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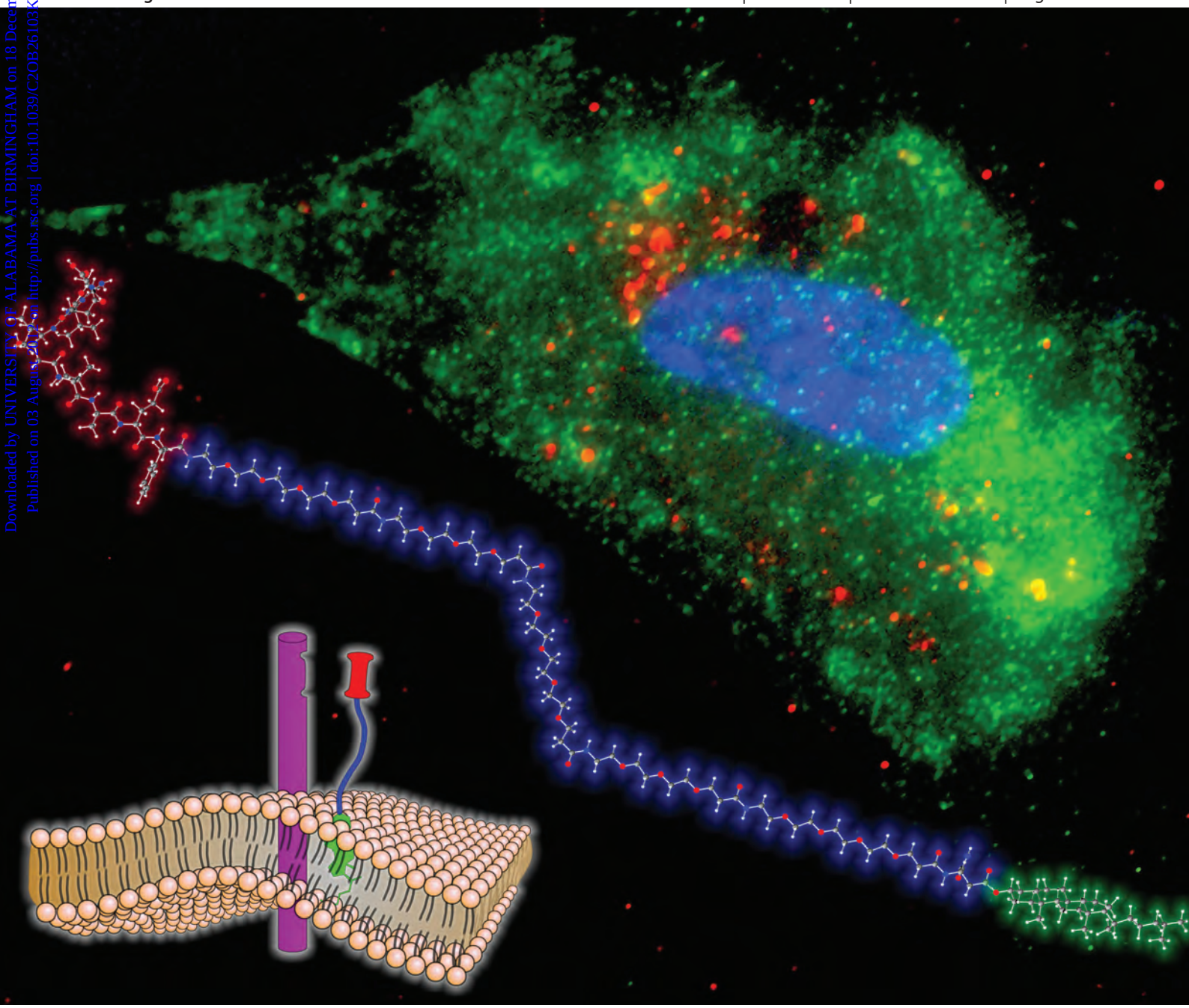

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PAPER

Optimisation of BACE1 inhibition of tripartite structures by modification of membrane anchors, spacers and pharmacophores – development of potential agents for the treatment of Alzheimer's disease†

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Systematic variation of membrane anchor, spacer and pharmacophore building blocks leads to an optimisation of the inhibitory effect of tripartite structures towards BACE1-induced cleavage of the amyloid precursor protein (APP).

Introduction

Alzheimer's disease (AD) is the most common cause of dementia and represents a growing burden for aging societies.[1] In 2006, 26.6 million people were estimated to be affected worldwide by this neurodegenerative disease and projection forecasts the number of AD patients to rise to over 100 million in 2050.[2]

According to the amyloid cascade hypothesis,[3,4] accumulation and aggregation of amyloid β-peptides (Aβ-peptides) in the brain plays a crucial role in the development and progression of AD. Aβ-peptides are produced by sequential proteolytic cleavages of the amyloid precursor protein (APP) executed by two membrane-associated enzymes called β- and γ-secretase. The transmembrane protein aspartyl protease β-site APP cleaving enzyme 1 (BACE1) (also called Asp2 or memapsin2) was discovered and cloned by several groups in 1999.[5–9] Subsequently, BACE1 was shown to represent the primary β-secretase activity in cultured cells and *in vivo*.[10] BACE1 cleaves APP in the lumenal/extracellular region to release a soluble, extracellular N-terminal fragment (sAPPβ).[11] The residual membrane-tethered C-terminal fragment C99 is subsequently cleaved by γ-secretase to generate different Aβ-peptides and the APP intracellular domain (AICD). BACE1 represents a potential therapeutic target for reducing the formation of Aβ-peptides. Several BACE1 inhibitors have been reported.[12–15] BACE1 appears to have diverse substrates and important physiological functions. Therefore, very careful dosing of BACE1-inhibiting drugs may be required to minimise possible adverse side effects.[10]

Mature BACE1 cycles between the plasma membrane, the endosomal compartment and the trans-Golgi network. The proteolytic activity of BACE1 on wild-type APP appears to be mainly confined to the endocytotic pathway[16,17] and can be increased by stimulating BACE1 internalisation.[18] Therefore, an efficient BACE1 inhibitor should be either membrane-permeable or targeted to the membrane to become available in the endosomes. Lipid rafts, liquid-ordered membrane domains enriched in cholesterol and sphingolipids,[19,20] appear to be important for BACE1 cleavage of APP. BACE1 has been detected in lipid raft fractions[21] and reduction of cholesterol was shown to decrease cellular Aβ-production.[22,23]

In order to achieve high local concentrations of BACE1 inhibitors in designated subcellular regions,[16] we have reported the "tripartite structure" concept in 2005.[24] Tripartite structures are assembled by covalent coupling of BACE1 inhibitors with membrane anchors *via* suitable spacers. The spacer is designed to keep the inhibitor at an appropriate distance from the plasma membrane. The membrane anchor should bind the tripartite structure preferentially to the lipid raft domains of the membrane. Thus, an enrichment of the tripartite structure in the lipid rafts is achieved with "raftophilic" membrane anchors (Fig. 1).

Tripartite structures are rapidly internalised by endocytosis and inhibit BACE1 much more efficiently than the corresponding free inhibitors in cell lines and primary neuronal cell culture.[25,26] Moreover, they were reported to be active *in vivo*

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†Electronic supplementary information (ESI) available: Analytical HPLC chromatograms, low resolution and high resolution ESI mass spectra (and ^{1}H and ^{13}C NMR spectra) of **6a–6j**, **7a–7q**, **9–11**, **13**, **14**, **16**, **18–20**; ^{1}H NMR spectra for **33** and **41**; ^{1}H and ^{13}C NMR spectra for **24**, **28**, **32**, **47** and **48**; log EC_{50} values and SD for all tested compounds; for tripartite structures with different membrane anchors: comparison of EC_{50} values calculated by averaging log EC_{50} values of three or more independent experiments and EC_{50} values calculated from non-linear regression of pooled data and pairwise comparison of log EC_{50} values by ANOVA and Tukey's post-test; detailed statistical comparison of the tripartite structures **19** and **20** with the pharmacophores **17**, **50** and **51**. See DOI: 10.1039/c2ob26103k

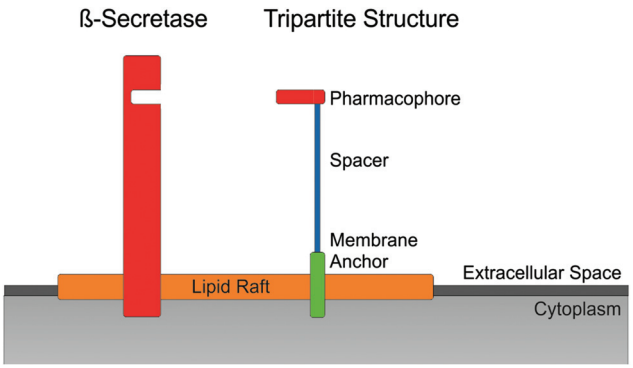


Fig. 1 Tripartite structure concept. Anchoring of the pharmacophore (BACE1 inhibitor) *via* an appropriate spacer to a raftophilic membrane anchor leads to an increase of the inhibitor concentration in the proximity of the active site of BACE1 as both are located in the lipid rafts.

when injected stereotactically into the hippocampus of transgenic APPsw/PSΔ9 mice.[25]

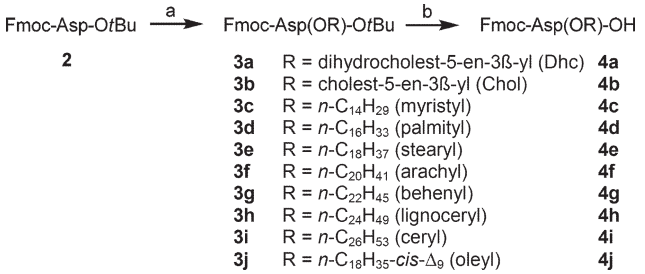
Herein, we describe the synthesis of novel tripartite structures and extensive studies regarding their structure–activity relations (SAR) in cell-based assays. In continuation of our previous work,[24–26] we report in detail the effect of different lipophilic membrane anchors and spacers on the activity of tripartite structures carrying the prototype peptidic pharmacophore GL189. Furthermore, we show that the potency of different non-peptidic transition state BACE1 inhibitors is substantially improved by incorporation into our tripartite structures.

Results and discussion

Tripartite structures were assembled from preformed building blocks by solid-phase peptide synthesis. The building blocks, used as pharmacophores, spacers and lipid anchors, were either obtained commercially or synthesised as described below. For a detailed SAR study by variation of lipid anchor and spacer modules, we used the same peptidic substrate-based inhibitor as before (GL189 = H-Glu-Val-Asn-Sta-Val-Ala-Glu-Phe-NH_2).[27] Modifications of further literature-known BACE1 inhibitors, obtained by *de novo* synthesis, provided additional building blocks for solid-phase peptide synthesis.

The membrane anchor building blocks were obtained by esterification of lipidic alcohols, *e.g.* dihydrocholesterol (Dhc-OH) with the protected amino acid Fmoc-Asp-O*t*Bu (**1**) using 1-(2-mesitylensulfonyl)-3-nitro-1*H*-1,2,4-triazole (MSNT) or *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl) (Scheme 1). Cleavage of the *tert*-butyl esters **2a–j** with trifluoroacetic acid afforded the Fmoc-protected building blocks **3a–j**, suitable for solid-phase peptide synthesis.

Ideally, the inhibitor should be positioned by the spacer close to the active site of BACE1 by keeping a similar distance from the plasma membrane. Moreover, the spacer should not affect the interaction between BACE1 and the inhibitor. We have chosen oligoethylene glycol units as spacer modules due to their low toxicity, high bio-compatibility, high stability and good solubility under physiological conditions. To investigate the potential influence of the chemical composition of spacer modules, the



Scheme 1 Synthesis of membrane anchor building blocks **3a–j**. *Reagents and conditions*: (a) 1.1 equiv. Fmoc-Asp-O*t*Bu (**1**), 1.0 equiv. Dhc-OH (or Chol-OH, $C_nH_{2n+1}OH$ (n = 14, 16, 18, 20, 22, 24, 26) or oleyl-OH), 1.1 equiv. MSNT and 1.1 equiv. MeIm (or 1.5 equiv. EDC·HCl cat. DMAP), CH_2Cl_2, rt, 4–52 h; (b) CF_3COOH, CH_2Cl_2 (1 : 0.8–1.5), rt, 90 min, **3a**: 71%, **3b**: 61%, **3c**: 67%, **3d**: 64%, **3e**: 76%, **3f**: 68%, **3g**: 92%, **3h**: 70%, **3i**: 80%, **3j**: 65% (yields over two steps).

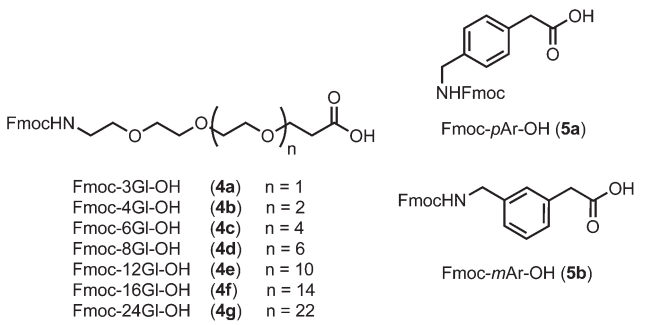


Fig. 2 Building blocks for the spacer moiety of the tripartite structures.

aminomethylphenylacetic acids **5a** and **5b** were tested as alternative building blocks. Using the commercially available *N*-Fmoc-ω-amino(oligoethylene glycol)carboxylic acids **4a–g** or the *N*-Fmoc-aminomethylphenylacetic acids **5a** and **5b** as building-blocks, spacers of various lengths were prepared. The *N*-Fmoc-protected carboxylic acids were directly suitable for solid-phase peptide synthesis (Fig. 2).

The tripartite structures were prepared by automated solid-phase peptide synthesis and purified by preparative HPLC (see the Experimental section). The structures were supported by mass spectrometry. Previous advanced NMR experiments confirmed that the peptide synthesis proceeded without epimerisation and single diastereoisomers were obtained.[26] According to HPLC analysis, the purities of tripartite structures generally were higher than 99.9% (Table 1).

As described previously, the biological activity of the tripartite structures was determined by measuring the concentration of Aβ40 peptides secreted by cultured SH-SY5Y cells overexpressing human wild-type APP695.[26]

Variation of the membrane anchor

The affinity of membrane anchors to lipid rafts depends on their structural features. Therefore, we have studied the effect of different membrane anchors on the efficiency of tripartite structures as BACE1 inhibitors in a cellular assay. Assuming that amyloidogenic APP cleavage by BACE1 is dependent on lipid

Table 1 Analytical data for the tripartite structures **6a–j**, **7a–q**, **9–11**, **13**, **14**, **16** and **18–20**

No.	Retention time[a] [min]	Purity[b] [%]	*m/z*[c] (calcd)	*m/z*[d] (found)
6a	27.5	>99.9	2595.5516	2595.5529
6b	26.5	>99.9	2593.5360	2593.5356
6c	20.4	99.1	2421.4108	2421.4113
6d	22.3[e]	>99.9	2449.4421	2449.4423
6e	24.7	>99.9	2477.4734	2477.4737
6f	27.2	>99.9	2505.5047	2505.5050
6g	29.4	99.9	2533.5360	2533.5346
6h	31.8	>99.9	2561.5673	2561.5669
6i	34.3	>99.9	2589.5986	2589.6005
6j	23.1[e]	99.9	2475.4577	2475.4522
7a	29.7	99.4	1783.0886	1783.0863
7b	29.2	99.9	1854.1257	1854.1267
7c	30.0	99.9	1871.1410	1871.1423
7d	27.8	>99.9	1898.1519	1898.1530
7e	27.9	>99.9	2057.2415	2057.2411
7f	29.8	97.8	2047.2459	2047.2499
7g	27.1	>99.9	2101.2677	2101.2650
7h	27.6	>99.9	2118.2830	2120.0[g]
7i	28.2	>99.9	2145.2939	2145.2938
7j	19.5[f]	>99.9	2295.3310	2297.4[g]
7k	20.1[f]	99.4	2295.3310	2295.3295
7l	29.1[e]	>99.9	2189.3201	2189.3208
7m	29.0	>99.9	2223.3507	2223.3509
7n	27.5	>99.9	2260.3572	2260.3574
7o	27.7	99.1	2436.4621	2436.4628
7p	28.8	99.8	2575.5605	2575.5616
7q	27.3	99.6	2683.6041	2683.6069
9	34.2	98.8	2210.3054	2211.8[g]
10	32.9	>99.9	2254.2952	2254.2970
11	30.4	>99.9	2180.2796	2180.2792
13	32.9	99.3	1970.0828	1970.0832
14	33.1	>99.9	1984.0985	1984.0994
16	30.8	99.9	2192.3966	2192.3962
18	27.3	>99.9	2105.3042	2105.3043
19	27.0	>99.9	2210.3620	2210.3630
20	27.6	>99.9	2195.3511	2195.3513

[a] HPLC retention time: column: Vydac 208TP104 (reverse-phase C_8, 4.6 × 250 mm). Flow rate: 1.0 mL min^{-1}. Eluent A: H_2O + 0.1% CF_3COOH, eluent B: MeCN + 0.1% CF_3COOH. Gradient from 20% to 90% B in 35 min. [b] Analysis by an evaporative light scattering detector. [c] Monoisotopic mass. [d] Reconstructed monoisotopic mass. [e] Eluent A: H_2O/MeCN (85 : 15) + 0.1% CF_3COOH, eluent B: MeCN + 0.1% CF_3COOH. Gradient from 10% to 100% B in 45 min. [f] Eluent A: H_2O/MeCN (85 : 15) + 0.1% CF_3COOH, eluent B: MeCN + 0.1% CF_3COOH. Gradient from 30% to 100% B in 35 min. [g] Most abundant mass observed for the adduct $[M + H]^+$.†

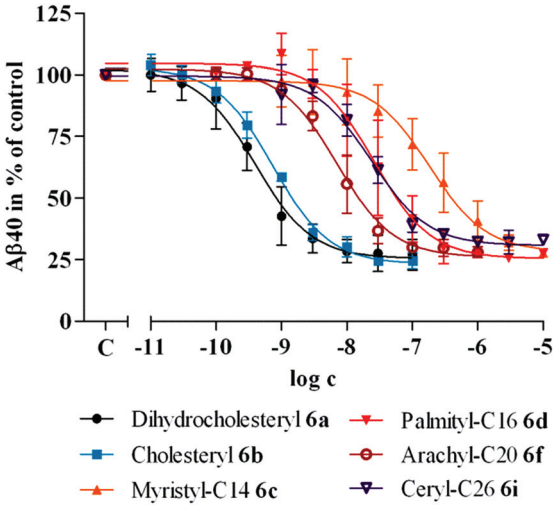


Fig. 3 Selected dose–response curves for quantifying the inhibition of Aβ40 secretion by tripartite structures with different membrane anchors. Aβ40 concentrations in cell culture supernatants are expressed as percent of the control condition C (1% DMSO). Compounds **6a** and **6b** were tested at concentrations ranging from 10 pM to 100 nM in SH-SY5Y APPwt cells. **6c** and **6i** were tested at concentrations from 1 nM to 10 μM; **6d** was tested with alternating concentration ranges (once: 100 pM to 1 μM; twice: 1 nM to 10 μM); **6f** was tested at concentrations from 100 pM to 1 μM. The dose–response curves were generated by pooling normalised data (means and SD) from three independent experiments (ESI, Table S2†). It should be noted that the concentration ranges were varied between experiments for some compounds. In cases where error bars are shown, they represent data from at least two independent experiments.

rafts, tripartite structures with very efficient raftophilic membrane anchors should exhibit the highest potency. The tripartite structures **6a–j** (Table 1 and 2) were synthesised using the membrane anchor building blocks **3a–j** (Scheme 1), the peptidic inhibitor GL 189 and the 4Gl-3Gl-4Gl-3Gl-4Gl spacer module.

The spacer length was kept constant (approx. 89 Å, assuming an extended conformation of the spacer) to have results comparable with existing data.[26] The effect of **6a**, **6c**, **6d** and **6j** on the generation of sAPPβ by HeLa cells overexpressing the Swedish-mutant form of human APP was studied before at two concentrations (20 and 200 nM).[25] The tripartite structure **6a** with the dihydrocholesteryl anchor was reported to be most potent. Compound **6j** with a *cis* double bond in the oleyl anchor was found to be essentially inactive. Overall, the efficiency of different tripartite structures in BACE1 inhibition appeared to resemble their degree of raft partitioning. In our previous study, cells expressing Swedish-mutant or wild-type APP showed differences with respect to the BACE1 inhibiting activity of tripartite structures and the secretion of Aβ-peptides.[26] Therefore, we conducted an extensive comparative study of the activity of tripartite structures with a range of different membrane anchors (long-chain lipid alcohols ranging from C_{14}–C_{26}, dihydrocholesterol and cholesterol) in SH-SY5Y cells overexpressing wild-type APP. To compare the tripartite inhibitors on the basis of reliable EC_{50} values, each compound was tested in serial dilutions in at least three independent experiments. In addition, for some compounds the range of tested concentrations was varied between different experiments to improve the accuracy of the determination of top and bottom plateaus of the dose–response curves. Consequently, not each single compound dilution depicted below was tested in all three experiments. The dose–response curves for tripartite compounds **6a–d**, **6f** and **6i** are shown in Fig. 3.

The tripartite structures **6a** and **6b** with cholesterol and dihydrocholesterol as raftophilic membrane anchors reduced very efficiently Aβ40 release into the cell culture medium. The EC_{50} values of 0.39 (log EC_{50} = −9.406 ± 0.182) and 0.74 nM (log EC_{50} = −9.130 ± 0.035) calculated for **6a** and **6b** were substantially lower than those of the tripartite structures **6c–i** with long-chain lipid alcohols as membrane anchors (Table 2). To evaluate the statistical significance of the observed differences, we compared the log EC_{50} values by one-way ANOVA and Tukey's multiple comparison post-test. The difference in log EC_{50} between **6a** (dihydrocholesteryl anchor, Dhc) and **6b** (cholesteryl anchor,

Table 2 Tripartite structures **6a–j** with different membrane anchors

No.	Sequence[a]	EC_{50} [b] [nM]
6a	Inhibitor-Spacer-Asp(ODhc)-NH_2	0.39
6b	Inhibitor-Spacer-Asp(OChol)-NH_2	0.74
6c	Inhibitor-Spacer-Asp(O-*n*$C_{14}H_{29}$)-NH_2	177.01
6d	Inhibitor-Spacer-Asp(O-*n*$C_{16}H_{33}$)-NH_2	24.60
6e	Inhibitor-Spacer-Asp(O-*n*$C_{18}H_{37}$)-NH_2	9.16
6f	Inhibitor-Spacer-Asp(O-*n*$C_{20}H_{41}$)-NH_2	6.82
6g	Inhibitor-Spacer-Asp(O-*n*$C_{22}H_{45}$)-NH_2	7.24
6h	Inhibitor-Spacer-Asp(O-*n*$C_{24}H_{49}$)-NH_2	7.66
6i	Inhibitor-Spacer-Asp(O-*n*$C_{26}H_{53}$)-NH_2	23.39
6j	Inhibitor-Spacer-Asp(O-oleyl)-NH_2	n.d.[c]

[a] Inhibitor: H-Glu-Val-Asn-Sta-Val-Ala-Glu-Phe; spacer: 4Gl-3Gl-4Gl-3Gl-4Gl. [b] Half maximal effect (EC_{50}) of inhibition of Aβ40 secretion from treated cells relative to control (1% DMSO). Log EC_{50} was determined by non-linear regression from dose–response curves. The value given is the delogarithmised mean value of three independent experiments (ESI†). [c] n.d. = not determined, **6j** was inactive at all concentrations tested (up to 100 nM).

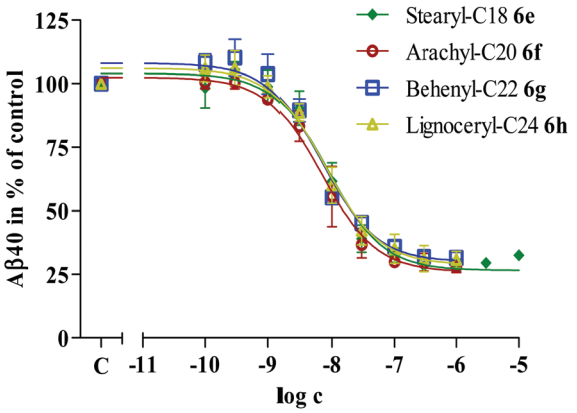


Fig. 4 Selected dose–response curves. Inhibition of Aβ40 secretion by tripartite structures with different lipid alcohol anchors. Aβ40 concentrations in cell culture supernatants are expressed as percent of the control condition C (1% DMSO). Compound **6e** was tested with alternating concentration ranges (once: 1 nM–10 μM; twice: 100 pM–1 μM); compounds **6f** and **6h** were tested at concentrations ranging from 100 pM to 1 μM in SH-SY5Y APPwt cells. Compound **6g** was tested twice at nine different concentrations from 100 pM–1 μM, while in the third experiment, a reduced number of only six concentrations was tested, ranging from 3 nM to 1 μM. Dose–response curves were generated by pooling normalised data (means and SD) from three independent experiments.†

Chol) did not reach statistical significance. However, both were significantly lower than each single log EC_{50} value obtained for **6c–6i** (for pairwise comparison of different anchors, see ESI, Table S1†). This result is explained by the different partitioning of sterol and lipid alcohol anchors between raft and non-raft phases. As dihydrocholesteryl anchors bind more efficiently to the raft phase,[25] the tripartite structures **6a** and **6b** should be available in higher concentrations at the subcellular site of amyloidogenic APP cleavage. Within the series of tripartite structures with lipid alcohol anchors, a chain length of 18 to 24 carbon atoms provided the best result. Compounds **6e–h** were equally potent (Fig. 4), whereas the log EC_{50} of **6c** (log EC_{50} = −6.752 ± 0.266) with the shortest lipid chain (C_{14}) was significantly higher than all other log EC_{50} values in this series ($p < 0.001$; ESI, Table S1†). Compared to compounds **6e–h**, the potency appeared to decrease for **6d** (log EC_{50} = −7.609 ± 0.342) and **6i** (log EC_{50} = −7.631 ± 0.124) with chain lengths of 16 and 26 atoms, albeit statistical significance was not reached. It is assumed that the observed influence of the chain length within lipid alcohol anchors may reflect subtle differences in the distribution between raft and non-raft domains.

Variations of the spacer module

According to the original tripartite structure concept,[24] the length of the spacer linking the pharmacophore to the membrane anchor was expected to play a crucial role for the inhibitory activity. The optimum spacer should position the pharmacophore at a distance from the plasma membrane approximately matching that of the BACE1 active site (or that of the β-secretase cleavage site within APP) (Fig. 1). Theoretical spacer lengths of our tripartite inhibitors have been estimated assuming an all-*anti*-conformation for the chain. Therefore, these lengths correspond to a maximal theoretical length irrespective of the actual conformation of the spacer. In a previous study, tripartite structures with theoretical spacer lengths ranging from 35 to 89 Å were shown to efficiently reduce cellular Aβ production (compound **6a** with an 89 Å spacer displayed the lowest EC_{50} value).[26] To extend our previous work and to investigate the influence of both, spacer length and chemical composition, we tested a series of tripartite structures with theoretical spacer lengths ranging from 26 to 96 Å. The subtle variations in length in this range were implemented by different combinations of the spacer building blocks **4a–c**, thus providing eleven tripartite structures: **6a**, **7b**, **7d**, **7e**, **7g–i**, **7l**, **7n**, **7o** and **7q** (Table 1 and 3).

Spacers with different chemical entities were tested. Previously, different spacer lengths ranging from 35 to 123 Å were assembled by amide coupling of the ω-aminooligoethylene glycol carboxylic acids **4a** and **4b**. In compounds **7a**, **7c**, **7f**, **7m** and **7p** one-block oligoethylene glycol units have been used as spacers. They derive from the ω-aminooligoethylene glycol carboxylic acids **4c–g** containing 6, 8, 12, 16 and 24 oligoethylene glycol units and thus have no amide bonds within the spacer module. The phenylacetic acids **5a** and **5b** were used to construct a further type of spacer and led to the tripartite structures **7j** and **7k** with more lipophilic aromatic moieties in the spacer module (Fig. 2).

In order to get data which are comparable to the previous ones, dihydrocholesterol was used as a lipid anchor and the peptidic transition state inhibitor GL189 as a pharmacophore. The tripartite structures **6a** and **7a–q** were shown to be highly active (Table 3). Irrespective of the chemical composition of the spacer and the theoretical length (ranging from 26–96 Å), **6a** and **7a–q** exhibited EC_{50} values below 7 nM. This finding indicates that within these limits the activities of our tripartite structures are relatively independent of the structure of the spacer module.

As shown in Fig. 5, the log EC_{50} values of the tripartite structures **6a** and **7a–q** did not show a single minimum. The compounds could be roughly divided into two groups with EC_{50}

Table 3 Tripartite structures **6a** and **7a–q** with different spacers

No.	Sequence[a]	Spacer length[b] [Å]	EC_{50} [c] [nM]
7a	Inhibitor-6Gl-Raftophile	26	0.56
7b	Inhibitor-3Gl-3Gl-Raftophile	31	0.53
7c	Inhibitor-8Gl-Raftophile	33	0.43
7d	Inhibitor-3Gl-4Gl-Raftophile	35	2.64[d]
7e	Inhibitor-3Gl-3Gl-3Gl-Raftophile	47	1.11[d]
7f	Inhibitor-12Gl-Raftophile	48	0.37
7g	Inhibitor-3Gl-4Gl-3Gl-Raftophile	51	0.51
7h	Inhibitor-6Gl-6Gl-Raftophile	53	0.52
7i	Inhibitor-4Gl-3Gl-4Gl-Raftophile	53	0.44
7j	Inhibitor-*p*Ar-3Gl- *p*Ar -3Gl-*p*Ar-Raftophile	53	0.45
7k	Inhibitor- *m*Ar-3Gl-*m*Ar-3Gl-*m*Ar-Raftophile	57	0.59
7l	Inhibitor-4Gl-4Gl-4Gl-Raftophile	58	0.81
7m	Inhibitor-16Gl-Raftophile	61	6.18
7n	Inhibitor-3Gl-3Gl-3Gl-3Gl-Raftophile	63	1.99[d]
7o	Inhibitor-4Gl-4Gl-4Gl-4Gl-Raftophile	77	0.67
6a	Inhibitor-4Gl-3Gl-4Gl-3Gl-4Gl-Raftophile	89	0.39[d]
7p	Inhibitor-24Gl-Raftophile	91	0.79
7q	Inhibitor-4Gl-4Gl-4Gl-4Gl-4Gl-Raftophile	96	1.55

[a] Inhibitor: H-Glu-Val-Asn-Sta-Val-Ala-Glu-Phe; raftophile: Asp(ODhc)-NH_2. [b] Maximal theoretical spacer length, assuming an all-*anti* conformation. [c] Half maximal effect (EC_{50}) of inhibition of Aβ40 secretion from treated cells relative to control (1% DMSO). The value given is the delogarithmised mean log EC_{50} of three independent experiments, except for **6a**, which was tested in eight independent experiments. All compounds were tested at concentrations from 10 pM to 100 nM, except for **7m**, which was tested at alternating ranges (once: 10 pM–100 nM; twice: 100 pM–1 μM). Log EC_{50} was determined by non-linear regression from dose–response curves (ESI, Table S2†). [d] EC_{50} values of compounds **6a**, **7d**, **7e** and **7n** were also reported in ref. 26. Subtle differences are due to adjustments in the statistical analysis of the data allowing direct comparison (ESI, Table S2†).

values below and above 1 nM, respectively. Interestingly, the difference in potency does not seem to depend on the chemical composition of the spacer. Among the structures with one-block oligoethylene glycol spacers, the four compounds **7a**, **7c**, **7f** and **7p** exhibit EC_{50} values < 1 nM (**7a**: EC_{50} = 0.56 nM; log EC_{50} = −9.248 ± 0.097; **7c**: EC_{50} = 0.43 nM; log EC_{50} = −9.368 ± 0.228; **7f**: EC_{50} = 0.37 nM; log EC_{50} = −9.436 ± 0.038; **7p**: EC_{50} = 0.79 nM; log EC_{50} = −9.104 ± 0.113), whereas the value of compound **7m** was much higher (EC_{50} = 6.18 nM, log EC_{50} = −8.209 ± 0.216). Spacer modules containing phenylacetic acid as in **7j** and **7k** did not affect the activity of the tripartite structures as compared to spacers consisting exclusively of oligoethylene glycol units with theoretical spacer lengths from 48 Å to 58 Å (**7j**: EC_{50} = 0.45 nM; log EC_{50} = −9.343 ± 0.080; **7k**: EC_{50} = 0.59 nM; log EC_{50} = −9.229 ± 0.282) (Table 3). For a statistical evaluation of the results, **6a** was chosen as the reference. The mean log EC_{50} values of each spacer variation were compared to the mean log EC_{50} of **6a** by one-way ANOVA followed by Dunnett's multiple comparison post-test. The mean log EC_{50} values of the tripartite structures **7d** (log EC_{50} = −8.578 ± 0.327), **7e** (log EC_{50} = −8.954 ± 0.318), **7m** (log EC_{50} = −8.209 ± 0.216), **7n** (log EC_{50} = −8.702 ± 0.252) and **7q** (logEC_{50} = −8.810 ± 0.055) were significantly different from that of **6a** (log EC_{50} = −9.406 ± 0.182).

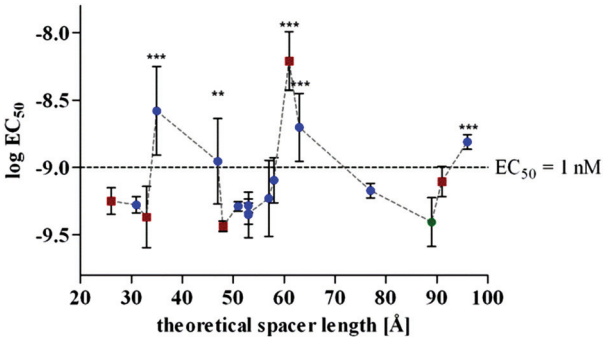


Fig. 5 Dependency of the log EC_{50} from the theoretical spacer length for tripartite structures with oligoethylene glycol spacers. Compounds **7b**, **7d**, **7e**, **7g–7l**, **7n**, **7o** and **7q** with assembled spacers are depicted as blue dots. Compounds **7a**, **7c**, **7f**, **7m** and **7p** with one-block spacers without internal amide bonds are depicted as red squares. To assess the statistical significance, the mean log EC_{50} values were compared to that of compound **6a** (green dot) by one way-ANOVA combined with Dunnett's multiple comparison post-test. The mean log EC_{50} values of compounds **7d**, **7e**, **7m**, **7n** and **7q** were significantly different from that of **6a** (**p < 0.01; ***p < 0.001) and higher than −9 (EC_{50} = 1 nM).

Apparent minima of the EC_{50} values were observed for tripartite structures with theoretical spacer lengths of 33 Å (**7c**: EC_{50} = 0.43 nM; log EC_{50} = −9.368 ± 0.228), 48 Å (**7f**: EC_{50} = 0.37 nM; log EC_{50} = −9.436 ± 0.038) and 89 Å (**6a**: EC_{50} = 0.39 nM; log EC_{50} = −9.406 ± 0.182). However, it should be noted that the subtle differences of activity of tripartite structures with EC_{50} values <1 nM did not reach statistical significance. In summary, our results indicate that the relative potency of the tripartite structures with theoretical spacer lengths ranging from 26 to 96 Å did neither solely depend on the number of amide groups nor on the chemical composition of the spacers. Oligoethylene glycols can form helical structures in water,[28] and thus it does not seem likely that the spacer molecules adopt a fully extended conformation in a physiological environment. Interactions with the aqueous medium and with cell membrane-related components may influence the conformation of the spacer and thus affect the potency of the tripartite structure.

Variation of the BACE1 inhibitor

Membrane anchoring considerably increases the efficacy of the peptidic BACE1 inhibitor GL189 in cellular assays.[24–26] In the present study, the tripartite structure concept has been tested on four literature-known BACE1 inhibitors: **8**, **12**, **15** and **17** (Fig. 6).[29–32] The inhibitors were adapted for solid-phase peptide synthesis to enable the attachment of a spacer and a raftophile. Thus, the structures had to be modified by adding a carboxyl group which served as a coupling partner for the N-terminus of the spacer. The modified pharmacophores were synthesised following literature procedures.[29–32] The additional carboxyl groups either derive from modified protected starting materials or from attachment of carboxyl group-bearing moieties at a later stage of

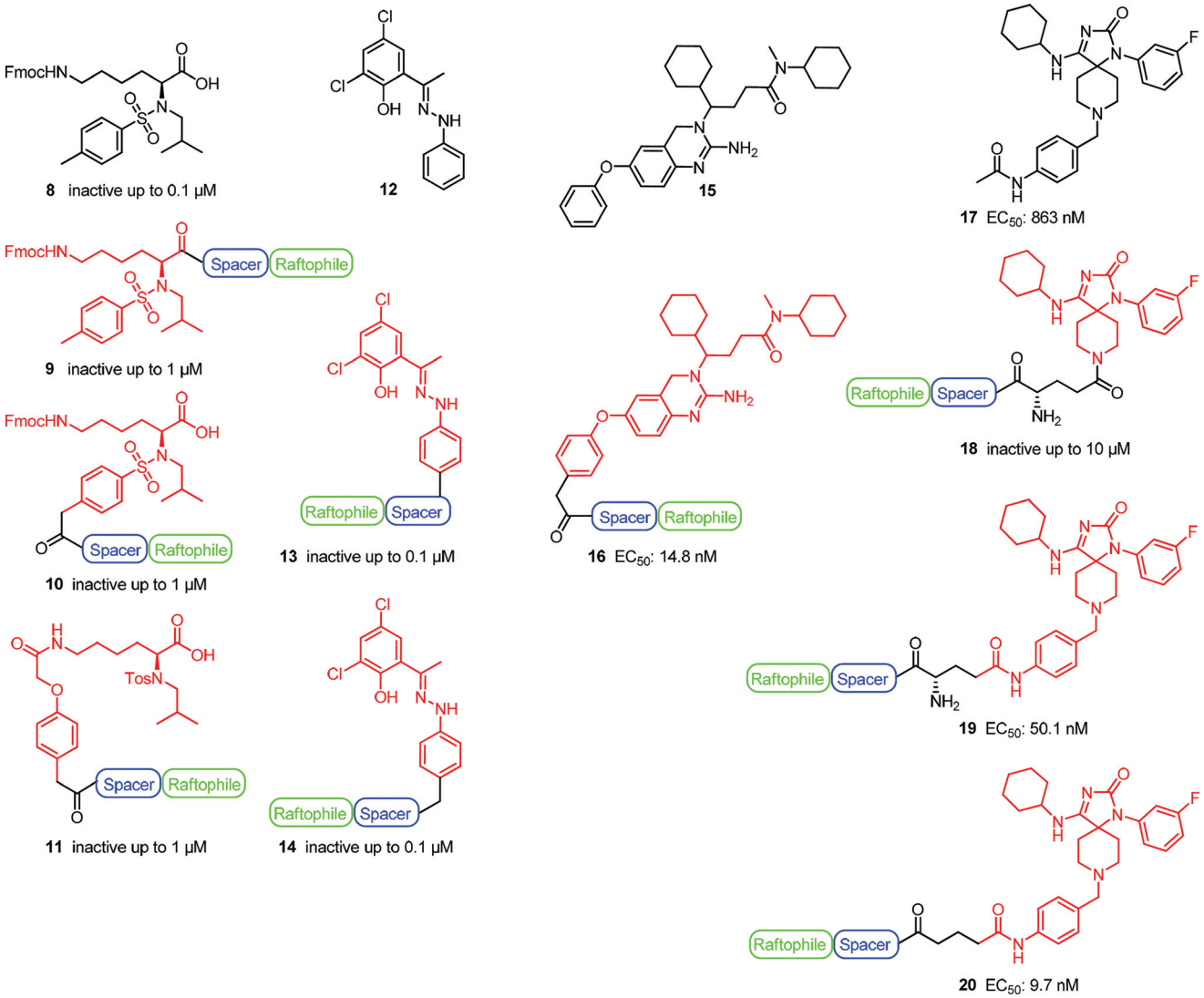
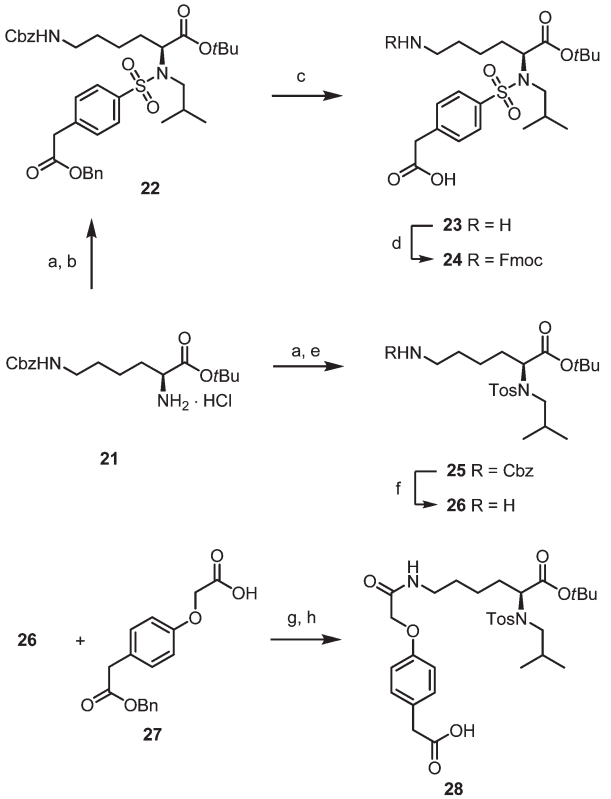


Fig. 6 BACE1 inhibitors **8**, **12**, **15**, **17** and tripartite analogues **9–11**, **13**, **14**, **16**, **18–20**. Spacer–Raftophile: -4Gl-3Gl-4Gl-3Gl-4Gl-Asp(ODhc)-NH_2. EC_{50}: inhibitor concentration at which inhibition of Aβ40 secretion from treated cells was half-maximal compared to the control condition (1% DMSO). The value given is the delogarithmised mean log EC_{50} of three independent experiments. Log EC_{50} was determined by non-linear regression from dose–response curves (ESI†).

the synthesis. Thus, the synthetic routes were kept similar to the original procedures and the building blocks for the solid-phase synthesis were obtained in a convergent manner.

The first small-molecule inhibitor examined was the lysine derivative **8** (Elan Pharmaceuticals/Pharmacia & Upjohn; Fig. 6).[13,29] A K_i value of 5 nM was reported for that compound. Tripartite structure **9** was obtained by coupling of the free carboxylic acid **8** to the spacer–membrane anchor module (-4Gl-3Gl-4Gl-3Gl-4Gl-Asp(ODhc)-NH_2). The tripartite structure **9** was reported to be an active inhibitor of BACE1 in cell-based assays.[26] This finding could not be reproduced and our present study demonstrated that the previous result was caused by an impurity.

In a previous structure–activity study, the isobutyl group at the sulfonamide and the free carboxylic acid were found to be crucial for the inhibitory effect of **8**.[29] Thus, the carboxylic acid group of compound **8** should not be functionalised. As a different coupling strategy might lead to active tripartite derivatives of **8**, we decided to connect the inhibitor and the spacer either *via* modification of the *p*-toluenesulfonamide or by replacement of the Fmoc-protecting group by a phenylacetic acid derivative. Both analogues could still exhibit inhibitory activity based on the structure–activity study.[29] The building blocks **24** and **28** for the solid-phase peptide synthesis were prepared from H-Lys(Cbz)-O*t*Bu (**21**) (Scheme 2). For the synthesis of compound **24** with the modified sulfonamide, amine **21** was reductively alkylated with isobutyraldehyde. The secondary amine was converted into the sulfonamide **22** using benzyl 2-(4-(chlorosulfonyl)phenyl)acetate, which was prepared from 4-iodophenylacetic acid *via* benzyl protection, copper-catalysed coupling with thiobenzoic acid[33] and subsequent oxidative chlorination with trichloroisocyanuric acid.[34] Deprotection of **22** by cleavage of the benzyl ester and the carbamate followed by Fmoc-protection of the amine **23** afforded building block **24**.

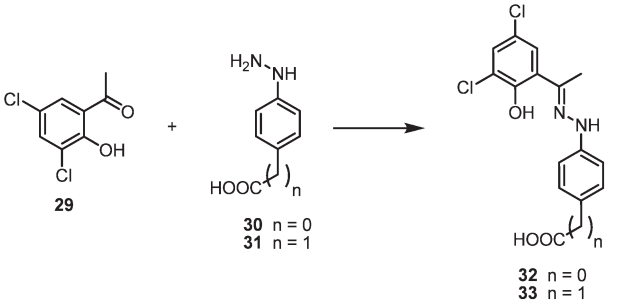


Scheme 2 Synthesis of the building blocks **24** and **28**. *Reagents and conditions*: (a) 1.14 equiv. isobutyraldehyde, 0.66 equiv. EtN(i-Pr)$_2$, 1.14 equiv. NaBH$_3$CN, 2.4 equiv. AcOH, MeOH, rt, 2 h, 92%; (b) 1.0 equiv. benzyl 2-(4-(chlorosulfonyl)phenyl)acetate, 1.0 equiv. EtN(i-Pr)$_2$, CH$_2$Cl$_2$, rt, 3 d, 63%; (c) cat. 10% Pd/C, H$_2$ (1 atm), EtOAc, rt, 23 h, 95%; (d) 1.5 equiv. *N*-(FmocO)succinimide, MeCN, THF, 1 M K$_2$CO$_3$ in H$_2$O (1 : 1 : 1), rt, 17 h, 51%; (e) 1.0 equiv. TosCl, 1.5 equiv. EtN(i-Pr)$_2$, CH$_2$Cl$_2$, rt, 5 d, 88%; (f) cat. 10% Pd/C, H$_2$ (1 atm), EtOAc, rt, 24 h, 99%; (g) 1.0 equiv. **26**, 1.5 equiv. **27**, 1.5 equiv. EDC·HCl, cat. DMAP, CH$_2$Cl$_2$, rt, 5 d, 56% (h) cat. 10% Pd/C, H$_2$ (1 atm), EtOAc, rt, 2 h, 100%.

Reductive amination of compound **21** and subsequent reaction with 4-toluenesulfonyl chloride afforded the sulfonamide **25**.[29] Deprotection of the primary amine led to compound **26**, which on amidation with the acid **27** and cleavage of the benzyl ester provided building block **28**. Acid **27** is accessible by chemoselective protection of 4-hydroxyphenylacetic acid (**34**) as benzyl ester,[35] reaction with *tert*-butyl bromoacetate and cleavage of the *tert*-butyl ester.

The free inhibitor **8** and the derived tripartite structures **9**, **10** and **11** were all unable to inhibit the Aβ40-secretion from SH-SY5Y APPwt cells at concentrations of up to 100 nM (**8**) and 1 μM (**9**, **10** and **11**), respectively.

As the second small-molecule inhibitor we have chosen the hydrazone **12** (The Genetics Company Inc., Switzerland; Fig. 6).[30] An IC$_{50}$ value of 14.4 μM for an *in vitro* assay and activity in a cell-based assay were reported.[30,36] Since no information on structure–activity relations was available, this inhibitor was attached *via* the mono-substituted aromatic ring using a benzoic or phenylacetic acid moiety (Scheme 3). The synthesis of the building blocks **32** and **33** started from commercially



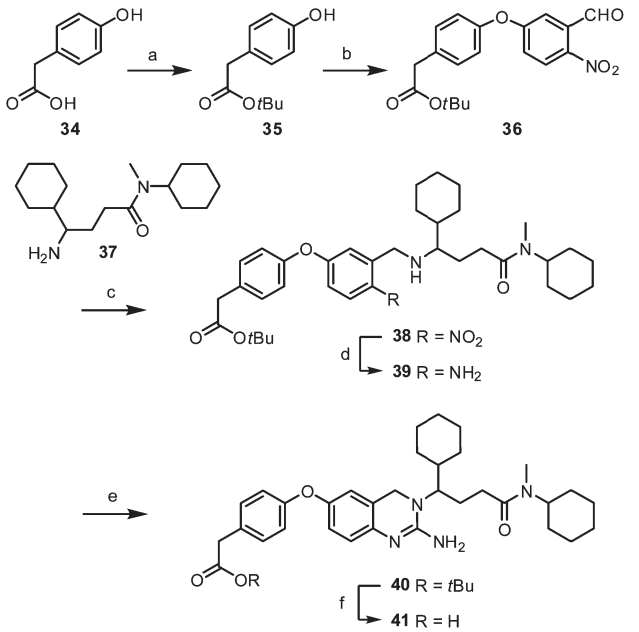
Scheme 3 Synthesis of the building blocks **32** and **33**. *Reagents and conditions*: 1.0 equiv. **30** or **31**, 1.0 equiv. **29**, 0.85 equiv. AcOH, EtOH, 78 °C, 2.5 h, 66% for **32**, 51% for **33**.

available 4-hydrazinobenzoic acid (**30**) and 4-hydrazinophenylacetic acid (**31**), prepared by diazotation and reduction of 4-aminophenylacetic acid.[37]

Acid-catalysed condensation of ketone **29** with the hydrazines **30** and **31** provided the building blocks **32** and **33** with no need for protecting group operations at the carboxylic acids.[38] The tripartite structures **13** and **14**, as well as the inhibitors **32** and **33** were unable to reduce the Aβ40-secretion from SH-SY5Y APPwt cells at concentrations of up to 100 nM (Fig. 6).

The third non-peptidic small-molecule inhibitor of BACE1 investigated in our present study was the 2-amino-3,4-dihydroquinazoline **15** (Johnson & Johnson; Fig. 6).[31] An X-ray analysis of a structurally related 2-amino-3,4-dihydroquinazoline bound to the active site of BACE1 showed that the primary amine interacts with the catalytic aspartates of BACE1.[31] A K_i value of 30 nM was found for *rac*-**15**, which was also active in a cell-based assay. Attachment of this inhibitor to the tripartite structure was achieved by substitution of the phenoxy group at the 6-position of the 3,4-dihydroquinazoline **15** by a 4-hydroxyphenylacetic acid. The primary amine and the cyclohexyl moieties are important for the activity of **15** because they interact with the catalytically active aspartates or the S_1 and S_1' pockets of BACE1, respectively. Therefore, these structural units were left untouched. Furthermore, the modified substituent at the 2-amino-3,4-dihydroquinazoline could easily be introduced at the beginning of the synthesis, keeping the remaining steps similar to the known procedure (Scheme 4).[31] 4-Hydroxyphenylacetic acid (**34**) was converted to the *tert*-butyl ester **35** using an excess of *tert*-butyl acetoacetate.[39] Nucleophilic aromatic substitution by reaction of 5-fluoro-2-nitrobenzaldehyde with the phenol **35** provided the diaryl ether **36**. Subsequent reductive amination with amine **37** afforded compound **38**.[31] Reduction of **38** to the aniline **39** followed by cyclisation using cyanogen bromide provided the 2-amino-3,4-dihydroquinazoline **40**. Cleavage of the *tert*-butyl ester led to the phenylacetic acid **41**.[40]

The tripartite structure **16** turned out to be a potent inhibitor of cellular Aβ release from SH-SY5Y APPwt cells. The EC$_{50}$ of 14.8 nM (log EC$_{50}$ = −7.831 ± 0.057) for **16** as compared to 5.4 μM (log EC$_{50}$ = −5.266 ± 0.060) for the pharmacophore **41** indicated a substantial improvement of potency (Fig. 7). A significant statistical difference for the mean log EC$_{50}$ values of **16** and **41** was revealed by Student's *t*-test ($p < 0.0001$). This result showed that membrane targeting of a non-peptidic compound,



Scheme 4 Synthesis of building block **41**. *Reagents and conditions*: (a) 6.4 equiv. *tert*-butyl acetoacetate, 0.08 equiv. conc. H_2SO_4, closed vessel, rt, 45 h, 41%; (b) 1.0 equiv. 5-fluoro-2-nitrobenzaldehyde, 1.2 equiv. K_2CO_3, DMF, 120 °C, 45 min, 63%; (c) 1.0 equiv. **36**, 1.0 equiv. **37**, 1.1 equiv. $NaBH_3CN$, 1.0 equiv. AcOH, CH_2Cl_2, rt, 17 h, 83%; (d) cat. 10% Pd/C, H_2 (1 atm), MeOH, rt, 2 h, 94%; (e) 1.5 equiv. BrCN, EtOH, 78 °C, 17 h, 84%; (f) 8.2 wt-equiv. silica gel, toluene, 111 °C, 3.5 h, 99%.

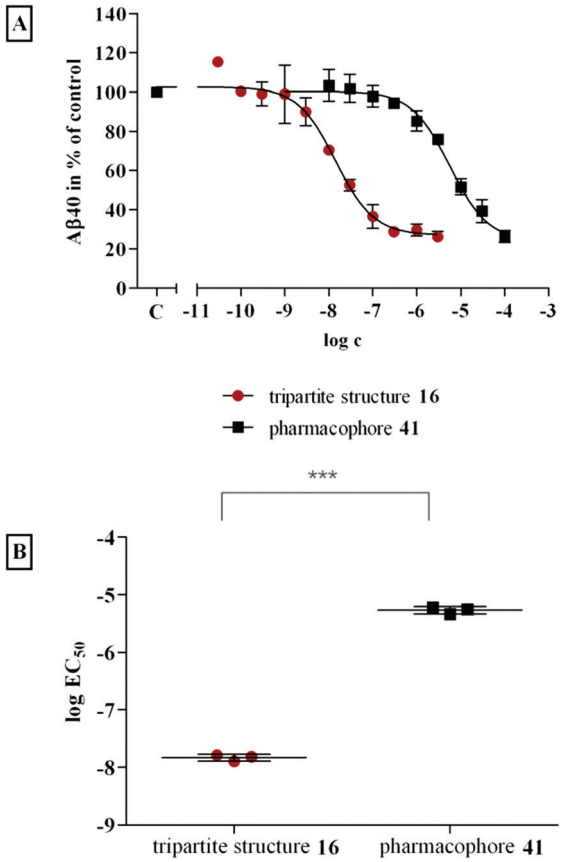
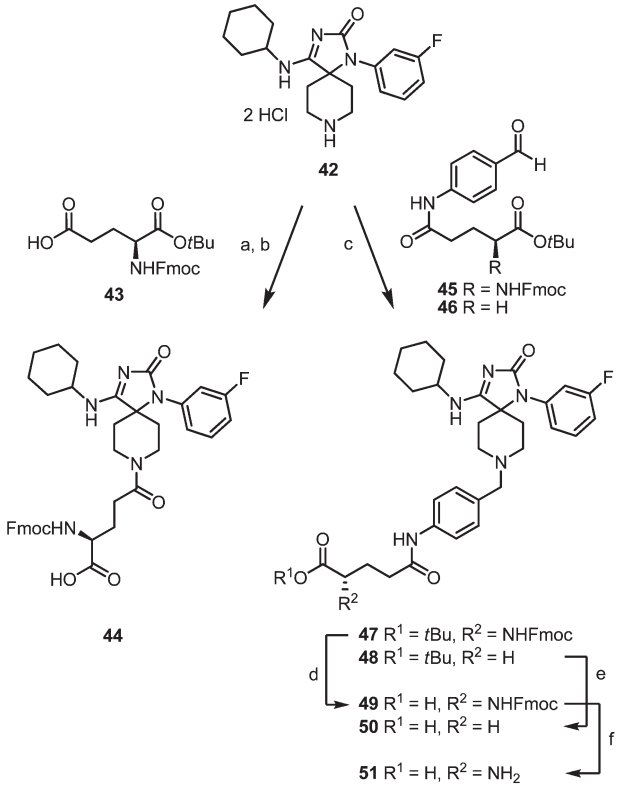


Fig. 7 (A) Dose–response curves: inhibition of Aβ40 secretion by tripartite structure **16** (red dots) and pharmacophore **41** (black squares). Aβ40 concentrations in cell culture supernatants are expressed as percent of the control condition C (1% DMSO). The curves were generated by non-linear regression on pooled normalised data from three independent experiments. Compound **16** was tested in alternating concentration ranges in SH-SY5Y APPwt cells (once: 30 pM–100 nM, 1 μM; twice: 300 pM–3 μM). Compound **41** was tested in two experiments at nine different concentrations ranging from 10 nM to 100 μM and for confirmation, in a third experiment at a reduced number of concentrations (10 nM, 100 nM, 1 μM, 10 μM, 100 μM). (B) Log EC_{50} values of tripartite structure **16** and pharmacophore **41** from three independent experiments were compared by an unpaired, two-tailed *t*-test. Mean log EC_{50} values were significantly different (***$p < 0.0001$). The lines and error bars represent means and standard deviations from three independent experiments.

which is only weakly active in a cellular assay, leads to a significant increase of the inhibitory activity.

As the fourth small-molecule BACE1 inhibitor we tested the spiropiperidine iminohydantoin **17** which was identified by high-throughput screening (Merck; Fig. 6).[32] An IC_{50} value of 2.8 μM on the isolated enzyme and an activity in a cellular assay at 8.2 μM have been reported.[32] The cyclohexyl and fluorophenyl rings of **17** were found to be essential for high potency *in vitro* and good activity in the cell-based assay, respectively. Therefore, attachment to the spacer was achieved by modification of the substituent at the piperidine nitrogen. The spiropiperidine **42** is readily available *via* an Ugi reaction (Scheme 5).[32] In a first attempt the piperidine nitrogen of **42** was directly acylated with Fmoc-Glu-O*t*Bu (**43**). Subsequent acidic cleavage of the *tert*-butyl ester afforded building block **44**. For the two pharmacophores **50** and **51**, attachment to the spacer was projected by substitution of the 4-acetamido group of **17** with a glutamic or glutaric acid linkage, respectively. Thus, the structure of the pharmacophores was kept similar to the one reported in the literature. Protection of 4-nitrobenzyl alcohol by reaction with *tert*-butylchlorodimethylsilane, reduction to the aniline,[41] amidation with either Fmoc-Glu-O*t*Bu (**43**) or mono-*tert*-butyl glutarate,[42] removal of the silyl protecting group and oxidation with manganese dioxide[43,44] afforded the benzaldehydes **45** and **46**. Reductive amination of the spiropiperidine **42** with the benzaldehydes **45** and **46** and subsequent cleavage of the *tert*-butyl ester led to compound **49** and the pharmacophore **50**. Cleavage of the Fmoc group in **49** provided the glutamic acid-derived pharmacophore **51**.

The tripartite structures **19** and **20** exhibited potent inhibition of Aβ40-secretion in a cellular assay with EC_{50} values of 50.1 nM (log EC_{50} = −7.300 ± 0.163) and 9.7 nM (log EC_{50} = −8.014 ± 0.048) (Fig. 6), whereas the reference substances **17**, **50** and **51** showed considerably higher EC_{50} values of 0.863 μM (log EC_{50} = −6.064 ± 0.178), 17.5 μM (log EC_{50} = −4.758 ± 0.212) and 4.3 μM (log EC_{50} = −5.368 ± 0.150), respectively (Scheme 5; ESI, Table S2†). The tripartite structure **18** failed to inhibit BACE1 at concentrations up to 10 μM. Pairwise comparison of the log EC_{50} values for the tripartite structures **19** and **20** and the free pharmacophores **17**, **50** and **51** by one-way ANOVA and Tukey's post-test showed that all five differed significantly from each other (ESI, Fig. S1 and Table S3†). In contrast to **18**, the tripartite structures **19** and **20**, which resemble structure **17**



Scheme 5 Synthesis of the building blocks **44**, **49** and **50**. *Reagents and conditions*: (a) 1.9 equiv. Fmoc-Glu-O*t*Bu (**43**), 1.9 equiv. HATU, 4.3 equiv. EtN(i-Pr)$_2$, CH_2Cl_2, rt, 18 h; (b) CF_3COOH, CH_2Cl_2 (1 : 1), rt, 90 min, 57% (two steps); (c) 1.4 equiv. **45** or 2.3 equiv. **46**, 3.0 equiv. NaBH(OAc)$_3$, 1.5 equiv. EtN(i-Pr)$_2$, 1,2-$C_2H_4Cl_2$, rt, 18–19 h, 70% for **47**, 73% for **48**; (d) CF_3COOH, CH_2Cl_2 (1 : 1), rt, 90 min, 78%; (e) 10.0 wt-equiv. silica gel, toluene, 111 °C, 6 h, 86%; (f) piperidine, DMF (1 : 5), rt, 3.5 h, 75%.

more closely, were highly active. The potency of **20**, the most active tripartite structure within this series, exceeded that of inhibitor **17** by almost two orders of magnitude. This outcome emphasises that the potency of intrinsically cell-permeable BACE1 inhibitors can be enhanced considerably by membrane anchoring. The tripartite structure **20** is significantly more active than **19**, which differs from the former only by the presence of an amino group in the linkage to the pharmacophore. Obviously, decreased polarity in the region of the hydrophobic S_3 binding pocket results in increased activity.

Conclusions

The present results provide strong evidence for our tripartite structure concept.[24] We have demonstrated that the cellular activity of small-molecule BACE1 inhibitors can be significantly increased by membrane targeting *via* a spacer with a raftophilic membrane anchor. A careful selection of the coupling strategy is crucial to avoid alteration of essential pharmacophoric groups of the inhibitor which may lead to a loss of inhibitory activity. The tripartite structure concept leads to an enrichment of the membrane-bound inhibitor in the lipid rafts which may reduce unwanted off-target side effects caused by the free BACE1 inhibitor. The ability of the membrane anchor to bind to lipid rafts strongly depends on its structure. This raftophilicity determines the concentration of the tripartite structure at the site of APP cleavage and therefore the potency of our compounds. Our observations indicate that a variation of the theoretical linker length from 26 to 96 Å has only a small impact on the inhibitory activity of the tripartite structure. This observation is tentatively explained by a spacer conformation which is not fully elongated in an aqueous environment. It is a further advantage of our concept that tripartite structures are relatively tolerant to structural changes in the spacer.

Experimental

General

The spacer building blocks **4a** and **4b**, and Fmoc-statine were purchased from Polypeptide, France. The spacer building blocks **4c–g** were purchased from Iris Biotech, Germany. The other amino acids and activation agents were bought from Novabiochem/Merck. All reactions were carried out using dry solvents in oven-dried glassware under an argon atmosphere. Solvents were dried using a solvent purification system (MBraun-SPS). Other chemicals were used as received from commercial sources. Flash chromatography was carried out using silica gel from Acros Organics (0.035–0.070 mm) and a Sepacore system (Büchi). The tripartite structures were assembled by automated solid-phase peptide synthesis on an ABI 433A peptide synthesiser with a UV detector (Fmoc-strategy, PAL-PEG-PS resin, HATU or HBTU activation). ATR FT-IR spectra were recorded on a Thermo Nicolet Avatar 360. NMR spectra were recorded on a Bruker Avance II 300, on a Bruker DRX 500 and an Avance III 600; δ in ppm with the non-deuterated solvent as an internal standard,[45] J in Hz. Mass spectra were measured on a Bruker Esquire LC Electrospray MS. The most abundant mass is given as theoretical mass. HRMS was measured on an LTQ ORBITRAP XL instrument (ThermoScientific) with a resolution of 60 000 and a mass accuracy within 3 ppm. Analytical HPLC was carried out on an Agilent Model 1100 with G1315B UV-DAD (detection at 215, 260 and 560 nm), G1321A fluorescence and an evaporative light scattering detector (ELS 1000, Polymer Laboratories). Column: Vydac 208TP104 (reverse-phase C_8, 4.6 × 250 mm). Flow rate: 1.0 mL min^{-1}. Eluent A: H_2O + 0.1% CF_3COOH, eluent B: MeCN + 0.1% CF_3COOH. Gradient from 20% to 90% B in 35 min. Preparative HPLC was carried out using a Varian PrepStar system, with a Varian ProStar Model 320 UV and an evaporative light scattering detector (ELS 1000, Polymer Laboratories) connected *via* a Sunchrom QuickSplit splitter. Column: Vydac 208TP1050 (reverse-phase C_8, 50 × 250 mm). Flow rate: 55 mL min^{-1}. Eluent A: H_2O + 0.1% CF_3COOH, eluent B: MeCN + 0.1% CF_3COOH. Gradient stated for the individual compounds.

Fmoc-Asp(ODhc)-OH (3a). For synthesis and spectroscopic data, see ref. 26.

Fmoc-Asp(OChol)-OH (3b). Cholesterol (1.29 g, 3.33 mmol) and a catalytic amount of DMAP were added to a solution of Fmoc-Asp-O*t*Bu (**1**) (1.99 g, 4.83 mmol) and EDC·HCl (0.938 g, 4.89 mmol) in CH_2Cl_2 (60 mL). After stirring for 52 h

at room temperature, the mixture was partitioned between 2% aqueous citric acid and CH_2Cl_2, washed with 2% citric acid, H_2O and brine. The organic layer was dried over Na_2SO_4 and evaporated to yield a colourless solid, which was dissolved in CH_2Cl_2 (30 mL). CF_3COOH (30 mL) was added at 0 °C, the mixture was stirred at room temperature for 90 min, diluted with CH_2Cl_2 and washed four times with water and once with brine. After drying over Na_2SO_4 and removal of the solvent, the residue was purified by flash chromatography (SiO_2; pentane/ethyl acetate [2 : 1] + 0.5% AcOH), affording **3b** as a colourless solid, yield: 1.46 g (61%), m.p. 157–158 °C. IR (ATR): ν = 2932, 2867, 2850, 1725, 1703, 1653, 1558, 1515, 1447, 1376, 1330, 1273, 1218, 1084, 1029, 1008, 957, 849, 801, 757, 735 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.67 (s, 3 H), 0.85–1.62 (m, 33 H), 1.79–1.87 (m, 3 H), 1.94–2.02 (m, 2 H), 2.32 (d, J = 7.9 Hz, 2 H), 2.86 (dd, J = 17.3, 4.7 Hz, 1 H), 3.06 (dd, J = 17.6, 4.1 Hz, 1 H), 4.24 (t, J = 7.3 Hz, 1 H), 4.31–4.39 (m, 1 H), 4.40–4.47 (m, 1 H), 4.57–4.72 (m, 2 H), 5.37 (d, J = 3.8 Hz, 1 H), 5.87 (d, J = 8.2 Hz, 1 H), 7.31 (t, J = 7.6 Hz, 2 H), 7.40 (t, J = 7.6 Hz, 2 H), 7.58–7.61 (m, 2 H), 7.76 (d, J = 7.6 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 11.99 (CH_3), 18.86 (CH_3), 19.42 (CH_3), 21.16 (CH_2), 22.71 (CH_3), 22.97 (CH_3), 23.99 (CH_2), 24.41 (CH_2), 27.79 (CH_2), 28.16 (CH), 28.37 (CH_2), 31.95 (CH), 32.02 (CH_2), 35.94 (CH), 36.32 (CH_2), 36.69 (C), 36.88 (CH_2), 37.03 (CH_2), 38.08 (CH_2), 39.65 (CH_2), 39.83 (CH_2), 42.44 (C), 47.20 (CH), 49.10 (CH), 50.34 (CH), 56.28 (CH), 56.80 (CH), 67.55 (CH_2), 75.57 (CH), 120.15 (2 CH), 123.13 (CH), 125.25 (CH), 125.29 (CH), 127.24 (2 CH), 127.90 (2 CH), 139.40 (C), 141.44 (2 C), 143.78 (C), 143.92 (C), 156.24 (C═O), 170.80 (C═O), 175.67 (C═O). ESI-MS: m/z = 724.5 $[M + H]^+$, 741.7 $[M + NH_4]^+$.

Fmoc-Asp(O-$C_{14}H_{29}$)-OH (3c). A solution of Fmoc-Asp-O*t*Bu (**1**) (5.56 g, 13.5 mmol), MSNT (4.00 g, 13.5 mmol) and MeIm (1.11 g, 13.5 mmol) in CH_2Cl_2 (60 mL) was stirred for 10 min and myristyl alcohol (2.63 g, 12.3 mmol) was added subsequently. After stirring for 4 h at room temperature, the mixture was partitioned between 2% aqueous citric acid and CH_2Cl_2, washed with 2% citric acid, H_2O and brine. After drying over Na_2SO_4 and removal of the solvent, the residue was purified by flash chromatography (SiO_2; petroleum ether/diethyl ether, 2 : 1), affording the protected ester as a colourless solid, yield: 5.56 g (76%). This product (5.00 g, 8.23 mmol) was dissolved in CH_2Cl_2 (15 mL). CF_3COOH (10 mL) was added at 0 °C, the mixture stirred at room temperature for 90 min, diluted with CH_2Cl_2 and washed four times with water and once with brine. After drying over Na_2SO_4 and removal of the solvent, the residue was purified by flash chromatography (SiO_2; petroleum ether/diethyl ether [2 : 1] + 0.5% AcOH), affording **3c** as a colourless solid, yield: 3.99 g (88%). IR (ATR): ν = 3355, 3247, 2920, 2851, 2057, 1724, 1652, 1543, 1470, 1457, 1404, 1372, 1280, 1237, 1184, 1083, 995, 832, 809, 770, 735 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.88 (t, J = 6.9 Hz, 3 H), 1.22–1.31 (m, 22 H), 1.59–1.65 (m, 2 H), 2.89 (d, J = 15.5 Hz, 1 H), 3.10 (d, J = 15.5 Hz, 1 H), 4.12 (t, J = 6.4 Hz, 2 H), 4.24 (t, J = 6.6 Hz, 1 H), 4.33–4.37 (m, 1 H), 4.42–4.46 (m, 1 H), 4.70 (br s, 1 H), 5.87 (br d, J = 7.7 Hz, 1 H), 7.31 (t, J = 7.3 Hz, 2 H), 7.40 (t, J = 7.4 Hz, 2 H), 7.57–7.61 (m, 2 H), 7.76 (d, J = 7.5 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 14.28 (CH_3), 22.84 (CH_2), 26.00 (CH_2), 28.61 (CH_2), 29.37 (CH_2), 29.51 (CH_2), 29.67 (CH_2), 29.73 (CH_2), 29.80 (3 CH_2), 29.84 (CH_2), 32.06 (CH_2), 36.54 (CH_2), 47.20 (CH), 50.31 (CH), 65.81 (CH_2), 67.60 (CH_2), 120.14 (2 CH), 125.26 (CH), 125.30 (CH), 127.23 (2 CH), 127.88 (2 CH), 141.43 (2 C), 143.76 (C), 143.89 (C), 156.26 (C═O), 171.43 (C═O), 175.69 (C═O). ESI-MS: m/z = 552.3 $[M + H]^+$, 569.3 $[M + NH_4]^+$.

Fmoc-Asp(O-$C_{16}H_{33}$)-OH (3d). A solution of Fmoc-Asp-O*t*Bu (**1**) (1.00 g, 2.43 mmol), MSNT (0.721 g, 2.43 mmol) and MeIm (0.204 g, 2.43 mmol) in CH_2Cl_2 (60 mL) was stirred for 10 min and palmityl alcohol (0.651 g, 2.68 mmol) was added subsequently. After stirring for 4 h at room temperature, the mixture was partitioned between 2% aqueous citric acid and CH_2Cl_2, washed with 2% citric acid, H_2O and brine. After drying over Na_2SO_4 and removal of the solvent, the residue was purified by flash chromatography (SiO_2; petroleum ether/diethyl ether, 2 : 1), affording the protected ester as a colourless solid, yield: 1.27 g (75%). This product (0.904 g, 1.42 mmol) was dissolved in CH_2Cl_2 (15 mL). CF_3COOH (10 mL) was added at 0 °C, the mixture stirred at room temperature for 90 min, diluted with CH_2Cl_2 and washed four times with water and once with brine. After drying over Na_2SO_4 and removal of the solvent, the residue was purified by flash chromatography (SiO_2; petroleum ether/diethyl ether [2 : 1] + 0.5% AcOH), affording **3d** as a colourless solid, yield: 0.702 g (85%). IR (ATR): ν = 3375, 3065, 2917, 2848, 2057, 1757, 1678, 1535, 1450, 1377, 1358, 1254, 1193, 1166, 1090, 1043, 988, 861, 736 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.88 (t, J = 6.9 Hz, 3 H), 1.20–1.35 (m, 26 H), 1.59–1.65 (m, 2 H), 2.89 (dd, J = 17.4, 4.6 Hz, 1 H), 3.10 (dd, J = 17.4, 4.1 Hz, 1 H), 4.12 (t, J = 6.7 Hz, 2 H), 4.24 (t, J = 7.2 Hz, 1 H), 4.33–4.37 (m, 1 H), 4.41–4.46 (m, 1 H), 4.66–4.72 (m, 1 H), 5.84 (br d, J = 8.5 Hz, 1 H), 7.31 (t, J = 7.3 Hz, 2 H), 7.40 (t, J = 7.4 Hz, 2 H), 7.56–7.61 (m, 2 H), 7.75 (d, J = 7.5 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 14.29 (CH_3), 22.85 (CH_2), 26.00 (CH_2), 28.61 (CH_2), 29.39 (CH_2), 29.52 (CH_2), 29.67 (CH_2), 29.74 (CH_2), 29.81 (CH_2), 29.84 (5 CH_2), 32.08 (CH_2), 36.52 (CH_2), 47.21 (CH), 50.28 (CH), 65.82 (CH_2), 67.58 (CH_2), 120.15 (2 CH), 125.25 (CH), 125.28 (CH), 127.24 (2 CH), 127.90 (2 CH), 141.44 (2 C), 143.76 (C), 143.90 (C), 156.25 (C═O), 171.46 (C═O), 175.33 (C═O). ESI-MS: m/z = 580.3 $[M + H]^+$, 597.3 $[M + NH_4]^+$.

Fmoc-Asp(O-$C_{18}H_{37}$)-OH (3e). A solution of Fmoc-Asp-O*t*Bu (**1**) (0.503 g, 1.22 mmol), MSNT (0.363 g, 1.21 mmol) and MeIm (99 mg, 1.22 mmol) in CH_2Cl_2 (20 mL) was stirred for 10 min and stearyl alcohol (301 mg, 1.11 mmol) was added subsequently. After stirring for 14 h at room temperature, the mixture was partitioned between 2% aqueous citric acid and CH_2Cl_2, washed with 2% citric acid, H_2O and brine. The organic layer was dried over Na_2SO_4 and evaporated to yield a colourless viscous oil, which was dissolved in CH_2Cl_2 (5 mL). After addition of CF_3COOH (6.25 mL) at 0 °C, the mixture was stirred at room temperature for 90 min, diluted with CH_2Cl_2 and washed four times with water and once with brine. After drying over Na_2SO_4 and removal of the solvent, the residue was purified by flash chromatography (SiO_2; petroleum ether/ethyl acetate [2 : 1] + 0.5% AcOH), affording **3e** as a colourless solid, yield: 0.568 g (76%), m.p. 97–99 °C. IR (ATR): ν = 3377, 3064,

2916, 2849, 1757, 1734, 1693, 1678, 1533, 1472, 1450, 1377, 1358, 1322, 1253, 1195, 1166, 1089, 1043, 990, 860, 736 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.88 (t, J = 6.9 Hz, 3 H), 1.17–1.34 (m, 30 H), 1.57–1.69 (m, 2 H), 2.89 (dd, J = 17.4, 4.5 Hz, 1 H), 3.09 (dd, J = 17.3, 3.9 Hz, 1 H), 4.12 (t, J = 6.7 Hz, 2 H), 4.23 (t, J = 7.2 Hz, 1 H), 4.32–4.40 (m, 1 H), 4.39–4.49 (m, 1 H), 4.64–4.75 (m, 1 H), 5.86 (d, J = 8.3 Hz, 1 H), 7.31 (t, J = 7.4 Hz, 2 H), 7.40 (t, J = 7.4 Hz, 2 H), 7.59 (d, J = 5.8 Hz, 2 H), 7.76 (d, J = 7.5 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 14.28 (CH_3), 22.84 (CH_2), 26.00 (CH_2), 28.60 (CH_2), 29.39 (CH_2), 29.51 (CH_2), 29.67 (CH_2), 29.74 (CH_2), 29.81 (CH_2), 29.85 (7 CH_2), 32.07 (CH_2), 36.55 (CH_2), 47.21 (CH), 50.30 (CH), 65.79 (CH_2), 67.55 (CH_2), 120.14 (2 CH), 125.25 (CH), 125.29 (CH), 127.24 (2 CH), 127.90 (2 CH), 141.44 (2 C), 143.78 (C), 143.90 (C), 156.26 (C═O), 171.51 (C═O), 174.46 (C═O). ESI-MS: m/z = 608.3 $[M + H]^+$, 625.4 $[M + NH_4]^+$.

Fmoc-Asp(O-$C_{20}H_{41}$)-OH (3f). A solution of Fmoc-Asp-O*t*Bu (**1**) (1.51 g, 3.68 mmol), MSNT (1.09 g, 3.67 mmol) and MeIm (0.311 g, 3.67 mmol) in CH_2Cl_2 (60 mL) was stirred for 10 min and arachidyl alcohol (1.00 g, 3.36 mmol) was added subsequently. After stirring for 17 h at room temperature, the mixture was partitioned between 2% aqueous citric acid and CH_2Cl_2, washed with 2% citric acid, H_2O and brine. The organic layer was dried over Na_2SO_4 and evaporated to yield a colourless viscous oil, which was dissolved in CH_2Cl_2 (60 mL). CF_3COOH (60 mL) was added at 0 °C, the mixture stirred at room temperature for 90 min, diluted with CH_2Cl_2 and washed four times with water and once with brine. After drying over Na_2SO_4 and removal of the solvent, the residue was purified by flash chromatography (SiO_2; petroleum ether/ethyl acetate [2 : 1] + 0.5% AcOH), affording **3f** as a colourless solid, yield: 1.45 g (68%), m.p. 97–99 °C. IR (ATR): ν = 3387, 3066, 2916, 2849, 1755, 1734, 1713, 1693, 1512, 1472, 1450, 1378, 1359, 1320, 1252, 1193, 1166, 1090, 1042, 992, 860, 775, 755, 736, 720 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.88 (t, J = 6.9 Hz, 3 H), 1.19–1.35 (m, 34 H), 1.60–1.66 (m, 2 H), 2.89 (dd, J = 17.4, 4.7 Hz, 1 H), 3.09 (dd, J = 17.4, 4.2 Hz, 1 H), 4.12 (t, J = 6.7 Hz, 2 H), 4.24 (t, J = 7.1 Hz, 1 H), 4.36 (s, 1 H), 4.41–4.48 (m, 1 H), 4.67–4.75 (m, 1 H), 5.85 (d, J = 8.4 Hz, 1 H), 7.31 (t, J = 7.2 Hz, 2 H), 7.40 (t, J = 7.4 Hz, 2 H), 7.59 (d, J = 5.4 Hz, 2 H), 7.76 (d, J = 7.5 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 14.28 (CH_3), 22.84 (CH_2), 25.99 (CH_2), 28.60 (CH_2), 29.38 (CH_2), 29.51 (CH_2), 29.67 (CH_2), 29.74 (CH_2), 29.81 (CH_2), 29.85 (9 CH_2), 32.07 (CH_2), 36.52 (CH_2), 47.21 (CH), 50.25 (CH), 65.82 (CH_2), 67.57 (CH_2), 120.15 (2 CH), 125.24 (CH), 125.28 (CH), 127.24 (2 CH), 127.90 (2 CH), 141.44 (2 C), 143.77 (C), 143.89 (C), 156.24 (C═O), 171.49 (C═O), 174.87 (C═O). ESI-MS: m/z = 636.6 $[M + H]^+$, 653.5 $[M + NH_4]^+$.

Fmoc-Asp(O-$C_{22}H_{45}$)-OH (3g). A solution of Fmoc-Asp-O*t*Bu (**1**) (1.68 g, 4.09 mmol), MSNT (1.21 g, 4.08 mmol) and MeIm (0.335 g, 4.09 mmol) in CH_2Cl_2 (60 mL) was stirred for 10 min and behenyl alcohol (1.22 g, 3.72 mmol) was added subsequently. After stirring for 24 h at room temperature, the mixture was partitioned between 2% aqueous citric acid and CH_2Cl_2, washed with 2% citric acid, H_2O and brine. The organic layer was dried over Na_2SO_4 and evaporated to yield a colourless solid, which was dissolved in CH_2Cl_2 (60 mL). CF_3COOH (60 mL) was added at 0 °C, the mixture stirred at room temperature for 90 min, diluted with CH_2Cl_2 and washed four times with water and once with brine. After drying over Na_2SO_4 and removal of the solvent, the residue was purified by flash chromatography (SiO_2; petroleum ether/ethyl acetate [2 : 1] + 0.5% AcOH), affording **3g** as a colourless solid, yield: 2.28 g (92%), m.p. 98–100 °C. IR (ATR): ν = 3387, 3066, 2916, 2849, 1755, 1732, 1712, 1539, 1515, 1472, 1450, 1380, 1359, 1320, 1252, 1206, 1091, 1041, 989, 965, 935, 860, 775, 756, 737, 718 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.88 (t, J = 6.8 Hz, 3 H), 1.19–1.30 (m, 38 H), 1.57–1.68 (m, 2 H), 2.89 (dd, J = 17.4, 4.5 Hz, 1 H), 3.09 (dd, J = 17.4, 4.2 Hz, 1 H), 4.11 (t, J = 6.7 Hz, 2 H), 4.23 (t, J = 7.2 Hz, 1 H), 4.31–4.48 (m, 2 H), 4.63–4.72 (m, 1 H), 5.89 (d, J = 8.5 Hz, 1 H), 7.31 (t, J = 7.3 Hz, 2 H), 7.40 (t, J = 7.4 Hz, 2 H), 7.55–7.63 (m, 2 H), 7.76 (d, J = 7.5 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 14.28 (CH_3), 22.84 (CH_2), 26.00 (CH_2), 28.60 (CH_2), 29.39 (CH_2), 29.51 (CH_2), 29.68 (CH_2), 29.75 (CH_2), 29.81 (CH_2), 29.86 (11 CH_2), 32.07 (CH_2), 36.52 (CH_2), 47.20 (CH), 50.30 (CH), 65.79 (CH_2), 67.57 (CH_2), 120.14 (2 CH), 125.25 (CH), 125.29 (CH), 127.24 (2 CH), 127.89 (2 CH), 141.43 (2 C), 143.77 (C), 143.90 (C), 156.26 (C═O), 171.42 (C═O), 175.61 (C═O). ESI-MS: m/z = 664.4 $[M + H]^+$, 681.5 $[M + NH_4]^+$.

Fmoc-Asp(O-$C_{24}H_{49}$)-OH (3h). A solution of Fmoc-Asp-O*t*Bu (**1**) (0.127 g, 0.314 mmol), MSNT (91.8 mg, 0.31 mmol) and MeIm (25.3 mg, 0.312 mmol) in CH_2Cl_2 (25 mL) was stirred for 10 min and lignoceryl alcohol (0.100 g, 0.284 mmol) was added subsequently. After stirring for 24 h at room temperature, the mixture was partitioned between 2% aqueous citric acid and CH_2Cl_2, washed with 2% citric acid, H_2O and brine. The organic layer was dried over Na_2SO_4 and evaporated to yield a colourless solid, which was dissolved in CH_2Cl_2 (5 mL). CF_3COOH (6.25 mL) was added at 0 °C, the mixture stirred at room temperature for 90 min, diluted with CH_2Cl_2 and washed four times with water and once with brine. After drying over Na_2SO_4 and removal of the solvent, the residue was purified by flash chromatography (SiO_2; petroleum ether/ethyl acetate [2 : 1] + 0.5% AcOH), affording **3h** as a colourless solid, yield: 0.137 g (70%), m.p. 97–99 °C. IR (ATR): ν = 3315, 2916, 2848, 1770, 1735, 1690, 1652, 1636, 1535, 1464, 1449, 1412, 1398, 1369, 1340, 1281, 1231, 1176, 1105, 1087, 1049, 1015, 921, 779, 756, 735, 637 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.88 (t, J = 7.0 Hz, 3 H), 1.23–1.36 (m, 42 H), 1.59–1.69 (m, 2 H), 2.88 (dd, J = 17.4, 4.7 Hz, 1 H), 3.08 (dd, J = 17.4, 4.3 Hz, 1 H), 4.12 (t, J = 6.7 Hz, 2 H), 4.23 (t, J = 7.1 Hz, 1 H), 4.34–4.40 (m, 1 H), 4.42–4.49 (m, 1 H), 4.69–4.72 (m, 1 H), 5.86 (d, J = 8.5 Hz, 1 H), 7.31 (t, J = 7.3 Hz, 2 H), 7.40 (t, J = 7.5 Hz, 2 H), 7.60 (d, J = 5.2 Hz, 2 H), 7.76 (d, J = 7.5 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 14.28 (CH_3), 22.84 (CH_2), 26.00 (CH_2), 28.61 (CH_2), 29.39 (CH_2), 29.51 (CH_2), 29.68 (CH_2), 29.75 (CH_2), 29.81 (CH_2), 29.86 (13 CH_2), 32.07 (CH_2), 36.52 (CH_2), 47.21 (CH), 50.36 (CH), 65.82 (CH_2), 67.57 (CH_2), 120.15 (2 CH), 125.25 (CH), 125.28 (CH), 127.24 (2 CH), 127.90 (2 CH), 141.44 (2 C), 143.77 (C), 143.89 (C), 156.25 (C═O), 171.49 (C═O), 174.91 (C═O). ESI-MS: m/z = 692.6 $[M + H]^+$, 709.7 $[M + NH_4]^+$.

Fmoc-Asp(O-$C_{26}H_{53}$)-OH (3i). A solution of Fmoc-Asp-O*t*Bu (**1**) (0.118 g, 0.288 mmol), MSNT (85.6 mg, 0.289 mmol) and MeIm (24.2 mg, 0.295 mmol) in CH_2Cl_2 (25 mL) was stirred for 10 min and ceryl alcohol (0.100 g, 0.261 mmol) was added subsequently. After stirring for 25 h at room temperature, the mixture was partitioned between 2% aqueous citric acid and CH_2Cl_2, washed with 2% citric acid, H_2O and brine. The organic layer was dried over Na_2SO_4 and evaporated to yield a colourless solid, which was dissolved in CH_2Cl_2 (5 mL). CF_3COOH (6.25 mL) was added at 0 °C, the mixture stirred at room temperature for 90 min, diluted with CH_2Cl_2 and washed four times with water and once with brine. After drying over Na_2SO_4 and removal of the solvent, the residue was purified by flash chromatography (SiO_2; petroleum ether/ethyl acetate [2 : 1] + 0.5% AcOH), affording **3i** as a colourless solid, yield: 0.151 g (80%), m.p. 99–101 °C. IR (ATR): ν = 3379, 2916, 2849, 2057, 1758, 1734, 1535, 1472, 1450, 1377, 1359, 1322, 1253, 1203, 1168, 1090, 1043, 989, 937, 860, 776, 755, 736, 718, 650 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.88 (t, J = 6.9 Hz, 3 H), 1.20–1.36 (m, 46 H), 1.59–1.69 (m, 2 H), 2.89 (dd, J = 17.4, 4.7 Hz, 1 H), 3.09 (dd, J = 17.4, 4.2 Hz, 1 H), 4.10–4.15 (m, 2 H), 4.24 (t, J = 7.1 Hz, 1 H), 4.34–4.38 (m, 1 H), 4.43–4.46 (m, 1 H), 4.68–4.71 (m, 1 H), 5.86 (d, J = 8.5 Hz, 1 H), 7.31 (t, J = 7.3 Hz, 2 H), 7.40 (t, J = 7.5 Hz, 2 H), 7.59 (d, J = 6.2 Hz, 2 H), 7.76 (d, J = 7.6 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 14.28 (CH_3), 22.84 (CH_2), 25.99 (CH_2), 28.60 (CH_2), 29.39 (CH_2), 29.51 (CH_2), 29.75 (CH_2), 29.81 (CH_2), 29.85 (16 CH_2), 32.07 (CH_2), 36.52 (CH_2), 47.21 (CH), 50.23 (CH), 65.83 (CH_2), 67.57 (CH_2), 120.15 (2 CH), 125.24 (CH), 125.28 (CH), 127.24 (2 CH), 127.91 (2 CH), 141.44 (2 C), 143.77 (C), 143.89 (C), 156.24 (C═O), 171.54 (C═O), 174.31 (C═O). ESI-MS: m/z = 720.7 $[M + H]^+$, 737.7 $[M + NH_4]^+$.

Fmoc-Asp(O-Oleyl)-OH (3j). A solution of Fmoc-Asp-O*t*Bu (**1**) (5.43 g, 13.2 mmol), MSNT (3.91 g, 13.2 mmol) and MeIm (1.08 g, 13.2 mmol) in CH_2Cl_2 (15 mL) was stirred for 10 min and oleyl alcohol (2.50 g, 12.0 mmol) was added subsequently. After stirring for 4 h at room temperature, the mixture was partitioned between 2% aqueous citric acid and CH_2Cl_2, washed with 2% citric acid, H_2O and brine. After drying over Na_2SO_4 and removal of the solvent, the residue was purified by flash chromatography (SiO_2; petroleum ether/diethyl ether, 2 : 1), affording the protected ester as a colourless solid, yield: 6.20 g (78%). This product (6.00 g, 9.07 mmol) was dissolved in CH_2Cl_2 (25 mL). CF_3COOH (15 mL) was added at 0 °C, the mixture stirred at room temperature for 90 min, diluted with CH_2Cl_2 and washed four times with water and once with brine. After drying over Na_2SO_4 and removal of the solvent, the residue was purified by flash chromatography (SiO_2; petroleum ether/diethyl ether [2 : 1] + 0.5% AcOH), affording **3j** as a colourless solid, yield: 4.56 g (83%). IR (ATR): ν = 3354, 3246, 3064, 3005, 2921, 2852, 2056, 1724, 1703, 1652, 1543, 1450, 1404, 1372, 1280, 1236, 1184, 1083, 994, 833, 810, 770, 734, 620 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.88 (t, J = 6.9 Hz, 3 H), 1.26–1.37 (m, 22 H), 1.62–1.65 (m, 2 H), 1.99–2.02 (m, 4 H), 2.89 (dd, J = 4.2, 17.5 Hz, 1 H), 3.10 (dd, J = 3.8, 17.4 Hz, 1 H), 4.13 (t, J = 6.6 Hz, 2 H), 4.24 (t, J = 7.0 Hz, 1 H), 4.34–4.38 (m, 1 H), 4.43–4.46 (m, 1 H), 4.69–4.71 (m, 1 H), 5.32–5.36 (m, 2 H), 5.85 (br d, J = 8.3 Hz, 1 H), 7.32 (t, J = 7.4 Hz, 2 H), 7.40 (t, J = 7.4 Hz, 2 H), 7.60 (d, J = 5.7 Hz, 2 H), 7.76 (d, J = 7.5 Hz, 2 H).^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 14.28 (CH_3), 22.84 (CH_2), 25.99 (CH_2), 27.34 (CH_2), 27.36 (CH_2), 28.60 (CH_2), 29.36 (2 CH_2), 29.47 (2 CH_2), 29.56 (CH_2), 29.68 (CH_2), 29.88 (CH_2), 29.91 (CH_2), 32.05 (CH_2), 36.51 (CH_2), 47.21 (CH), 50.25 (CH), 65.82 (CH_2), 67.58 (CH_2), 120.16 (2 CH), 125.28 (CH), 125.48 (CH), 127.24 (2 CH), 127.91 (2 CH), 129.91 (CH), 130.14 (CH), 141.44 (2 C), 143.77 (C), 143.89 (C), 156.24 (C═O), 171.52 (C═O), 174.81 (C═O). ESI-MS: m/z = 606.3 $[M + H]^+$.

i-Bu-Lys(Cbz)-O*t*Bu. Acetic acid (77 μL, 1.4 mmol), a solution of Lys(Cbz)-O*t*Bu·HCl (**21**) (0.209 g, 0.561 mmol) and $EtN(i\text{-}Pr)_2$ (64 μL, 0.37 mmol) in methanol (4 mL), and finally isobutyraldehyde (58 μL, 0.64 mmol) were added one after the other to a solution of $NaBH_3CN$ (40.3 mg, 0.642 mmol) in methanol (10 mL). The mixture was stirred at room temperature for 2 h. The major part of methanol was removed and the mixture was partitioned between ethyl acetate and K_2CO_3 (half-sat. aqueous). The organic layer was washed with brine and dried over Na_2SO_4. Removal of the solvent and flash chromatography (SiO_2; petroleum ether/ethyl acetate, 2 : 3) afforded i-Bu-Lys(Cbz)-O*t*Bu as a colourless oil, yield: 0.194 g (92%). IR (ATR): ν = 3329, 2948, 2933, 2867, 1771, 1723, 1709, 1653, 1531, 1452, 1395, 1367, 1338, 1243, 1150, 1081, 1048, 1027, 845, 777, 738, 696, 651 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.89 (d, J = 6.6 Hz, 6 H), 1.36–1.42 (m, 1 H), 1.46 (s, 9 H), 1.48–1.61 (m, 5 H), 1.64–1.71 (m, 1 H), 2.26 (dd, J = 11.1, 6.1 Hz, 1 H), 2.35 (dd, J = 11.1, 7.4 Hz, 1 H), 3.02 (t, J = 6.7 Hz, 1 H), 3.19 (q, J = 6.6 Hz, 2 H), 4.82 (br s, 1 H), 5.08 (s, 2 H), 7.29–7.37 (m, 5 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 20.61 (CH_3), 20.96 (CH_3), 23.15 (CH_2), 28.36 (3 CH_3), 28.81 (CH), 29.82 (CH_2), 33.28 (CH_2), 41.01 (CH_2), 56.32 (CH_2), 62.41 (CH), 66.70 (CH_2), 81.03 (C), 128.20 (2 CH), 128.24 (CH), 128.63 (2 CH), 136.76 (C), 156.48 (C═O), 175.14 (C═O). ESI-MS: m/z = 393.4 $[M + H]^+$.

Benzyl 4-iodophenylacetate. Benzyl bromide (58 μL, 0.49 mmol) was added dropwise to a mixture of 4-iodophenylacetic acid (0.101 g, 0.385 mmol) and Cs_2CO_3 (0.162 g, 0.497 mmol) in DMF (10 mL). The reaction mixture was stirred at room temperature for 1 h and then partitioned between ethyl acetate and H_2O. The organic layer was washed twice with H_2O, once with brine and dried over Na_2SO_4. Removal of the solvent and flash chromatography (SiO_2; petroleum ether/ethyl acetate, 50 : 1 to 3 : 1) afforded benzyl 4-iodophenylacetate as a colourless solid, yield: 0.124 g (92%), m.p. 49–50 °C. IR (ATR): ν = 3031, 2955, 2893, 2056, 2030, 2010, 1918, 1844, 1771, 1722, 1684, 1635, 1485, 1457, 1416, 1399, 1330, 1216, 1165, 1058, 1006, 974, 911, 847, 796, 754, 699 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 3.61 (s, 2 H), 5.14 (s, 2 H), 7.03 (d, J = 8.3 Hz, 2 H), 7.30–7.38 (m, 5 H), 7.65 (d, J = 8.3 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 40.93 (CH_2), 66.96 (CH_2), 92.83 (C), 128.35 (2 CH), 128.49 (CH), 128.73 (2 CH), 131.45 (2 CH), 133.61 (C), 135.75 (C), 137.79 (2 CH), 171.01 (C═O). MS (EI): m/z (%) = 352 (28) $[M^+]$, 217 (84), 91 (100), 90 (20), 89 (17). Elemental analysis calcd for $C_{15}H_{13}IO_2$: C 51.16, H 3.72; found: C 51.24, H 3.70.

Benzyl 4-benzoylthiophenylacetate. Thiobenzoic acid (0.8 mL, 6.8 mmol), EtN(i-Pr)$_2$ (1.46 g, 2.83 mmol) and copper(I) iodide (0.108 g, 0.567 mmol) were added to a solution of benzyl 4-iodophenylacetate (1.99 g, 5.66 mmol) and 1,10-phenanthroline (0.203 g, 1.13 mmol) in toluene (20 mL). The reaction mixture was stirred at reflux for 18 h and then filtered through a short path of Celite®. Removal of the solvent and flash chromatography (SiO_2; petroleum ether/ethyl acetate, 20 : 1 to 2 : 1) afforded benzyl 4-benzoylthiophenylacetate as a light brown solid, yield: 1.73 g (84%), m.p. 130–131 °C. IR (ATR): ν = 3348, 3094, 3057, 2988, 2913, 2842, 2056, 2030, 1844, 1771, 1734, 1704, 1692, 1618, 1585, 1516, 1475, 1457, 1437, 1409, 1369, 1340, 1308, 1274, 1246, 1213, 1164, 1144, 1058, 963, 934, 856, 843, 812, 756, 722, 680, 640, 612 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.73 (s, 2 H), 5.15 (s, 2 H), 7.31–7.42 (m, 7 H), 7.46–7.52 (m, 4 H), 7.62 (t, J = 7.4 Hz, 1 H), 8.03 (d, J = 7.2 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 41.24 (CH_2), 66.96 (CH_2), 126.28 (C), 127.63 (2 CH), 128.35 (2 CH), 128.46 (CH), 128.74 (2 CH), 128.90 (2 CH), 130.41 (2 CH), 133.84 (CH), 135.39 (2 CH), 135.60 (C), 135.81 (C), 136.72 (C), 171.01 (C═O), 190.27 (C═O). ESI-MS: m/z = 742.4 [2 M + NH_4]$^+$. Elemental analysis calcd for $C_{22}H_{18}O_3S$: C 72.90, H 5.01, S 8.85; found: C 72.96, H 5.10, S 8.84.

Benzyl 2-(4-(chlorosulfonyl)phenyl)acetate. A solution of benzyltrimethylammonium chloride (0.993 g, 4.97 mmol) and trichloroisocyanuric acid (0.527 g, 2.27 mmol) in acetonitrile (9 mL) was stirred for 30 min and added to a solution of benzyl 4-benzoylthiophenylacetate (0.500 g, 1.42 mmol) in acetonitrile (5 mL). The reaction mixture was stirred at 0 °C for 17 min, then diluted with ethyl acetate, washed with a dil. aqueous solution of K_2CO_3, brine and dried over Na_2SO_4. Removal of the solvent and instantaneous flash chromatography (SiO_2; petroleum ether/ethyl acetate, 50 : 1 to 3 : 1) afforded benzyl 2-(4-(chlorosulfonyl)phenyl)acetate as white crystals, yield: 0.364 g (79%), m.p. 57–58 °C. IR (ATR): ν = 3090, 3028, 2893, 2826, 2674, 2559, 1736, 1683, 1597, 1496, 1473, 1458, 1417, 1369, 1333, 1295, 1238, 1215, 1164, 1079, 1017, 989, 930, 859, 829, 806, 741, 703, 676 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.80 (s, 2 H), 5.16 (s, 2 H), 7.31–7.39 (m, 5 H), 7.54 (d, J = 8.6 Hz, 2 H), 8.00 (d, J = 8.6 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 41.22 (CH_2), 67.40 (CH_2), 127.41 (2 CH), 128.51 (2 CH), 128.72 (CH), 128.81 (2 CH), 130.85 (2 CH), 135.39 (C), 142.05 (C), 143.32 (C), 169.91 (C═O). ESI-MS: m/z = 322.7 [M − H]$^-$.

22: EtN(i-Pr)$_2$ (0.111 mL, 0.653 mmol) was added to a solution of iBu-Lys(Cbz)-O*t*Bu (0.255 g, 0.65 mmol) and benzyl 2-(4-(chlorosulfonyl)phenyl)acetate (0.211 g, 0.65 mmol) in CH_2Cl_2 (20 mL). The mixture was stirred at room temperature for 3 d and then partitioned between CH_2Cl_2 and 2% aqueous citric acid. The aqueous layer was extracted three times with CH_2Cl_2 and the combined organic layers were washed with brine and dried over Na_2SO_4. Removal of the solvent and flash chromatography (SiO_2; pentane/ethyl acetate, 2 : 3) afforded **22** as a colourless oil, yield: 0.280 g (63%). IR (ATR): ν = 3389, 2959, 2929, 2870, 1724, 1651, 1529, 1454, 1395, 1368, 1337, 1242, 1149, 1089, 998, 845, 813, 735, 697, 677 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.85 (d, J = 6.6 Hz, 3 H), 0.90 (d, J = 6.6 Hz, 3 H), 1.29 (s, 9 H), 1.44–1.62 (m, 5 H), 1.85–1.99 (m, 2 H), 2.95 (dd, J = 14.5, 7.4 Hz, 1 H), 3.06–3.17 (m, 3 H), 3.70 (s, 2 H), 4.23 (t, J = 7.3 Hz, 1 H), 4.86 (br s, 1 H), 5.10 (s, 2 H), 5.12 (s, 2 H), 7.29–7.40 (m, 12 H), 7.78 (d, J = 8.3 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 20.33 (CH_3), 20.39 (CH_3), 23.65 (CH_2), 27.78 (3 CH_3), 28.03 (CH), 29.34 (CH_2), 30.28 (CH_2), 40.71 (CH_2), 40.93 (CH_2), 53.86 (CH_2), 60.76 (CH), 66.53 (CH_2), 66.99 (CH_2), 82.02 (C), 127.77 (2 CH), 128.06 (CH), 128.08 (CH), 128.26 (2 CH), 128.43 (2 CH), 128.49 (2 CH), 128.61 (2 CH), 129.84 (2 CH), 135.40 (C), 136.62 (C), 138.61 (C), 139.12 (C), 156.36 (C═O), 169.62 (C═O), 170.34 (C═O). ESI-MS: m/z = 698.6 [M + NH_4]$^+$.

23: Compound **22** (89.3 mg, 0.132 mmol) and palladium on activated carbon (90 mg, 10 wt%) in ethyl acetate (10 mL) were stirred under hydrogen (1 atm) at room temperature for 23 h. Filtration of the reaction mixture through a short path of Celite® and removal of the solvent afforded **23** as a colourless, viscous oil, yield: 56.9 mg (95%). IR (ATR): ν = 3055, 2959, 2929, 2870, 2056, 2030, 1917, 1770, 1728, 1631, 1577, 1460, 1431, 1366, 1336, 1256, 1152, 1089, 1025, 992, 920, 846, 788, 723, 701, 675 cm^{-1}. ^{1}H NMR (500 MHz, methanol-d_4): δ = 0.92 (d, J = 6.6 Hz, 3 H), 0.95 (d, J = 6.6 Hz, 3 H), 1.11–1.19 (m, 1 H), 1.23–1.32 (m, 2 H), 1.38 (s, 9 H), 1.42–1.54 (m, 2 H), 1.83–2.00 (m, 2 H), 2.65–2.77 (m, 2 H), 3.00 (dd, J = 14.4, 7.4 Hz, 1 H), 3.13 (dd, J = 14.5, 7.3 Hz, 1 H), 3.63 (s, 2 H), 4.13 (t, J = 7.3 Hz, 1 H), 7.52 (d, J = 8.3 Hz, 2 H), 7.78 (d, J = 8.3 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, methanol-d_4): δ = 20.76 (CH_3), 20.82 (CH_3), 24.65 (CH_2), 28.10 (CH_2), 28.16 (3 CH_3), 29.11 (CH), 30.57 (CH_2), 40.50 (CH_2), 44.24 (CH_2), 55.86 (CH_2), 62.06 (CH), 83.31 (C), 128.53 (2 CH), 131.23 (2 CH), 139.89 (C), 143.66 (C), 171.16 2 C═O. ESI-MS: m/z = 457.3 [M + H]$^+$, 479.3 [M + Na]$^+$, 913.6 [2 M + H]$^+$, 935.6 [2 M + Na]$^+$.

24: *N*-(FmocO)succinimide (0.239 g, 0.708 mmol) was added to a solution of **23** (0.216 g, 0.473 mmol) in acetonitrile (4 mL), THF (4 mL) and K_2CO_3 (1 M aqueous, 4 mL). The reaction mixture was stirred at room temperature for 17 h and then partitioned between CH_2Cl_2 and 2% aqueous citric acid. The organic layer was washed with 2% citric acid, H_2O and brine and dried over Na_2SO_4. Removal of the solvent and flash chromatography (SiO_2; petroleum ether/[CH_2Cl_2/MeOH 10 : 1] + 0.5% AcOH, 20 : 1 to 1 : 1) afforded **24** as a colourless, viscous oil, yield: 0.163 g (51%). IR (ATR): ν = 3369, 3067, 2958, 2929, 2869, 1844, 1723, 1530, 1450, 1416, 1393, 1367, 1336, 1247, 1152, 1089, 999, 938, 845, 813, 759, 740, 651 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.92 (t, J = 7.7 Hz, 6 H), 1.02–1.11 (m, 1 H), 1.24–1.34 (m, 4 H), 1.38 (s, 9 H), 1.78–1.86 (m, 1 H), 1.89–1.96 (m, 1 H), 2.90–3.06 (m, 4 H), 3.66 (s, 2 H), 4.12 (t, J = 7.2 Hz, 1 H), 4.20 (t, J = 6.7 Hz, 1 H), 4.41 (d, J = 6.8 Hz, 2 H), 4.84 (br t, J = 5.3 Hz, 1 H), 7.31 (t, J = 7.4 Hz, 2 H), 7.38–7.44 (m, 4 H), 7.58 (d, J = 7.4 Hz, 2 H), 7.75–7.79 (m, 4 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 20.55 (CH_3), 20.57 (CH_3), 24.00 (CH_2), 27.97 (3 CH_3), 28.05 (CH), 29.42 (2 CH_2), 40.90 (CH_2), 41.24 (CH_2), 47.30 (CH), 53.73 (CH_2), 60.63 (CH), 66.95 (CH_2), 82.30 (C), 120.13 (2 CH), 125.15 (2 CH), 127.20 (2 CH), 127.70 (2 CH), 127.84 (2 CH), 130.11 (2 CH), 139.02 (C), 139.45 (C), 141.44 (2 C), 143.99 (2 C), 157.00 (C═O), 170.01 (C═O), 173.79 (C═O). ESI-MS: m/z = 679.5 [M + H]$^+$, 696.3 [M + NH_4]$^+$, 701.6 [M + Na]$^+$.

25: EtN(i-Pr)$_2$ (1.25 g, 9.61 mmol) and 4-toluenesulfonyl chloride (1.22 g, 6.42 mmol) were added to a solution of i-Bu-Lys(Cbz)-O*t*Bu (2.52 g, 6.42 mmol) in CH_2Cl_2 (60 mL). The mixture was stirred at room temperature for 5 d and then partitioned between CH_2Cl_2 and 2% aqueous citric acid. The aqueous layer was extracted three times with CH_2Cl_2, the combined organic layers were washed with brine and dried over Na_2SO_4. Removal of the solvent and flash chromatography (SiO_2; petroleum ether/ethyl acetate, 1 : 1) afforded **25** as a colourless solid, yield: 3.07 g (88%), m.p. 54–56 °C. IR (ATR): ν = 3398, 2952, 2872, 1727, 1710, 1686, 1653, 1617, 1596, 1558, 1528, 1456, 1395, 1367, 1336, 1308, 1244, 1230, 1147, 1111, 1088, 1013, 951, 866, 841, 816, 762, 718, 706, 661 cm^{-1}. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 0.84 (d, J = 6.6 Hz, 3 H), 0.90 (d, J = 6.6 Hz, 3 H), 1.30 (s, 9 H), 1.36–1.40 (m, 1 H), 1.46–1.63 (m, 4 H), 1.85–1.99 (m, 2 H), 2.40 (s, 3 H), 2.94 (dd, J = 14.6, 7.7 Hz, 1 H), 3.10 (dd, J = 14.6, 7.7 Hz, 1 H), 3.17 (q, J = 6.7 Hz, 2 H), 4.25 (t, J = 7.3 Hz, 1 H), 4.79 (br s, 1 H), 5.09 (s, 2 H), 7.27 (d, J = 7.2 Hz, 2 H), 7.29–7.38 (m, 5 H), 7.71 (d, J = 8.3 Hz, 2 H). ^{13}C NMR and DEPT (150 MHz, $CDCl_3$): δ = 20.52 (2 CH_3), 21.61 (CH_3), 23.82 (CH_2), 27.95 (3 CH_3), 28.37 (CH), 29.53 (CH_2), 30.62 (CH_2), 40.91 (CH_2), 53.91 (CH_2), 60.84 (CH), 66.73 (CH_2), 82.08 (C), 127.75 (2 CH), 128.22 (CH), 128.24 (CH), 128.66 (2 CH), 129.58 (3 CH), 136.79 (C), 137.44 (C), 143.30 (C), 156.51 (C═O), 169.89 (C═O). ESI-MS: m/z = 547.8 $[M + H]^+$, 564.7 $[M + NH_4]^+$.

26: Compound **25** (0.101 g, 0.185 mmol) and palladium on activated carbon (50 mg, 10 wt%) in ethyl acetate (15 mL) were stirred under hydrogen (1 atm) at room temperature for 24 h. Filtration of the reaction mixture through a short path of Celite® and removal of the solvent afforded **26** as a light yellow oil, yield: 75.4 mg (99%). IR (ATR): ν = 2987, 2932, 2870, 1727, 1673, 1654, 1598, 1534, 1493, 1464, 1451, 1394, 1367, 1338, 1250, 1151, 1089, 996, 937, 845, 814, 719, 664, 656 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$/methanol-d_4, 3 : 1): δ = 0.66 (d, J = 6.6 Hz, 3 H), 0.71 (d, J = 6.7 Hz, 3 H), 1.11 (s, 9 H), 1.14–1.20 (m, 2 H), 1.22–1.34 (m, 2 H), 1.36–1.46 (m, 1 H), 1.65–1.80 (m, 2 H), 2.22 (s, 3 H), 2.44 (t, J = 7.2 Hz, 2 H), 2.75 (dd, J = 14.7, 7.4 Hz, 1 H), 2.90 (dd, J = 14.7, 7.6 Hz, 1 H), 4.04 (t, J = 7.4 Hz, 1 H), 7.11 (d, J = 8.2 Hz, 2 H), 7.51 (d, J = 8.2 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$/methanol-d_4, 3 : 1): δ = 20.48 (CH_3), 20.53 (CH_3), 21.54 (CH_3), 24.14 (CH_2), 27.92 (3 CH_3), 28.43 (CH), 30.91 (CH_2), 32.71 (CH_2), 41.56 (CH_2), 54.17 (CH_2), 61.21 (CH), 82.55 (C), 127.84 (2 CH), 129.88 (2 CH), 137.25 (C), 143.93 (C), 170.18 (C═O). ESI-MS: m/z = 413.4 $[M + H]^+$.

(*S*)-*tert*-Butyl 6-(2-(4-(2-benzyloxy-2-oxoethyl)phenoxy)acetamido)-2-(*N*-isobutyl-4-methylphenylsulfonamido)hexanoate. A catalytic amount of DMAP was added to a solution of **26** (92.3 mg, 0.224 mmol), **27** (0.100 g, 0.333 mmol) and EDC·HCl (64.3 mg, 0.335 mmol) in CH_2Cl_2 (10 mL). The reaction mixture was stirred at room temperature for 5 d, then diluted with CH_2Cl_2, washed three times with H_2O, once with brine and dried over Na_2SO_4. Removal of the solvent and flash chromatography (SiO_2; pentane/ethyl acetate, 5 : 1 to 2 : 3) afforded (*S*)-*tert*-butyl 6-(2-(4-(2-benzyloxy-2-oxoethyl)phenoxy)acetamido)-2-(*N*-isobutyl-4-methylphenylsulfonamido)hexanoate as a colourless solid, yield: 87.4 mg (56%), m.p. 72–74 °C. IR (ATR): ν = 3304, 2931, 2864, 1726, 1674, 1595, 1537, 1512, 1450, 1395, 1368, 1337, 1305, 1243, 1152, 1088, 1057, 1001, 843, 815, 740, 721, 697 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.84 (d, J = 6.6 Hz, 3 H), 0.89 (d, J = 6.6 Hz, 3 H), 1.29 (s, 9 H), 1.37–1.47 (m, 1 H), 1.51–1.67 (m, 4 H), 1.85–1.99 (m, 2 H), 2.40 (s, 3 H), 2.94 (dd, J = 14.7, 7.4 Hz, 1 H), 3.10 (dd, J = 14.7, 7.6 Hz, 1 H), 3.33 (q, J = 6.7 Hz, 2 H), 3.62 (s, 2 H), 4.28 (t, J = 7.4 Hz, 1 H), 4.46 (s, 2 H), 5.13 (s, 2 H), 6.66 (br t, J = 5.6 Hz, 1 H), 6.89 (d, J = 8.7 Hz, 2 H), 7.23 (d, J = 8.7 Hz, 2 H), 7.27 (d, J = 8.5 Hz, 2 H), 7.30–7.37 (m, 5 H), 7.71 (d, J = 8.3 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 20.51 (CH_3), 20.57 (CH_3), 21.61 (CH_3), 23.89 (CH_2), 27.92 (3 CH_3), 28.27 (CH), 29.10 (CH_2), 30.61 (CH_2), 38.93 (CH_2), 40.49 (CH_2), 53.88 (CH_2), 60.74 (CH), 66.81 (CH_2), 67.55 (CH_2), 82.08 (C), 114.96 (2 CH), 127.65 (C), 127.74 (2 CH), 128.31 (2 CH), 128.41 (CH), 128.70 (2 CH), 129.57 (2 CH), 130.81 (2 CH), 135.90 (C), 137.34 (C), 143.32 (C), 156.51 (C), 168.25 (C═O), 169.83 (C═O), 171.67 (C═O). ESI-MS: m/z = 695.4 $[M + H]^+$, 712.6 $[M + NH_4]^+$.

28: (*S*)-*tert*-Butyl 6-(2-(4-(2-benzyloxy-2-oxoethyl)phenoxy)-acetamido)-2-(*N*-isobutyl-4-methylphenylsulfonamido)hexanoate (0.143 g, 0.206 mmol) and palladium on activated carbon (14 mg, 10 wt%) in ethyl acetate (10 mL) were stirred under hydrogen (1 atm) at room temperature for 2 h. Filtration of the reaction mixture through a short path of Celite® and removal of the solvent afforded **28** as a colourless solid, yield: 0.125 g (100%), m.p. 47–49 °C. IR (ATR): ν = 3364, 2957, 2933, 2870, 1725, 1641, 1611, 1569, 1555, 1512, 1449, 1394, 1367, 1337, 1291, 1239, 1152, 1089, 1062, 1000, 940, 843, 813, 720, 664 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$/methanol-d_4, 3 : 1): δ = 0.69 (d, J = 6.6 Hz, 3 H), 0.75 (d, J = 6.6 Hz, 3 H), 1.15 (s, 9 H), 1.21–1.31 (m, 2 H), 1.37–1.51 (m, 3 H), 1.71–1.83 (m, 2 H), 2.26 (s, 3 H), 2.79 (dd, J = 14.7, 7.4 Hz, 1 H), 2.94 (dd, J = 14.7, 7.6 Hz, 1 H), 3.15 (td, J = 7.0, 2.5 Hz, 2 H), 3.41 (s, 2 H), 4.09 (t, J = 7.4 Hz, 1 H), 4.30 (s, 2 H), 6.76 (d, J = 8.7 Hz, 2 H), 7.08 (d, J = 8.7 Hz, 2 H), 7.15 (d, J = 8.2 Hz, 2 H), 7.55 (d, J = 8.2 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$/methanol-d_4, 3 : 1): δ = 19.90 (CH_3), 19.97 (CH_3), 21.00 (CH_3), 23.45 (CH_2), 27.34 (3 CH_3), 27.81 (CH), 28.46 (CH_2), 30.10 (CH_2), 38.49 (CH_2), 39.95 (CH_2), 53.55 (CH_2), 60.43 (CH), 66.89 (CH_2), 81.95 (C), 114.47 (2 CH), 127.23 (2 CH), 127.75 (C), 129.27 (2 CH), 130.33 (2 CH), 136.60 (C), 143.28 (C), 156.03 (C), 168.74 (C═O), 169.50 (C═O), 174.08 (C═O). ESI-MS: m/z = 603.1 $[M - H]^-$.

Benzyl 4-(2-*tert*-butoxy-2-oxoethoxy)phenylacetate. *tert*-Butyl bromoacetate (4.00 mL, 27.1 mmol) was added dropwise to a mixture of benzyl 4-hydroxyphenylacetate (6.57 g, 27.1 mmol) and Cs_2CO_3 (13.3 g, 40.7 mmol) in DMF (60 mL). The reaction mixture was stirred at room temperature for 5 h. The reaction mixture was diluted with ethyl acetate and washed four times with water and once with brine. The organic layer was dried over Na_2SO_4. Removal of the solvent and flash chromatography (SiO_2; pentane/ethyl acetate, 20 : 1 to 3 : 2) afforded benzyl 4-(2-*tert*-butoxy-2-oxoethoxy)phenylacetate as a colourless oil, yield: 9.15 g (95%). IR (ATR): ν = 3065, 3031, 2978, 2934, 2056, 1918, 1869, 1844, 1828, 1727, 1651, 1611, 1558, 1541, 1512, 1454, 1369, 1303, 1216, 1145, 1079, 982, 842, 737, 697 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.49 (s, 9 H), 3.61 (s, 2 H),

4.50 (s, 2 H), 5.12 (s, 2 H), 6.85 (d, J = 8.7 Hz, 2 H), 7.20 (d, J = 8.7 Hz, 2 H), 7.30–7.37 (m, 5 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 28.19 (3 CH_3), 40.59 (CH_2), 65.89 (CH_2), 66.74 (CH_2), 82.52 (C), 114.85 (2 CH), 127.01 (C), 128.28 (2 CH), 128.36 (CH), 128.69 (2 CH), 130.53 (2 CH), 135.98 (C), 157.21 (C), 168.16 (C═O), 171.76 (C═O). ESI-MS: m/z = 374.2 $[M + NH_4]^+$, 730.5 $[2\ M + NH_4]^+$.

27: Trifluoroacetic acid (60 mL) was added slowly at 0 °C to a solution of benzyl 4-(2-*tert*-butoxy-2-oxoethoxy)phenylacetate (1.28 g, 3.57 mmol) in CH_2Cl_2 (60 mL). The mixture was stirred at room temperature for 90 min, diluted with CH_2Cl_2 and washed four times with water and once with brine. Drying over Na_2SO_4 and removal of the solvent afforded **27** as a light yellow solid, yield: 0.815 g (76%), m.p. 103–104 °C. IR (ATR): ν = 3058, 3035, 2922, 2890, 2774, 1917, 1870, 1844, 1726, 1651, 1610, 1588, 1511, 1421, 1379, 1347, 1308, 1253, 1224, 1158, 1083, 984, 940, 907, 838, 807, 774, 741, 694 cm^{-1}. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 3.62 (s, 2 H), 4.67 (s, 2 H), 5.13 (s, 2 H), 6.88 (d, J = 8.6 Hz, 2 H), 7.23 (d, J = 8.6 Hz, 2 H), 7.30–7.37 (m, 5 H). ^{13}C NMR and DEPT (150 MHz, $CDCl_3$): δ = 40.54 (CH_2), 65.04 (CH_2), 66.84 (CH_2), 114.94 (2 CH), 127.73 (C), 128.31 (2 CH), 128.41 (CH), 128.71 (2 CH), 130.76 (2 CH), 135.91 (C), 156.67 (C), 171.74 (C═O), 173.12 (C═O). ESI-MS: m/z = 318.1 $[M + NH_4]^+$, 618.0 $[2\ M + NH_4]^+$, 623.1 $[2\ M + Na]^+$. Elemental analysis calcd for $C_{17}H_{16}O_5$: C 67.99, H 5.37; found: C 68.13, H 5.34.

32: A solution of 3,5-dichloro-2-hydroxyacetophenone (**29**) (1.00 g, 4.88 mmol) and 4-hydrazinobenzoic acid (**30**) (0.742 g, 4.88 mmol) in ethanol (90 mL) and AcOH (0.25 mL) was stirred at reflux for 2.5 h. During the reaction, brown crystals precipitated and were collected by filtration of the hot reaction mixture. The filtrate was concentrated, ethanol (30 mL) was added, the mixture was heated at reflux and filtered immediately. These steps were repeated (10 mL ethanol). After drying **32** was obtained as brown crystals, yield: 1.09 g (66%), m.p. 299–301 °C. IR (ATR): ν = 3379, 2078, 2790, 2530, 2053, 1661, 1600, 1577, 1518, 1451, 1420, 1290, 1251, 1163, 1120, 939, 839, 766, 737, 684 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 2.43 (s, 3 H), 7.12 (d, J = 8.7 Hz, 2 H), 7.55 (s, 1 H), 7.60 (s, 1 H), 7.91 (d, J = 8.7 Hz, 2 H), 10.21 (s, 1 H), 12.50 (br s, 1 H), 13.56 (s, 1 H). ^{13}C NMR and DEPT (125 MHz, DMSO-d_6): δ = 13.63 (CH_3), 111.88 (2 CH), 121.33 (C), 122.03 (C), 122.47 (C), 125.99 (CH), 129.03 (CH), 131.38 (2 CH), 147.62 (C), 148.00 (C), 152.02 (C), 167.06 (C═O, C═N). ESI-MS: m/z = 339.1 $[M + H]^+$. Elemental analysis calcd for $C_{15}H_{12}Cl_2N_2O_3$: C 53.12, H 3.57, N 8.26; found: C 52.86, H 3.54, N 8.18.

33: A solution of 3,5-dichloro-2-hydroxyacetophenone (**29**) (1.00 g, 4.88 mmol) and 4-hydrazinophenylacetic acid[36] (**31**) (0.811 g, 4.87 mmol) in ethanol (90 mL) and AcOH (0.25 mL) was stirred and heated at reflux for 2.5 h. The solvent was removed and the residue recrystallised from ethyl acetate (100 mL) and i-hexane (600 mL). After drying **33** was obtained as orange crystals, yield: 0.881 g (51%), m.p. 231–233 °C. IR (ATR): ν = 3371, 3077, 3031, 2906, 2731, 2635, 2566, 2056, 1733, 1704, 1652, 1619, 1585, 1517, 1454, 1424, 1366, 1301, 1244, 1198, 1174, 1116, 954, 853, 822, 799, 736 cm^{-1}. ^{1}H NMR (500 MHz, acetone-d_6): δ = 2.46 (s, 3 H), 3.58 (s, 2 H), 7.09 (d, J = 8.5 Hz, 2 H), 7.30 (d, J = 8.5 Hz, 2 H), 7.38 (d, J = 2.4 Hz, 1 H), 7.52 (d, J = 2.4 Hz, 1 H), 9.16 (s, 1 H), 10.71 (br s, 1 H), 13.67 (s, 1 H). ^{13}C NMR and DEPT (125 MHz, acetone-d_6): δ = 12.79 (CH_3), 40.47 (CH_2), 113.81 (2 CH), 115.85 (C), 122.67 (C), 123.50 (C), 126.15 (CH), 128.31 (C), 129.47 (CH), 131.31 (2 CH), 136.20 (C), 153.30 (C), 172.93 (C═O), one carbon signal missing. ESI-MS: m/z = 353.2 $[M + H]^+$.

***tert*-Butyl 4-hydroxyphenylacetate (35).** A mixture of 4-hydroxyphenylacetic acid (**34**) (2.00 g, 13.2 mmol), *tert*-butyl acetoacetate (14 mL, 84.4 mmol) and conc. H_2SO_4 (0.104 g, 1.06 mmol) was stirred in a sealed tube at room temperature for 45 h and then diluted with diethyl ether. The organic layer was washed twice with H_2O, twice with $NaHCO_3$ (dil. aqueous), once with brine and dried over $MgSO_4$. Removal of the solvent and flash chromatography (SiO_2; pentane/ethyl acetate, 8 : 1 to 6 : 1) afforded *tert*-butyl 4-hydroxyphenylacetate (**35**) as a colourless solid, yield: 1.14 g (41%), m.p. 92–93 °C. IR (ATR): ν = 3306, 3007, 2980, 2934, 2056, 2030, 2009, 1917, 1871, 1844, 1771, 1734, 1717, 1693, 1653, 1613, 1593, 1559, 1515, 1446, 1369, 1313, 1269, 1221, 1166, 1129, 954, 859, 826, 808, 767, 711, 665 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.44 (s, 9 H), 3.45 (s, 2 H), 5.15 (s, 1 H), 6.74 (d, J = 8.5 Hz, 2 H), 7.11 (d, J = 8.5 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 28.17 (3 CH_3), 41.86 (CH_2), 81.13 (C), 115.54 (2 CH), 126.67 (C), 130.50 (2 CH), 154.81 (C), 171.97 (C═O). MS (EI): m/z (%) = 208 (13) [M^+], 107 (93), 77 (16), 57 (100), 41 (19).

36: A mixture of *tert*-butyl 4-hydroxyphenylacetate (**35**) (1.12 g, 5.38 mmol) and 5-fluoro-2-nitrobenzaldehyde (0.915 g, 5.38 mmol), K_2CO_3 (0.901 g, 6.45 mmol) in DMF (46 mL) was stirred at 120 °C for 45 min. The reaction mixture was partitioned between 2% aqueous citric acid and ethyl acetate. The organic layer was washed three times with H_2O, once with brine and dried over $MgSO_4$. Removal of the solvent and flash chromatography (SiO_2; pentane/ethyl acetate, 30 : 1 to 6 : 1) afforded **36** as a yellow solid, yield: 1.20 g (63%), m.p. 69–70 °C. IR (ATR): ν = 3104, 3072, 2978, 2929, 2056, 2031, 1771, 1727, 1697, 1653, 1624, 1581, 1541, 1510, 1469, 1417, 1365, 1326, 1296, 1270, 1230, 1203, 1140, 1067, 1019, 966, 936, 878, 849, 808, 757, 681, 643 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.46 (s, 9 H), 3.57 (s, 2 H), 7.04 (d, J = 8.5 Hz, 2 H), 7.20 (d, J = 9.0 Hz, 1 H), 7.34–7.36 (m, 3 H), 8.15 (d, J = 9.0 Hz, 1 H), 10.43 (s, 1 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 28.19 (3 CH_3), 41.95 (CH_2), 81.38 (C), 116.91 (CH), 120.70 (2 CH), 127.49 (CH), 131.53 (2 CH), 131.67 (CH), 132.66 (C), 134.56 (C), 143.35 (C), 153.13 (C), 163.09 (C), 170.75 (C), 188.20 (CHO). ESI-MS: m/z = 375.2 $[M + NH_4]^+$, 732.3 $[2\ M + NH_4]^+$. Elemental analysis calcd for $C_{19}H_{19}NO_6$: C 63.86, H 5.36, N 3.92; found: C 63.92, H 5.37, N 3.91.

38: $NaBH_3CN$ (0.137 g, 2.18 mmol) was added to a solution of **36** (0.688 g, 1.93 mmol) and **37** (0.540 g, 1.93 mmol) in AcOH (114 µL, 1.99 mmol) and CH_2Cl_2 (20 mL). The reaction mixture was stirred at room temperature for 17 h and then treated with 1 M HCl (12 mL) for 10 min. After addition of 1 M NaOH (16 mL), the layers were separated, and the aqueous layer was extracted twice with CH_2Cl_2. The combined organic layers were washed with brine and dried over $MgSO_4$. Removal of the solvent and flash chromatography (SiO_2; pentane/ethyl acetate, 10 : 1 to 2 : 3) afforded **38** as a yellow, viscous oil, yield: 0.998 g

(83%). IR (ATR): ν = 3432, 2924, 2852, 2056, 2030, 1843, 1729, 1611, 1581, 1558, 1540, 1515, 1473, 1448, 1419, 1365, 1339, 1256, 1226, 1141, 1101, 1017, 957, 892, 830, 754, 694 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.98–1.85 (m, 32 H), 2.24–2.36 (m, 2 H), 2.42–2.53 (m, 1 H), 2.78 (s, 0.4 × 3 H), 2.80 (s, 0.6 × 3 H), 3.54 (s, 2 H), 3.57–3.60 (m, 0.6 H), 3.96–4.05 (m, 2 H), 4.40–4.44 (m, 0.4 H), 6.80–6.84 (m, 1 H), 7.02 (d, J = 8.5 Hz, 2 H), 7.23 (d, J = 7.6 Hz, 1 H), 7.30 (d, J = 8.5 Hz, 2 H), 7.94 (d, J = 9.0 Hz, 1 H). ESI-MS: m/z = 622.6 $[M + H]^+$, 1243.8 $[2 M + H]^+$.

39: Compound **38** (0.859 g, 1.38 mmol) and palladium on activated carbon (0.127 g, 10 wt%) in methanol (14 mL) were stirred under hydrogen (1 atm) at room temperature for 2 h. Filtration of the reaction mixture through a short path of Celite® and removal of the solvent afforded **39** as a yellow oil, yield: 0.772 g (94%). IR (ATR): ν = 3415, 3321, 2924, 2852, 2056, 2026, 2007, 1727, 1684, 1624, 1576, 1558, 1541, 1447, 1366, 1295, 1222, 1138, 1101, 1015, 954, 888, 810, 755 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.99–1.85 (m, 34 H), 2.24–2.37 (m, 2 H), 2.38–2.55 (m, 1 H), 2.78 (s, 0.4 × 3 H), 2.79 (s, 0.6 × 3 H), 3.47 (s, 2 H), 3.48–3.51 (m, 0.4 H), 3.68–3.76 (m, 2 H), 4.40–4.45 (m, 0.6 H), 6.62 (dd, J = 8.2, 3.4 Hz, 1 H), 6.76–6.81 (m, 2 H), 6.85 (d, J = 8.6 Hz, 2 H), 7.16 (d, J = 8.6 Hz, 2 H). ESI-MS: m/z = 592.5 $[M + H]^+$.

40: A solution of **39** (0.888 g, 1.50 mmol) and cyanogen bromide (0.240 g, 2.27 mmol) in ethanol (30 mL) was stirred at reflux for 17 h. After removal of the solvent, the residue was purified by flash chromatography (SiO_2; pentane/[CH_2Cl_2/methanol 10 : 1], 5 : 1 to 2 : 3) to afford **40** as a light brown solid, yield: 0.775 g (84%), m.p. 112–113 °C. IR (ATR): ν = 3127, 2972, 2926, 2853, 1844, 1726, 1658, 1618, 1557, 1496, 1448, 1414, 1366, 1314, 1236, 1216, 1140, 1102, 1015, 955, 875, 825 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.85–1.87 (m, 32 H), 2.02–2.18 (m, 2 H), 2.59–2.71 (m, 1 H), 2.80 (s, 0.4 × 3 H), 2.86 (s, 0.6 × 3 H), 3.51 (s, 2 H), 4.11–4.21 (m, 2.4 H), 4.34–4.45 (m, 0.6 H), 6.70 (d, J = 3.1 Hz, 1 H), 6.02–6.94 (m, 3 H), 7.19 (d, J = 8.7 Hz, 1 H), 7.22 (d, J = 8.5 Hz, 2 H), 8.17 (br s, 2 H). ESI-MS: m/z = 617.6 $[M + H]^+$.

41: A mixture of **40** (0.245 g, 0.397 mmol), silica gel (2.0 g) and toluene (13 mL) was stirred at reflux for 3.5 h. The reaction mixture was diluted with CH_2Cl_2/methanol (10 : 1) and filtered through a short path of Celite®. After removal of the solvent, compound **41** was obtained as a light yellow solid, yield: 0.220 g (99%), m.p. 145–146 °C. IR (ATR): ν = 3306, 3121, 2924, 2853, 2029, 1724, 1658, 1616, 1558, 1495, 1447, 1410, 1314, 1235, 1214, 1166, 1146, 1099, 1017, 955, 891, 803 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.86–1.87 (m, 23 H), 2.01–2.06 (m, 0.5 × 2 H), 2.18–2.22 (m, 0.5 × 2 H), 2.59–2.65 (m, 1 H), 2.79 (s, 0.5 × 3 H), 2.85 (s, 0.5 × 3 H), 3.47–3.53 (m, 0.5 H), 3.61 (s, 2 H), 4.10–4.24 (m, 2 H), 4.32–4.35 (m, 0.5 H), 6.70–6.74 (m, 1 H), 6.86–6.93 (m, 3 H), 7.13 (d, J = 8.4 Hz, 1 H), 7.22–7.25 (m, 2 H), 8.11 (br s, 2 H), 11.58 (br s, 1 H). ESI-MS: m/z = 561.5 $[M + H]^+$.

44: To a solution of Fmoc-Glu-O*t*Bu (**43**) (0.430 g, 1.01 mmol) and HATU (0.384 g, 1.01 mmol) in CH_2Cl_2 (15 mL) was added EtN(i-Pr)$_2$ (172 μL, 1.01 mmol). After stirring at room temperature for 10 min, **42** (0.224 g, 0.537 mmol) and EtN(i-Pr)$_2$ (223 μL, 1.31 mmol) were added. After stirring for 18 h at room temperature, the mixture was partitioned between 2% aqueous citric acid and CH_2Cl_2, washed with 2% citric acid, H_2O and brine. The organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by flash chromatography (SiO_2; pentane/[CH_2Cl_2/methanol 10 : 1], 10 : 1 to 2 : 3) to yield a colourless solid, yield: 0.249 g (61%). This solid (0.139 g, 0.185 mmol) was dissolved in CH_2Cl_2 (5 mL). CF_3COOH (5 mL) was added at 0 °C, and the mixture stirred at room temperature for 90 min, diluted with CH_2Cl_2 and washed four times with water and once with brine. After drying over Na_2SO_4 and removal of the solvent, compound **44** was obtained as a colourless solid, yield: 0.118 g (93%), m.p. 152–154 °C. IR (ATR): ν = 3272, 3067, 2931, 2857, 1770, 1694, 1632, 1583, 1533, 1490, 1475, 1447, 1387, 1340, 1226, 1156, 1050, 951, 891, 823, 778, 757, 739, 676, 648 cm^{-1}. ^1H NMR (500 MHz, methanol-d_4): δ = 1.11–1.20 (m, 1 H), 1.26–1.40 (m, 4 H), 1.63–2.05 (m, 8 H), 2.11–2.16 (m, 2 H), 2.19–2.28 (m, 1 H), 2.31–2.44 (m, 2 H), 2.47–2.55 (m, 1 H), 2.85–2.91 (m, 1 H), 3.65–3.76 (m, 2 H), 4.15–4.40 (m, 5 H), 7.15–7.23 (m, 3 H), 7.28–7.32 (m, 2 H), 7.37–7.41 (m, 2 H), 7.47 (q, J = 6.6 Hz, 1 H), 7.63–7.69 (m, 2 H), 7.79–7.83 (m, 2 H). ^{13}C NMR and DEPT (125 MHz, methanol-d_4): δ = 26.07 (2 CH_2), 26.42 (CH_2), 28.11 (CH_2), 28.34 (CH_2), 29.68 (CH_2), 29.89 (CH_2), 33.13 (CH_2), 34.92 (CH_2), 38.88 (CH and CH_2), 42.57 (CH_2), 54.07 (CH), 54.77 (CH), 66.27 (C), 68.22 (CH_2), 117.22 (d, J = 21.2 Hz, CH), 119.65 (d, J = 22.0 Hz, CH), 121.00 (CH), 121.05 (CH), 126.17 (CH), 126.38 (CH), 128.18 (CH), 128.24 (CH), 128.46 (CH), 128.88 (2 CH), 132.01 (CH), 140.54 (C), 142.57 (C), 142.62 (C), 144.97 (C), 145.44 (C), 158.80 (C═O), 164.40 (d, J = 247.3 Hz, C), 168.09 (C═N), 172.49 (2 C═O), 175.41 (C═O). ESI-MS: m/z = 696.5 $[M + H]^+$.

45: EtN(i-Pr)$_2$ (375 μL, 2.21 mmol) was added to a solution of Fmoc-Glu-O*t*Bu (**43**) (0.910 g, 2.14 mmol) and HATU (0.814 g, 2.14 mmol) in CH_2Cl_2 (50 mL). The mixture was stirred at room temperature for 10 min and then 4-(*tert*-butyldimethylsilyloxymethyl)aniline (0.495 g, 2.08 mmol) and EtN(i-Pr)$_2$ (375 μL, 2.21 mmol) were added. After stirring at room temperature for 28 h, the mixture was partitioned between 2% aqueous citric acid and CH_2Cl_2, washed with 2% citric acid, H_2O and brine. The organic layer was dried over Na_2SO_4. Removal of the solvent and flash chromatography (SiO_2; pentane/ethyl acetate, 10 : 1 to 3 : 7) afforded the amide as a colourless solid, yield: 1.07 g (80%), m.p. 99–100 °C. IR (ATR): ν = 3326, 2953, 2930, 2888, 2856, 1771, 1733, 1694, 1662, 1601, 1557, 1528, 1452, 1410, 1369, 1338, 1250, 1155, 1081, 1056, 938, 835, 775, 758, 737, 664, 621 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.08 (s, 6 H), 0.93 (s, 9 H), 1.46 (s, 9 H), 1.87–1.95 (m, 1 H), 2.27–2.41 (m, 3 H), 4.20 (t, J = 6.8 Hz, 1 H), 4.26–4.30 (m, 1 H), 4.39–4.50 (m, 2 H), 4.68 (s, 2 H), 5.61 (br d, J = 8.2 Hz, 1 H), 7.25–7.33 (m, 4 H), 7.38–7.42 (m, 2 H), 7.55 (d, J = 8.3 Hz, 2 H), 7.60 (t, J = 6.5 Hz, 2 H), 7.77 (dd, J = 7.5, 2.4 Hz, 2 H), 8.29 (br s, 1 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = −5.09 (2 CH_3), 18.54 (C), 26.09 (3 CH_3), 28.11 (3 CH_3), 30.33 (CH_2), 34.10 (CH_2), 47.33 (CH), 53.85 (CH), 64.78 (CH_2), 67.23 (CH_2), 83.08 (C), 119.67 (2 CH), 120.14 (CH), 120.18 (CH), 125.15 (CH), 125.26 (CH), 126.87 (2 CH), 127.23 (2 CH), 127.91 (2 CH), 137.11 (C), 137.36 (C), 141.46 (C), 141.49 (C), 143.69 (C), 143.92 (C), 157.03 (C═O), 170.39 (C═O), 171.14 (C═O). ESI-MS: m/z = 645.5 $[M + H]^+$, 662.5 $[M + NH_4]^+$.

HF–pyridine (5.0 mL) was added at 0 °C to a solution of this silyl ether (0.203 g, 0.315 mmol) in THF (10 mL). The reaction mixture was stirred at 0 °C for 75 min, diluted with ethyl acetate and neutralised with Na_2CO_3 (sat. aqueous). The organic layer was washed with 2% aqueous citric acid, H_2O, brine and dried over Na_2SO_4. After removal of the solvent, the residue was purified by flash chromatography (SiO_2; petroleum ether/[CH_2Cl_2/methanol 10 : 1], 10 : 1 to 1 : 1), affording the alcohol as a colourless solid, yield: 0.145 g (87%), m.p. 140–142 °C. IR (ATR): ν = 3311, 3253, 3121, 3058, 2931, 2856, 1695, 1662, 1595, 1534, 1447, 1414, 1367, 1337, 1257, 1227, 1153, 1081, 1054, 1033, 960, 892, 827, 784, 755, 733, 666 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 1.46 (s, 9 H), 1.87–1.95 (m, 1 H), 2.27–2.41 (m, 3 H), 4.18 (t, J = 6.8 Hz, 1 H), 4.25–4.30 (m, 1 H), 4.38–4.49 (m, 2 H), 4.62 (s, 2 H), 5.60 (br d, J = 8.0 Hz, 1 H), 7.29–7.33 (m, 4 H), 7.38–7.42 (m, 2 H), 7.57–7.61 (m, 4 H), 7.77 (dd, J = 7.5, 2.9 Hz, 2 H), 8.35 (br s, 1 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 28.12 (3 CH_3), 30.39 (CH_2), 34.16 (CH_2), 47.33 (CH), 53.86 (CH), 65.15 (CH_2), 67.26 (CH_2), 83.15 (C), 119.92 (2 CH), 120.16 (CH), 120.20 (CH), 125.15 (CH), 125.36 (CH), 127.24 (2 CH), 127.94 (4 CH), 136.74 (C), 137.84 (C), 141.46 (C), 141.48 (C), 143.69 (C), 143.90 (C), 157.07 (C═O), 170.53 (C═O), 171.09 (C═O). ESI-MS: m/z = 531.5 $[M + H]^+$, 553.5 $[M + Na]^+$.

A mixture of this alcohol (0.599 g, 1.13 mmol) and manganese dioxide (0.981 g, 11.3 mmol) in CH_2Cl_2 (60 mL) was stirred at room temperature for 22 h. The reaction mixture was filtered through a short path of Celite®. After removal of the solvent, the residue was purified by flash chromatography (SiO_2; petroleum ether/[CH_2Cl_2/methanol 10 : 1], 10 : 1 to 3 : 1), affording **45** as a colourless solid, yield: 0.484 g (81%), m.p. 104–105 °C. IR (ATR): ν = 3302, 3061, 2978, 2936, 1718, 1692, 1670, 1590, 1533, 1447, 1414, 1368, 1306, 1260, 1216, 1151, 1102, 1086, 1057, 1031, 963, 829, 734, 642 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ = 1.46 (s, 9 H), 1.82–1.95 (m, 1 H), 2.28–2.43 (m, 3 H), 4.19–4.29 (m, 2 H), 4.42–4.55 (m, 2 H), 5.63 (br d, J = 8.0 Hz, 1 H), 7.32 (t, J = 7.0 Hz, 2 H), 7.41 (td, J = 7.4, 2.8 Hz, 2 H), 7.59 (d, J = 7.4 Hz, 2 H), 7.76–7.85 (m, 6 H), 8.98 (br s, 1 H), 9.90 (s, 1 H). ^{13}C NMR and DEPT (75 MHz, $CDCl_3$): δ = 28.11 (3 CH_3), 30.81 (CH_2), 34.34 (CH_2), 47.34 (CH), 53.68 (CH), 67.41 (CH_2), 83.43 (C), 119.39 (2 CH), 120.24 (2 CH), 125.08 (CH), 125.16 (CH), 127.23 (CH), 127.27 (CH), 128.01 (2 CH), 131.26 (2 CH), 132.28 (C), 141.49 (C), 141.54 (C), 143.54 (C), 143.83 (C), 143.99 (C), 157.39 (CO), 170.86 (C═O), 171.04 (C═O), 191.17 (CHO). ESI-MS: m/z = 529.4 $[M + H]^+$, 546.4 $[M + NH_4]^+$.

46: EtN(i-Pr)$_2$ (468 µL, 2.75 mmol) was added to a solution of mono-*tert*-butyl glutarate (0.622 g, 3.30 mmol) and HATU (1.26 g, 3.31 mmol) in CH_2Cl_2 (25 mL). The mixture was stirred at room temperature for 10 min and then 4-(*tert*-butyldimethylsilyloxymethyl)aniline (0.523 g, 2.21 mmol) and EtN(i-Pr)$_2$ (468 µL, 2.75 mmol) were added. After stirring at room temperature for 24 h, the mixture was partitioned between 2% aqueous citric acid and CH_2Cl_2. The organic layer was washed with 2% citric acid, H_2O and brine. The organic layer was dried over Na_2SO_4. Removal of the solvent and flash chromatography (SiO_2; pentane/ethyl acetate, 20 : 1 to 3 : 7) afforded the amide as a colourless, viscous oil, yield: 0.702 g (78%). IR (ATR): ν = 3296, 2954, 2930, 2856, 1727, 1699, 1661, 1602, 1534, 1472, 1458, 1411, 1367, 1309, 1255, 1145, 1083, 1007, 939, 834, 775, 665 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.08 (s, 6 H), 0.93 (s, 9 H), 1.46 (s, 9 H), 2.01 (quin, J = 7.2 Hz, 2 H), 2.34 (t, J = 7.0 Hz, 2 H), 2.41 (t, J = 7.3 Hz, 2 H), 4.69 (s, 2 H), 7.27 (d, J = 8.4 Hz, 2 H), 7.48 (d, J = 8.4 Hz, 2 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = −5.08 (2 CH_3), 18.55 (C), 21.25 (CH_2), 26.09 (3 CH_3), 28.26 (3 CH_3), 34.55 (CH_2), 36.72 (CH_2), 64.78 (CH_2), 80.80 (C), 119.74 (2 CH), 126.91 (2 CH), 136.85 (C), 137.46 (C), 170.72 (C═O), 173.01 (C═O). ESI-MS: m/z = 408.3 $[M + H]^+$, 815.7 $[2 M + H]^+$.

TBAF (1 M in THF, 180 µL, 1.80 mmol) was added at 0 °C to a solution of this amide (0.503 g, 1.23 mmol) in THF (25 mL). The reaction mixture was stirred at room temperature for 1 h and then diluted with ethyl acetate. The organic layer was washed with H_2O, brine and dried over Na_2SO_4. After removal of the solvent, the residue was purified by flash chromatography (SiO_2; petroleum ether/ethyl acetate, 4 : 1 to 1 : 1), affording the alcohol as a colourless, viscous oil, yield: 0.348 g (96%). ^1H NMR (500 MHz, $CDCl_3$): δ = 1.45 (s, 9 H), 1.97 (quin, J = 7.2 Hz, 2 H), 2.15 (br s, 1 H), 2.31 (t, J = 7.1 Hz, 2 H), 2.38 (t, J = 7.4 Hz, 2 H), 4.62 (s, 2 H), 7.27 (d, J = 8.4 Hz, 2 H), 7.47 (d, J = 8.4 Hz, 2 H), 7.81 (br s, 1 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 21.20 (CH_2), 28.23 (3 CH_3), 34.56 (CH_2), 36.62 (CH_2), 64.97 (CH_2), 80.89 (C), 120.11 (2 CH), 127.91 (2 CH), 136.88 (C), 137.49 (C), 171.03 (C═O), 173.11 (C═O). ESI-MS: m/z = 294.2 $[M + H]^+$.

A mixture of this alcohol (0.348 g, 1.19 mmol) and manganese dioxide (1.02 g, 11.7 mmol) in CH_2Cl_2 (60 mL) was stirred at room temperature for 22 h. The reaction mixture was filtered through a short path of Celite®. After removal of the solvent, the residue was purified by flash chromatography (SiO_2; petroleum ether/ethyl acetate, 4 : 1 to 2 : 1), affording **46** as a colourless solid, yield: 0.257 g (74%), m.p. 131–132 °C. IR (ATR): ν = 3438, 3326, 3064, 3035, 2952, 2896, 2056, 2030, 1771, 1727, 1699, 1673, 1653, 1581, 1493, 1449, 1401, 1387, 1340, 1223, 1206, 1172, 1017, 1001, 972, 898, 853, 807, 771, 702, 682 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 1.46 (s, 9 H), 2.02 (quin, J = 6.7 Hz, 2 H), 2.36 (t, J = 6.9 Hz, 2 H), 2.47 (t, J = 7.3 Hz, 2 H), 7.72 (d, J = 8.6 Hz, 2 H), 7.84 (d, J = 8.6 Hz, 2 H), 8.06 (br s, 1 H), 9.91 (s, 1 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 21.04 (CH_2), 28.24 (3 CH_3), 34.43 (CH_2), 36.82 (CH_2), 81.10 (C), 119.29 (2 CH), 131.32 (2 CH), 132.27 (C), 143.78 (C), 171.34 (C═O), 173.16 (C═O), 191.23 (CHO). ESI-MS: m/z = 292.1 $[M + H]^+$. Elemental analysis calcd for $C_{16}H_{21}NO_2$: C 65.96, H 7.27, N 4.81; found: C 66.19, H 7.40, N 5.04.

47: $NaBH(OAc)_3$ (0.306 g, 1.44 mmol) and a solution of **45** (0.381 g, 0.721 mmol) in 1,2-$C_2H_4Cl_2$ (8 mL) were added to a solution of **42** (0.200 g, 0.480 mmol) and EtN(i-Pr)$_2$ (122 µL, 0.717 mmol) in 1,2-$C_2H_4Cl_2$ (8 mL). The reaction mixture was stirred for 18 h at room temperature and then partitioned between Na_2CO_3 (sat. aqueous) and CH_2Cl_2. The organic layer was washed with H_2O, brine and dried over Na_2SO_4. Removal of the solvent and purification of the residue by flash chromatography (SiO_2; pentane/[CH_2Cl_2/methanol 10 : 1], 10 : 1 to 2 : 3) afforded **47** as a colourless solid, yield: 0.289 g (70%), m.p. 114–116 °C. IR (ATR): ν = 3278, 3061, 2930, 2855, 1696, 1687, 1531, 1449, 1368, 1339, 1307, 1243, 1152, 1078, 1046, 893, 844, 824, 781, 738, 696 cm^{-1}. ^1H NMR (500 MHz,

$CDCl_3$): δ = 1.14–1.23 (m, 3 H), 1.32–1.40 (m, 2 H), 1.46 (s, 9 H), 1.58–1.64 (m, 1 H), 1.68–1.72 (m, 2 H), 1.87–1.95 (m, 4 H), 1.99–2.08 (m, 4 H), 2.26–2.43 (m, 4 H), 2.70–2.75 (m, 2 H), 3.47 (s, 2 H), 3.91–3.98 (m, 1 H), 4.17 (t, J = 6.9 Hz, 1 H), 4.26 (t, J = 7.5 Hz, 1 H), 4.36–4.47 (m, 2 H), 5.71 (br d, J = 8.0 Hz, 1 H), 6.94–7.03 (m, 3 H), 7.14 (d, J = 8.3 Hz, 2 H), 7.27–7.41 (m, 5 H), 7.57–7.59 (m, 4 H), 7.76 (d, J = 7.5 Hz, 2 H), 8.04 (br d, J = 7.4 Hz, 1 H), 8.67 (br s, 1 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 24.86 (2 CH_2), 25.55 (CH_2), 28.09 (3 CH_3), 30.28 (CH_2), 30.66 (2 CH_2), 32.88 (2 CH_2), 34.08 (CH_2), 47.26 (CH), 47.46 (2 CH_2), 51.68 (CH), 53.87 (CH), 61.96 (CH_2), 64.74 (C), 67.28 (CH_2), 83.07 (C), 114.78 (d, J = 20.7 Hz, CH), 117.26 (d, J = 21.9 Hz, CH), 119.88 (2 CH), 120.16 (2 CH), 120.19 (CH), 125.13 (CH), 125.21 (CH), 125.64 (CH), 127.21 (2 CH), 127.93 (2 CH), 129.92 (CH), 130.41 (d, J = 9.4 Hz, CH), 137.90 (d, J = 9.8 Hz, C), 138.18 (C), 141.42 (2 C), 143.63 (C), 143.87 (2 C), 157.08 (C═O), 162.99 (d, J = 247.8 Hz, C), 167.09 (C═N), 170.74 (C═O), 171.04 (C═O), 180.07 (C═O). ESI-MS: m/z = 857.8 $[M + H]^+$.

48: $NaBH(OAc)_3$ (91 mg, 0.429 mmol) and portionwise **46** (63 mg, 0.216 mmol) were added to a solution of **42** (62.5 mg, 0.15 mmol) and $EtN(i\text{-}Pr)_2$ (36 µL, 0.212 mmol) in 1,2-$C_2H_4Cl_2$ (2 mL). The reaction mixture was stirred for 19 h at room temperature and then partitioned between Na_2CO_3 (sat. aqueous) and CH_2Cl_2. The organic layer was washed with H_2O, brine and dried over Na_2SO_4. Removal of the solvent and purification of the residue by flash chromatography (SiO_2; pentane/[CH_2Cl_2/methanol 10 : 1], 20 : 1 to 1 : 1) afforded compound **48** as a colourless solid, yield: 68.3 mg (73%), m.p. 102–104 °C. IR (ATR): ν = 3263, 3183, 3114, 3058, 2930, 2855, 1723, 1692, 1585, 1530, 1491, 1455, 1396, 1367, 1339, 1308, 1241, 1139, 1079, 1047, 957, 892, 824, 781, 756, 691 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.15–1.23 (m, 3 H), 1.32–1.40 (m, 2 H), 1.44 (s, 9 H), 1.61–1.67 (m, 1 H), 1.69–1.74 (m, 2 H), 1.95–2.10 (m, 8 H), 2.30–2.40 (m, 6 H), 2.73–2.78 (m, 2 H), 3.48 (s, 2 H), 3.90–3.96 (m, 1 H), 6.93–7.02 (m, 3 H), 7.15 (d, J = 8.4 Hz, 2 H), 7.30–7.35 (m, 1 H), 7.52 (d, J = 8.3 Hz, 2 H), 7.99 (br s, 1 H), 8.09 (br d, J = 8.0 Hz, 1 H). ^{13}C NMR and DEPT (125 MHz, $CDCl_3$): δ = 21.17 (2 CH_2), 24.88 (CH_2), 25.57 (CH_2), 28.22 (3 CH_3), 30.72 (2 CH_2), 32.88 (2 CH_2), 34.58 (CH_2), 36.54 (CH_2), 47.48 (2 CH_2), 51.74 (CH), 61.94 (CH_2), 64.75 (C), 80.74 (C), 114.83 (d, J = 20.8 Hz, CH), 117.30 (d, J = 21.8 Hz, CH), 119.91 (2 CH), 125.69 (d, J = 2.7 Hz, CH), 129.92 (2 CH), 130.42 (d, J = 9.3 Hz, CH), 131.61 (C), 137.89 (d, J = 9.7 Hz, C), 138.04 (C), 162.98 (d, J = 247.4 Hz, C), 167.15 (C═N), 171.08 (C═O), 172.98 (C═O), 180.11 (C═O). ESI-MS: m/z = 620.9 $[M + H]^+$.

49: Trifluoroacetic acid (5 mL) was added slowly at 0 °C to a solution of **47** (50.1 mg, 58.4 µmol) in CH_2Cl_2 (5 mL). The mixture was stirred at room temperature for 90 min, diluted with CH_2Cl_2 and washed four times with water and once with brine. Drying over Na_2SO_4 and removal of the solvent afforded **49** as a light yellow solid, yield: 36.5 mg (78%), m.p. 156–159 °C. IR (ATR): ν = 3325, 2930, 2857, 1694, 1662, 1601, 1576, 1529, 1473, 1450, 1411, 1369, 1338, 1249, 1155, 1081, 1055, 1007, 938, 835, 775, 758, 737, 664 cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 1.07–1.16 (m, 1 H), 1.23–1.32 (m, 5 H), 1.61 (d, J = 12.0 Hz, 1 H), 1.69–1.80 (m, 3 H), 1.84–1.94 (m, 3 H), 1.98–2.09 (m, 4 H), 2.17–2.41 (m, 3 H), 2.68–2.77 (m, 3 H), 3.45–3.51 (m, 1 H), 3.60–3.69 (m, 2 H), 3.94–4.00 (m, 1 H), 4.14–4.30 (m, 2 H), 7.18–7.33 (m, 6 H), 7.39–7.49 (m, 4 H), 7.63 (t, J = 7.1 Hz, 2 H), 7.69–7.72 (m, 2 H), 7.89 (d, J = 7.6 Hz, 2 H), 8.14 (m, 1 H), 12.62 (br s, 1 H). HPLC ret. time = 14.9 min, 99.9%. ESI-MS: m/z = 801.7 $[M + H]^+$.

50: A mixture of **48** (57.3 mg, 92.5 µmol), silica gel (0.570 g) and toluene (15 mL) was stirred at reflux for 6 h. The reaction mixture was diluted with CH_2Cl_2/methanol (10 : 1) and filtered through a short path of Celite®. After removal of the solvent **50** was obtained as a light yellow solid, yield: 44.8 mg (86%), m.p. 143–146 °C. IR (ATR): ν = 3261, 3051, 2926, 2854, 1700, 1587, 1530, 1490, 1402, 1336, 1249, 1134, 1092, 1072, 1005, 938, 893, 833, 767 cm^{-1}. HPLC ret. time = 6.6 min, 97.3%. ESI-MS: m/z = 562.2 $[M - H]^-$.

51: Compound **49** (51.4 mg, 64.2 µmol) was dissolved in DMF (5 mL), piperidine (1 mL) was added and the reaction mixture was stirred at room temperature for 3.5 h. After complete removal of the solvent, the residue was washed twice with diethyl ether (1 mL) and taken up in the minimal volume of methanol. The product was precipitated by dropwise addition to diethyl ether (100 mL), filtered and dried. **51** was obtained as a colourless solid, yield: 27.7 mg (75%), m.p. 178–180 °C. IR (ATR): ν = 3316, 3064, 2930, 2856, 1693, 1600, 1531, 1515, 1473, 1451, 1410, 1366, 1338, 1308, 1249, 1155, 1080, 1051, 1006, 937, 892, 836, 776, 758, 739, 664 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$/methanol-d_4, 3 : 1): δ = 1.03–1.26 (m, 6 H), 1.45–1.52 (m, 2 H), 1.56–1.64 (m, 3 H), 1.79 (br d, J = 10.1 Hz, 2 H), 1.89–2.07 (m, 8 H), 2.40–2.43 (m, 2 H), 2.64 (br s, 2 H), 3.15 (s, 2 H), 3.31 (br s, 2 H), 3.40 (t, J = 5.8 Hz, 1 H), 3.61 (br s,1 H), 6.74 (dt, J = 9.5, 2.2 Hz, 1 H), 6.80 (d, J = 7.9 Hz, 1 H), 6.88–6.94 (m, 3 H), 7.15–7.20 (m, 1 H), 7.33 (d, J = 8.5 Hz, 2 H). HPLC ret. time = 3.2 min, 99.9%. ESI-MS: m/z = 579.5 $[M + H]^+$.

Typical procedure for the preparation of the peptides

H-Glu-Val-Asn-Sta-Val-Ala-Glu-Phe-4Gl-3Gl-4Gl-3Gl-4Gl-Asp-(ODhc)-NH_2 (**6a**), where Sta is (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid. Loading and automated assembly of Asp(ODhc)[14] to Glu[1]: the PAL-PEG-PS resin (0.38 mmol g^{-1}, 0.30 mmol) was subjected to automated deprotection, and loaded with Fmoc-Asp(ODhc)-OH, which was automatically activated and coupled. While the majority of the amino acids was coupled using the standard cycle as described above, the building blocks **4d–g**, **24**, **28**, **32**, **33**, **41**, **44**, **49** and **50** were manually activated: the amino acid (2 equiv. based on the resin) was dissolved in DMF (2 mL). HATU (2 equiv. based on the resin) and $EtN(i\text{-}Pr)_2$ (2 equiv. based on the resin) were added and the mixture was stirred at room temperature for 10 min. Then $EtN(i\text{-}Pr)_2$ (2 equiv. based on the resin) was added and the mixture was transferred to the synthesiser, where coupling, washing and deprotection took place. Cleavage and post-cleavage processing: after washing with CH_2Cl_2 and drying *in vacuo*, the peptide was cleaved by stirring in CF_3COOH/$(i\text{-}Pr)_3SiH/H_2O$ (95 : 2.5 : 2.5) at room temperature for 90 min. After removal of the resin by filtration, the crude peptide was collected by precipitation from pentane/diethyl ether (1 : 1) at

−78 °C and subsequent centrifugation. The centrifugate was dissolved in methanol/acetonitrile/H_2O/AcOH (20 : 20 : 10 : 1). Preparative HPLC with a gradient from 50% to 85% of eluent B in 20 min (product ret. time: 18.4 min) afforded after drying **6a** as a colourless lacquer, yield: 0.174 g (57%). HPLC ret. time = 27.5 min, 99.9%. ESI-MS: *m/z* = 2596.5 $[M + H]^+$, 2614.4 $[M + NH_4]^+$, 2619.5 $[M + Na]^+$.

Cell culture

An SH-SY5Y cell line stably overexpressing wild-type APP695 with an N-terminal Myc tag and a C-terminal Flag tag[46] was kindly provided by L. Münter and G. Multhaup (Freie Universität Berlin). The cells were cultured in D-MEM/F12, supplemented with 10% fetal calf serum, 2 mM L-glutamine, non-essential amino acids and 50 µg mL^{-1} hygromycin B. Cultures were grown at 37 °C, 5% CO_2.

Inhibitor treatments

The test compounds were initially dissolved in DMSO at concentrations of 5 or 10 mM and stored in aliquots at −20 °C. For the cell culture experiments, compound dilutions (100× of the desired final concentration in the test) were prepared in 100% DMSO, and were freshly diluted 1 : 100 with cell culture medium. The final concentration of DMSO in the experiments and controls was 1%. The culture medium in the cell culture plates was replaced with the fresh medium containing the compounds or 1% DMSO (blank test). For the ECL measurements SH-SY5Y cells were seeded 24 h before treatment in 96 well cell culture plates at a cell density of 2×10^5 cells cm^{-2}. The compounds were diluted in the medium and added to the cells as described above. For each condition six wells were used and pooled after an incubation time of 4–5 h.

Electrochemiluminescence (ECL) assay

Aβ-peptides in cell supernatants were measured with the MSD® Human (6E10) Abeta 40 Kit according to the manufacturer's protocol on a Sector™ Imager 6000 (Meso Scale Discovery). The synthetic reference peptide dilutions were prepared in 1% blocker A. The fetal calf serum present in the SH-SY5Y cell culture medium contains bovine Aβ-peptides which are detected by the ECL assay. The amount of the bovine Aβ40 in an unconditioned culture medium was determined to be ~50 pg mL^{-1}, and this background value was subtracted from the calculated Aβ concentrations in the SH-SY5Y cell culture experiments.

Dose–response curves and EC_{50} determinations

For the calculation of dose–response curves and EC_{50} values for SH-SY5Y APPwt cells, all compounds were tested in three independent experiments in approximately half-logarithmic serial dilutions. For six compounds (**6d**, **6e**, **7m**, **16**, **19** and **20**), ranges of inhibitor concentrations were altered in different experiments in order to define both top and bottom plateaus of the dose–response curve. Therefore, not each single concentration was analysed in all three experiments. Error bars in the corresponding graphs represent data from at least two experiments. For four other compounds (free pharmacophores **41**, **17**, **51** and anchor variant **6g**), at least two experiments were conducted over the full concentration range (nine inhibitor dilutions and DMSO control), while in a third experiment a reduced number of concentrations was used to confirm the EC_{50}. After 4–5 h incubation at 37 °C and 5% CO_2, the cell culture supernatants from 6 wells of a 96 well culture plate were pooled and measured in duplicate with the Aβ40 ECL assay. The signals were normalised to % of the DMSO controls. Means of the normalised duplicate values were plotted against inhibitor concentration and the log EC_{50} values were calculated by non-linear regression using GraphPad Prism 5.01 software (GraphPad Software Inc., La Jolla, USA).

Statistical analysis

Statistical analysis was performed with the GraphPad Prism Software, version 5.01. The initial result obtained from curve fitting by non-linear regression is the log EC_{50} of the compound (not the EC_{50}, which is calculated in a second step). To compare the potency of different compounds, the respective log EC_{50} values from at least 3 independent experiments were averaged and subjected to statistical tests. For two compounds, Student's *t*-test (two-tailed, unpaired) was used to compare means and variations of log EC_{50} values, while a one-way ANOVA was employed for comparison of three or more compounds. In this case, either Dunnett's (comparison of all compounds to one reference compound) or Tukey's (pairwise comparison of all compounds) post-test was added to correct for multiple testing. In our previous publication,[26] we pooled normalised values from three independent measurements to calculate the EC_{50} by non-linear regression from the pooled dataset. This method leads to slightly different EC_{50} values. In this study, pooled datasets were used only for the representation of dose–response curves in graphs, allowing a more clear visualisation. For all statistical analyses, log EC_{50} means and standard deviations from three independent measurements were used, because this method facilitates comparison of multiple compounds. A comparison of log EC_{50} and de-logarithmised EC_{50} values calculated by averaging values from three or more experiments with EC_{50} values obtained by non-linear regression analysis of pooled datasets revealed very small differences (ESI, Table S2†).

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