the presence of a double concentration of **1** with PA (75 μM), stronger inhibition was observed, with only 35% of the enzyme activity maintained.

Thus, it remained to be investigated whether fragment **1** and peptide aldehyde **V** indeed formed a ligation product, such as an imine or hemiaminal, as the active species responsible for decreasing the enzymatic activity in the assay. Isolation

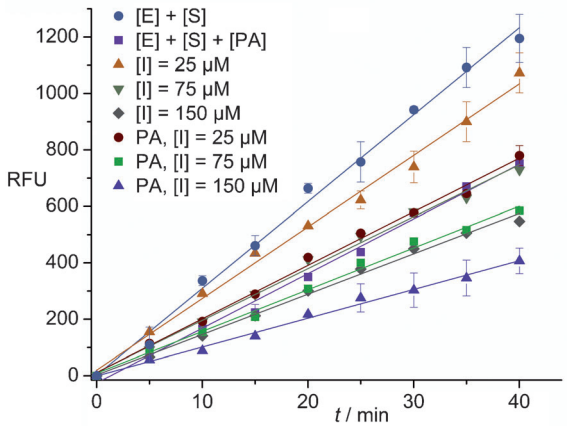
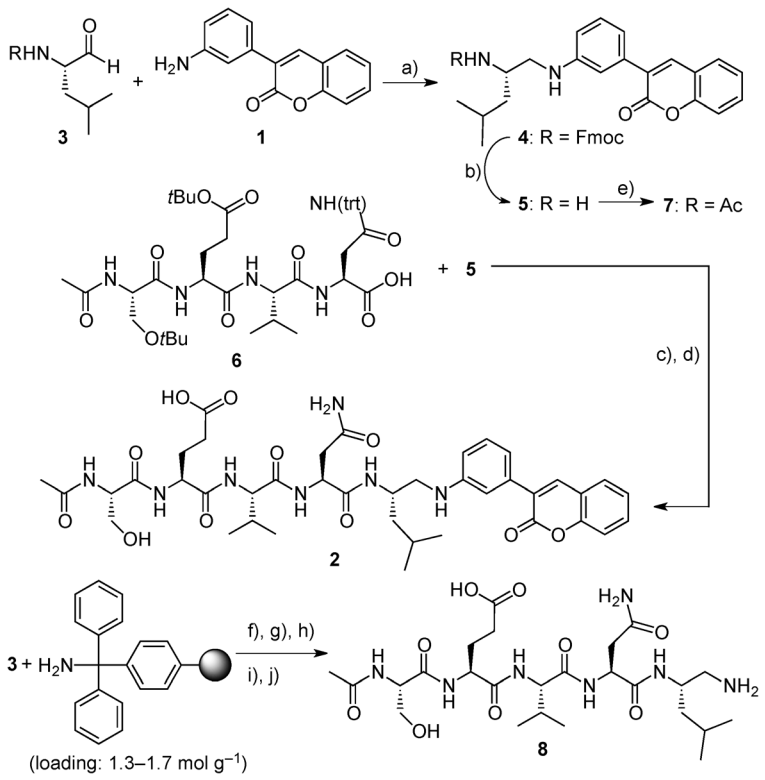


Figure 3. The turnover of substrate **I** ([S] = 25 μM) was recorded with BACE-1 ([E] = 18.6 nM) alone (●, positive control), in the presence of PA (■, 75 μM), or in the presence of increasing concentrations of inhibitor **1** (▲, 25 μM; ▼, 75 μM; ◆, 150 μM). The substrate turnover was decreased in the mixtures containing PA (75 μM) and inhibitor **1** (●, 25 μM; ■, 75 μM; ▲], 150 μM). These changes in activity suggest that the formation of an intermediate between both inhibitors (PA and **1**) is responsible for the observed changes.



Scheme 1. *Reagents and conditions*: a) N_2, molecular sieves, DMF, $NaCNBH_3$ (2 equiv), 10% AcOH, 18 h, RT; b) 20% piperidine/DMF, 30 min, RT; c) N_2, HOBt (1.2 equiv), DIC (1.2 equiv), CH_2Cl_2, 12 h, RT; d) N_2, CH_2Cl_2/TFA/EDT (80:17.5:2.5), 2 h, RT; e) N_2, Ac_2O (1 equiv), CH_2Cl_2, 2 h, RT; f) DMF, $NaCNBH_3$ (2.5 equiv), 24 h, RT; g) Fmoc SPPS, $(AA)_n$ (5 equiv), HOBt (5 equiv), DIC (5 equiv), DMF, 2 h, RT; h) 20% piperidine/DMF (1×1 min, 2×20 min); i) Ac_2O (5 equiv; 2×20 min), DMF, RT; j) TFA/TIS/H_2O/EDT (94:1:2.5:2.5), 2 h, RT. DMF: *N*,*N*-dimethylformamide; HOBt: *N*-hydroxybenzotriazole; DIC: *N*,*N*′-diisopropylcarbodiimide; TFA: trifluoroacetic acid; EDT: ethanedithiol; AA: amino acid; TIS: triisopropylsilane; trt: triphenylmethyl (trityl).

of these reversibly formed intermediates was not possible, so secondary amine **2** was synthesized as a stable ligation product instead (Scheme 1). The hit fragment, **1**, was synthesized by using a reported procedure.[47] Fmoc-leucinal, **3**, was prepared in two steps from Fmoc-leucine.[29a] First, treatment with isobutylchloroformate (IBCF), *N*-methylmorpholine (NMM), and sodium borohydride in situ furnished Fmoc-leucinol. Subsequently, oxidation of the intermediary alcohol with Dess–Martin periodinane afforded aldehyde **3** in almost quantitative yield (95%).[20] By using this protocol, racemization of aldehyde **3** could be avoided, as reported.[37] Treatment of **3** with **1** in the presence of sodium cyanoborohydride afforded the secondary amine **4** in moderate yield (65%). Deprotection of **4** with 20% piperidine in *N*,*N*′-dimethylformamide furnished 3-(3-((*S*)-2-amino-4-methylpentylamino)phenyl)-2*H*-chromen-2-one (**5**), which was finally coupled with the fully protected tetrapeptide AcS(*t*Bu)E(*Ot*Bu)VN(trt)-OH **6** in the presence of HOBt and DIC. Deprotection of the side chains with TFA and EDT afforded the final product **2** in moderate yield. The protected tetrapeptide Ac-S(*t*Bu)E(*Ot*Bu)VN(trt)-OH **6** was synthesized by solid-phase peptide synthesis in good yield (75%) by using a 2-chlorotrityl chloride resin as the solid support. The N-acetylation product **7** of diamino derivative **5** was obtained by treatment with acetic anhydride in the absence of base at room temperature.

Inhibition of the enzyme BACE-1 by these compounds was determined by using the FRET assay described above.[30,32] The IC_{50} values obtained for each compound are represented in Table 2. Reductive amination product **2** displayed a K_i value of 15 μM, which indicated a significantly stronger inhibition than both the initial directing probe **V** and the amine hit fragment **1**. For comparison, the N-acetylated pentapeptide amine AcSEVNL-CH_2NH_2 (**8**), the formal reductive amination product of aldehyde **V** with ammonia, was synthesized. The compound was obtained in 71% yield from SPPS with tritylamine resin as the solid support (Scheme 1). The inhibitory capacity of **8** against BACE-1 was tested and the compound was inactive, with an IC_{50} value higher than 250 μM. Evidently, the amino group of the reduced amide alone was not sufficient to establish binding to the active site but required the contribution of either the APC fragment or the aldehyde functionality for activity. These results confirmed our initial hypothesis and indicated that the hit fragment is capable of interacting with an additional binding site of the enzyme next to the binding site of the peptide aldehyde.

The potential binding mode of compound **2** with the catalytic active site of the protein target was investigated by molecular modeling (Figure 4). For the purpose, the X-ray co-crystal structure of BACE-1 protein and a peptidic statine inhibitor (PDB ID: 1FKN) available in the Protein Data Bank was analyzed.[33] Binding simulations were carried out with the Surflex docking program within SYBYL (see the Experimental Section for details).[48–51] All resulting docking conformations were inspected visually and the most probable docking conformations were chosen.

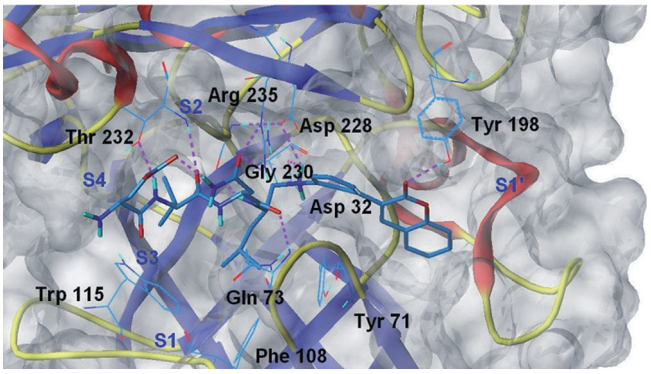


Figure 4. Proposed binding mode of the pentapeptide amine **2** (carbon atoms in bold blue) docked at the active site of BACE-1 (cyan). The hydrogen bonds are depicted as dashed line (magenta). Amino acids are indicated by three-letter codes.

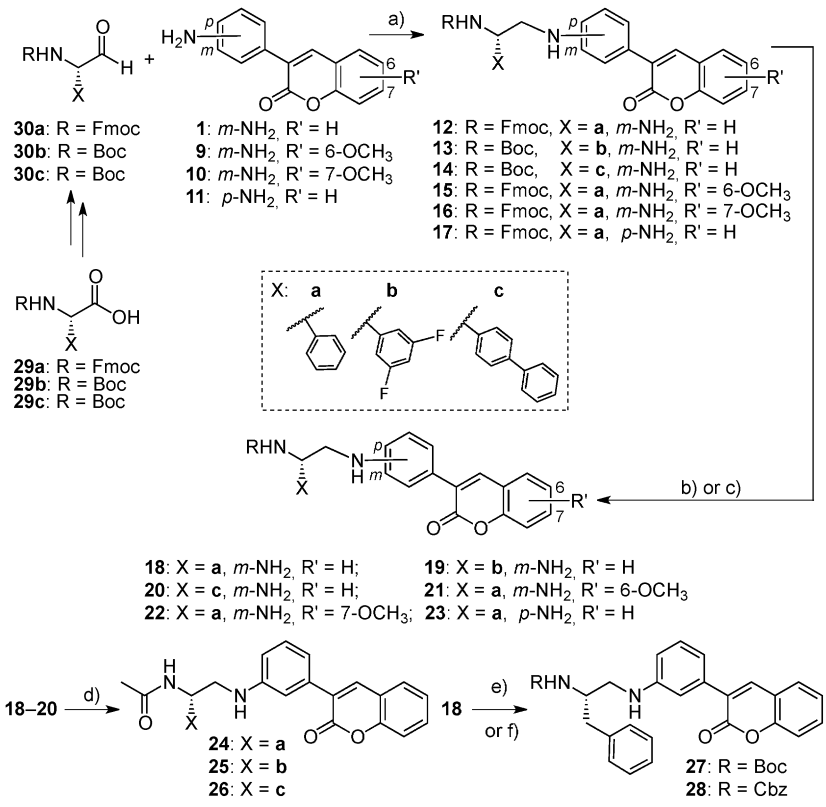
In Figure 4, the potential binding mode of compound **2** in the active site of BACE-1 is visualized. The pentapeptide-derived secondary amine **2** fitted well into the active site of the enzyme. Firstly, the acylated ethane-1,2-diamine isostere was able to interact with aspartic acid residues Asp32 and Asp228, present in the catalytic site. The nitrogen atom of the aniline is protonated, so hydrogen bonds interaction with both amino acids were supplemented by a Coulomb attraction. This result constituted an extension of earlier findings by us and others, which had described ethane-1,2-diamine isosteres incorporated in peptides as potent inhibitors of BACE-1.[52] Secondly, the leucine residue at the P1 position of **2** was located in the large and hydrophobic pocket S1, establishing CH–π interactions with aromatic residues such as Phe108, Trp115, and Tyr71, whereas the rest of the amino acid residues establish an hydrogen bond network with the S2–S5 pockets of the enzyme, such as Arg235, Gly230, Thr232, and Gln73. Thirdly, the coumarin moiety was always exposed to the protein surface, acquiring different orientations, and its carbonyl group interacted with Tyr198, located in the S2′ pocket, through a hydrogen bond.

Next, compounds **5** and **7**, the entirely nonpeptidic combinations of hit fragment **1** and the ethane-1,2-diamine isostere, were tested for BACE-1 inhibition. Compound **5**, the isobutyl-substituted ethane-1,2-diamine derivative, which was obtained by reductive amination of L-leucinal with fragment **1** and contained a free primary amine, did not show any inhibitory activity. This observation was in agreement with our earlier findings indicating that the ethane-1,2-diamine isosteres needed a peptide extension for activity.[52] To our great surprise, however, compound **7**, formally the reductive amination product of *N*-acetyl-leucinal and fragment **1**, almost maintained the affinity of the tetrapeptide-derived fragment ligation product, with an IC_{50} value of 96 μM (K_i value of 20 μM). The better inhibitory affinity observed for the acetyl derivative **7** could be explained by a postulated hydrogen bond interaction of the acetyl carbonyl group with the side chain of Gln73, found in the catalytic site. This interaction cannot be formed by the nonacylated diamine **5**. Furthermore, compound **7** establishes one extra hydrogen bond with Thr231. This finding indicated that compound **7** was a favorable starting point for further optimization of a nonpeptidic structure.

Hit optimization

In the next phase, the fragment combination product **7** was optimized by fragment variations by employing input derived from the docking experiments with the target protein. The active site of BACE-1 is composed by the catalytic dyad formed by Asp32 and Asp228. Directly adjacent to this catalytic site, the S1 pocket is located. This S1 pocket is a large hydrophobic site lined by the side chains of Phe108, Trp115, Ile118, and Leu30.[12] In close proximity to the S1 pocket, the flap region can be found, which is composed of (among others) the amino acid residue Tyr71. The presence of these aromatic residues suggested the introduction of an aromatic side chain, which might be able to establish π-stacking interactions, into the fragment-based inhibitor **7**.[53] This strategy was supported by the size of the S1 pocket, which was not completely filled by the leucine residue (Figure 4) and should be able to accommodate an aromatic moiety. Additional interactions might be contributed by fluorine substituents.[54–56] Thus, by starting from inhibitor **7**, we planned the synthesis of derivatives **12**–**28** (Scheme 2). In these new derivatives, the leucine side chain in the P1 position was replaced by benzyl, 3,5-difluorobenzyl, and biphenyl groups. In addition, substituents at the terminal amino group were varied and some modifications in the coumarin scaffold were introduced.[54,55]

The syntheses of the designed compounds started from the commercially available *N*-Fmoc- or *N*-Boc-protected L-α-amino



Scheme 2. *Reagents and conditions*: a) N_2, DMF, AcOH, $NaCNBH_3$ (2 equiv), 30 min, RT; b) 20%piperidine/DMF, 30 min, RT; c) 4 M HCl/dioxane, 2 h, RT; d) Ac_2O (1 equiv), CH_2Cl_2, 3 h, RT; e) $(Boc)_2O$, CH_2Cl_2, 4 h, RT; f) CbzCl, CH_2Cl_2, 2 h, RT. Boc: *tert*-butoxycarbonyl; Cbz: benzyloxycarbonyl.

acids **29a–c**, which contained benzyl, biphenyl, or 3,5-difluorobenzyl side chains, respectively. Activation of the protected amino acids **29a–c** with IBCF and NMM, followed by reduction with $NaBH_4$ in situ afforded the corresponding alcohols in good yields. Subsequent oxidation of these intermediates with Dess–Martin periodinane furnished the aldehydes **30a–c** in quantitative yield.[57,52] Treatment of **30a–c** with the 3-(3-aminophenyl)coumarin derivatives **1**, **9** (6-MeO), or **10** (7-MeO), or with 3-(4-aminophenyl)coumarin (**11**) under reductive amination conditions provided the derivatives **12–17** in moderate yields (45–70%). The aminophenylcoumarin derivatives **1** and **9–11** were prepared by following the experimental conditions described in the literature.[47,58] Treatment of the Fmoc derivatives **12** and **15–17** with 20% piperidine in DMF yielded the deprotected products **18** and **21–23** in good yields, whereas deprotection of Boc derivatives **13** and **14** with a solution 4 M HCl in dioxane afforded diamines **19** and **20**. N-Acetylation of the amines **18–20** with acetic anhydride in the absence of base at room temperature provided the acetylated derivatives **24–26** in good yields. The Boc derivative **27** was obtained by treatment of **18** with Boc anhydride. Acylation of **18** with benzyloxycarbonyl chloride in the absence of base afforded the Cbz derivative **28** in moderate yield.

The activity of these synthesized compounds against BACE-1 was evaluated in the cell-free inhibition assay in a concentration-dependent manner. Inhibition constants (K_i values) were calculated from the IC_{50} values by using the equation of Cheng and Prusoff ($K_i = IC_{50}/1 + [S]/K_M$),[59] which is valid for competitive inhibitors. Results for the IC_{50} and K_i values are summarized in Table 3. In general, fragment combinations of the hit 3-(3-aminophenyl)-coumarin fragment **1** ($K_i = 146$ μM) with amino acid aldehydes were found to be significantly more potent inhibitors of human BACE-1 (K_i values of 5–50 μM) than the starting fragment alone or in combination with the complete peptide aldehyde **V** (compound **5**).

Table 3. IC_{50} determination for 3-(3-aminophenyl)coumarin derivatives with aromatic rings in the P1 position (see Scheme 2 for structures).[a,b]

Compd	R	X	R′	IC_{50} [μM]	K_i [μM][c]
13	Boc	**b**	H	25.1 ± 1	8.8
14	Boc	**c**	H	15.1 ± 0.5	5.3
18	H	**a**	H	114.9 ± 10	40.1
19	H	**b**	H	96.4 ± 3.5	33.7
20	H	**c**	H	31.5 ± 1.8	11.0
21	H	**a**	6-OCH_3	55.2 ± 3.3	19.3
22	H	**a**	7-OCH_3	33 ± 1	11.5
23	H	**a**	H	31.6 ± 1.7	11.0
24	Ac	**a**	H	14.7 ± 0.5	5.1
25	Ac	**b**	H	47.2 ± 1.3	16.5
26	Ac	**c**	H	10.7 ± 0.4	3.7
27	Boc	**a**	H	55.0 ± 3	19.2
28	Cbz	**a**	H	> 200	> 200

[a] For the IC_{50} determination, full-length protein BACE-1 (His*Tag, human, recombinant NSO cells, PF 125, [E] = 18.6 nM, 25 ng), RhoEVNLDAEFR-Quencher ([S] = 250 nM), and 100 mM sodium acetate (pH 4) were used. [b] The IC_{50} values are the mean ($n = 3$) ± SD; $\lambda_{abs} = 535$ nm, $\lambda_{emi} = 590$ nm. The assay was carried out with 5% DMSO (final concentration). [c] The K_i values for these inhibitors were calculated by using the equation of Cheng and Prusoff ($K_i = IC_{50}/(1+[S]/K_M)$), taking into account competitive binding mode.[59] [S] = 250 nM and $K_M = 0.134$ μM (see the Supporting Information).

In agreement with our hypothesis, the introduction of aromatic rings at P1, the extended and lipophilic biphenyl group, and the fluorine atoms as electron-acceptor groups into the phenyl ring increased the activity of these new compounds against BACE-1 and decreased their K_i values.[53] In a comparison of the different fragments in the P1 position of compounds **18–20** containing the nonacylated, primary amine, the biphenyl derivative **20** was the most active compound, with a K_i value of 11 μM, whereas the benzyl- and the 3,5-difluorobenzyl derivatives showed little difference in their K_i values (**18**: 40.1 μM; **19**: 33.7 μM). A similar tendency for the three side chains was observed if the N terminus of the fragment combinations was modified, for example, in the Boc derivatives **27** (benzyl, 19.2 μM), **13** (3,5-difluorobenzyl, 8.8 μM), and **14** (biphenyl, 5.3 μM). The most active N-terminal substituent was the *N*-acetyl group (compounds **24–26**). The latter three derivatives were the most potent inhibitors with K_i values of 5.1 (**24**: benzyl), 16.5 (**25**: 3,5-difluorobenzyl), and 3.7 μM (**26**: biphenyl). The potency of these three compounds against BACE-1 was two- to sevenfold higher than that of the corresponding products without N-acylation. Introduction of a Cbz group (compound **28**; $IC_{50} > 200$ μM), however, was not tolerated by the protein and decreased the inhibitory capacity of this compound. Variations or additional substitutions on the aminophenylcoumarin moiety also led to a distinct improvement of the inhibitory activities. First, the new initial fragments **9–11** were investigated as inhibitors of BACE-1 and showed activities in the same μM range as the initial hit **1** (data not shown). Incorporation of the fragment derivatives in the ethane-1,2-diamine structure provided fragment combinations with a significant increase in affinity (decrease in K_i values). Relative to the activity of compound **18** ($K_i = 40.1$ μM), the introduction of the 6-methoxy group in **21** increased the activity twofold ($K_i = 19.3$ μM). This enhancement in the activity could be explained by formation of one additional hydrogen bond with Arg128 present on the protein surface (Figure 5). The 7-methoxy derivative **22** displayed an almost fourfold increase in activity ($K_i = 11.5$ μM) relative to that of reference compound **18**, which can be rationalized by the formation of two additional hydrogen bonds between the carbonyl group of the chromone ring and the hydroxy group of Tyr198 and between the methoxy oxygen atom and Arg128, respectively. The shift of the amino substituent from the *meta* to the *para* position in compound **23** also led to an almost fourfold rise in activity ($K_i = 11$ μM) and the docking of the inhibitor (Figure 6) indicated a good fit into the S1 pocket, including hydrogen bonding with the catalytic dyad (Asp32 and Asp228), as well as with Gly230 and Thr72. Additional studies are required to investigate whether these modifications lead to further improved inhibitors if they are combined with each other and with N-acylation of the terminal amino group.

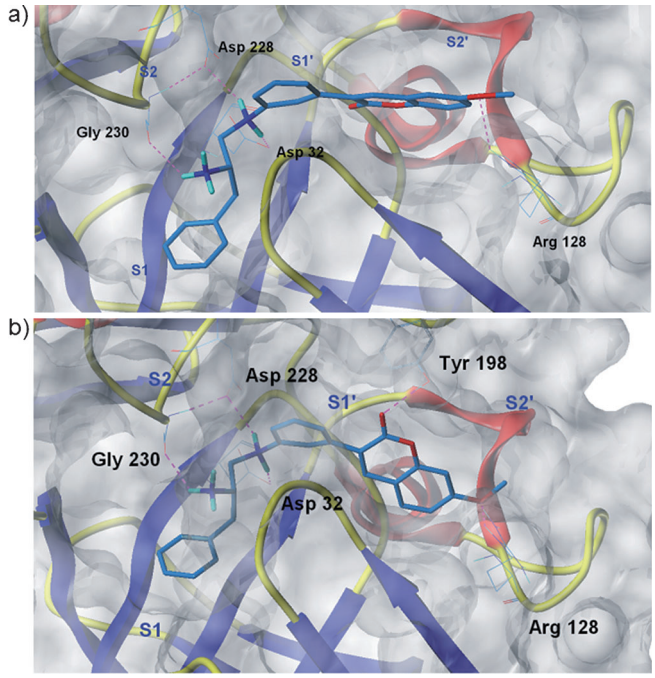


Figure 5. Cartoon representation of the BACE-1 active site. Key residues, belonging to the different subsites, are highlighted in black for clarity. a) Docking pose of compound **21**. b) Docking pose of compound **22**. Hydrogen bonds are depicted as dashed lines (magenta). The binding mode was derived from the Surflex program within the SYBYL software.

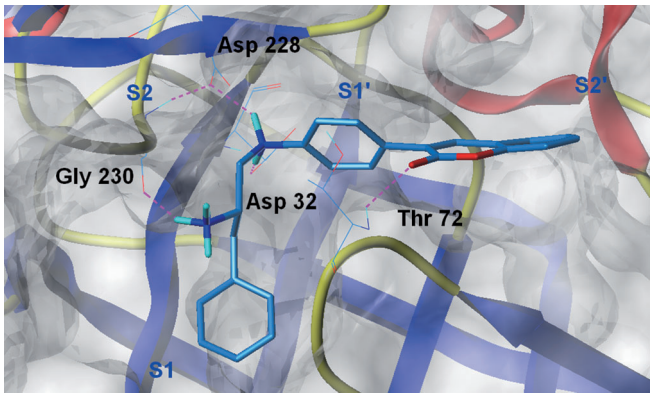


Figure 6. Most reasonable docked pose of compound **23** in the BACE-1 binding site. The amino acid residues, belonging to the different subsides, are labeled in black for clarity. The hydrogen bonds are depicted as dashed lines (magenta).

Conclusions

In this work, we have investigated the application of dynamic ligation screening, a method for fragment-based drug discovery, to the identification of active-site binding fragments of the aspartic protease BACE-1 and their development into nonpeptidic, small-molecule inhibitors of this protein. The implementation of DLS for BACE-1 involved the design and synthesis of new FRET substrates **I**–**III**, with strongly enhanced sensitivity, as well as the development of a peptide aldehyde inhibitor of BACE-1 as a chemically reactive directing probe. FRET substrate **I** ($V_{max} = 0.142 \times 10^{-2}$ μM s^{-1}, $K_M = 4.97$ μM, and $k_{cat}/K_M = 15\,687$ s^{-1} M^{-1}) was selected due to a fivefold increase in sensitivity compared with similar, published substrates. The use of the pentapeptide aldehyde as a directing probe in the dynamic ligation assay in presence of the protein target (BACE-1) was established. The assay was used to screen a library of nucleophilic fragments, which had been chosen by using a maximum common substructure concept. The best fragment hit, 3-(3-aminophenyl)coumarin (APC, **1**; $K_i = 146$ μM), was selected for validation. Competitive binding of **1** to the active site of the enzyme BACE-1 was confirmed by kinetic analysis and by the synthesis and investigation of the irreversible fragment combination product with the directing probe.

For hit-to-lead optimization, combinations of fragment **1** with various nonpeptidic fragments targeting the S1 site of the protein were prepared. Entirely nonpeptidic fragment combinations containing the *N*-acyl-ethane-1,2-diamine motif were found to up to 30-fold more active than the initial fragment hit. Considering earlier findings, which had indicated that the ethane-1,2-diamine motif required an extended peptide sequence for activity, these observations were completely unexpected.[56] Clearly, fragment **1** is capable of favorably replacing the amino acids in the S′ direction. As secondary fragments targeting the S1 site of BACE-1, 2-methylpropyl, benzyl, 3,5-difluorobenzyl, and biphenyl were found to enhance binding, with K_i values in the low micromolar range. Preference for the S1 position depended on the *N*-acyl substituent. With *N*-Boc substitution, the 3,5-difluorobenzyl (**13**, 8.8 μM) and the biphenyl fragments (**14**, 5.3 μM) were preferred, whereas with *N*-acetyl substitution, the unsubstituted benzyl derivative (**24**, 5.1 μM) was found to be superior to the substituted 3,5-difluorobenzyl (**25**, 16.5 μM) and isobutyl fragments (**7**, 20.3 μM). The strongest activity for a compound with an acylated, primary aliphatic amine was detected for the biphenyl derivative **26** ($K_i = 3.7$ μM). Further improvement is indicated for a modification of the hit fragment itself (compare, for example, compounds **18** and **23**) and by variation of the *N*-acyl substituent. The chemical optimization process was supported by the calculated binding modes of the fragment combinations to the protein. According to molecular modeling and the recorded structure–activity relationships, the compounds can interact favorably with Asp32 and Asp228, which define the active catalytic site of the enzyme, through a protonated aromatic amine. Binding is further sustained by additional hydrogen bonds and hydrophobic interactions of the molecules. Exchange of an isobutyl residue in the P1 position by several aromatic residues was found to be advantageous due to better space-filling of the hydrophobic S1 pocket.

In summary, we have demonstrated in this work that dynamic ligation screening can be applied successfully to aspartic proteases such as BACE-1 as targets and represents a valuable approach for the site-directed discovery of inhibitory fragments. Peptide aldehydes can be used as directing probes for this enzyme class and hit fragments can be extended to fragment-based compounds containing the N-acylated ethane-1,2-diamine motif, which can act as potent inhibitors. Herein, the identified 3-(aminophenyl)coumarin fragment has been estab-

lished as a successful starting point for the development of entirely nonpeptidic, drug-like inhibitors of BACE-1 with low-micromolar activity and further potential for optimization.

Experimental Section

Synthesis

General: Chemicals and dry solvents were purchased from Sigma–Aldrich, Fluka, or Novabiochem and used without further purification or distillation unless otherwise stated. Fmoc solid-phase peptide syntheses were performed in polypropylene 2–20 mL syringes fitted with polyethylene porous filter disks. Solvents and soluble reagents were removed by suction. The Fmoc group was removed with piperidine/DMF (2:8; 1×1 min, 2×10 min). Washings between coupling and deprotection steps were performed with DMF (5× 0.5 min), tetrahydrofuran (THF; 5×0.5 min), and CH_2Cl_2 (5×0.5 min) by using at least 10 mL of solvent per gram of resin each time. For elemental analyses or Fmoc determination, the resins were washed as described above with additional washings with MeOH (5× 0.5 min) and Et_2O (5×0.5 min) and with brief drying under suction of air and finally under high vacuum. Loadings were quantitatively determined by using a Jasco V-550 UV/Vis spectrophotometer with Hellma Suprasil cuvettes with a path length of 1 mm. Solid-phase reactions were monitored by FTR-IR spectrometry of the resin by using a Bruker Tensor 27 FTR-IR instrument equipped with a Pike MIRacle ATR apparatus fitted with a ZnSe crystal plate. Frequencies ($\tilde{\nu}$) are expressed in cm^{-1}. Solution-phase reactions were monitored either by LC–MS techniques or by thin layer chromatography (TLC) with Analtech silica gel plates (60 F_{254}) and the spots were examined under UV light at 254 nm or stained with developing reagents. LC–MS data were recorded on an Agilent 1100 series chromatography workstation (Agilent Technologies) equipped with a single quadrupole mass spectrometer and electrospray ionization (ESI). Solvents A (0.1% TFA in Millipore water), B (0.1% TFA in acetonitrile), and C (0.1% TFA in MeOH) were used in a linear gradient (5–99% B or C in 5 min or 30 min for preparative runs). Peptides were purified either on a semipreparative HPLC column (VP 250/10 Nucleodur 100-5 C_{18} ec Macherey–Nagel) by employing individual gradients derived from analytical runs (eluents A–C). HRMS measurements were conducted on an Agilent 6220 ESI-TOF mass spectrometer. Nuclear magnetic resonance (NMR) spectra (^1H and ^{13}C NMR) were recorded on a Bruker AVANCE 300 MHz instrument and chemical shifts (δ) were measured in parts per million (ppm) relative to tetramethylsilane (TMS) used as an internal reference. Coupling constants (J) are expressed in Hertz (Hz). The following abbreviations are used to describe peak patterns when appropriate: s: singlet; d: doublet; t: triplet; qt: quintet; m: multiplet; br: broad. The commercial FRET substrates **IV** ((MOCAc)SEVN**LD**AEFRK-(DNP)RR) and RhoEVNLDAEFR-Quencher were purchased from R&D Systems, Inc. (ES004) and Invitrogen (P2985), respectively. Triton X-100 was purchased from Aldrich Chemical Co. Inc.

(MOCAc)SEVNLDAEFK(DNPA)RR (I) and (MOCAc)SEVNLDAEFK-(DNB)RR (II): A Rink amide resin (300 mg, $f=0.7$ mmol g^{-1}) placed in a 25 mL polypropylene syringe fitted with a polyethylene filter disk was treated with Fmoc-Arg(Pbf)-OH (Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; 5 equiv) in the presence of HOBt (5 equiv) and DIC (5 equiv) for 4 h at room temperature, until the ninhydrin test for primary amines was negative. When the reaction was finished, the resin was filtered off, washed several times (five times) with DMF, MeOH, THF, and CH_2Cl_2, and dried under high vacuum. The loading was calculated by UV spectroscopy ($f=$ 0.60 mmol g^{-1}). The chain was elongated by sequentially coupling Fmoc-Arg(Pbf)-OH and Fmoc-Lys(Mtt)-OH (Mtt: 4-methyltrityl) by using HOBt and DIC as coupling reagents (fivefold molar excess) in DMF for 2 h at room temperature. The completion of all coupling was confirmed with a negative ninhydrin test result. The resin was then split into two parts (100 mg) and the *N*-Mtt group on the lysine side chain was removed by using a cocktail of TFA/TIS/CH_2Cl_2 (2:5:93) for 2 h at room temperature. After the washing, the quencher groups 2-(2,4-dinitrophenyl)acetic acid (substrate **I**) or 2,4-dinitrobenzoic acid (substrate **II**) were coupled with a fivefold molar excess of HOBt and DIC in a mixture of DMF/THF (2:1) for 10 h at room temperature. After that, the peptide was elongated by SPPS coupling of Fmoc-Phe-OH, Fmoc-Glu(*Ot*Bu)-OH, Fmoc-Ala-OH, Fmoc-Asp(*Ot*Bu)-OH, Fmoc-Leu-OH, Fmoc-Asn(trt)-OH, Fmoc-Val-OH, Fmoc-Glu(*Ot*Bu)-OH, and Fmoc-Ser(*t*Bu)-OH by using the same coupling reagents (fivefold molar excess) in DMF for 2 h at room temperature. After SPPS, the substrate was then N-terminally labeled with the fluorophore MOCAc. Thus, the resin was treated with 7-methoxycoumarin-4-acetic acid (fivefold molar excess) in the presence of HOBt (5 equiv) and DIC (5 equiv) in DMF for 10 h at room temperature. Finally, the peptide was cleaved from the resin and the protective amino acid side chains were removed with a cocktail TFA/TIS/H_2O/EDT (94:1:2.5:2.5) for 3 h at room temperature. The filtrate was collected and the solvents were removed under reduced pressure. The residue was purified by reverse HPLC (solvent A and solvent B, from 5% B to 99% B over 45 min, at a flow of 20 mL min^{-1} with a linear gradient). Thus, substrate **I** (60 mg, 52%) was isolated as a yellow powder after lyophilization: HPLC purity: 98% (UV, $t_R=2.62$ min); HRMS (electrospray ionization, positive mode): m/z calcd for $C_{82}H_{117}N_{24}O_{29}$: 1901.8418 $[M+H]^+$; found: 1902.8376. Substrate **II** was isolated as a yellow powder after lyophilization (53 mg, 47%): HPLC purity: 99% (UV, $t_R=2.54$ min); HRMS (electrospray ionization, positive mode): m/z calcd for $C_{81}H_{114}N_{23}O_{29}$: 1872.8153 $[M+H]^+$; found: 1873.8212.

(DNPA)SEVNLDAEFRRG(AMCA) (III): A Rink amide resin (500 mg, $f=0.70$ mmol g^{-1}) placed in a 25 mL polypropylene syringe fitted with a polyethylene filter disk was treated with 20% piperidine in DMF (5 mL; 1×1 min, 2×20 min). After that, the resin was filtered off, washed several times (five times) with DMF, MeOH, THF, and CH_2Cl_2, and dried under high vacuum. Subsequently, it was treated with 2-{7-[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-4-methyl-2-oxo-2*H*-chromen-3-yl)acetic acid (5 equiv) in presence of HOBt (5 equiv) and DIC (5 equiv) in DMF for 15 h at room temperature, until the ninhydrin test result was negative. When the reaction was finished, the loading was calculated by UV spectroscopy ($f=$ 0.62 mmol g^{-1}). The chain was elongated by sequentially coupling Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Phe-OH, Fmoc-Glu(*Ot*Bu)-OH, Fmoc-Ala-OH, Fmoc-Asp(*Ot*Bu)-OH, Fmoc-Leu-OH, Fmoc-Asn(trt)-OH, Fmoc-Val-OH, Fmoc-Glu(*Ot*Bu)-OH, and Fmoc-Ser(*t*Bu)-OH with HOBt and DIC as a coupling reagents (fivefold molar excess) in DMF for 2 h at room temperature. The completion of all couplings was confirmed with negative ninhydrin test results. After the last amino acid coupling, the Fmoc protective group was removed with 20% piperidine in DMF. The amino terminus was modified by the introduction of the fluorescence quencher by treatment with 2-(2,4-dinitrophenylamino)acetic acid (5 equiv), HOBt (5 equiv), and DIC (5 equiv), in THF/DMF (3:2) for 2 h at room temperature. The resin was filtered off, the peptide was cleaved from the resin, and the protective amino acid side chains were removed with a cocktail TFA/TIS/H_2O/EDT (94:1:2.5:2.5) for 3 h at room temperature. The filtrate was collected and the solvents were removed under reduced pressure. The residue was purified by reverse HPLC (solvent A and solvent B, from 5% B to 99% B over 45 min, at a flow of 20 mL min^{-1} with a linear gradient).

Thus, substrate **III** (336 mg, 53%) was isolated as a yellow powder after lyophilization: HPLC purity: 98% (UV, $t_R = 3.01$ min); HRMS (electrospray ionization, positive mode): *m/z* calcd for $C_{79}H_{110}N_{24}O_{27}$: 915.3955 $[M+2H]^{2+}/2$; found: 915.3972.

AcSEVNL–CHO (**V**): An H-Leu-H-NovaSyn TG resin (500 mg, $f = 0.23$ mmol g^{-1}) placed in a 10 mL polypropylene syringe fitted with a polyethylene filter disk was treated with 20% piperidine in DMF (5 mL; 1×1 min, 2×20 min). After that, the resin was filtered off, washed with several times (five times) with DMF, MeOH, THF, and CH_2Cl_2, and dried under high vacuum. The chain was elongated by sequentially coupling Fmoc-Asn(trt)-OH, Fmoc-Val-OH, Fmoc-Glu(*Ot*Bu)-OH, and Fmoc-Ser(*t*Bu)-OH (fourfold molar excess) by using HOBt and DIC as coupling reagents (fourfold molar excess) in DMF for 2 h at room temperature. The completion of all coupling was confirmed with a negative ninhydrin test result. The Fmoc protective group for the last amino acid was removed by 20% piperidine in DMF. The N terminus was acetylated with acetic anhydride (4 equiv) in DMF (2 times, until the ninhydrin test was negative). Finally, the peptide was cleaved from the resin and the protective groups at the amino acid side chains were removed with a cocktail of TFA/CH_2Cl_2 (80:20) for 3 h at room temperature. The filtrate was then treated with cool Et_2O and the precipitate was filtered off and washed three times with Et_2O to yield compound **V** (24.3 mg, 36%) as a white powder after lyophilization: HPLC purity: 98% (UV, $t_R = 1.61$ min); HRMS (electrospray ionization, positive mode): *m/z* calcd for $C_{25}H_{43}N_6O_{11}$: 587.3035 $[M+H]^+$; found: 587.3053.

AcS(*t*Bu)E(*Ot*Bu)VN(trt)-OH (**6**): 2-Chlorotrityl chloride resin (1.5 g, $f = 1.2$–1.5 mmol g^{-1}) placed in a 10 mL polypropylene syringe fitted with a polyethylene filter disk was treated with Fmoc-Asn-(trt)-OH (1.3 g, 1.8 mmol, 1.0 equiv) and with diisopropylethylamine (DIPEA; 1.2 mL, 7.2 mmol, 4.0 equiv) in DMF for 15 min at room temperature. Subsequently, an additional portion of DIPEA (2.5 mL, 14.4 mmol, 8.0 equiv) was added and the reaction mixture was stirred for 3 h at room temperature. After that, the resin was treated with MeOH (1.18 mL) and the reaction mixture was stirred for another 15 min. The resin was filtered off, washed five times with DMF, MeOH, THF, and CH_2Cl_2, and dried under high vacuum. When the reaction was finished, the loading was calculated by UV spectroscopy ($f = 0.9$ mmol g^{-1}). The chain was elongated by sequentially coupling Fmoc-Val-OH, Fmoc-Glu(*Ot*Bu)-OH, and Fmoc-Ser(*t*Bu)-OH by using HOBt and DIC as coupling reagents (fourfold molar excess) in DMF for 2 h at room temperature. The completion of all couplings was confirmed with a negative ninhydrin test result. After the last amino acid coupling, the Fmoc protective group was removed with 20% piperidine in DMF and the amino terminus was acetylated with Ac_2O (679 μL, 7.2 mmol, 4 equiv; 2 times, until the ninhydrin test was negative). Finally, the peptide was cleaved from the resin, and the protected amino acid side chains were maintained with a cocktail of TFE/AcOH (1:1; 30%) and CH_2Cl_2 (70%) for 1 h at room temperature and successive washings with CH_2Cl_2 (5 times). The filtrates were collected and the solvents were evaporated. The resulting residue was treated with cool Et_2O and the precipitate was filtered off and washed three times with cold Et_2O to afford compound **6** (685 mg, 90%) as a white powder after lyophilization: HPLC purity: 99% (UV, $t_R = 4.59$ min); HRMS (electrospray ionization, positive mode): *m/z* calcd for $C_{46}H_{62}N_5O_{10}$: 844.4491 $[M+H]^+$; found: 844.4478.

AcSEVNL-CH$_2$NH$_2$ (**8**): Fmoc-leucinal (860.4 mg, 2.55 equiv), $NaCNBH_3$ (160.3 mg, 2.55 equiv), and DMF were added to an amino trityl resin (500 mg, $f = 1.7$ mmol g^{-1}) placed in a 10 mL polypropylene syringe fitted with a polyethylene filter disk. The resulting slurry was stirred for 24 h at room temperature. When the reaction was finished, the loading was calculated by UV spectroscopy ($f = 0.8$ mmol g^{-1}; IR: $\tilde{\nu} = 1727.91$, 1663.08, 1118.07, 737.31 cm^{-1}). The chain was elongated by sequentially coupling Fmoc-Asn(trt)-OH, Fmoc-Val-OH, Fmoc-Glu(*Ot*Bu), and Fmoc-Ser(*t*Bu)-OH by using HOBt and DIC as coupling reagents (fourfold molar excess) in DMF for 2 h at room temperature. The completion of all couplings was confirmed with a negative ninhydrin test result. After the last amino acid coupling, the Fmoc protective group was removed with 20% piperidine in DMF. The amino terminus was acetylated with acetic anhydride (400 μL, 4.25 mmol, 5 equiv; 2 times, until the ninhydrin test was negative). Finally, the peptide was cleaved from the resin and the protective groups at the amino acid side chains were removed with a cocktail of TFA/TIS/H_2O/EDT (94:1:2.5:2.5) for 3 h at room temperature, followed by successive washings with CH_2Cl_2 (five times). The filtrates were collected, the solvents were evaporated, and the resulting residue was then treated with cool Et_2O. The precipitate was filtered off and washed three times with cold Et_2O to yield compound **8** (235 mg, 71%) as a white powder after lyophilization: HPLC purity: 99% (UV, $t_R = 1.49$ min); HRMS (electrospray ionization, positive mode): *m/z* calcd for $C_{25}H_{42}N_6O_{10}$: 588.3352 $[M+H]^+$; found: 588.3367.

General procedure for the synthesis of derivatives 4 and 12–17: In a round-bottom flask, the molecular sieves were activated under high vacuum and heating. After that, under an N_2 atmosphere, a solution of 3-(3-aminophenyl)-2*H*-chromen-2-one derivatives **1**, **9**, or **10** or 3-(4-aminophenyl)-2*H*-chromen-2-one (**11**; 1 equiv) and the appropriate aldehyde (1.4 equiv) in a mixture of DMF/AcOH (1 mL:100 μL) was added. The reaction mixture was stirred for 1 h at room temperature and then $NaCNBH_3$ (2 equiv) was added in one portion. The resulting mixture was stirred for an additional 12 h at room temperature. The reaction mixture was then diluted with CH_2Cl_2 (75 mL) and washed sequentially with brine (3×50 mL) and H_2O (3×50 mL). The organic phases were dried over Na_2SO_4 and filtered. The solvents were removed under reduced pressure. The mixture was purified by flash column chromatography with the solvent mixture indicated for each case.

(9*H*-Fluoren-9-yl)-methyl-(*S*)-1-(3-(2*H*-chromen-2-one-3-yl)phenylamino)-4-methylpentan-2-ylcarbamate (4): Reagents were 3-(3-aminophenyl)-2*H*-chromen-2-one (**1**; 28.5 mg, 0.12 mmol, 1 equiv), Fmoc-leucinal **3** (60.7 mg, 0.18 mmol, 1.4 equiv), and $NaCNBH_3$ (15.8 mg, 0.25 mmol, 2 equiv). Flash chromatography was conducted with hexane/EtOAc (3:1). From the fractions with $R_f = 0.5$ (hexane/EtOAc, 2:1), compound **4** (40 mg, 57%) was isolated as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 0.93$ (d, $J = 5.5$ Hz, 3H), 0.96 (d, $J = 5.5$ Hz, 3H), 1.42 (m, 1H), 1.65 (m, 2H), 3.28–3.17 (m, 2H), 3.98 (br s, 1H), 4.20 (m, 1H), 4.53–4.43 (m, 2H), 4.73 (d, $J = 8.3$ Hz, 1H), 6.68 (d, $J = 7.8$ Hz, 1H), 6.99 (s, 1H), 7.20–7.42 (m, 9H), 7.49 (d, $J = 7.3$ Hz, 2H), 7.57 (d, $J = 7.8$ Hz, 2H), 7.75 ppm (t, $J = 7.3$ Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 21.0$, 22.0, 23.2, 24.6, 42.3, 47.2, 49.5, 60.4, 112.9, 113.3, 117.7, 119.7, 119.9 (2C), 120.0, 124.5, 124.9, 127.0 (2C), 127.6 (2C), 127.8, 128.5 (2C), 129.3, 131.2, 135.6, 139.7, 141.2 (2C), 141.3 (2C), 148.1, 153.3, 156.7, 160.6 ppm; HRMS (electrospray ionization, positive mode): *m/z* calcd for $C_{36}H_{35}N_2O_4$: 559.2591 $[M+H]^+$; found: 559.2597; HPLC purity: 100% (UV, $t_R = 3.50$ min).

(9*H*-Fluoren-9-yl)methyl-(*S*)-1-(3-(2*H*-chromen-2-one-3 yl)phenylamino)-3-phenylpropyl-2-ylcarbamate (12): Reagents were 3-(3-aminophenyl)-2*H*-chromen-2-one (**1**; 23.7 mg, 0.10 mmol, 1 equiv), (9*H*-fluoren-9-yl)methyl-(*S*)-1-formyl-2-phenylethylcarbamate (**30a**; 43.8 mg, 0.13 mmol, 1.2 equiv), and $NaCNBH_3$ (13.2 mg, 0.21 mmol, 2 equiv). Flash chromatography was conducted with hexane/EtOAc (3:1). From the fractions with $R_f = 0.6$ (hexane/EtOAc, 3:1), com-

pound **12** was isolated as a yellow oil (41.8 mg, 67%): ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.93 (m, 2H), 3.30 (m, 2H), 4.48–4.36 (m, 3H), 4.80 (m, 1H), 6.62 (brd, J = 7.5 Hz, 1H), 6.96 (brs, 1H), 7.06 (d, J = 7.5 Hz, 1H), 7.20–7.43 (m, 12H), 7.51 (m, 5H), 7.75 ppm (t, J = 7.5 Hz, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 38.8, 47.1, 52.1, 60.3, 66.6, 112.9, 113.2, 116.2, 117.7, 119.5, 119.8, 124.3, 124.9, 126.6, 126.9, 127.6, 127.7 (2C), 128.4, 128.6, 128.7, 128.8, 128.9, 129.1, 129.2, 131.1, 135.5, 136.3, 137.2, 131.1, 135.5, 136.3, 137.2, 139.7, 141.2 (2C), 143.6 (2C), 147.8, 153.3, 156.2, 160.5, 171.1 ppm; HRMS (electrospray ionization, positive mode): m/z calcd for $C_{39}H_{33}N_2O_4$: 593.2435 $[M+H]^+$; found: 593.2470; HPLC purity: 100% (UV, t_R = 3.76 min).

(*S*)-1-(3-(2*H*-Chromen-2-one-3-yl)phenylamino)-*tert*-butyl-3-(3,5-difluorophenylpropyl)-2-ylcarbamate (13): Reagents were 3-(3-aminophenyl)-2*H*-chromen-2-one (**1**; 19.0 mg, 0.08 mmol, 1 equiv), *tert*-butyl-(*S*)-2-(3,5-difluorophenyl)-1-formylethylcarbamate (**30b**; 39.1 mg, 0.10 mmol, 1.2 equiv), and $NaCNBH_3$ (10.5 mg, 0.17 mmol, 2 equiv). Flash chromatography was conducted with hexane/EtOAc (4:1 to 2:1). From the fractions with R_f = 0.3 (hexane/EtOAc, 4:1), compound **13** (30 mg, 70%) was isolated as a pale oil: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.41 (s, 9H), 2.89 (m, 2H), 3.20 (m, 1H), 3.30 (dd, J = 12.7, 4.3 Hz, 1H), 4.07 (m, 1H), 4.54 (brs, 1H), 5.30 (brs, 1H), 6.65 (dt, J = 8.4, 2.2 Hz, 2H), 6.76 (d, J = 6.5 Hz, 2H), 6.98 (m, 1H), 7.02 (d, J = 7.6 Hz, 1H), 7.22–7.32 (m, 2H), 7.37 (d, J = 8.4 Hz, 1H), 7.53 (t, J = 8.0 Hz, 2H), 7.82 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 28.3 (3C), 43.4, 47.7, 53.5, 77.2, 102.2, 112.0 (d, $^2J_{C,F}$ = 23.0 Hz), 113.0 (d, $^2J_{C,F}$ = 23 Hz), 116.4 (2C), 119.7, 124.4 (2C), 127.1, 127.8 (2C), 128.6, 129.4, 131.2, 135.7, 137.6, 138.3, 139.8, 141.7, 153.5 (2C), 155.4 ppm; HRMS (electrospray ionization, positive mode): m/z calcd for $C_{29}H_{29}F_2N_2O_4$: 507.2090 $[M+H]^+$; found: 507.1925; HPLC purity: 100% (UV, t_R = 2.70 min).

(*S*)-1-(3-(2*H*-Chromen-2-one-3-yl)phenylamino)-*tert*-butyl-3-(3,5-biphenylpropyl)-2-ylcarbamate (14): Reagents were 3-(3-aminophenyl)-2*H*-chromen-2-one (**1**; 23.7 mg, 0.10 mmol, 1 equiv), 2-biphenyl-*tert*-butyl-(*S*)-1-formylethylcarbamate (**30c**; 39.2 mg, 0.12 mmol, 1.2 equiv), and $NaCNBH_3$ (13.2 mg, 0.20 mmol, 2 equiv). Flash chromatography was conducted with hexane/EtOAc (4:1 to 2:1). From the fractions with R_f = 0.3 (hexane/EtOAc, 3:1), compound **14** (34.5 mg, 60%) was isolated as a white powder after lyophilization: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.41 (brs, 9H), 2.92 (d, J = 6.0 Hz, 2H), 3.36 (m, 1H), 3.45 (dd, J = 12.7, 4.4 Hz, 1H), 4.19 (m, 1H), 4.87 (brs, 2H), 6.97 (s, 1H), 7.24–7.36 (m, 7H), 7.41 (t, J = 7.5 Hz, 3H), 7.50–7.56 (m, 6H), 7.82 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 28.1 (2C), 45.4, 48.6, 53.5, 77.0, 116.3, 116.5, 119.0, 119.4, 123.2, 123.4, 124.4, 124.7, 126.8 (2C), 127.1, 127.3, 127.9, 128.1, 128.2, 128.6 (2C), 129.5, 131.4, 132.2, 134.5, 136.0, 136.1, 139.7, 140.5, 141.1, 153.4, 153.6, 160.3 ppm; HRMS (electrospray ionization, positive mode): m/z calcd for $C_{35}H_{35}N_2O_4$: 547.2591 $[M+H]^+$; found: 547.2601; HPLC purity: 100% (UV, t_R = 5.20 min).

(9*H*-Fluoren-9-yl)methyl-(*S*)-1-(3-(6-methoxy-2*H*-chromen-2-one-3-yl)phenylamino)-3-phenylpropyl-2-ylcarbamate (15): Reagents were 3-(3-aminophenyl)-6-methoxy-2*H*-chromen-2-one (**9**; 29.4 mg, 0.11 mmol, 1 equiv), (9*H*-fluoren-9-yl)methyl (*S*)-1-formyl-2-phenylethylcarbamate (**30a**; 48.3 mg, 0.13 mmol, 1.2 equiv), and $NaCNBH_3$ (8.50 mg, 0.22 mmol, 2 equiv). Flash chromatography was conducted with hexane/EtOAc (3:1). From the fractions with R_f = 0.5 (hexane/EtOAc, 3:1), compound **15** (45 mg, 64%) was isolated as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.98 (brs, 1H), 3.28 (m, 2H), 3.83 (s, 3H), 4.16 (m, 2H), 4.39 (m, 2H), 4.84 (m, 1H), 6.62 (m, 1H), 6.93 (dd, J = 8.5, 3.0 Hz, 2H), 7.19–7.30 (m, 10H), 7.38 (t, J = 7.4 Hz, 3H), 7.75 (d, J = 7.4 Hz, 2H), 7.74 (d, J = 8.5 Hz, 4H), 8.02 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 31.4, 36.47, 47.2, 55.8, 66.7, 77.2, 109.6, 112.9, 113.2, 115.1, 117.2, 117.3, 117.7, 118.8, 119.7 (2C), 119.9, 124.8, 126.6, 126.9, 127.5 (2C), 128.5 (2C), 128.7 (2C), 129.1 (2C), 129.2, 135.7, 137.3, 139.6, 141.3, 143.8, 147.9, 153.7, 156.0, 156.3, 160.7, 162.5 ppm; HRMS (electrospray ionization, positive mode): m/z calcd for $C_{40}H_{35}N_2O_5$: 623.2540 $[M+H]^+$; found: 623.2558; HPLC purity: 100% (UV, t_R = 2.84 min).

(9*H*-Fluoren-9-yl)methyl-(*S*)-1-(3-(7-methoxy-2*H*-chromen-2-one-3-yl)phenylamino)-3-phenylpropyl-2-ylcarbamate (16): Reagents were 3-(3-aminophenyl)-7-methoxy-2*H*-chromen-2-one (**10**, 48.1 mg, 0.18 mmol, 1 equiv), (9*H*-fluoren-9-yl)methyl-(*S*)-1-formyl-2-phenylethylcarbamate (**30a**, 83.2 mg, 0.22 mmol, 1.2 equiv), and $NaCNBH_3$ (14.4 mg, 0.37 mmol, 2 equiv). Flash chromatography was conducted with hexane/EtOAc (3:1). From the fractions with R_f = 0.5 (hexane/EtOAc, 3:1), compound **16** (75 mg, 64%) was isolated as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.92 (brs, 1H), 3.26 (m, 2H), 3.89 (s, 3H), 4.16 (m, 2H), 4.40 (m, 2H), 4.85 (m, 2H), 6.59 (brs, 1H), 6.84–7.01 (m, 4H), 7.13–7.55 (m, 14H), 8.02 (s, 1H), 7.73 ppm (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 31.4, 36.4, 47.2, 55.8, 66.6, 77.2, 100.3, 112.6, 112.9, 113.4, 115.3, 117.7, 119.9 (3C), 125.0, 126.7, 127.0, 127.6 (2C), 128.6 (2C), 128.7 (2C), 129.2 (2C), 129.3, 135.9, 137.3, 139.8, 141.3, 143.8, 153.7, 155.2, 156.3, 160.8, 162.5 ppm; HRMS (electrospray ionization, positive mode): m/z calcd for $C_{40}H_{35}N_2O_5$: 623.2540 $[M+H]^+$; found: 623.2563; HPLC purity: 100% (UV, t_R = 2.84 min).

(9*H*-Fluoren-9-yl)-methyl-(*S*)-1-(4-(2*H*-chromen-2-one-3-yl)phenylamino)-3-phenylpropyl-2-ylcarbamate (17): Reagents were 3-(4-aminophenyl)-2*H*-chromen-2-one (**11**, 35.6 mg, 0.15 mmol, 1 equiv), (9*H*-fluoren-9-yl)methyl-(*S*)-1-formyl-2-phenylethylcarbamate (**30a**; 66.8 mg, 0.18 mmol, 1.2 equiv), and $NaCNBH_3$ (11.2 mg, 0.29 mmol, 2 equiv). Flash chromatography was conducted with hexane/EtOAc (3:1). From the fractions with R_f = 0.4 (hexane/EtOAc, 3:1), compound **17** (58.0 mg, 66%) was isolated as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.97 (m, 2H), 3.26 (brs, 1H), 3.96 (m, 1H), 4.17 (m, 2H), 4.42 (m, 2H), 4.78 (m, 1H), 6.62 (d, J = 6.7 Hz, 1H), 6.74 (d, J = 7.8 Hz, 2H), 7.20 (brs, 1H), 7.23–7.41 (m, 8H), 7.45–7.58 (m, 7H), 7.65 (s, 1H), 7.72–7.76 ppm (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 31.5, 35.8, 47.3, 52.0, 66.6, 77.3, 102.5, 112.5, 116.3, 120.0, 124.2, 124.9, 126.8, 127.0, 127.2, 127.5, 127.7, 127.8 (2C), 128.1, 128.7 (2C), 128.8, 128.9 (2C), 129.1, 129.2, 129.7 (2C), 130.5, 134.5, 136.9, 137.1, 141.3, 143.8, 153.1, 153.6, 154.0, 161.0 ppm; HRMS (electrospray ionization, positive mode): m/z calcd for $C_{39}H_{33}N_2O_4$: 593.2435 $[M+H]^+$; found: 593.2421; HPLC purity: 100% (UV, t_R = 5.17 min).

General procedure for Fmoc deprotection: synthesis of derivatives 5, 18, and 21–23: Under an N_2 atmosphere, the appropriate Fmoc derivative (1 equiv) was treated with a 20% piperidine in DMF solution for 30 min at room temperature. After that, the reaction mixture was diluted with CH_2Cl_2 (50 mL) and washed sequentially with brine (2×30 mL) and H_2O (2×20 mL). The organic phases were dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure. The residue was purified by flash column chromatography with the solvents indicated in each case.

3-(3-((*S*)-2-Amino-4-methylpentylamino)phenyl)-2*H*-chromen-2-one (5): Reagents were ((9*H*-fluoren-9-yl)methyl-(*S*)-1-(3-(2*H*-chromen-2-one-3-yl)phenylamino)-4-methylpentan-2-yl-carbamate (**4**; 40 mg, 0.07 mmol, 1 equiv) and 20% piperidine in DMF (750 μL). Flash chromatography was conducted with hexane/EtOAc (3:1) to CH_2Cl_2/CH_3OH/30% $NH_{3(aq)}$ (8:1:0.1). From the fractions with R_f = 0.6 (CH_2Cl_2/CH_3OH/30% $NH_{3(aq)}$, 8:1:0.2), amine derivative **5** was isolated as a yellow oil (16 mg, 66%): ^{1}H NMR (300 MHz, CD_3OD): δ = 0.94 (d, J = 6.5 Hz, 3H), 0.97 (d, J = 6.5 Hz, 3H), 1.37 (m, 2H), 1.64

(m, 2H), 1.80 (hept, J=6.5 Hz, 1H), 3.00 (m, 1H), 3.13 (m, 1H), 3.22 (dd, J=12.8, 4.9 Hz, 1H), 6.70 (dd, J=8.0, 1.9 Hz, 1H), 6.94 (dd, J=8.0, 1.9 Hz, 1H), 7.01 (t, J=1.9 Hz, 1H), 7.18 (t, J=8.0 Hz, 1H), 7.30–7.36 (m, 2H), 7.57 (dt, J=7.5, 1.5 Hz, 1H), 7.67 (d, J=7.5 Hz, 1H), 8.00 ppm (s, 1H); ^{13}C NMR (75 MHz, CD_3OD): δ=20.6, 21.6, 23.8, 42.8, 47.9, 48.8, 112.0, 112.4, 115.0, 116.4, 119.2, 123.8, 127.4, 127.7, 128.1, 130.6, 134.9, 139.5, 148.1, 152.7, 160.4 ppm; HRMS (electrospray ionization, positive mode): m/z calcd for $C_{21}H_{25}N_2O_2$: 337.1911 $[M+H]^+$; found: 337.1943; HPLC purity: 100% (UV, t_R=2.58 min).

3-(3-((*S*)-2-Amino-3-phenylpropylamino)phenyl)-2*H*-chromen-2-one (18): Reagents were ((9*H*-fluoren-9-yl)methyl-(*S*)-1-(3-(2*H*-chromen-2-one-3-yl)phenylamino)-3-phenylpropyl-2-yl-carbamate (**12**; 32.0 mg, 0.05 mmol) and 20% piperidine in DMF solution (500 μL). Flash chromatography was conducted with CH_2Cl_2/CH_3OH/30% $NH_{3\,(aq)}$ (from 20:1:0 to 8:1:0.1). From the fractions with R_f=0.3 (CH_2Cl_2/CH_3OH/aqueous 30% NH_3, 10:1:0.2), **18** (19 mg, 95%) was isolated as a yellow solid after lyophilization: IR (DMSO): $\tilde{\nu}$=2360.0, 2341.9, 1717.7, 1601.8, 1507.1, 1489.8, 1456.8, 1331.8, 1204.0, 1110.1, 986.5, 923.2, 787.8, 752.9, 695.5 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.62 (dd, J=8.3, 13.7 Hz, 1H), 2.91 (1H, m), 3.03 (dd, J=8.5, 13.7 Hz, 1H), 3.31 (2H, m), 3.47 (br s, 3H), 6.65 (d, J=8.1 Hz, 1H), 6.98 (d, J=6.4 Hz, 2H), 7.20–7.37 (m, 8H), 7.52 (2H, m), 7.78 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=42.5, 49.7, 52.0, 113.1, 113.4, 116.3, 117.6, 119.7, 124.4, 126.5, 127.8, 128.5 (2C), 128.7, 129.2 (2C), 129.3, 131.2, 135.6, 138.5, 139.6, 148.3, 153.42, 160.6 ppm; HRMS (electrospray ionization, positive mode): m/z calcd for $C_{24}H_{23}N_2O_2$: 371.1754 $[M+H]^+$; found: 371.1763; HPLC purity: 100% (UV, t_R=2.58 min).

3-(3-((*S*)-2-Amino-3-phenylpropylamino)phenyl)-6-methoxy-2*H*-chromen-2-one (21): Reagents were ((9*H*-fluoren-9-yl)methyl-(*S*)-1-(3-(6-methoxy-2*H*-chromen-2-one-3-yl)phenylamino)-3-phenylpropyl-2-yl-carbamate (**15**; 14.9 mg, 0.02 mmol) and 20% piperidine in DMF solution (400 μL). Flash chromatography was conducted with CH_2Cl_2/CH_3OH/30% $NH_{3\,(aq)}$ (from 8:1:0 to 8:1:0.1). From the fractions with R_f=0.2 (CH_2Cl_2/CH_3OH/30% $NH_{3\,(aq)}$, 10:1:0.1), compound **21** (8 mg, 83%) was isolated as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.50 (br s, 3H), 2.77 (dd, J=13.8, 7.8 Hz, 1H), 3.13 (dd, J=13.8, 7.8 Hz, 1H), 3.29–3.41 (m, 2H), 3.49 (m, 1H), 3.86 (s, 3H), 6.60 (d, J=7.7 Hz, 1H), 6.95 (m, 2H), 7.03–7.11 (m, 2H), 7.16–7.30 (m, 6H), 7.73 (d, J=8.0 Hz, 1H), 8.02 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=42.4, 49.6, 52.2, 55.8, 100.4, 112.7, 113.0, 113.1, 113.4, 117.5, 125.2, 126.5, 128.6 (2C), 128.7, 129.2 (2C), 135.9, 138.4, 139.8, 148.3, 153.7, 155.2, 160.8, 162.5 ppm; HRMS (electrospray ionization, positive mode): m/z calcd for $C_{25}H_{25}N_2O_2$: 401.1860 $[M+H]^+$; found: 401.1870; HPLC purity: 100% (UV, t_R=1.96 min).

3-(3-((*S*)-2-Amino-3-phenylpropylamino)phenyl)-7-methoxy-2*H*-chromen-2-one (22): Reagents were ((9*H*-fluoren-9-yl)methyl-(*S*)-1-(3-(7-methoxy-2*H*-chromen-2-one-3-yl)phenylamino)-3-phenylpropyl-2-yl-carbamate **16** (37.4 mg, 0.06 mmol) and 20% piperidine in DMF solution (500 μL). Flash chromatography was conducted with CH_2Cl_2/CH_3OH/30% $NH_{3\,(aq)}$ (from 8:1:0 to 8:1:0.1). From the fractions with R_f=0.2 (CH_2Cl_2/CH_3OH/30% $NH_{3\,(aq)}$, 10:1:0.1), compound **22** (15 mg, 65%) was isolated as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.46 (br s, 3H), 2.66 (dd, J=14.0, 7.7 Hz, 1H), 2.99 (m, 1H), 3.06 (dd, J=14.0, 7.8 Hz, 1H), 3.32 (m, 2H), 3.89 (s, 3H), 6.62 (d, J=8.1 Hz, 1H), 6.85–6.88 (m, 2H), 6.96 (m, 2H), 7.18–7.33 (m, 6H), 7.42 (d, J=9.2 Hz, 1H), 7.74 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=42.4, 49.6, 52.2, 55.8, 100.4, 112.7, 113.0, 113.1, 113.4, 117.5, 125.2, 126.5, 128.6 (2C), 128.7, 129.2 (2C), 135.9, 138.4, 139.8, 148.3, 153.7, 155.2, 160.8, 162.5 ppm; HRMS (electrospray ionization, positive mode): m/z calcd for $C_{25}H_{25}N_2O_2$: 401.1860 $[M+H]^+$; found: 401.1862; HPLC purity: 100% (UV, t_R=1.96 min).

3-(4-((*S*)-2-Amino-3-phenylpropylamino)phenyl)-2*H*-chromen-2-one (23): Reagents were ((9*H*-fluoren-9-yl)methyl-(*S*)-1-(4-(2*H*-chromen-2-one-3-yl)phenylamino)-3-phenylpropyl-2-yl-carbamate (**17**; 30.0 mg, 0.05 mmol) and 20% piperidine in DMF solution (500 μL). Flash chromatography was conducted with CH_2Cl_2/CH_3OH/30% $NH_{3\,(aq)}$ (from 20:1:0 to 8:1:0.1). From the fractions with R_f=0.2 (CH_2Cl_2/CH_3OH/30% $NH_{3\,(aq)}$, 10:1:0.2), compound **23** (13.8 mg, 74%) was isolated as a yellow solid after lyophilization: ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.88 (br s, 3H), 2.64 (dd, J=13.2, 7.8 Hz, 1H), 2.99 (m, 1H), 3.04 (dd, J=13.2, 8.6 Hz, 1H), 3.31 (d, J=9.0 Hz, 2H), 6.66 (d, J=8.4 Hz, 2H), 7.21–7.35 (m, 7H), 7.44–7.51 (m, 2H), 7.59 (d, J=7.2 Hz, 2H), 7.70 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=41.8, 48.7, 52.2, 112.6 (2C), 116.3, 120.2, 123.4, 124.3, 126.7, 127.5, 128.2, 128.7 (2C), 129.3 (2C), 129.7 (2C), 130.5, 136.9, 138.0, 148.7, 153.1, 161.0 ppm; HRMS (electrospray ionization, positive mode): m/z calcd for $C_{24}H_{23}N_2O_2$: 371.1754 $[M+H]^+$; found: 371.1801; HPLC purity: 100% (UV, t_R=1.95 min).

3-(3-((*S*)-2-Amino-AcSEVN-4-methylpentylamino)phenyl)-2*H*-chromen-2-one (2): Under an N_2 atmosphere, a mixture of 3-(3-((*S*)-2-amino-4-methylpentylamino)phenyl)-2*H*-chromen-2-one (**5**; 6.73 mg, 0.02 mmol), AcS(*t*Bu)E(*Ot*Bu)VN(trt)-OH (**6**; 20.9 mg, 0.02 mmol), HOBt (4.82 mg, 0.04 mmol), and DIC (5.5 μL, 4.50 mg, 0.04 mmol) in CH_2Cl_2 (20 mL) was treated with DIPEA (8.9 μL, 6.15 mg, 0.047 mmol) at room temperature overnight. After that, the reaction mixture was diluted with CH_2Cl_2 (50 mL) and washed with 10% aq $NaHCO_3$ solution (3×30 mL), brine (3×30 mL), and H_2O (3×30 mL). The organic phase was dried over Na_2SO_4 and filtered, then the solvent was removed under reduced pressure. The resulting residue was purified by flash chromatography on a silica gel column with the mixture CH_2Cl_2/MeOH (16:1) as an eluent. From the fractions with R_f=0.6 (CH_2Cl_2/MeOH, 16:1), the fully protected peptide intermediate (22.7 mg, 82%) was isolated as a white solid after lyophilization: HRMS (electrospray ionization, positive mode): m/z calcd for $C_{67}H_{84}N_7O_{11}$: 1162.6223 $[M+H]^+$; found: 1162.6218; HPLC purity: 99% (UV, t_R=3.60 min). After that, the intermediate (18 mg, 0.015 mmol) was treated with a mixture of CH_2Cl_2/TFA/EDT (25:6:1; 1 mL) for 2 h at room temperature. The mixture was then treated with cool Et_2O and the precipitate was filtered off and washed three times with Et_2O. Compound **2** was isolated as a white powder after lyophilization (7 mg, 56%): HRMS (electrospray ionization, positive mode): m/z calcd for $C_{40}H_{54}N_7O_{11}$: 808.3876 $[M+H]^+$; found: 808.3867; HPLC purity: 99% (UV, t_R=3.02 min).

General procedure for Boc deprotection: synthesis of chromen-2-ones 19 and 20: Under an N_2 atmosphere, the appropriate *tert*-butylcarbamate derivatives **10b** or **10c** (1 equiv) were treated with 4 M HCl/dioxane solution for 2 h at room temperature. After that, the solvents were removed under reduced pressure, the resulting residue was precipitated in cool Et_2O, and the product was isolated by filtration as the hydrochloride form.

3-(3-((*S*)-2-Amino-3-(3,5-difluorophenyl)propylamino)phenyl)-2*H*-chromen-2-one (19): Reagents were 3-(3-((*S*)-2-amino-*tert*-butylcarbamato-3-(3,5-difluorophenyl)propylamino)phenyl)-2*H*-chromen-2-one (**13**; 10.1 mg, 0.02 mg, 1 equiv) and 4 M HCl/dioxane (0.8 mL). Compound **19** (6.80 mg, 85%) was obtained as a yellow powder: ^{1}H NMR (300 MHz, $CDCl_3$): δ=3.04 (d, J=7.6 Hz, 2H), 3.29 (m, 2H), 3.68 (m, 1H), 3.85 (br s, 1H), 6.87–6.89 (m, 3H), 7.21–6.99 (m, 4H), 7.37 (dt, J=8.3, 1.3 Hz, 1H), 7.43 (d, J=8.3 Hz, 1H), 7.62 (dt, J=8.3, 1.4 Hz, 1H), 7.79 (dd, J=7.6, 1.3 Hz, 1H), 8.18 (s, 1H), 8.43 ppm (br s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=43.6, 50.9, 60.0, 102.3, 112.3, 112.5, 112.8, 113.1, 115.8, 117.1, 119.4 (2C), 124.5, 127.4 (2C), 128.5, 128.8, 131.6, 135.3, 140.8, 147.6, 152.9, 159.5 ppm; HRMS

(electrospray ionization, positive mode): *m/z* calcd for $C_{24}H_{21}F_2N_2O_2$: 407.1566 $[M+H]^+$; found: 407.1577; HPLC purity: 100% (UV, t_R = 1.96 min).

3-(3-((*S*)-2-Amino-3-biphenylpropylamino)phenyl)-2*H*-chromen-2-one (20): Reagents were (*S*)-1-(3-(2*H*-chromen-2-one-3-yl)phenylamino)-*tert*-butyl-3-(3,5-biphenylpropyl)-2-yl-carbamate (**14**; 8.20 mg, 0.01 mmol, 1 equiv) and 4 M HCl/dioxane (0.8 mL). Compound **20** was obtained as a white powder after lyophilization (5.87 mg, 90%): ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 2.94 (dd, *J* = 13.3, 8.1 Hz, 1H), 3.11 (dd, *J* = 13.3, 5.7 Hz, 1H), 3.28 (m, 2H), 6.63 (d, *J* = 7.9 Hz, 1H), 3.60 (m, 2H), 6.90 (d, *J* = 7.9 Hz, 1H), 7.18–7.12 (m, 1H), 7.47–7.30 (m, 8H), 7.66–7.55 (m, 5H), 7.75 (dd, *J* = 7.9, 1.3 Hz, 1H), 8.15 (s, 1H), 8.39 ppm (br s, 2H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 38.0, 44.3, 53.6, 117.6, 121.5, 126.4, 128.3 (2C), 128.4, 128.9, 129.0, 129.1, 130.1, 130.3 (2C), 130.4, 131.0, 131.3, 131.4, 133.3, 136.2, 136.6, 137.9, 142.1, 142.2, 144.4, 143.5, 155.2, 155.6, 162.8 ppm; HRMS (electrospray ionization, positive mode): *m/z* calcd for $C_{30}H_{27}N_2O_2$: 447.2067 $[M+H]^+$; found: 447.2082; HPLC purity: 100% (UV, t_R = 2.06 min).

General procedure for the synthesis of acetyl derivatives 7 and 24–26: Under an N_2 atmosphere, a solution of the amino derivatives **5** or **18–20** (1 equiv) in CH_2Cl_2 (2 mL) was treated with Ac_2O (1 equiv) in the absence of base for 4 h at room temperature. After that, the reaction mixture was diluted with CH_2Cl_2 (30 mL) and washed sequentially with brine (3×20 mL) and H_2O (3×20 mL). The organic phase was dried over Na_2SO_4 and filtered, then the solvent was removed under reduced pressure. The residue was purified by silica gel flash column chromatography with solvent mixtures indicated in each case as eluents.

3-(3-((*S*)-2-Acetylamino-4-methylpentylamino)phenyl)-2*H*-chromen-2-one (7): Reagents were 3-(3-((*S*)-2-amino-4-methylpentylamino)phenyl)-2*H*-chromen-2-one (**5**; 5.72 mg, 0.02 mmol) and Ac_2O (1.6 μL, 0.02 mmol). Flash chromatography was conducted with CH_2Cl_2/MeOH (8:0.2). From the fractions with R_f = 0.4 (CH_2Cl_2/MeOH, 8:0.2), compound **7** (10 mg, 87%) was isolated as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.87 (t, *J* = 6.0 Hz, 6H), 1.37 (m, 2H), 1.94 (s, 3H), 1.63 (m, 1H), 3.00 (m, 1H), 3.13 (dd, *J* = 13.8, 3.8 Hz, 1H), 4.27 (m, 1H), 5,90 (d, *J* = 8.9 Hz, 1H), 7.29–7.40 (m, 4H), 7.50–7.60 (m, 3H), 7.63 (d, *J* = 7.8 Hz, 1H), 7.81 (d, *J* = 7.8 Hz, 1H), 8.05 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 22.3, 22.7, 23.6, 24.9, 42.5, 47.3, 52.5, 116.5, 119.6, 124.6, 128.3 (2C), 128.5, 130.1, 131.8, 134.9, 136.5, 140.9, 142.9, 153.6, 154.2, 160.4, 170.3 ppm; HRMS (electrospray ionization, positive mode): *m/z* calcd for $C_{23}H_{27}N_2O_3$: 379.2016 $[M+H]^+$; found: 379.2011; HPLC purity: 100% (UV, t_R = min).

3-(3-((*S*)-2-Acetylamino-3-phenylpropylamino)phenyl)-2*H*-chromen-2-one (24): Reagents were 3-(3-((*S*)-2-amino-3-phenylpropylamino)phenyl)-2*H*-chromen-2-one (**18**; 10.0 mg, 0.03 mmol) and Ac_2O (2.5 μL, 0.03 mmol). Flash chromatography was conducted with CH_2Cl_2/MeOH (8:0.2). From the fractions with R_f = 0.4 (CH_2Cl_2/MeOH, 8:0.2), compound **24** (10 mg, 87%) was isolated as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.90 (s, 3H), 2.91 (d, *J* = 6.8 Hz, 2H), 3.20 (dd, *J* = 12.6, 7.4 Hz, 1H), 3.30 (m, 1H), 4.40 (m, 1H), 5.62 (d, *J* = 5.6 Hz, 1H), 6.62 (dd, *J* = 8.2, 2.2 Hz, 1H), 6.97 (d, *J* = 7.7 Hz, 2H), 7.19–7.24 (m, 4H), 7.28–7.38 (m, 5H), 7.54 (d, *J* = 7.7 Hz, 2H), 7.81 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 23.5, 38.8, 47.6, 50.4, 112.8, 113.3, 116.4, 117.6, 119.7, 124.4, 126.7, 127.8, 128.5, 128.7 (2C), 129.1 (2C), 129.4, 131.4, 135.6, 137.4, 139.8, 148.1, 153.4, 160.7, 170.8 ppm; HRMS (electrospray ionization, positive mode): *m/z* calcd for $C_{26}H_{25}N_2O_3$: 413.1865 $[M+H]^+$; found: 413.1879; HPLC purity: 100% (UV, t_R = 2.39 min).

3-(3-((*S*)-2-Acetylamino-3-(3,5-difluorophenyl)propylamino)phenyl)-2*H*-chromen-2-one (25): Reagents were 3-(3-((*S*)-2-amino-3-(3,5-difluorophenyl)propylamino)phenyl)-2*H*-chromen-2-one (**19**; 10.9 mg, 0.03 mmol) and Ac_2O (2.5 μL, 0.027 mmol). Flash chromatography was conducted with CH_2Cl_2/hexane (10:1). From the fractions with R_f = 0.4 (CH_2Cl_2/hexane, 10:1), compound **25** (9.5 mg, 77%) was isolated as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.95 (s, 3H), 2.90 (d, *J* = 7.5 Hz, 2H), 3.29 (m, 1H), 3.65 (m, 1H), 4.43 (m, 1H), 5.67 (d, *J* = 7.6 Hz, 1H), 6.75–6.78 (m, 3H), 6.97–7.02 (m, 2H), 7.23 (d, *J* = 8.8 Hz, 1H), 7.30–7.41 (m, 4H), 7.55 (d, *J* = 7.6 Hz, 1H), 7.84 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 20.5, 43.3, 46.7, 50.3, 112.5 (2C), 114.3, 115.2, 116.5, 118.9, 124.0, 126.9, 128.0, 128.3, 128.7, 131.0, 134.9, 139.6, 140.3, 143.1, 147.2, 152.3, 159.1, 160.2 (d, $^1J_{C,F}$ = 206 Hz, 2C), 171.4 ppm; HRMS (electrospray ionization, positive mode): *m/z* calcd for $C_{26}H_{23}F_2N_2O_3$: 449.4687 $[M+H]^+$; found: 449.1673; HPLC purity: 100% (UV, t_R = 2.43 min).

3-(3-((*S*)-2-Acetylamino-3-biphenylpropylamino)phenyl)-2*H*-chromen-2-one (26): Reagents were 3-(3-((*S*)-2-amino-3-biphenylpropylamino)phenyl)-2*H*-chromen-2-one (**20**; 9.82 mg, 0.02 mmol) and Ac_2O (2.1 μL, 0.022 mmol). Flash chromatography was conducted with CH_2Cl_2/hexane (10:1). From the fractions with R_f = 0.6 (CH_2Cl_2/hexane, 10:1), compound **26** (8.5 mg, 79%) was isolated as a yellow oil: ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 1.84 (s, 1H), 2.88 (dd, *J* = 13.7, 8.1 Hz, 1H), 3.01 (dd, *J* = 13.7, 5.4 Hz, 1H), 3.51 (m, 1H), 3.77 (dd, *J* = 14.6, 4.2 Hz, 1H), 4.04 (dd, *J* = 14.6, 7.6 Hz, 1H), 7.19 (d, *J* = 7.9 Hz, 2H), 7.31–7.42 (m, 5H), 7.46 (t, *J* = 7.20 Hz, 2H), 7.55 (t, *J* = 7.20 Hz, 5H), 7.65 (t, *J* = 7.9 Hz, 2H), 7.75 (t, *J* = 7.8 Hz, 2H), 8.18 (br s, 1H), 8.31 ppm (s, 1H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 22.9, 44.4, 49.8, 50.9, 115.9, 119.3, 124.7, 125.8, 126.4 (2C), 126.5, 126.7 (2C), 127.3, 128.0, 128.6, 128.8 (2C), 128.9, 129.5, 129.7 (2C), 129.9, 132.0, 135.1, 138.6, 139.6, 141.2, 142.6, 153.0, 159.6, 171.0 ppm; HRMS (electrospray ionization, positive mode): *m/z* calcd for $C_{32}H_{29}N_2O_3$: 489.2173 $[M+H]^+$; found: 489.2209; HPLC purity: 100% (UV, t_R = 2.03 min).

3-(3-((*S*)-Amino-*tert*-butylcarbamate-3-phenylpropylamino)phenyl)-2*H*-chromen-2-one (27): Under an N_2 atmosphere, 3-(3-((*S*)-2-amino-3-phenylpropylamino)phenyl)-2*H*-chromen-2-one (**18**; 10.0 mg, 0.03 mmol) was treated with di-*tert*-butyldicarbonate (6.33 mg, 0.03 mmol, 1 equiv) in CH_2Cl_2 (2 mL) for 4 h at room temperature. The reaction was diluted with CH_2Cl_2 (40 mL) and washed with saturated aq. NaCl (3×30 mL) and H_2O (3×30 mL). The organic phase was dried over Na_2SO_4 and filtered, then the solvent was removed under reduced pressure. The residue was purified by flash chromatography on a silica gel column by using CH_2Cl_2/MeOH (4:0.2) as the eluent mixture. From the fractions with R_f = 0.7 (CH_2Cl_2/MeOH, 4:0.2), compound **27** (11 mg, 86%) was isolated as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.41 (br s, 9H), 2.88 (br d, *J* = 5.9 Hz, 2H), 3.13 (m, 1H), 3.29 (dd, *J* = 12.7, 5.5 Hz, 1H), 4.10 (m, 1H), 4.58 (br, 1H), 6.61 (dd, *J* = 7.9, 1.9 Hz, 1H), 6.93 (br s, 1H), 6.99 (br d, *J* = 7.9 Hz, 1H), 7.02 (d, *J* = 7.5 Hz, 1H), 7.20–7.37 (m, 8H), 7.54 (d, *J* = 7.9 Hz, 1H), 7.81 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 28.3 (3C), 40.6, 46.8, 47.6, 77.2, 112.9, 113.2, 116.4, 117.7, 119.7, 124.3, 126.6, 127.9, 128.6, 128.7 (2C), 129.1 (2C), 129.3, 131.3, 135.6, 137.5, 139.7, 146.8, 153.4, 155.9, 160.6 ppm; HRMS (electrospray ionization, positive mode): *m/z* calcd for $C_{29}H_{31}N_2O_4$: 471.2278 $[M+H]^+$; found: 471.2333; HPLC purity: 100% (UV, t_R = 2.71 min).

3-(3-((*S*)-Amino-benzoylcarbamate-3-phenylpropylamino)phenyl)-2*H*-chromen-2-one (28): Under an N_2 atmosphere, a solution of 3-(3-((*S*)-2-amino-3-phenylpropylamino)phenyl)-2*H*-chromen-2-one (**18**; 10.2 mg, 0.03 mmol) in CH_2Cl_2 (2 mL) was treated with benzyl chloroformate (3.8 μL, 0.03 mmol, 1 equiv) for 4 h at room temper-

ature. After that, the reaction mixture was diluted with CH_2Cl_2 (50 mL) and washed with H_2O (3×25 mL). The organic phase was dried over Na_2SO_4 and filtered off, then the solvent was removed under reduced pressure. The residue was purified by flash chromatography on silica gel by employing CH_2Cl_2/MeOH (4:0.2). From the fractions with $R_f = 0.4$ (CH_2Cl_2/MeOH, 4:0.2) compound **28** (9 mg, 66%) was isolated as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta =$ 2.81 (d, $J = 6.3$ Hz, 2H), 3.31 (m, 1H), 3.57 (m, 1H), 4.00 (m, 1H), 4.16 (br s, 1H), 5.03 (s, 2H), 7.09 (d, $J = 6.8$ Hz, 2H), 7.18–7.42 (m, 13H), 7.49–7.52 (m, 3H), 7.69 (d, $J = 6.8$ Hz, 1H), 7.78 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 51.3$, 51.9, 52.9, 66.3, 116.3, 119.4, 124.0, 126.4, 127.5 (2C), 127.7, 127.9, 128.2 (3C), 128.3, 128.3 (3C), 128.6, 128.7, 128.8, 129.0, 129.1 (2C), 131.4, 135.5, 136.7, 140.3, 153.4, 155.9, 160.2 ppm; HRMS (electrospray ionization, positive mode): *m/z* calcd for $C_{32}H_{29}N_2O_4$: 505.2121 $[M+H]^+$; found: 505.2167; HPLC purity: 100% (UV, $t_R = 2.68$ min).

Biological evaluation

BACE-1 kinetics: The increase of fluorescence during the substrate cleavage was monitored by using a Tecan Safire spectrofluorimeter (working at λ_{exc} 328 nm and λ_{emi} 398 nm wavelengths) in black with clear bottom microtiter plates (Corning 3711, 384 wells). Typically, 100 mM sodium acetate buffer (pH 4.0; 9 μL) with 0.001% Triton X-100 and BACE-1 full protein (His*Tag, human, recombinant, NSO cells, purchased from Calbiochem (PF 125); 10 μL, 25 ng) were pre-incubated for 5 min at room temperature. Finally, the reaction was initiated by addition of the FRET substrate (**I**–**IV**; 1 μL) in DMSO. The final substrate concentration range was 1–100 μM (final concentration) and the final DMSO concentration range was 5–10% for all assays. The enzymatic activity was measured by monitoring the increase of fluorescence intensity at the emission maximum of 398 nm. The initial velocity, measured as fluorescence intensity per time (V_f), was calculated from the slope during the linear phase of cleavage, usually during the first 20 min. Initial velocities were converted from units of fluorescence (V_f) into concentration per unit time (V_c). Initial velocity data were fitted to the Michaelis–Menten equation by using GraphPad Prism 4.0 (GraphPad Software Inc.) to calculate the V_{max} and K_M values.

BACE-1 inhibition assay: BACE-1 full protein (His*Tag, human, recombinant, NSO cells) was purchased from Calbiochem (PF 125) and the rhodamine-derivative substrate, which contains the peptide quencher sequence, RhoRVNLDAEFK (Panvera peptide, P2985), was acquired from Invitrogen (Milan, Italy). Sodium acetate and DMSO were obtained from common commercial suppliers. Purified water from a Mili-RX system (Millipore, Milford, USA) was used to prepare buffers and standard solutions. Spectrofluorimetric analyses were carried out on Tecan Safire spectrofluorimeter (working at λ_{exc} 544 and λ_{emi} 590 nm wavelengths) by using black with clear bottom microtiter plates (Corning 3711, 384 wells). Stock solutions of tested compounds were prepared at 10 mM in DMSO and diluted with 100 mM sodium acetate buffer (pH 4) containing 0.001% Triton X-100. For each reaction, BACE-1 enzyme (10 μL; 18.6 nM, final concentration) was incubated with the tested compound (5 μL) for 60 min. The reaction was then started by addition of the peptidic FRET substrate (Panvera peptide; 1 μL; 0.25 μM, final concentration). The final volume in each reaction is 20 μL. The mixture was incubated at 28 °C for 60 min. To stop the reaction, BACE-1 stock solution (20 μL; sodium acetate, 2.5 M) was added to each well. The fluorimetric assay was followed by monitoring the increase of the fluorescence signal at 590 nm with time. The DMSO concentration in the final reaction volume was maintained at 5% to guarantee no significant loss of enzymatic activity. The fluorescence intensities in the presence and absence of inhibitor were compared, and the percent inhibition due to the presence of tested compounds was calculated. The background signal, measured in control wells containing all of the reagents except BACE-1, was subtracted for each reaction mixture. The relative percent inhibition due to the presence of eight increasing concentrations of test compounds was calculated from the equation: $100-(IF_i/IF_0 \times 100)$, in which IF_i and IF_0 are the fluorescence intensities obtained for BACE-1 in the presence and absence of inhibitor, respectively. The inhibition curves were obtained by plotting the relative inhibition or activity (%) versus the logarithm of concentration of the inhibitor. The regression parameters were determined and the IC_{50} value was extrapolated (GraphPad Prism 4.0, GraphPad Software Inc.; dose–response inhibition, log [*I*] versus normalized response).

Docking studies

All docking studies were carried out by using the Surflex docking program within the SYBYL-X1.3 program. As the receptor, the X-ray structure of β-secretase complexed with a statine peptide inhibitor, available in the Protein Data Bank (PDB ID: 1FKN), was used. The ligands were prepared by using the ligand preparation module of the SYBYL-X1.3 program. The ligand 3D input files (**2**, **5**, **7**, and **13**–**28**) were generated with the SYBYL-X1.3 program and optimized by energy minimization with the Conjugate Gradients method and the Tripos force field until the convergence criterion of 0.05 kcal (mol^{-1} $Å^{-1}$) was reached; 20 docked poses were obtained and examined visually for their interactions with the active-site residues through hydrogen and aromatic bonding interactions. The most probable docking conformations were chosen.

Acknowledgements

The authors thank the European Union for the Postdoctoral Intra-European Fellowship (IEF) Marie Curie to M.I.F.-B. in the 7th Framework (Call: FP7-PEOPLE-IEF-2008).

Keywords: aspartic proteases · drug discovery · FRET · inhibitors · peptides

[1] a) M. Tobias, L. C. Yeh, E. Johnson, *Aust. N. Z. J. Psychiatry* **2008**, *42*, 828–836; b) C. Dufouil, A. Alpérovitch, *Rev. Prat.* **2005**, *55*, 1869–1878.
[2] H. Fillit, J. Hill, *Am. J. Geriatr. Pharmacother.* **2005**, *3*, 39–49.
[3] J. T. Jarrett, E. P. Berger, P. T. Lansbury, *Biochemistry* **1993**, *32*, 4693–4697.
[4] J. Hardy, *Curr. Alzheimer Res.* **2006**, *3*, 71–73.
[5] D. Leung, G. Abbenante, D. P. Fairlie, *J. Med. Chem.* **2000**, *43*, 305–341.
[6] T. B. Durham, T. A. Shepherd, *Curr. Opin. Drug Discovery Dev.* **2006**, *9*, 776–791.
[7] B. De Strooper, W. Annaert, P. Cupers, P. Saftig, K. Craessaerts, J. S. Mumm, E. H. Schroeter, V. Schrijvers, M. S. Wolfe, W. J. Ray, A. Goate, R. A. Kopan, *Nature* **1999**, *398*, 518–522.
[8] D. W. Klaver, M. C. Wilce, H. Cui, A. C. Hung, R. Gasperini, L. Foa, D. H. Small, *Biol. Chem.* **2010**, *391*, 849–859.
[9] X. Luo, R. Yan, *Int. J. Clin. Exp. Pathol.* **2010**, *3*, 618–628.
[10] A. K. Ghosh, N. Kumaragurubaran, J. Tang, *Curr. Top. Med. Chem.* **2005**, *5*, 1609–1622.
[11] A. K. Ghosh, N. Kumaragurubaran, L. Hong, S. S. Kulkarni, X. Xu, W. Chang, V. Weerasena, R. Turner, G. Koelsch, G. Bilcer, J. Tang, *J. Med. Chem.* **2007**, *50*, 2399–2407.
[12] M. C. Maillard, R. K. Hom, T. E. Benson, J. B. Moon, S. Mamo, M. Bienkowski, A. G. Tomasselli, D. D. Woods, D. B. Prince, D. J. Paddock, T. L.

Emmons, J. A. Tucker, M. S. Dappen, L. Brogley, E. D. Thorsett, N. Jewett, S. Sinha, V. John, *J. Med. Chem.* **2007**, *50*, 776 – 781.
[13] a) M. S. Malamas, J. Erdei, I. Gunawan, J. Turner, Y. Hu, E. Wagner, K. Fan, R. Chopra, A. Olland, J. Bard, S. Jacobsen, R. L. Magolda, M. Pangalos, A. J. Robichaud, *J. Med. Chem.* **2010**, *53*, 1146 – 1158; b) M. S. Malamas, J. Erdei, I. Gunawan, K. Barnes, M. Johnson, Y. Hui, J. Turner, Y. Hu, E. Wagner, K. Fan, A. Olland, J. Bard, A. J. Robichaud, *J. Med. Chem.* **2009**, *52*, 6314 – 6323.
[14] Z. Zhu, Z. Y. Sun, Y. Ye, J. Voigt, C. Strickland, E. M. Smith, J. Cumming, L. Wang, J. Wong, Y. S. Wang, D. F. Wyss, X. Chen, R. Kuvelkar, M. E. Kennedy, L. Favreau, E. Parker, B. A. McKittrick, A. Stamford, M. Czarniecki, W. Greenlee, J. C. Hunter, *J. Med. Chem.* **2010**, *53*, 951 – 965.
[15] E. W. Baxter, K. A. Conway, L. Kennis, F. Bischoff, M. H. Mercken, H. L. Winter, C. H. Reynolds, B. A. Tounge, C. Luo, M. K. Scott, Y. Huang, M. Braeken, S. M. Pieters, D. J. Berthelot, S. Masure, W. D. Bruinzeel, A. D. Jordan, M. H. Parker, R. E. Boyd, J. Qu, R. S. Alexander, D. E. Brenneman, A. B. Reitz, *J. Med. Chem.* **2007**, *50*, 4261 – 4264.
[16] W. H. Huang, R. Sheng, Y. Z. Hu, *Curr. Med. Chem.* **2009**, *16*, 1806 – 1820.
[17] Y. S. Wang, C. Strickland, J. H. Voigt, M. E. Kennedy, B. M. Beyer, M. M. Senior, E. M. Smith, T. L. Nechuta, V. S. Madison, M. Czarniecki, B. A. McKittrick, A. W. Stamford, E. M. Parker, J. C. Hunter, W. J. Greenlee, D. F. Wyss, *J. Med. Chem.* **2010**, *53*, 942 – 950.
[18] R. Godemann, J. Madden, J. Krämer, M. Smith, U. Fritz, T. Hesterkamp, J. Barker, S. Höppner, D. Hallett, A. Cesura, A. Ebneth, J. Kemp, *Biochemistry* **2009**, *48*, 10743 – 10751.
[19] W. Yang, R. Fucini, B. T. Fahr, M. Randal, K. E. Lind, M. B. Lam, W. Lu, Y. Lu, D. R. Cary, M. J. Romanowski, D. Colussi, B. Pietrak, T. J. Allison, J. M. Munshi, D. M. Penny, P. Pham, J. Sun, A. E. Thomas, J. M. Wilkinson, J. W. Jacobs, R. S. McDowell, M. D. Balliger, *Biochemistry* **2009**, *48*, 4488 – 4496.
[20] P. D. Edwards, J. S. Albert, M. Sylvester, D. Aharony, D. Andisik, O. Callaghan, J. B. Campbell, R. A. Carr, G. Chessari, M. Congreve, M. Frederickson, R. H.-A. Folmer, S. Geschwindner, G. Koether, K. Kolmodin, J. Krumrine, R. C. Mauger, C. W. Murray, L. L. Olsson, S. Patel, N. Spear, G. Tian, *J. Med. Chem.* **2007**, *50*, 5912 – 5925.
[21] M. Congreve, G. Chessari, D. Tisi, A. J. Woodhead, *J. Med. Chem.* **2008**, *51*, 3661 – 3680.
[22] P. J. Hajduk, J. Greer, *Nat. Rev. Drug Discovery* **2007**, *6*, 211 – 219.
[23] C. W. Murray, D. C. Rees, *Nat. Chem.* **2009**, *1*, 187 – 192.
[24] C. W. Murray, T. L. Blundell, *Curr. Opin. Struct. Biol.* **2010**, *20*, 497 – 507.
[25] a) J. Barker, S. Courtney, T. Hesterkamp, D. Ullmann, M. Whittaker, *Exp. Opin. Drug Discovery* **2006**, *1*, 225 – 236; b) T. Hesterkamp, J. Barker, A. Davenport, M. Whittaker, *Curr. Top. Med. Chem.* **2007**, *7*, 1582 – 1591.
[26] M. F. Schmidt, J. Rademann, *Trends Biotechnol.* **2009**, *27*, 512 – 521.
[27] a) M. F. Schmidt, A. Isidro-Llobet, M. Lisurek, A. El-Dahshan, J. Tan, R. Hilgenfeld, J. Rademann, *Angew. Chem.* **2008**, *120*, 3319 – 3323; *Angew. Chem. Int. Ed.* **2008**, *47*, 3275 – 3278; b) M. F. Schmidt, A. El-Dahshan, S. Keller, J. Rademann, *Angew. Chem.* **2009**, *121*, 6464 – 6467; *Angew. Chem. Int. Ed.* **2009**, *48*, 6346 – 6349; c) M. F. Schmidt, M. Groves, J. Rademann, *ChemBioChem* **2011**, *12*, 2640 – 2646.
[28] L. Zhu, S. George, M. F. Schmidt, S. I. Al-Gharabli, J. Rademann, R. Hilgenfeld, *Antiviral Res.* **2011**, *92*, 204 – 212.
[29] a) S. Cosgrove, L. Rogers, C. M. Hewage, J. P. G. Malthouse, *Biochemistry* **2007**, *46*, 11205 – 11215; b) L. Toulokhonova, W. J. Metzler, M. R. Witmer, R. A. Copeland, J. Marcinkeviciene, *J. Biol. Chem.* **2003**, *278*, 4582 – 4589.
[30] F. Grüninger-Leitch, D. Schlatter, E. Küng, P. Nelböck, H. Döbeli, *J. Biol. Chem.* **2002**, *277*, 4687 – 4693.
[31] J. Ermolieff, J. A. Loy, G. Koelsch, J. Tang, *Biochemistry* **2000**, *39*, 12450 – 12456.
[32] V. Porcari, L. Magnoni, G. C. Terstappen, W. Fecke, *Assay Drug Dev. Technol.* **2005**, *3*, 287 – 297.
[33] L. Hong, G. Koelsch, X. Lin, S. Wu, S. Terzyan, A. K. Ghosh, X. C. Zhang, J. Tang, *Science* **2000**, *290*, 150 – 153.
[34] J. Marcinkeviciene, Y. Luo, N. R. Graciani, A. P. Combs, R. A. Copeland, *J. Biol. Chem.* **2001**, *276*, 23790 – 23794.
[35] P. Colantonio, L. Leboffe, A. Bolli, M. Marino, P. Ascenzi, G. Luisi, *Biochem. Biophys. Res. Commun.* **2008**, *377*, 757 – 762.
[36] N. Micale, A. P. Kozikowski, R. Ettari, S. Grasso, M. Zappala, J. J. Jeong, A. Kumar, M. Hanspal, A. H. Chishti, *J. Med. Chem.* **2006**, *49*, 3064 – 3067.
[37] J. C. Kappel, G. Barany, *J. Pept. Sci.* **2005**, *11*, 525 – 535.
[38] E. Sarubbi, P. F. Seneci, M. R. Angelastro, N. P. Peet, M. Denaro, K. Islam, *FEBS Lett.* **1993**, *319*, 253 – 256.
[39] S. I. Al-Gharabli, S. T. Shah, S. Weik, M. F. Schmidt, J. R. Mesters, D. Kuhn, G. Klebe, R. Hilgenfeld, J. Rademann, *ChemBioChem* **2006**, *7*, 1048 – 1055.
[40] a) M. Lisurek, B. Rupp, J. Wichard, M. Neuenschwander, J. P. v. Kries, R. Frank, J. Rademann, R. Kühne, *Mol. Diversity* **2010**, *14*, 401 – 408; b) Y. Cao, T. Jiang, T. Girke, *Bioinformatics* **2008**, *24*, i366 – i374.
[41] L. Piazzi, A. Cavalli, F. Colizzi, F. Belluti, M. Bartolini, F. Mancini, M. Recanatini, V. Andrisano, A. Rampa, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 423 – 426.
[42] C. Garino, T. Tomita, N. Pietrancosta, Y. Laras, R. Rosas, G. Herbette, B. Maigret, G. Quéléver, T. Iwatsubo, J. L. Kraus, *J. Med. Chem.* **2006**, *49*, 4275 – 4285.
[43] S. Marumoto, M. Miyazawa, *Bioorg. Med. Chem.* **2012**, *20*, 784 – 788.
[44] Y. H. Choi, G. H. Yon, K. S. Hong, D. S. Yoo, C. W. Choi, W. K. Park, J. Y. Kong, Y. S. Kim, S. Y. Ryu, *Planta Med.* **2008**, *74*, 1405 – 1408.
[45] Y. H. Choi, M. Y. Yoo, C. W. Choi, M. R. Cha, G. H. Yon, D. Y. Kwon, Y. S. Kim, W. K. Park, S. Y. Ryu, *Planta Med.* **2009**, *75*, 537 – 540.
[46] G. Kemmer, S. Keller, *Nat. Protoc.* **2010**, *5*, 267 – 281.
[47] W. Lin, L. Yuan, Z. Cao, Y. Feng, J. Song, *Angew. Chem.* **2010**, *122*, 385 – 389; *Angew Chem. Int. Ed.* **2010**, *49*, 375 – 379.
[48] SYBYL-X1.3: Tripos Inc., St. Louis, MO 63144 (USA).
[49] A. N. Jain, *J. Med. Chem.* **2003**, *46*, 499 – 511.
[50] A. N. Jain, *J. Comput.-Aided Mol. Des.* **2007**, *21*, 281 – 306.
[51] A. N. Jain, *J. Comput.-Aided Mol. Des.* **2009**, *23*, 355 – 374.
[52] G. Velmourougane, M. B. Harbut, S. Dalal, S. McGowan, C. A. Oellig, N. Meinhardt, J. C. Whisstock, M. Klemba, D. C. Greenbaum, *J. Med. Chem.* **2011**, *54*, 1655 – 1666.
[53] V. Limongelli, L. Marinelli, S. Cosconati, H. A. Braun, B. Schmidt, E. Novellino, *ChemMedChem* **2007**, *2*, 667 – 678.
[54] J. N. Freskos, Y. M. Fobian, T. E. Benson, M. J. Bienkowski, D. L. Brown, T. L. Emmos, R. Heintz, A. LaBorde, J. J. McDonald, B. V. Mischke, J. M. Molyneaux, J. B. Moon, P. B. Mullins, D. B. Prince, D. J. Pakkock, A. G. Tomaselli, G. Winterrowd, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 73 – 77.
[55] J. N. Freskos, Y. M. Fobian, T. E. Benson, J. B. Moon, M. J. Bienkowski, D. L. Brown, T. L. Emmons, R. Heintz, A. Laborde, J. J. McDonald, B. V. Mischke, J. M. Molyneaux, P. B. Mullins, D. B. Prince, D. J. Paddock, A. G. Tomasselli, G. Winterrowd, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 78 – 81.
[56] S. Weik, T. Luksch, A. Evers, J. Böttcher, C. A. Sotriffer, A. Hasilik, H. G. Löffler, G. Klebe, J. Rademann, *ChemMedChem* **2006**, *1*, 445 – 457.
[57] a) K. Bornemann, H. Nar, M. Bauer, S. Handschuh, M. Kostka, C. Dorner-Ciossek, W. Stransky, K. Fuchs, C. Eickmeier, S. Peters, K. Klinder, J. Rademann, S. Weik, *PCT Int. Appl.* WO2005113582 A1, **2005**; b) C. Eickmeier, K. Fuchs, S. Peters, C. Dorner-Crossek, N. Heine, S. Handschuh, K. Klinder, M. Kostka, *PCT Int. Appl.* WO2006103038 A1, **2006**.
[58] L. Piazzi, A. Cavalli, F. Belluti, A. Bisi, S. Gobbi, S. Rizzo, M. Bartolini, V. Andrisano, M. Recanatini, A. Rampa, *J. Med. Chem.* **2007**, *50*, 4250 – 4254.
[59] Y.-C. Cheng, W. H. Prusoff, *Biochem. Pharmacol.* **1973**, *22*, 3099 – 3108.

Received: February 20, 2013
Revised: April 25, 2013
Published online on June 11, 2013