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Supplementary Material

Peptidomimetic Modulators of BACE

John Paul Juliano,[A] David H. Small,[B] and Marie-Isabel Aguilar[A,C]

[A]Department of Biochemistry & Molecular Biology, Monash University, Clayton, Vic. 3800, Australia.

[B]Menzies Research Institute, University of Tasmania, Hobart, Tas. 7005, Australia.

[C]Corresponding author. Email: mibel.aguilar@monash.edu

MATERIALS AND METHODS

MANUAL SOLID PHASE SYNTHESIS METHODS

Fmoc Deprotection

To the resin, a solution of 25 % piperidine in DMF was added for 20 min, stirring at 5 min intervals. In cases of known problematic deprotection, a sample of resin was removed and the Kaiser test performed to test for the extent of deprotection. If the deprotection was incomplete (negative Kaiser test), the solvent was drained and the deprotection procedure repeated. Following complete deprotection the solvent was drained and the resin flow-washed sequentially with DMF (x3), DCM (x1) and DMF (x1).

Wang Resin Preparation & Amino Acid Loading

Wang resin was prepared at 1 mmol equivalent scale (pre-weighed) by pre-swelling in 1.5 resin-bed volumes of DMF for 30 mins in a 15 mL polypropylene tube, followed by primary carboxylic amino acid loading. Equimolar amounts of Fmoc-α-amino acid and HBTU, at 3 molar equivalents with respect to the resin loading, were dissolved in DMF to an α-amino acid/HBTU concentration of 0.5 M. Following complete dissolution, 4.5-fold molar equivalents of DIPEA was added to the solution and gently stirred. A separate solution of 0.1 molar equivalents of DMAP dissolved in DMF was prepared. The activated amino acid solution was then added to the resin, and while stirring, DMAP solution was added in 1/5 volume aliquots and the resin/coupling mixture placed on a rotator overnight. The solution was transferred to a sintered glass reaction vessel, flow-washed with DMF (x4) and free amines capped by acetylation. Following capping, the resin was flow-washed with DMF (x4), DCM (x1), ether (x1), then dried under vacuum for 15 min and air-dried overnight. The resulting resin was weighed to determine loading efficiency and a Kaiser test performed.

Rink Amide-AM Resin Preparation & Primary Coupling

Rink Amide-AM resin was prepared at 1/0.5/0.1 mmol equivalent scales (pre-weighed) by pre-swelling in 1.5 resin-bed volumes of DMF for 30 mins in a sintered glass reaction vessel. Once sufficiently swollen, the resin was deprotected with 25 % piperidine in DMF for 1 hour and washed. Equimolar amounts of Fmoc-α-amino acid and HBTU were dissolved in DMF to an α-amino acid/HBTU concentration of 0.5 M, at 3 molar equivalents with respect to the

resin loading. Following complete dissolution, a 4.5-fold molar equivalent of DIPEA was added to the solution and gently stirred. The activated amino acid solution was then added to the resin and coupling performed for 3 hours.

Generalised Amino Acid Activation & Coupling

Equimolar amounts of Fmoc-α-amino acid, HBTU and HOBt (or equivalent ie: HAtU/HOAt), at 6-fold molar equivalents with respect to the resin loading, were dissolved in DMF to an α-amino acid/HBTU/HOBt concentration of 0.5 M. Following complete dissolution, a 9-fold molar equivalent of DIPEA was added to the solution. For β-amino acid couplings, a 3-fold molar equivalent of Fmoc-β-amino acid, HBTU and HOBt was used and dissolved in DMF and 4.5-fold molar equivalents of DIPEA added. The activated amino acid solution was then added to the resin and coupling performed for 40 min. In the case of problematic couplings, a sample of resin was removed and the Kaiser test performed to determine the extent of coupling. If the coupling was incomplete (positive Kaiser test), a double coupling was performed using the above quantities. Alternatively, the coupling time was extended until a negative Kaiser test was obtained. Following complete coupling, the solution was drained and the resin flow-washed with DMF (x3).

Selective N-Methylation of Peptides

N-methylation of N-terminal free amines was performed at 1 mmol scale during solid phase synthesis at selected residues utilising the optimised reaction scheme as described by Biron *et al* [1]. The methods were based on those previously described by Miller & Scanlon [2] and are compatible with solid phase Fmoc based peptide synthesis techniques utilised in this study. The Fmoc protecting group of the selected residue for N-methylation was removed and the resin washed thoroughly in DMF (x3). Firstly, the free amine underwent o-NBS protection. A solution of o-NBS-Cl (4 eq.) and Collidine (10 eq.) in NMP (3 mL) was prepared and added to the resin. After 15 mins, the resin was drained under vacuum and washed with NMP (3 mL, x5). Secondly, N-methylation was carried out as a two-step reaction. To the resin a solution of DBU (3 eq.) in NMP (1.5 mL) was added for 3 mins immediately followed by the addition of DMS (10 eq.) in NMP (1.5 mL) for a further 2 mins after which the resin was drained under vacuum and washed with NMP (3 mL, x5). Thirdly, the o-NBS protecting group was removed by the addition of βME (10 eq.), DBU (5 eq.) in NMP (3 mL) over 5 mins. The resin

was then washed with NMP (3 mL, x5). Finally, efficient coupling to the free N-methylated N-terminus was achieved with the addition of 3-fold molar equivalents of the required Fmoc-amino acid in HATU (3 eq.), HOAT (3 eq.) and DIPEA (6 eq.) in NMP (3 mL) over 3 hours. The remaining sequence residues were synthesised as previously described.

N-terminal Acetylation of Free Amine Groups

During synthesis, an N-terminal acetylation procedure was used to block any free amine groups in the event of incomplete coupling to terminate deletion peptides. Following final deprotection, N-terminal acetylation was employed to neutralise the N-terminal charge of completed peptides. The resin was flow-washed 4 times with DMF and to it a 50:5:1 solution of DMF/acetic anhydride/DIPEA was added. The reaction vessel was placed on a rotator for 20 mins after which the resin was flow-washed with DMF (x4).

Peptide Cleavage from a Solid Support

On completion of peptide synthesis and final deprotection the resin was flow-washed sequentially with DMF (x3), DCM (x3), ether (x3) and MeOH (x3) and dried under vacuum for 20 min, followed by air drying overnight. The dried resin was then transferred to a pre-weighed 5 mL glass vial and the dry weight determined. Crude peptide was liberated from the resin and side chain protecting groups removed utilising one of two cleavage/scavenger reagent cocktails. Peptides that did not contain Met residues were cleaved using a solution of 95 % TFA / 2.5 % H_2O / 2.5 % TIPS. Peptides containing Met residues were cleaved with 95 % TFA / 2 % H_2O / 2 % TIPS / 1 % EDT. An appropriate cleavage solution (500 µL / 0.1 mmol resin) was added to the vial which was then placed on a rotator at low speed for 6-8 hours. Following cleavage, the TFA was evaporated by application of a nitrogen stream.

Crude Peptide Recovery by Ether Precipitation

To precipitate the peptide, ether (500 µL / 0.1 mmol resin) was added to the tube and thoroughly agitated. The suspension was then transferred to a 20 mL glass vial and the volume made up to 5 mL with additional ether. A stirring bar was added to the vial and the solution was subjected to mixing at a moderate speed for 30-60 mins. To remove the ether and protecting groups, the suspension was filtered through a sintered-glass funnel with clean ether (3 x 5 mL). The funnel with filtered peptide was then placed into a small side-arm flask.

Aliquots of 50 % ACN / 0.1 % TFA buffer (500 µL) was added to the peptide and mixed gently with a spatula. The liquid was then drawn into the side-arm flask using suction, and additional dissolutions performed until only a gritty resin remained in the funnel. If the peptide failed to dissolve following 3 applications of the above solvent, an extra dissolution step was performed with the addition of 3 M urea (100 µL) aliquots. Following filtration, the peptide solution was transferred to a pre-weighed 20 mL glass vial, and the side-arm flask washed with 50 % ACN / 0.1 % TFA buffer to ensure efficient transfer. A small sample of crude solution (10 µL) was set-aside for LC-MS analysis. The vial was sealed with Parafilm, frozen on dry ice / acetone, and lyophilised for 48 hours to obtain dry crude peptide. Following lyophilisation, the vial was weighed to determine crude peptide weight and was stored at 4 °C.

PEPTIDE CHARACTERISATION & PURIFICATION

Following synthesis, the crude peptides were characterised by MS and purified by gradient semi-preparative RP-HPLC to isolate the target peptide. MS was used to evaluate purity of the resulting fraction and successful separation of the target from the crude sample. Following lyophilisation, the target peptide was analysed by isocratic LC-MS and gradient analytical RP-HPLC. Synthesis was repeated in the event that the crude analysis indicated poor synthesis of the target peptide. Purification was repeated under altered gradient conditions if the resultant purity of target peptide was inadequate.

Target Identification by LC-MS

Crude and purified dry peptide and fractions were analysed by MS (*LC-MSD Trap Series 1100 system*). Sample analysis (2-8 µL injection volume) was performed over 4 min in 20 % Buffer A, 80 % Buffer B at a flow rate of 15 µL/min. Prior to injection, lyophilised dry crude and pure peptide samples were prepared as a ~0.2 mg/mL solution, solubilised in 30 % Buffer A in Buffer B. Mass spectra were obtained via ESI-Trap-MS over a 100-2,000 mass/charge (m/z) scan range in positive ion mode with a capillary voltage of 3,000 V. Target mass/charge ratio, accumulation time, trap-drive level and compound stability settings were adjusted per peptide. Peptide composition was determined by resolving the sum of mass spectra across the corresponding total ion chromatogram peak, and were identified based on the calculated target peptide mass and the expected m/z values in various charged ion states (z) governed by

$$m/z = \frac{mass\ (Da) +\ z\ (charge)}{z\ (charge)}$$

Purification by RP-HPLC

Efficient separation of target peptides from crude samples was achieved at a semi-preparative scale using either a semi-preparative Vydac Protein C-4 column (10 µm, 300 Å, 10 mm ID x 250 mm L), or a preparative Brownlee Aquapore Prep-10 C-18 column (15 µm, 300 Å, 10 mm ID x 100 mm L). Dry crude peptides (5-20 mg) were dissolved in 3 M urea / 200 mM ammonium hydrogen carbonate (< 5 mL) and sterile filtered (*Pall*, 0.22 µm) prior to manual injection. Crude preparations were separated by gradient elution over a 5-65 % Buffer B gradient over 60 min at a flow rate of 4 mL/min and detection at 214 nm. Fractions were manually collected in 10 mL clean glass tubes. Fractions were then analysed by MS. Fractions abundant in target whilst free of contaminants or deletion products were deemed most pure and pooled. Subsequent purifications of same crude material were performed over optimised gradient conditions, derived from the initial RP-HPLC purification chromatogram and resulting MS fractional analysis.

Purity Analysis

The pooled fractions were transferred to pre-weighed 20 mL glass vials, shell frozen on dry-ice/acetone, and lyophilised for 48 hours to obtain dry purified peptide. Vials were weighed and the final yield determined. Final purity and component composition was determined by MS and analytical capillary RP-HPLC using a Agilent XDB-C18 reverse-phase column (5 µm, 300 Å, 4.6 mm ID x 150 mm L). Sample analysis (10-25 µL injection volume) was performed at a 10 µL/min flow rate under gradient conditions as described for RP-HPLC purification. Prior to injection, lyophylised purified peptide for analysis was prepared as a ~0.5 mg/mL solution, solubilised in 95 % Buffer A in Buffer B. Retention times for each target peak were recorded.

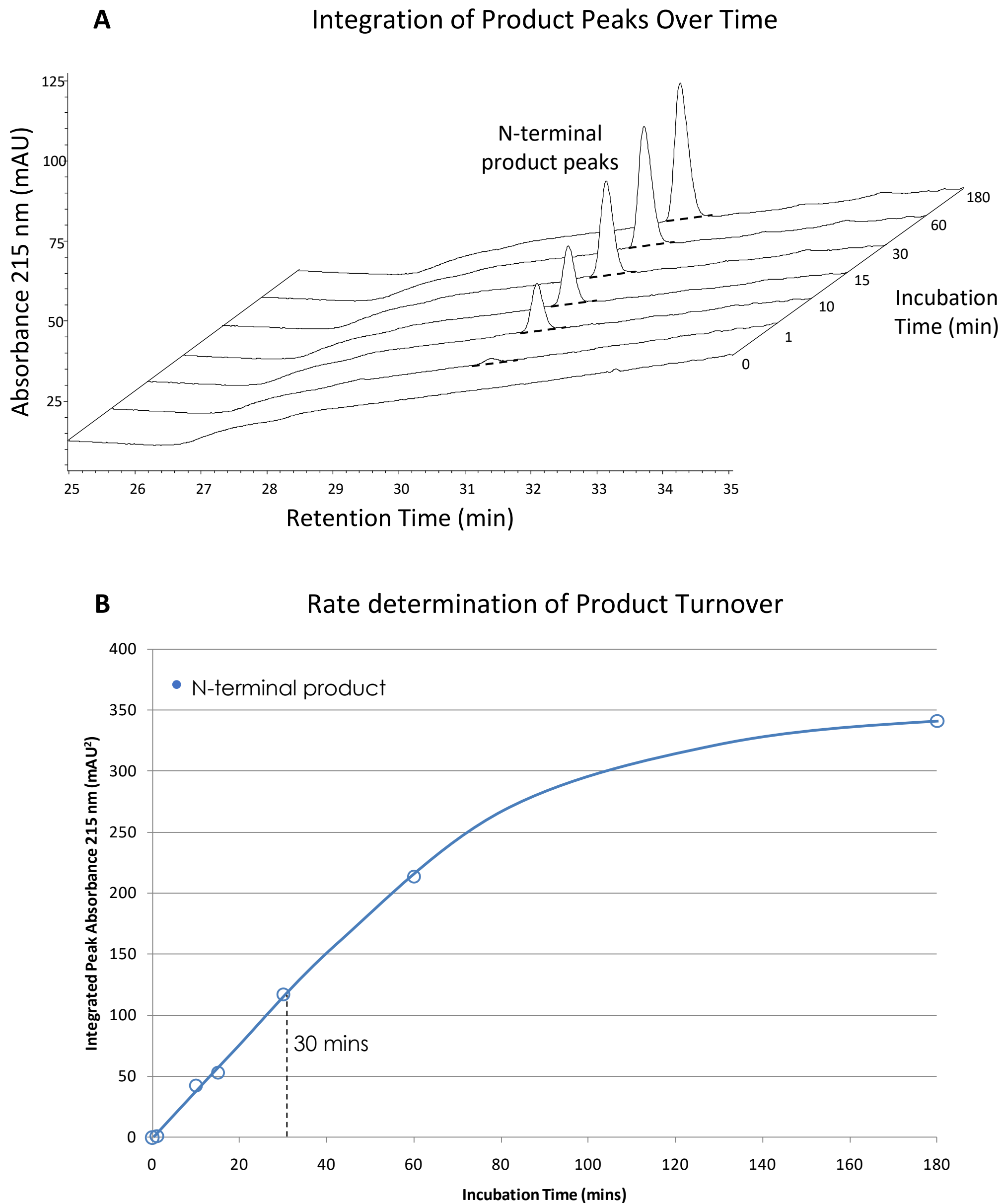

FIGURE S1: TYPICAL ANALYSIS OF SUBSTRATE TURNOVER BY LC-MS TIME-COURSE

Cleavage product rate determination by gradient LC-MS time-course. Peptides (10 μM) were subject to the rhBACE assay over defined time-points followed by gradient capillary LC-MS. The change in product formed over time is expressed as a percentage rate with respect to the QFS reference rate (the rate of QFS turnover). **A:** Integration of N-terminal product peaks by chromatographic (Abs: 215 nm) analysis at defined time points. **B:** Rate determination of product turnover - Blue(•): N-terminal product.

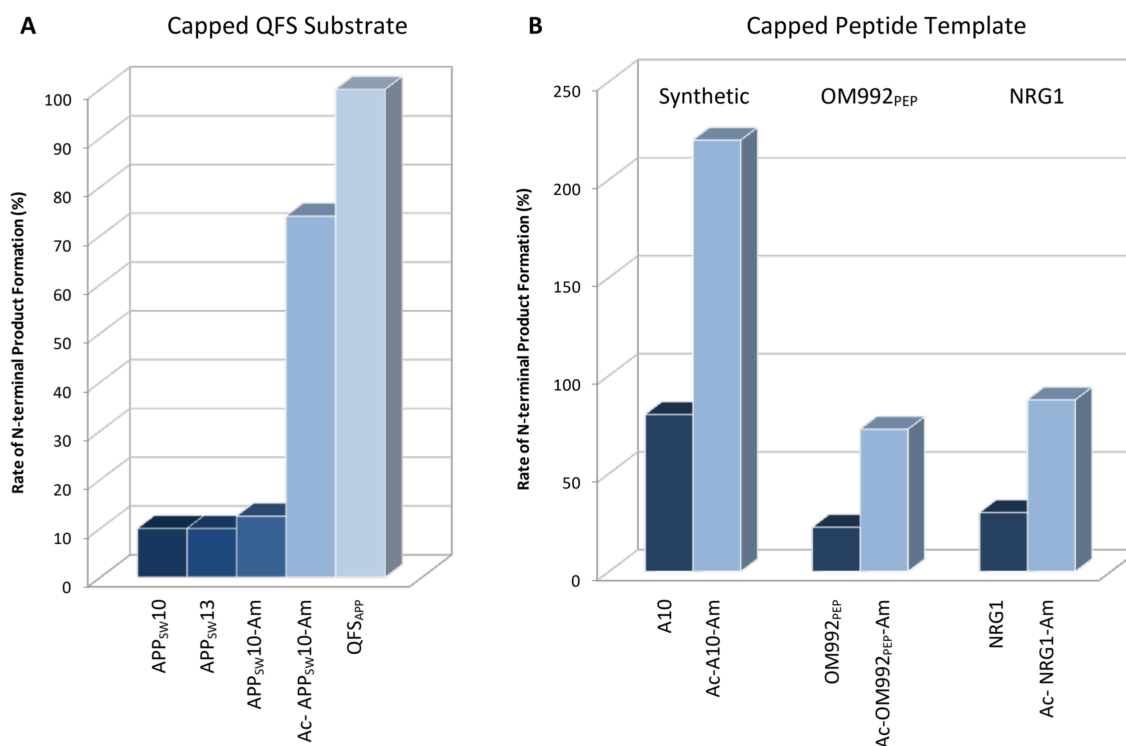

FIGURE S2: EFFECT OF PEPTIDE CAPPING ON SUBSTRATE TURNOVER

A: Product turnover of QFS_{APP} substrate core sequence and capping. **B:** Product turnover of peptide templates with and without capping. Rates of N-terminal product turnover were determined by LC-MS time-course. All assays were performed utilising rhBACE with 10 μM peptide. Product turnover expressed as a percentage of the determined QFS_{APP} rate (100%). See **Table 1 – Series 1** for peptide sequences.

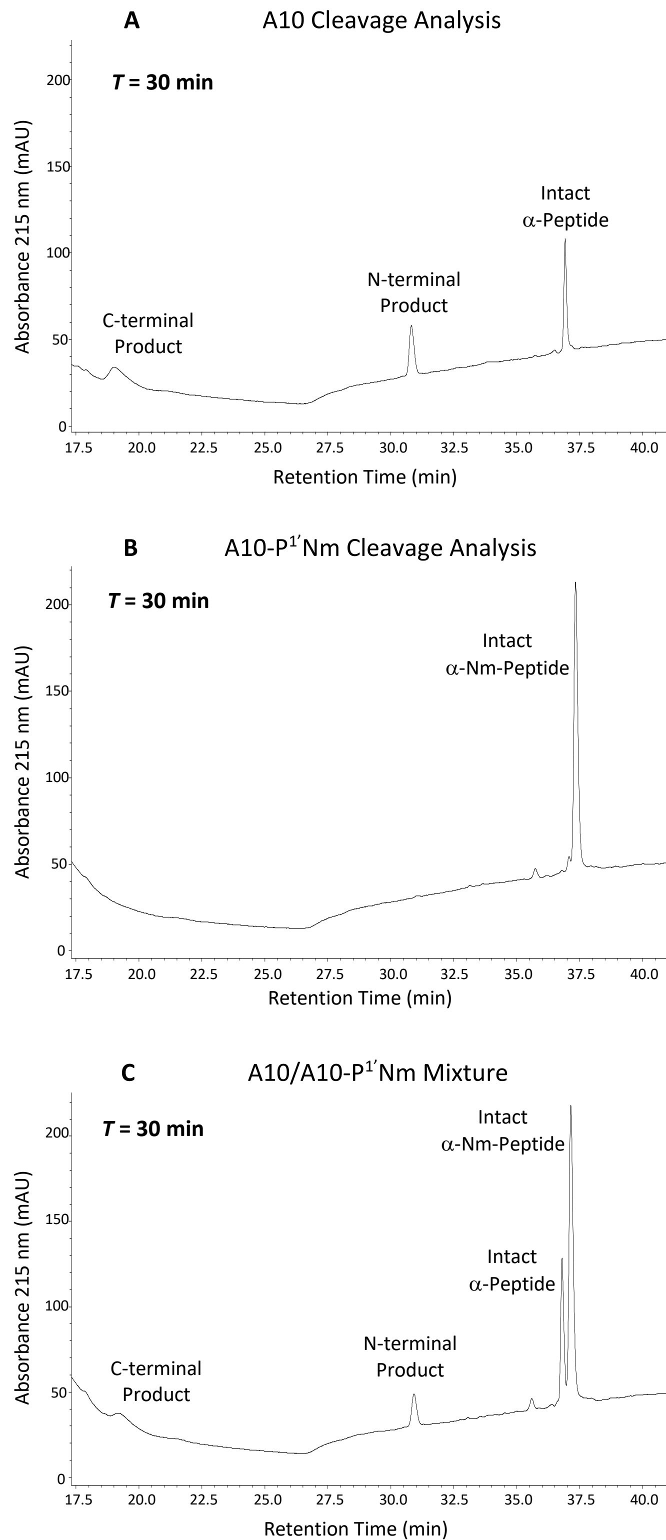

FIGURE S3: SCISSILE-SITE N-METHYLATION CLEAVAGE ANALYSIS BY LC-MS

Cleavage analysis of A10-α and P^1-$P^{1'}$ N-methylated peptide by LC-MS time-course (30 min time-point). **A:** Chromatogram of A10 peptide and resulting cleavage products. **B:** Chromatogram of A10-$P^{1'}$Nm peptide – scissile site N-methylation protects the peptide from proteolysis. **C:** Chromatogram of A10/A10-$P^{1'}$Nm peptide mixture (10 μM each – 20 μM total) – a significant reduction in A10 N-terminal product is noted in the presence of A10-$P^{1'}$Nm peptide. Intact, N-terminal and C-terminal products were identified. All assays performed utilising rhBACE.

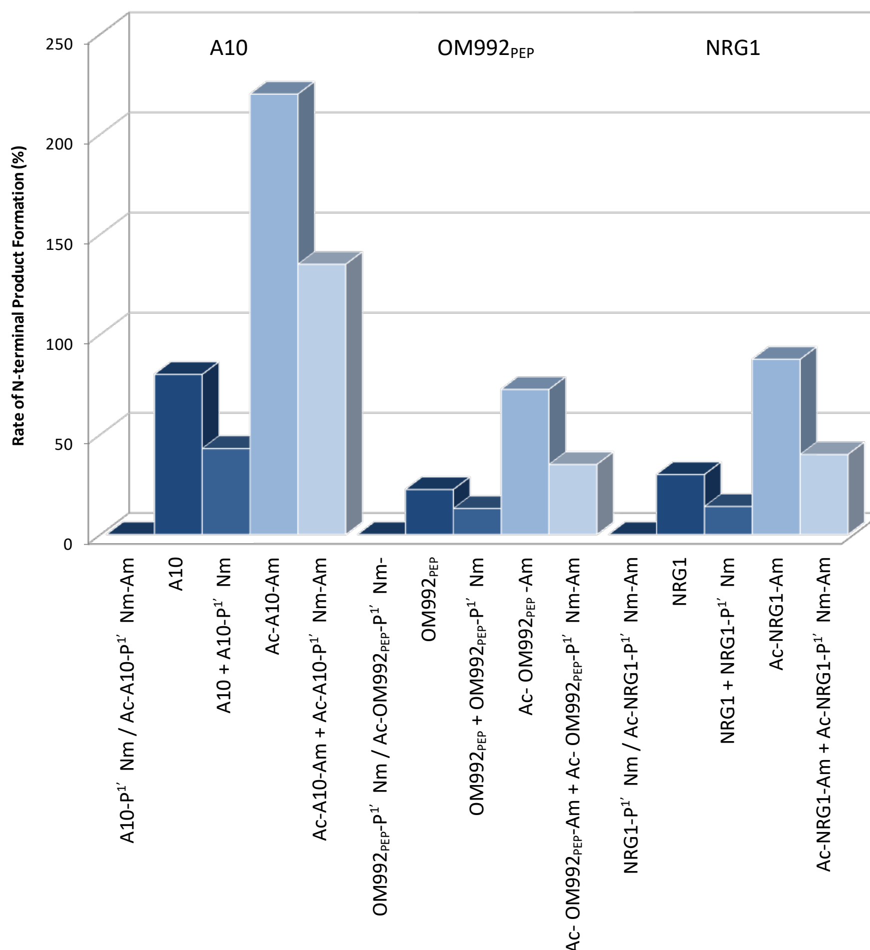

FIGURE S4: EFFECT OF CAPPING & SCISSILE-SITE N-METHYLATION ON SUBSTRATE TURNOVER

Product turnover analysis of peptide templates with and without capping and N-methlyation. Rates of N-terminal product turnover were determined by LC-MS time-course. All assays were performed utilising rhBACE with 10 μM each peptide (mixtures 20 μM total). As N-methylated peptides do not produce cleavage products, rates are for N-terminal products resulting from the cleavage of non-N-methylated peptides. Product turnover was expressed as a percentage of the determined QFS_{APP} rate (100%). See **Table 1 – Series 1 & 2** for peptide sequences.

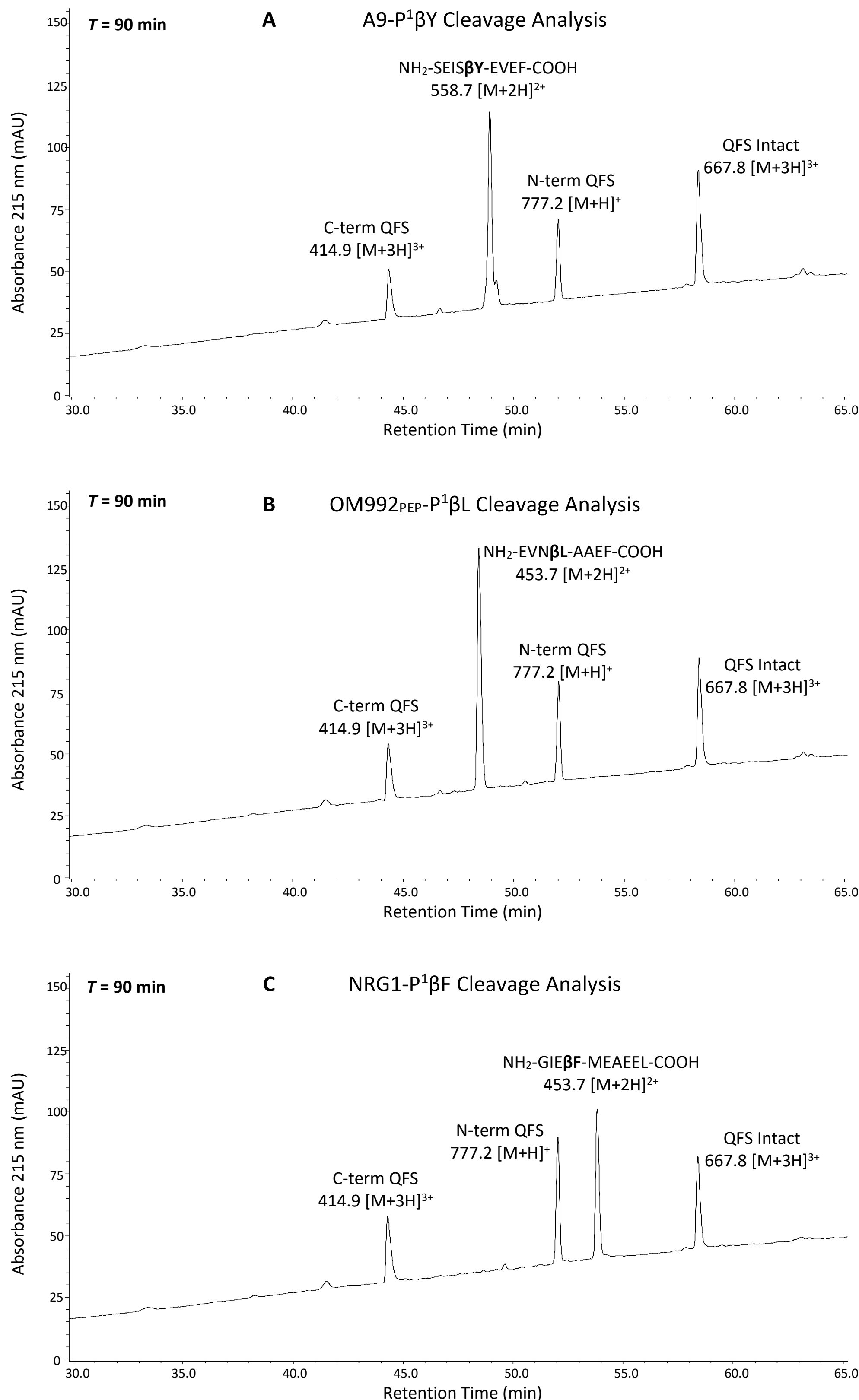

FIGURE S5: P^1 SCISSILE-SITE β-AMINO ACID SUBSTITUTION CLEAVAGE ANALYSIS BY LC-MS

Cleavage analysis of P^1 β-amino acid substituted peptide templates followed by the rhBACE QFS_{APP} modulation assay (90 min incubation). Cleavage products were identified by gradient LC-MS. Intact, N-terminal and C-terminal products are indicated. All assays were performed utilising rhBACE.

[1] E. Biron, J. Chatterjee, H. Kessler, Optimized selective N-methylation of peptides on solid support, J Pept Sci 12 (2006) 213-9.
[2] S.C. Miller, T.S. Scanlon, Site-selective N-methylation of peptides on a solid support, J Am Chem Soc 119 (1997) 2301-2.