# Supporting Information for

# "Imidazole-derived 2-[N-carbamoylmethyl-alkylamino]acetic acids , substrate-dependent modulators of insulin-degrading enzyme in amyloid-β hydrolysis."

Dr. Julie Charton, Dr. Marion Gauriot, Dr. Qing Guo, Dr. Nathalie Hennuyer, Dr. Xavier Marechal, Julie Dumont, Dr. Malika Hamdane, Valerie Landry, Dr. Olivier Sperandio, Dr. Marion Flipo, Prof. Luc Buee, Prof. Bart Staels, Dr. Florence Leroux, Prof. Wei-Jen Tang, Prof. Benoit Deprez*, Prof. Rebecca Deprez-Poulain*

**Suppl. Figures:**



**Suppl. Tables:**



**Methods:**



**NMR Spectra for compounds 1-8**

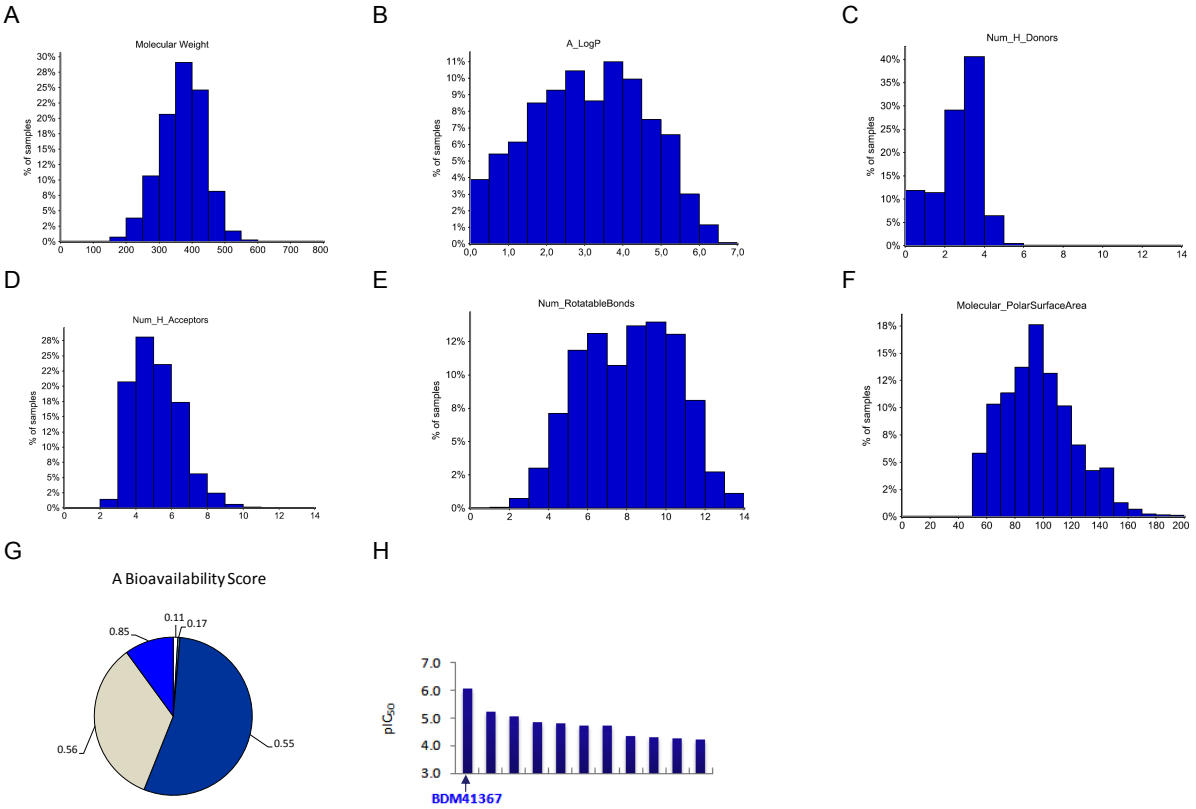



**Figure S1. Drug-like properties of the 2040-members library and screening results.**
Drug-like properties were evaluated using Pipeline Pilot v6.0 from Accelrys. Parameters evaluated in the Lipinski's rule of 5 : (A) Molecular weight; (B) ALogP; (C) H-Bond Donor Atoms; (D) H-Bond Acceptor Atoms. Other parameters: (E) Number of rotatable bonds and (F) Polar Surface area (Veber et al.); ionisable atoms; (G) Bioavailability score (Martin et al.) Screening results (H) pIC50 values of eleven confirmed hits. **1 (BDM41367)** proved to be the most active inhibitor of $A\beta$-$_{16\text{-}23}$ hydrolysis by IDE, *(hit rate 0.54%).*

Lipinski, C.A.; Lombardo, F.; Dominy, B.W.; Feeney, P.J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Del Rev* **23**. 3–25 (1997).
Veber, D. F. et al. Molecular Properties That Influence the Oral Bioavailability of Drug Candidates. *J. Med. Chem.* **45**, 2615-2623 (2002). Veber et al showed that an inverse relationship between oral bioavailability and the number of rotatable bonds, independently of molecular weight.
Martin, Y. A bioavailability score *J. Med. Chem.* **48**, 3164-3170 (2005). For anions the important property for bioavailability is polar surface area (PSA) whereas for the other compounds the rule-of-five has predictive ability. A Bioavailability Score, ABS, is formulated as the probability that a compound will have >10% bioavailability in rat or measurable Caco-2 permeability. ABS is 0.11 for anions for which PSA is >150 $Å^2$, 0.56 if PSA is between 75 and 150 $Å^2$, and 0.85 if PSA is <75 $Å^2$.

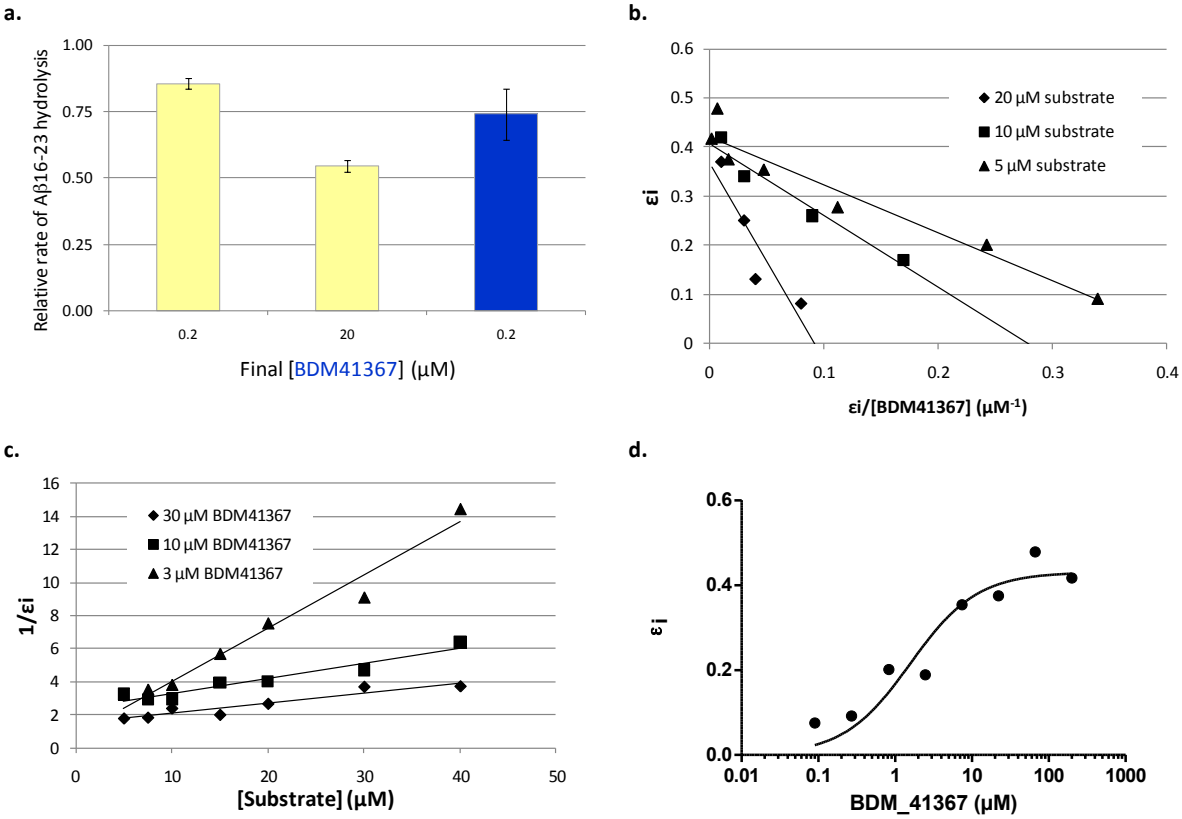



**Figure S2. Inhibition mechanism of IDE dependent amyloid-β hydrolysis of 1 (BDM41367) by IDE.** (**a**) **1** is a reversible inhibitor of Aβ hydrolysis by IDE. IDE (3.75 µg/mL) was pre-incubated 20 minutes with **1** (0.2 or 20 µM) or vehicle (DMSO 1%) before adding the substrate (5 µM). Relative rates are shown as yellow bars. Pre-incubate at 20µM was diluted 100-fold (Blue bar) before adding the substrate (5 µM). Observed relative rates were mean of triplicates. Recovery of IDE activity after large dilution (Blue bar) shows that **1** is a reversible inhibitor. (**b**) **1** induces a hyperbolic inhibition of Aβ hydrolysis by IDE (graphical approach described by Antunes et al. *BBA* 2003): Functional dependence of εi (degree of inhibition) of Aβ in function of [I] for **1** shows straight lines ($r^2$=0.94 for 5 µM of substrate) with a y-axis intercept $\varepsilon i_{max} < 1$ characteristic of a hyperbolic inhibition. (**c**) **1** is a competitive inhibitor of Aβ hydrolysis by IDE (graphical approach described by Antunes et al. *BBA* 2003): the nature of inhibition (competitive, uncompetitive, mixed or pure non-competitive) is determined by 1/ εi vs [S]. The correlation was found to be linear ($r^2$=0.97 for **1** at 3 µM) showing **1** is a competitive inhibitor of $A\beta_{16\text{-}23}$ hydrolysis by IDE. (**d**) Fitting into a mathematical model associated to hyperbolic and competitive inhibition for **1**:

$$\varepsilon i = \frac{\frac{[I](\alpha\text{-}1)}{[S]/(Ks+\alpha)}}{[I]+Kic^{*}\alpha^{*}\frac{[S]/(Ks+1)}{[S]/(Ks+\alpha)}}$$

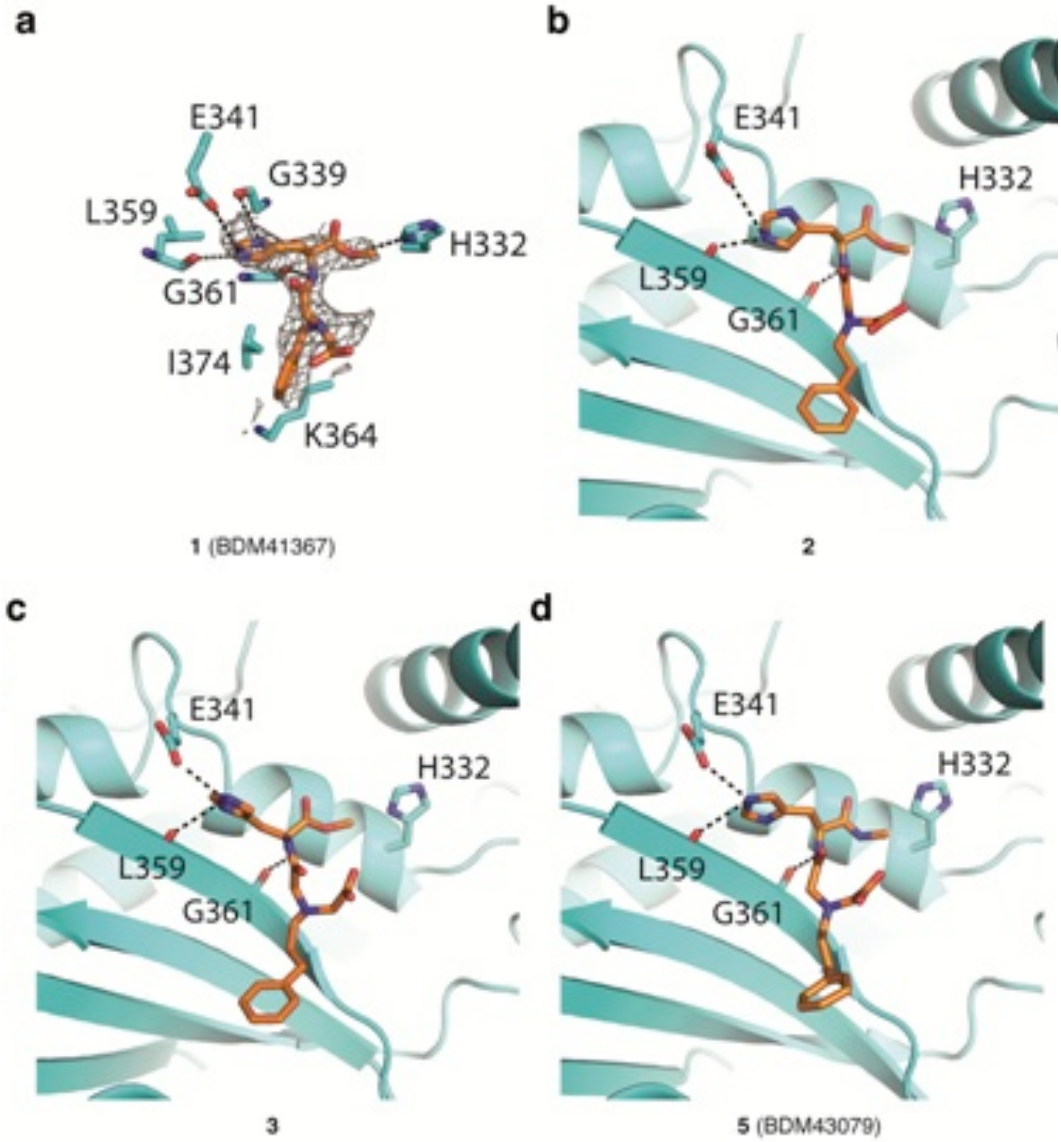



**Figure S3. Crystal structures of IDE-CF-E111Q in complex with 1 (BDM41367) and analogues.** Detailed interaction of **1** (**BDM41367**) (Protein Data Bank accession code 2YPU), **2** (Protein Data Bank accession code 4GSC); **3** (Protein Data Bank accession code 4DWK) and **5** (BDM43079) (Protein Data Bank accession code 4DTV) with IDE-CF-E111Q exosite. **1** is located parallel to beta strand (359-364). E341 and backbone of L359 interact with the imidazole ring and methyl ester with H332. Amide bond interacts with G361 backbone. The binding mode of the series scaffold is conserved in all complexes.
In IDE-CF-E111Q, the catalytic site is mutated, preventing both catalytic activity and binding of the series. Conversely, the exosite is intact and the compounds still bind to it.

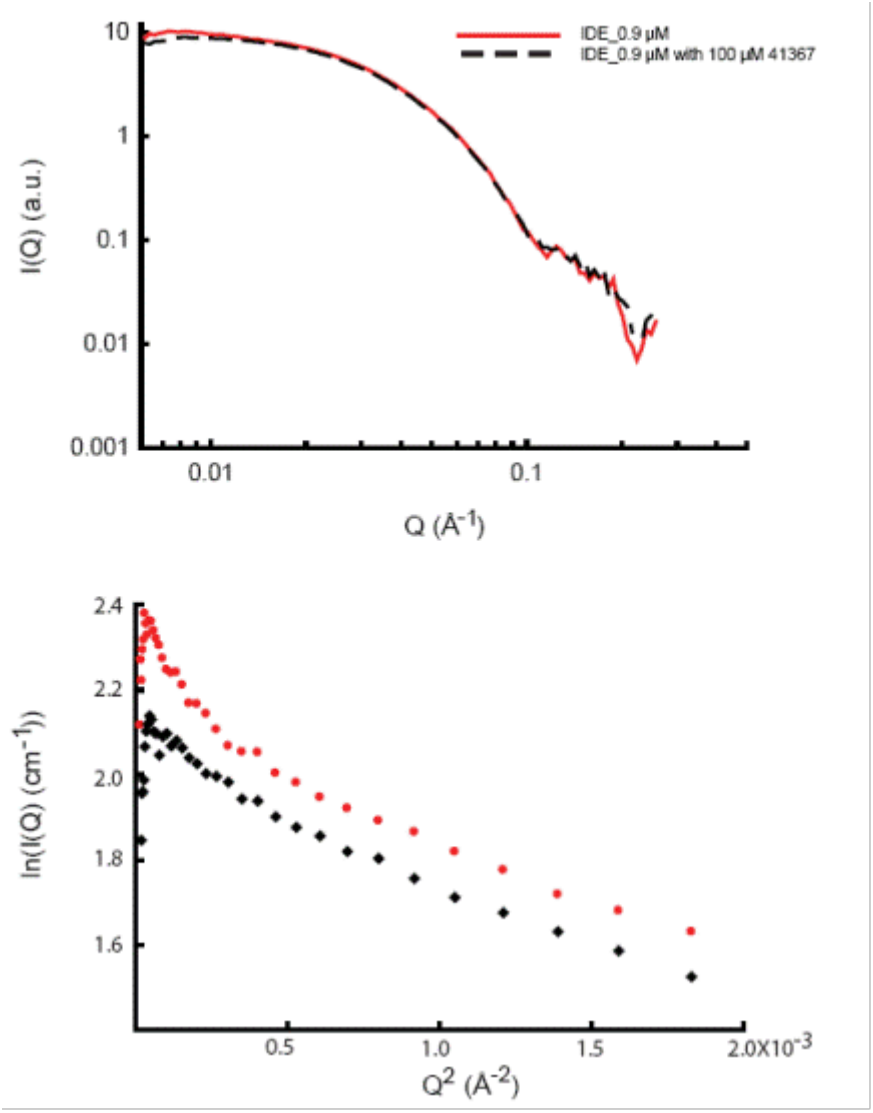



**Figure S4. Effect of 1 (BDM41367) on the SAXS profile of IDE.**

(**a**) Experimental scattering profile of 9 µM IDE (red) and 9 µM IDE with 100 µM of **1** (black). (**b**) The Guinier plot of the scattering profile of IDE in the presence (black) or absence (red) of **1**. The SAXS profile of IDE (radius of gyration (Rg) = 51.2 Å) is consistent with a monomer/dimer equilibrium. The addition of 100 µM **1** did not significantly alter the SAXS profile (Rg = 50.8 Å). However, the scattering profile at the low q range in the Guinier plot showed that IDE had some aggregation in the absence of **1** depicted by the biphasic pattern. The addition of **1** reduced such aggregation, which may in part explain how **1** can increase the catalytic activity of IDE.


**Figure S5. Expression of Insulin-Degrading Enzyme in SY5Y cells.** SY5Y cell lines either APPswe or APPwt determined by Western Blot.

**a**

| | IHH | Min6 |
|---|---|---|
| IDE mRNA (Ct value) | 25 | 26 |

**b**

| PEPCK mRNA expression in IHH (%)[a] | |
|---|---|
| **DMSO** | 58 ± 4 |
| **(8) BDM43124** | 62 ± 5 |

[a] induced by insulin at 1 nM

**c**

| Insulin secretion in Min6 cells (µg/L) [a] | | | |
|---|---|---|---|
| **[Glucose] (mM)** | 2.8 | 11 | 20 |
| **DMSO** | 30.9 ± 10.8 | 61.5 ± 5.1 | 127.5 ± 9.1 |
| **(8) BDM43124** | 21.2 ± 1.4 | 78.8 ± 14.6 | 132.5 ± 15.5 |

[a] Compound incubated 24h with cells before glucose stimulation

**Figure S6. Absence of effect of 8 (BDM43124) in insulin-dependent cell models.** (**a**) IDE mRNA is expressed in the different cell lines (Ct values). (**b**) PEPCK mRNA expression under insulin stimulation during 24h in IHH cells (Immortalized human hepatocytes) induced by 1 nM of insulin in the presence or not of **8** (10 µM). Results are normalized to cyclophiline. (**c**) Glucose stimulated insulin secretion in Min6 cells. Secretion of insulin induced by 2.8, 11 or 20 mM glucose stimulation in the presence or not of **8** (10 µM) pre-incubated with cells (24 h).

In both assays, **8** shows no significant effect on either PECK expression or insulin secretion. Reported values obtained are the mean ± s.d. of at least three independent experiments.

**Suppl. Table 1.** XRay data collection and refinement statistics.

| | IDE-CF-(**1**) BDM41367 | IDE-CF-E111Q-(**1**) BDM41367 | IDE-CF | IDE-CF-E111Q-(**2**) | IDE-CF-E111Q-(**3**) | IDE-CF-E111Q-(**5**) BDM43079 |
|---|---|---|---|---|---|---|
| Data Collection | | | | | | |
| Beamline | APS 19ID | APS 14ID | APS 19ID | APS 19ID | APS 14ID | APS 19ID |
| Wavelength (Å) | 0.9795 | 0.9793 | 0.9795 | 0.9795 | 0.9793 | 0.9795 |
| Space group | $P6_5$ | $P6_5$ | $P6_5$ | $P6_5$ | $P6_5$ | $P6_5$ |
| Cell dimension(Å) | | | | | | |
| a | 263.6 | 263.1 | 262.1 | 263.8 | 263.2 | 262.8 |
| b | 263.6 | 263.1 | 262.1 | 263.8 | 263.2 | 262.8 |
| c | 91.1 | 90.5 | 90.8 | 91.1 | 90.9 | 90.8 |
| Resolution (Å) | 50-3.2 | 50-2.8 | 50-3.2 | 50-2.9 | 50-3.05 | 50-3.11 |
| Rsym (%)[a] | 12.5(41.3)[e] | 11.4(34.2)[e] | 14.9(42.2)[e] | 16.4(45.3)[e] | 17.7(57.6)[e] | 17.4(50.1)[e] |
| I/sigma | 18.4(4.0)[e] | 13.5(2.0)[e] | 11.6(3.7)[e] | 8.6(2.3)[e] | 9.2(2.0)[e] | 7.3(3.2)[e] |
| Redundancy[b] | 4.5(4.2)[e] | 3.7(3.7)[e] | 3.5(3.3)[e] | 3.5(2.1)[e] | 3.7(3.6)[e] | 4.6(4.7)[e] |
| Completeness (%) | 99.6(99.6)[e] | 98.4(99.4)[e] | 99.8(99.1)[e] | 99.9(99.9)[e] | 99.8(99.8)[e] | 99.8(100.0)[e] |
| FOM(Figure of Merit) | 0.8713 | 0.8835 | 0.8795 | | | |
| Unique reflections | 55194 | 82996 | 55620 | 75029 | 68272 | 62394 |
| Refinement | | | | | | |
| $R_{work}$ [c] | 0.172 | 0.172 | 0.175 | 0.195 | 0.177 | 0.178 |
| $R_{free}$ [d] | 0.239 | 0.225 | 0.241 | 0.250 | 0.227 | 0.235 |
| No. atoms | | | | | | |
| Protein | 15526 | 15526 | 15563 | 15651 | 15644 | 15635 |
| Water | 135 | 296 | 177 | 183 | 105 | 77 |
| B-factors | | | | | | |
| IDE | 43.2 | 28.9 | 42.8 | 38.2 | 33.4 | 33.7 |
| Ligand | 85.3 | 63.5 | - | 79.5 | 78.5 | 82.7 |
| Water | 49.9 | 34.8 | 48.3 | 41.4 | 27.8 | 28.2 |
| r.m.s. deviations | | | | | | |
| Bond lengths (Å) | 0.018 | 0.017 | 0.018 | 0.019 | 0.019 | 0.019 |
| Bond angles (°) | 1.785 | 1.564 | 1.852 | 1.875 | 1.899 | 1.823 |
| Ramachandran plot (%) | | | | | | |
| Favorable region | 88.4/ 87.6 | 90.3 / 90.5 | 89.1 / 87.0 | 89.7 / 89.8 | 90.2 / 89.6 | 89.8 / 90.0 |
| Allowed region | 11.2 / 12.3 | 9.7 / 9.5 | 10.7 / 12.9 | 10.1 /10.2 | 9.8 / 10.4 | 10.2 / 9.9 |
| Generously allowed region | 0.4 / 0.1 | 0 / 0 | 0.2 / 0.1 | 0.1 / 0 | 0 / 0 | 0 / 0.1 |
| Disallowed region | 0 /0 | 0 / 0 | 0 /0 | 0 / 0 | 0 / 0 | 0 / 0 |
| PDE code | 4DTT | 2YPU | 3QZ2 | 4GSC | 4DWK | 4GS8 |

[a] $R_{merge} = \Sigma (I - \langle I \rangle) / \Sigma \langle I \rangle$
[b] $N_{obs}/N_{unique}$
[c] $R_{work} = \Sigma_{hkl} ||F_{obs}| - k |F_{calc}|| / \Sigma_{hkl} |F_{obs}|$
[d] $\boldsymbol{R}_{free}$, calculated the same as for $\mathbf{R}_{work}$ but on the 5% data excluded from the refinement calculation.
[e] the outer resolution shell. Values in parentheses indicate the highest resolution shell

**Suppl. Table 2.** Antibodies used in immunoblotting studies for amyloid β cell-based assays.

| Antibody | Species | Specificity | Dilution | References |
|---|---|---|---|---|
| APP C1-3 | Mice | human and mice APP C-ter | 1:2 000[a] | Innogenetics, Ghent, Belgium |
| IDE (A-23) | Rabbit | human specific | 1:2 000[b] | Sc-130784, Santa Cruz Biotechnology |
| GAPDH | Rabbit | Human and mice | 1:10 000[a] | Sc-25778, Santa Cruz Biotechnology |

[a]Blocking and antibody incubation: Tris-buffered saline, pH 8, 0.05% Tween 20 + 5% skim milk
[b]Blocking and antibody incubation: Tris-buffered saline, pH 8, 0.05% Tween 20 + 1% BSA

**Suppl. methods:**

**Preparation of the fluorogenic substrate.**

12.05 mg of Ac-Cys-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Trp-NH2 (1.2 eq.) from NeOMPS were dissolved in 4620 µL water. 5 mg of fluorophore (7.716 µmol) ATTO655 maleimide (Sigma-Aldrich Inc) were dissolved in 770 µL of DMF and directly added to the peptide solution. NaOH 0.1 N was added to the solution until pH = 7.4 (careful not to have pH >7.5, the maleimide function of the fluorophore will be hydrolyzed by OH-). The substitution reaction is flowed by LCMS at 215 nm. When completed, the Ac-Cys-(ATTO655)-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Trp-NH2 substrate is aliquoted, lyophilized to use directly in the assay.

**Proteolytic profile (full substrates)**

The enzymatic activity of IDE was assayed using human amyloid-β (1-40), insulin, IGF-II, somatostatin or glucagon as follows.

1/ Insulin, IGF-II, somatostatin or glucagon were dissolved in Hepes 50 mM with 100 mM NaCl, pH 7.4. Then, 20 µL of IDE at 30 µg/mL were pre-incubated 10 minutes at 37 °C with 20 µL of vehicle or modulator in microtiter plates (black, low-binding). The reaction was started with the addition of 40 µL of substrate at 4 µM. Final concentrations of IDE and substrate, were 7.51 µg/mL and 1 µM respectively. The reaction was quenched at a suitable time point with 20 µL of a solution of TFA/ACN 0.1%. Quantification of residual substrate was performed by HPLC analysis (UV 215 nm) using a UltiMate™ nano LC system apparatus, equipped with a Famos® autosampler and a Switchos™ precolumn switching device all from LC_packings (Dionex.Corp). 10µL of sample were loaded on a precolumn 5*0.3 mm equipped with a guard cartridge Q95961 10*1.0 mm from Interchim at a flowrate of 50 µL/min for preconcentration. Separation was performed using an Uptisphere UP5WTF-A10 C18wtf 5-µm particle size column, dimensions 0.3 * 100 mm from Interchim at 50 °C. A gradient starting from 75% $H_2O$/0.1% TFA and reaching 75% $CH_3CN$ /0.09% TFA within 10 min at a flow rate of 0.3 µL/min was used and quantification was done at 215/254 nm.

2/ Amyloid-β (1-40) was obtained from Bachem (Bubendorf, Switzerland). It was solubilized to 5 mg/mL in HFIP [1] and incubated overnight at room temperature. Aliquots of 25 µg were prepared and solvent was removed using a spin-vacuum system. Aliquots were then stored at -80°C until using. We measured amyloid-β using a commercial kit from Perkin Elmer: Human Amyloid β 1-40 (Aβ 1-40) AlphaLISA Kit (catalog#AL202C). Assay buffer was Hepes at 50 mM with 100 mM NaCl, pH 7.4. 5 µL of IDE (30 µg/mL) were pre-incubated 10 minutes at ambient temperature with 5 µL of test compound at 4X final concentration or vehicle in 96 half-area wells plates (dark, non-binding surface). The reaction was then started with the addition of 10 µL of amyloid-β at 2µM. The incubation was maintained 5 minutes at ambient temperature and stopped with 20µL of EDTA at 200mM. Samples were diluted with immunoassay buffer and the content of amyloid-β was determined by AlphaLISA according to the manufacturer recommendations. AlphaLISA signal is detected with a Mithras (Berthold) using a excitation at 680 nm and a emission at 620 nm(bandwidth 10 nm)

| Substrate | [substrate] (µM) | Hydrolysis Time (min) |
|---|---|---|
| Aβ | 2 | 5 |
| insulin | 1 | 10 |
| IGF-II | 1 | 30 |
| Somatostatin | 1 | 30 |
| Glucagon | 1 | 1 |

[1] Wood SJ et al., (**1996**), *J. Mol. Biol. 256*, 870-877

**Protein expression and purification**

The expression vectors for cysteine free human IDE (IDE-CF; C110L, C171S, C178A, C257V, C414L, C573N, C590S, C789S, C812A, C819A, C904S, C966N, C974A) and the catalytically inactive IDE-CF-E111Q mutant were created as described [1]. Cysteine free IDE and inactive IDE-CF-E111Q were expressed in *E. coli* Rosetta (DE3) cells (at 25 °C and 19 hours, 0.5 mM IPTG induction). IDE-CF-E111Q was purified by Ni-NTA, Source-Q, and Superdex S-200 columns as described [2]. To avoid the contamination of co-purified peptides that could bind the exosite and catalytic site to interfere the binding of 41367, IDE-CF was purified by Source-Q column first, followed by Ni-NTA and Superdex S-200 columns [3].

[1] Malito E, Ralat LA, Manolopoulou M, Tsay JL, Wadlington NL, & Tang W-J (**2008**) Molecular bases for the recognition of the short peptide substrate and cysteine-directed modifications of human insulin-degrading enzyme. *Biochemistry* 47:12822-12834.

[2] Manolopoulou M, Guo Q, Malito E, Schilling AB, & Tang WJ (**2009**) Molecular basis of catalytic chamber-assisted unfolding and cleavage of human insulin by human insulin-degrading enzyme. *J Biol Chem* 284:14177-14188.

[3] Im H, Manolopoulou M, Malito E, Shen Y, Zhao J, Neant-Fery M, Sun C-Y, Meredith SC, Sisodia SS, Leissring MA, & Tang W-J (**2007**) Structure of substrate-free human insulin-degrading enzyme (IDE) and biophysical analysis of ATP-induced conformational switch of IDE. *J. Biol. Chem.* 282:25453-25463.

**Crystallization and data process**

IDE-CF and IDE-CF-E111Q were first purified by 3 runs of Superdex S-200 chromatography. The purified IDE was then mixed with compound **1-3, 5** in 1 to 10 molar ratio at room temperature for 30 minutes and then compound bound IDE was fractionated by Superdex S-200 column and collected. The incubation and fractionation was repeated again before setting up the crystallization. Such extensive purification of IDE using gel filtration chromatography was a proven key step to ensure the formation of diffracting quality crystals of IDE. The complex of IDE-CF-E111Q with compound **1-3, 5** was crystallized by hanging drop vapor diffusion at 18 °C, using 1 µl of 10 mg/ml IDE and 1 µl of mother liquor (10-13% PEG MME 5000, 100 mM HEPES pH 7.0, 4-14% Tacsimate, 10% dioxane). IDE-CF in complex with **1** (BDM41367) was crystallized under the same condition except the addition of 200 µM of given compound in the crystallization drop. IDE crystals were equilibrated in cryo-protective buffer containing mother liquor with 30% glycerol and flash frozen in liquid nitrogen. Diffraction data were collected at 100K at the Advance Photon Source 19-ID beamline at Argonne National Laboratory. The data sets were processed using HKL2000 [1] and the structures were solved by molecular replacement using software Phaser [2] and the IDE-CF-E111Q portion of insulin-bound IDE-CF-E111Q structure as a search model (PDB:2WBY). Structure refinement and rebuilding were performed using software REFMAC and Coot [1,3]. The extra electron density at the catalytic chamber of IDE-CF in the structures of IDE in complex with compound were clearly visible based on $\sigma_A$-weighted Fo-Fc map calculated by software CNS [4] and manually built. The refinement statistics are summarized in supplemental information Table S1. Figures were generated using software PyMol [5].

[1] Murshudov GN, Vagin AA, & Dodson EJ (**1997**) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* 53:240-255.

[2] Potterton E, McNicholas S, Krissinel E, Cowtan K, & Noble M (**2002**) The CCP4 molecular-graphics project. *Acta Crystallogr D Biol Crystallogr* 58:1955-1957.
[3] Emsley P & Cowtan K (**2004**) Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 60:2126-2132.
[4] Brunger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T, & Warren GL (**1998**) Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr D Biol Crystallogr* 54:905-921.
[5] WL. D (**2002**) Pymol.

**SAXS analysis of IDE**

The purified IDE (9 µM) was mixed with 100 µM **1** (BDM41367) before the data collection. SAXS data were collected at the 18-ID (BioCAT) beamline using the Mar 165 CCD detector at 20° using 1.033 Å as the incident X-ray wavelength. All data processing was carried out using Igor Pro (WaveMetrics Inc.) with macros written by the BioCAT staff.

**Selectivity**

1/ *In vitro* NEP activity was measured with a quenched substrate N-Dansyl-D-Ala-Gly-p-nitro-Phe-Gly (Km = 350 µM). Briefly, human NEP (R&D Systems) (200 ng/mL) was incubated 10 min at room temp. with compound in Hepes 50 mM, NaCl 100 mM, pH 7.4 and the enzymatic reaction started by adding the substrate (final conc. 200 µM). After 2 h, samples (1% DMSO final) are excited at 340 nm and fluorescence emission at 535 nm is measured on a Victor3 V1420 Perkin Elmer spectrophotometer. All measurements are reported as the average of at least three independent measurements. (DL-thiorphan reference inhibitor ($IC_{50}$ = 1 (±0.3) nM)).

2/ *In vitro* ACE activity was measured with a quenched substrate Abz-Gly-p-nitro-Phe-Pro-OH (Km = 180 µM). Briefly, human ACE (R&D Systems) was incubated 10 min at room temperature with compound in Tris 50 mM 1% NaCl pH7.4 and the enzymatic reaction is started by adding the substrate (final concentration 300 µM). After 17 h, samples (1% DMSO final) are excited at 340 nm and fluorescence emission at 420 nm is measured on a Victor3 V1420 Perkin Elmer spectrophotometer. All measurements are reported as the average of at least three independent measurements. (Captopril reference inhibitor ($IC_{50}$ = 11 nM)).

3/ Inhibition of hACE2, hECE, hTACE, hMMP1 and hMMP13. *In vitro* activity was assayed at CEREP.SA. Assays are summarized in the table below.

| Assay | Source | Substrate | Incubation | Measured Component | Detection Method | Bibl. |
|---|---|---|---|---|---|---|
| **ACE-2** ***(h)*** | h recombinant (murine cells) | Mca-Tyr-Val-Ala-Asp-Pro-Ala-Lys-(DNP)-OH (10 µM) | 20 min RT | Mca peptides | Fluorimetry | [1] |
| **ECE-1** ***(h)*** | h recombinant (NSO cells) | ECE-1 fluorescent substrate (15 µM) | 45 min RT | Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala | Fluorimetry | [2] |
| **MMP-1 (h)** | h recombinant (*E. coli*) | DNP-Pro-Cha-Gly- Cys(Me)-His-Ala- Lys(n-Me-Abz)-NH2 (10 µM) | 40 min 37°C | Cys(Me)-His-Ala-Lys(n-Me-Abz)-NH2 | Fluorimetry | [3] |
| **MMP-13 (h)** | h recombinant (*Spodoptera frugiperda*) | MMP-13 substrate (2 µM) | 10 min RT | Mca-Pro-Cha-Gly | Fluorimetry | [4] |
| **TACE** ***(h)*** | h recombinant (insect cells) | TACE substrate II (4 µM) | 5 min RT | Mca-Pro-Leu-Ala-Gln-Ala | Fluorimetry | [5] |

[1]. Huang et al. (**2003**), *J. Biol. Chem., 278: 15532-15540*
[2]. Ahn et al. (**1998**), *Arch. Biochem. Biophys., 359: 258-268.*
[3]. Bickett et al. (**1993**), *Anal. Biochem., 212: 58-64*.
[4]. Knauper et al. (**1996**), *J. Biol. Chem., 271: 1544-1550.*
[5]. Van Dyk et al. (**1997**), *Bioorg. Med. Chem. Lett., 7: 1219-1224.*

**Solubility/LogD measurements.**

The analysis was performed using a LC-MS/MS system (Varian 1200L) under SIM detection using the parameters optimized for each compounds. HPLC analysis was performed using a Luna C18 (50*2.1 mm, 5 µm); the gradient and the mobile phase (flow rate 600 µL.min$^{-1}$) used are determined in order to detect the compound of interest with satisfying retention time and peak shape. Acquisition and analysis of data were performed with MS WorkstationTM software (version 6.3.0 or higher).

10µL of a 10 mM solution in DMSO of the compound are diluted either in 490µL of PBS pH 7.4 or in organic solvent MeOH in a 700µL-microtube (in triplicate). The tubes are gently shaken 24 h at room temperature, then centrifuged for 5 minutes at 4000 rpm. The mixtures are filtered over 0.45 µm filters (Millex-LH Millipore). 20 µL of sample are diluted in 180 µL of MeOH. The solubility is determined by the ratio of mass signal area PBS/ organic solvent.

40µL of a 10 mM solution in DMSO of the compound were diluted in 1.960 mL of a 1/1 octanol /PBS at pH 7.4 mixture. The mixture was gently shaken 2 h at room temperature. 20 µL of each phase was diluted in 480 µL of MeOH and analyzed by LC-MS. Each compound is tested in triplicate. Log D was determined as the logarithm of the ratio of concentration of product in octanol and PBS respectively, determined by mass signals.

**Stability in mouse plasma or IHH cell culture medium:**

Incubations were performed in duplicate in Eppendorf tubes. The mouse plasma (Mouse Plasma Lithium Heparine from Sera Laboratories International Ltd) or the William's E medium supplemented with 10 % fetal calf serum was pre-incubated 5 min at 37°C before the addition of test compounds to a final concentration of 10µM (1% DMSO maximum). At the defined time points, 50 µL from each tube were removed to another tube containing 450µL of cold $CH_3CN$ + internal standard (1µM). After centrifugation (10 min at 10000 rpm), supernatants are analyzed. Analysis and quantification used a LC-MS/MS triple-quadrupole system (Varian 1200L) under MRM detection using the parameters optimized for each compounds. HPLC analysis was performed using a Luna $C_{18}$ (50*2.1 mm, 5µm); the gradient and the mobile phase (flow rate 600µL/min-1) used are determined in order to detect the compound of interest with satisfying retention time and peak shape. Acquisition and analysis of data were performed with MS Workstation$^{TM}$ software (version 6.3.0 or higher). The degradation half-life (t1/2) values were calculated using the following equation: t1/2 = 0.693/k where k is the first-order degradation rate constant. The degradation rate constant (k) was estimated by one-phase exponential decay non-linear regression analysis of the degradation time course data using XlfitTM software (version 2.1.2 or higher) from IDBS.Ltd.

**Microsomal stability:**

Pooled human (male and female) liver microsomes were purchased from BD Gentest (Le Pont de Claix, France). All incubations were performed in triplicates in a shaking water bath at 37°C. The incubation mixture were prepared in polypropylene tubes and contained 1 µM test compound (1% acetonitrile), human liver microsomes (0.6 mg of microsomal protein / ml), 5 mM $MgCl_2$, 1 mM NADP, 5 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase and 50 mM potassium phosphate buffer pH 7.4 in a final volume of 0.5 mL. Sampling points were taken at 10, 20, 30 and 40 min and reactions were terminated by adding ice-cold acetonitrile containing 1 µM internal standard (4 vol). The samples were centrifuged for 10 min at 10000*g*, 4°C to pellet precipitated microsomal protein, and the supernatant was subjected to LC-MS/MS analysis. Control incubations were performed with

denaturated microsomes with acetonitrile containing 1µM internal standard and sampling points were taken at 0 min and 40 min (to evaluate the compound chemical stability in the experimental conditions).

**Cell permeability**

Permeability was assessed in duplicate at CEREP. SA on caco2 cell monolayer (pH: 7.4-7.4 on both compartments). Fluoresceine was used as a cell integrity marker. Permeability is estimated by LCMS/MS and expressed in $x10^{-6}$ cm. $s^{-1}$. Compound **8** was tested at 10 µM and incubated for 1 hr on and displayed a slow Papp of 0.2.

**Cell Culture**

**Cell culture of Min6 (mouse pancreatic beta cell) cell lines.**

Min 6 cells were cultured in a humidified atmosphere containing 5% $CO_2$ in DMEM glutamax medium (containing 25mmol/L glucose) supplemented with 10% FCS, β-mercapto-Ethanol 50 µM, and gentamycin 1%.

**Cell culture of IHH (Immortalized Human Hepatocytes) cell lines**

IHH cells were maintained in standard culture conditions: William's E medium supplemented with 10 % fetal calf serum, glutamine, insulin, dexamethasone and Penicillin/Streptomycin (P/S) at 37°C in a humidified atmosphere of 5% $CO_2$/95% air. Medium was changed every 3 days.

**Glucose Stimulated Insulin Secretion by beta-pancreatic cells (GSIS)**

Min6 were seeded in 24 well plate (300 000 cells/well) and cultured for 7 days. The cells are pre-treated for 24h before or 1h during GSIS. Each compound is dissolved in DMSO. Before the GSIS experiment the cells were washed and preincubated for 1 hour at 37°C in glucose-free Krebs-Ringer bicarbonate HEPES buffer (KRBH) of the following composition : 115 mM NaCl, 4.7 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 20 mM $NaHCO_3$, 16mM HEPES, BSA free fatty acid 0.5%. Next the cells are stimulated in KRBH buffer containing 2.8mM of glucose or 20mM of glucose for 1 hour. Incubation was stopped by putting the plate on the ice, the supernatant was collected for insulin secretion. Insulin secretion was measured by ELISA (Mercodia). Compounds activity on insulin secretion in Min6 stimulated by glucose was measured in 2 conditions of treatment: during the GSIS (1 h of stimulation) or 24 h of treatment before GSIS. The data was obtained in duplicate from three independent experiments and normalized to untreated cells.

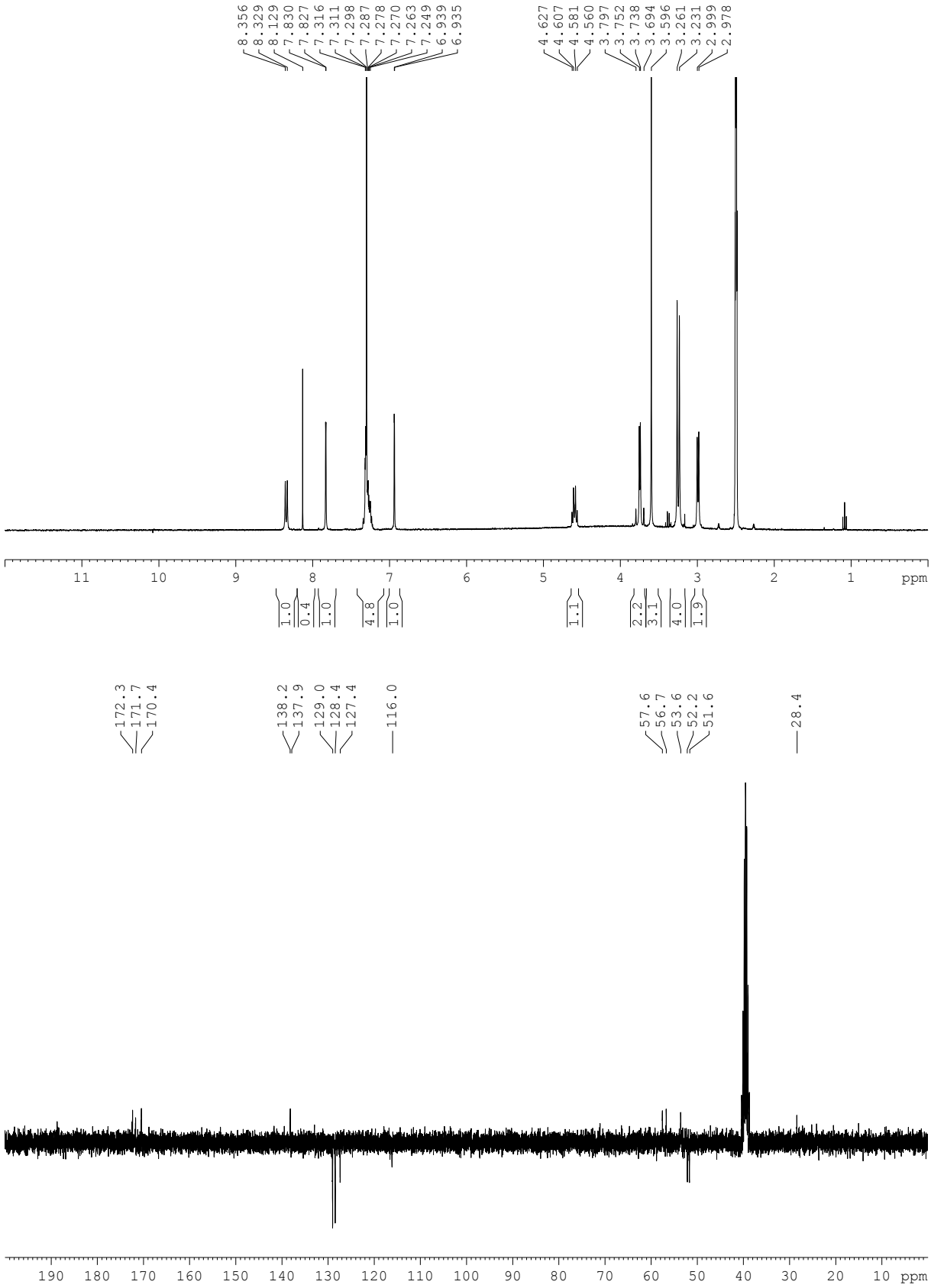
**Insulin inhibition of PEPCK expression in IHH cells**

IHH cells were seeded in 6-well plates (gelatin coated plate) at a density of 0.8 x $10^6$ cells/dish in William's E medium supplemented with 10% FCS insulin, dexamethasone and P/S, and incubated at 37°C overnight prior to insulin starvation in DMEM (Dulbecco's modified Eagle's minimal essential medium) supplemented with 0.2% FCS, glucose 11mM, P/S without insulin and dexamethasone for 10 h. Cells were pretreated in this medium with compounds (10 µM) overnight. Cells were then incubated 24 h in DMEM containing 0.2% FCS, 11 mM glucose and insulin (1 nM) and compounds (10 µM) or vehicle (DMSO). At the end of the experiment, cells were washed once with ice-cold PBS before mRNA extraction. mRNA levels of PEPCK were measured by quantitative RT- PCR 24 h after stimulation. The data was obtained in duplicate from three independent experiments and normalized to untreated cells.

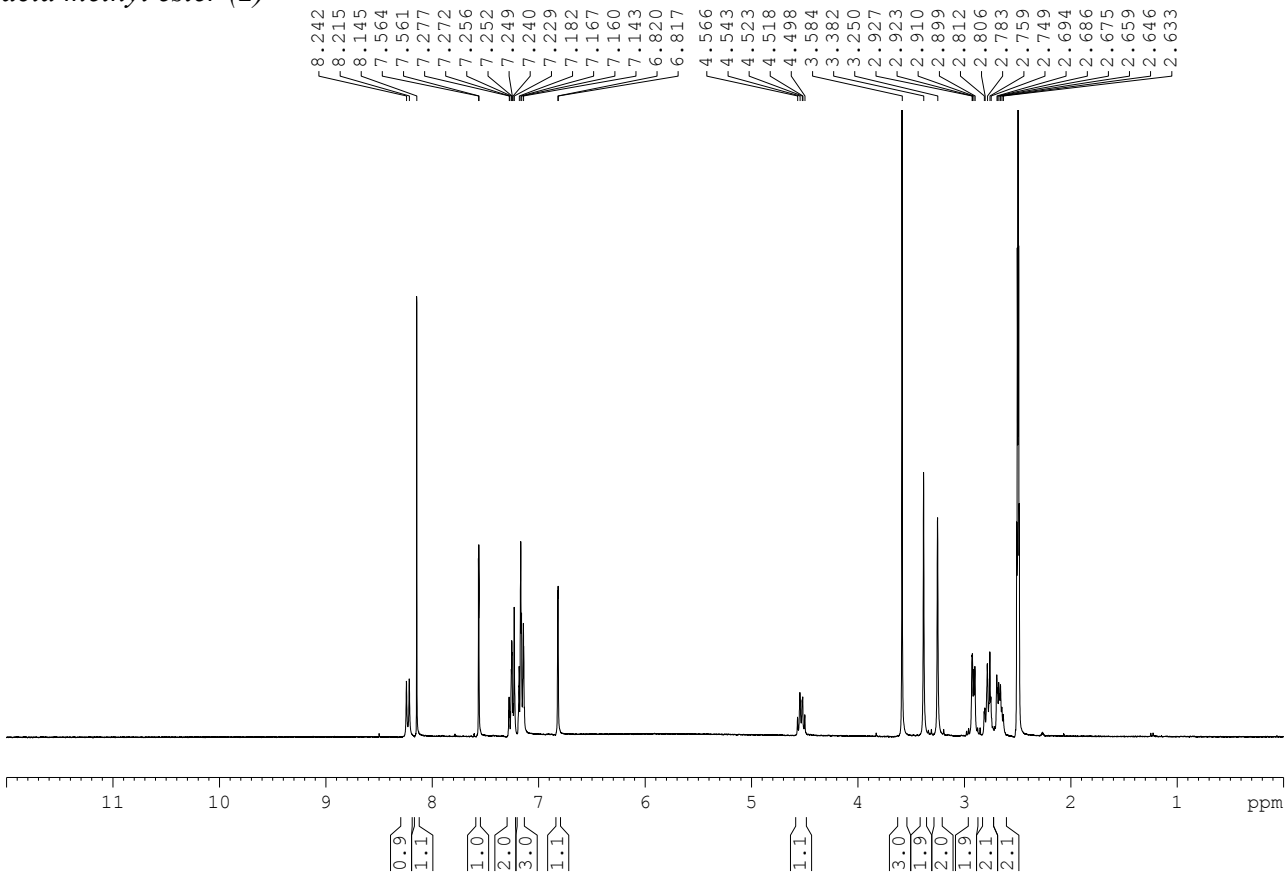
**Data and statistical analysis**

Data analysis was performed using Xlfit v 5.0 and GraphPad Prism v 4.0. Nonlinear curve fitting and statistical analysis was done using built-in functions. Data are plotted as mean ± s.e.m. unless otherwise stated.

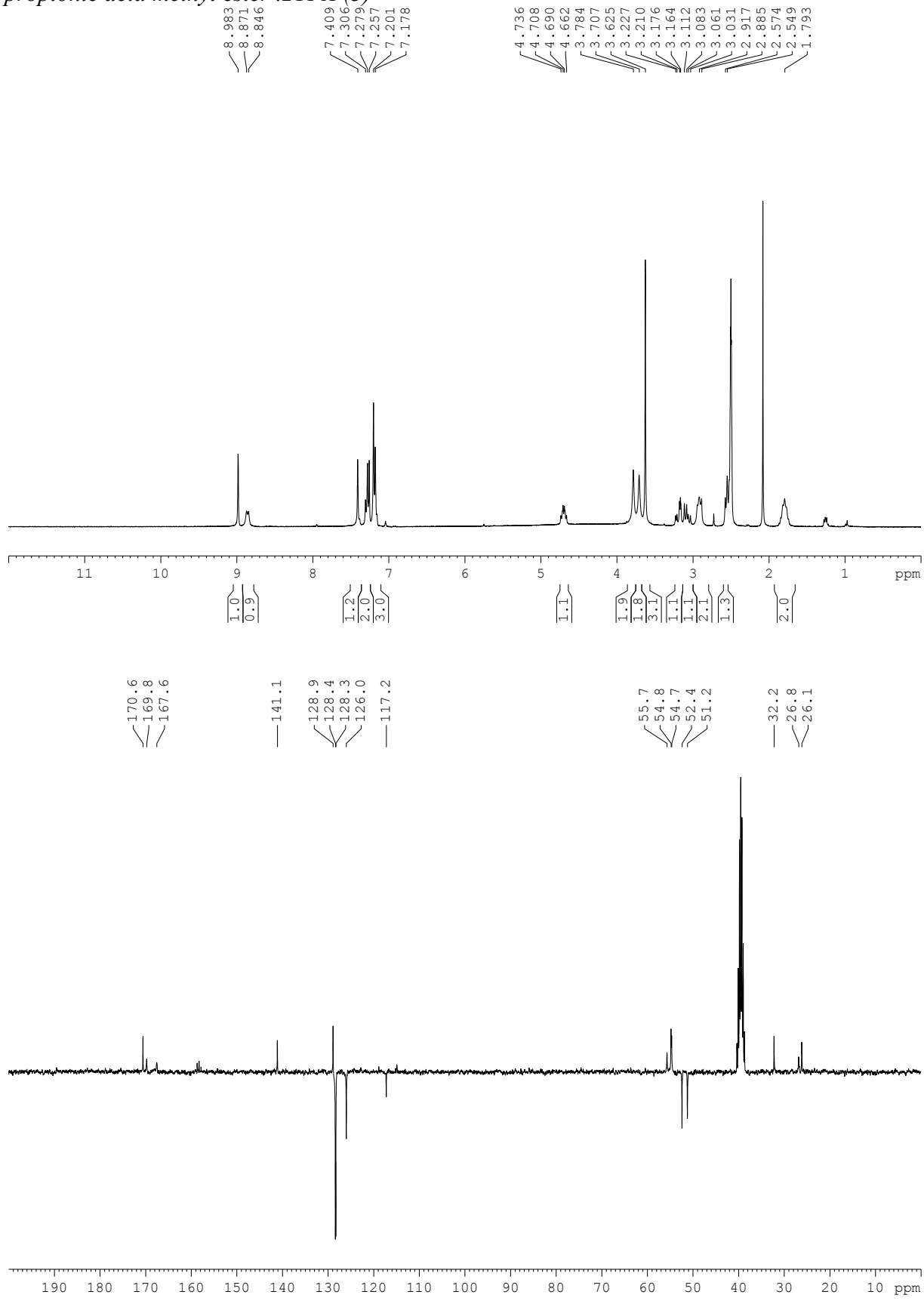
*(S)-2-[2-(Benzyl-carboxymethyl-amino)-acetylamino]-3-(1H-imidazol-4-yl)-propionic acid methyl ester (**1**) (BDM41367)*



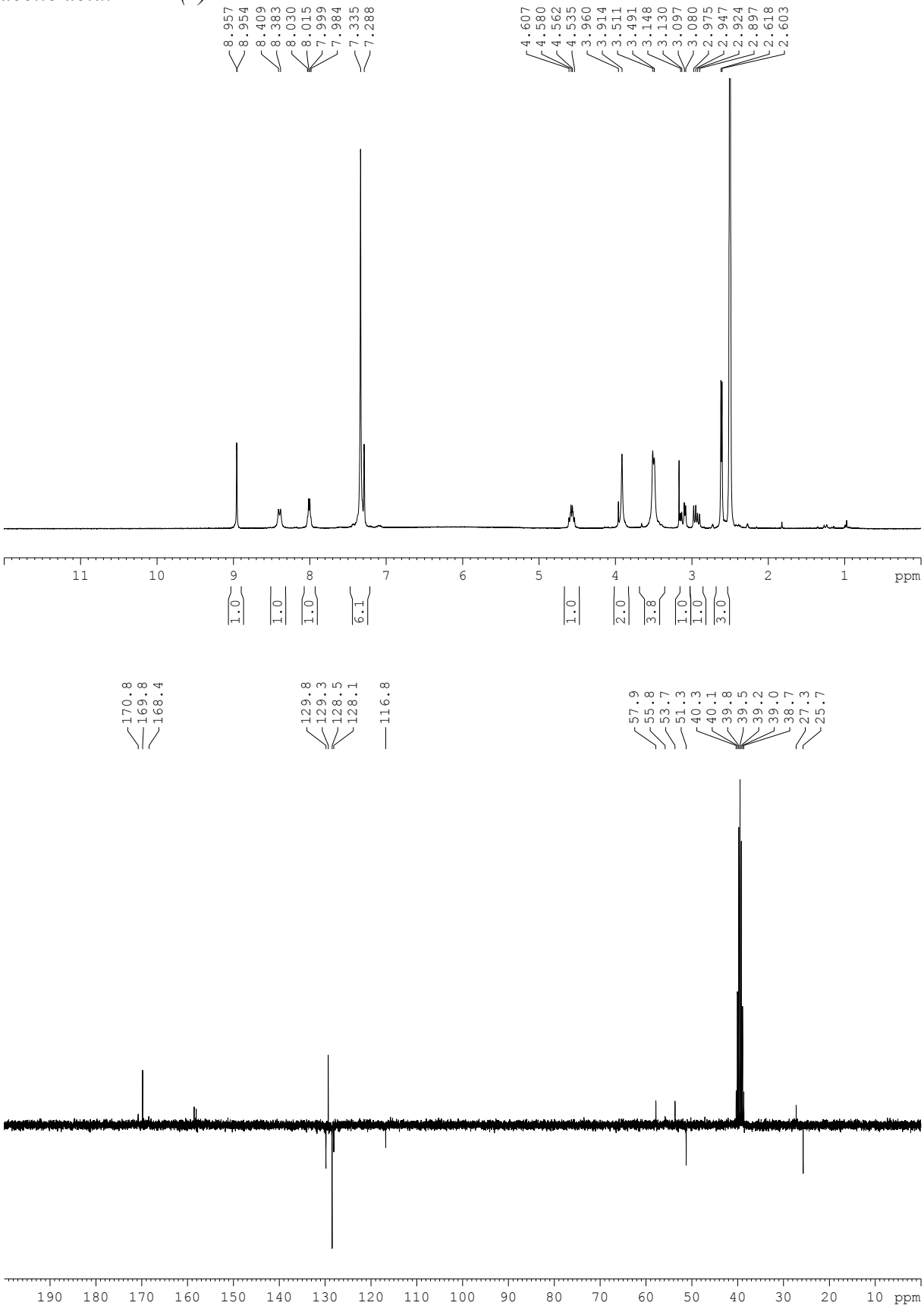
*(S)-2-[2-(Carboxymethyl-phenethyl-amino)-acetylamino]-3-(1H-imidazol-4-yl)-propionic acid methyl ester (**2**)*



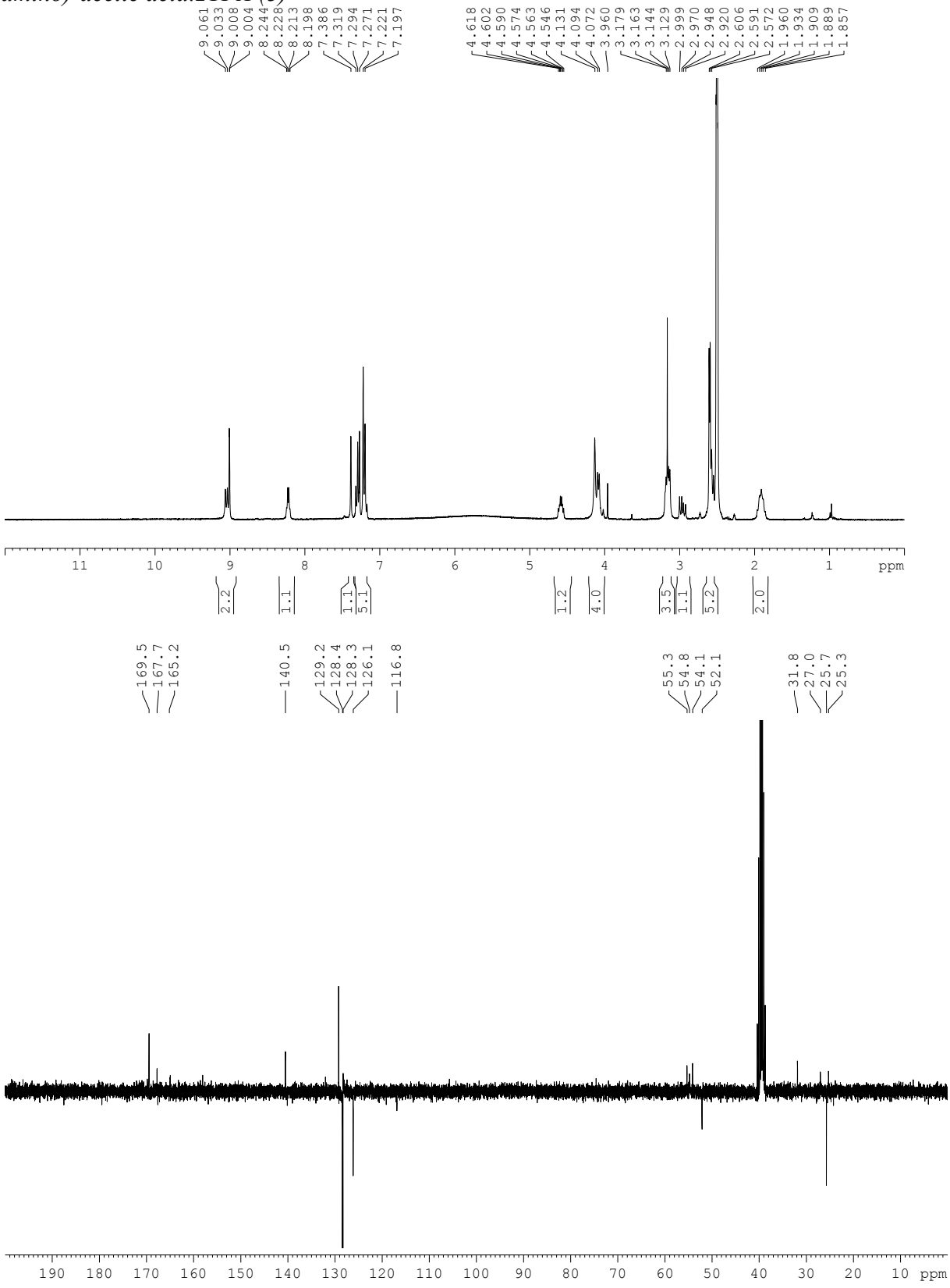
*(S)-2-{2-[Carboxymethyl-(3-phenyl-propyl)-amino]-acetylamino}-3-(1H-imidazol-4-yl)-propionic acid methyl ester .2TFA* (**3**)



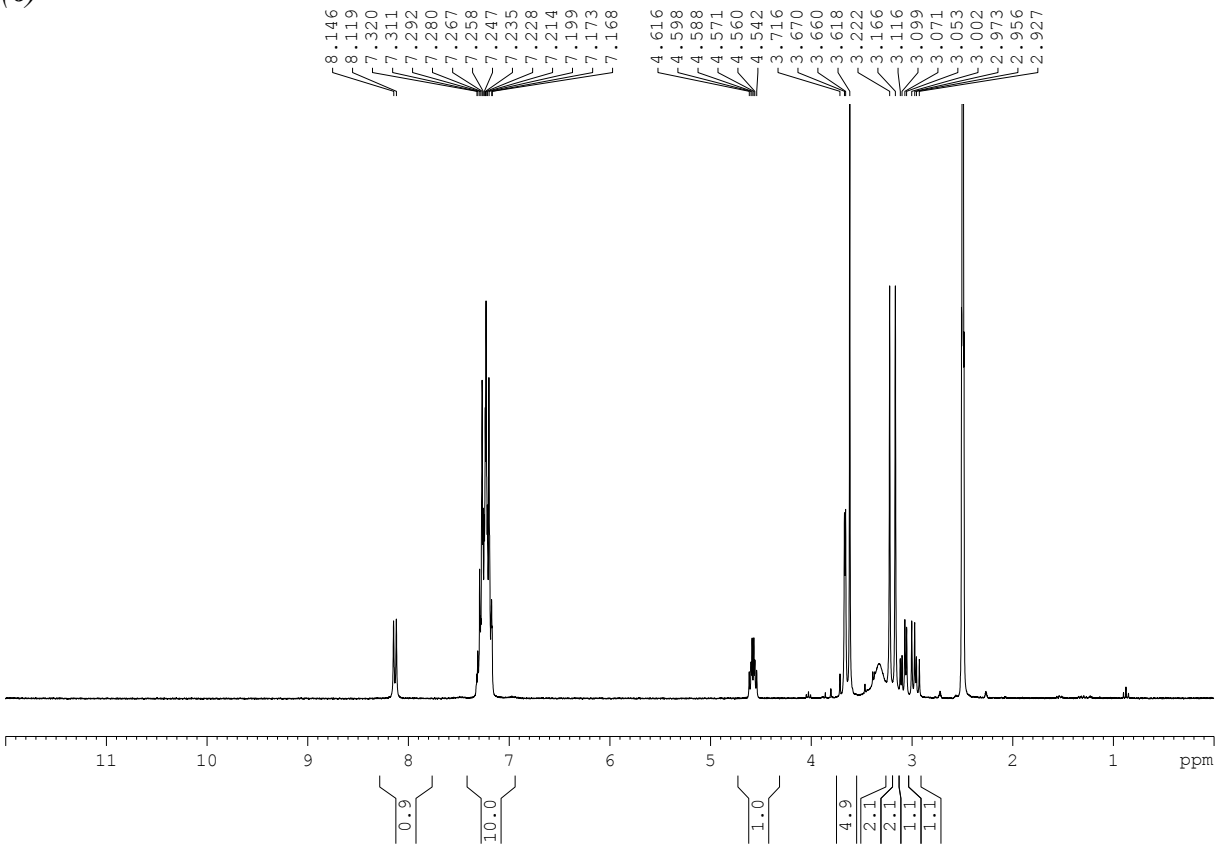
*(Benzyl-{[(S)-2-(1H-imidazol-4-yl)-1-methylcarbamoyl-ethylcarbamoyl]-methyl}-amino)-acetic acid. 2TFA (**4**)*

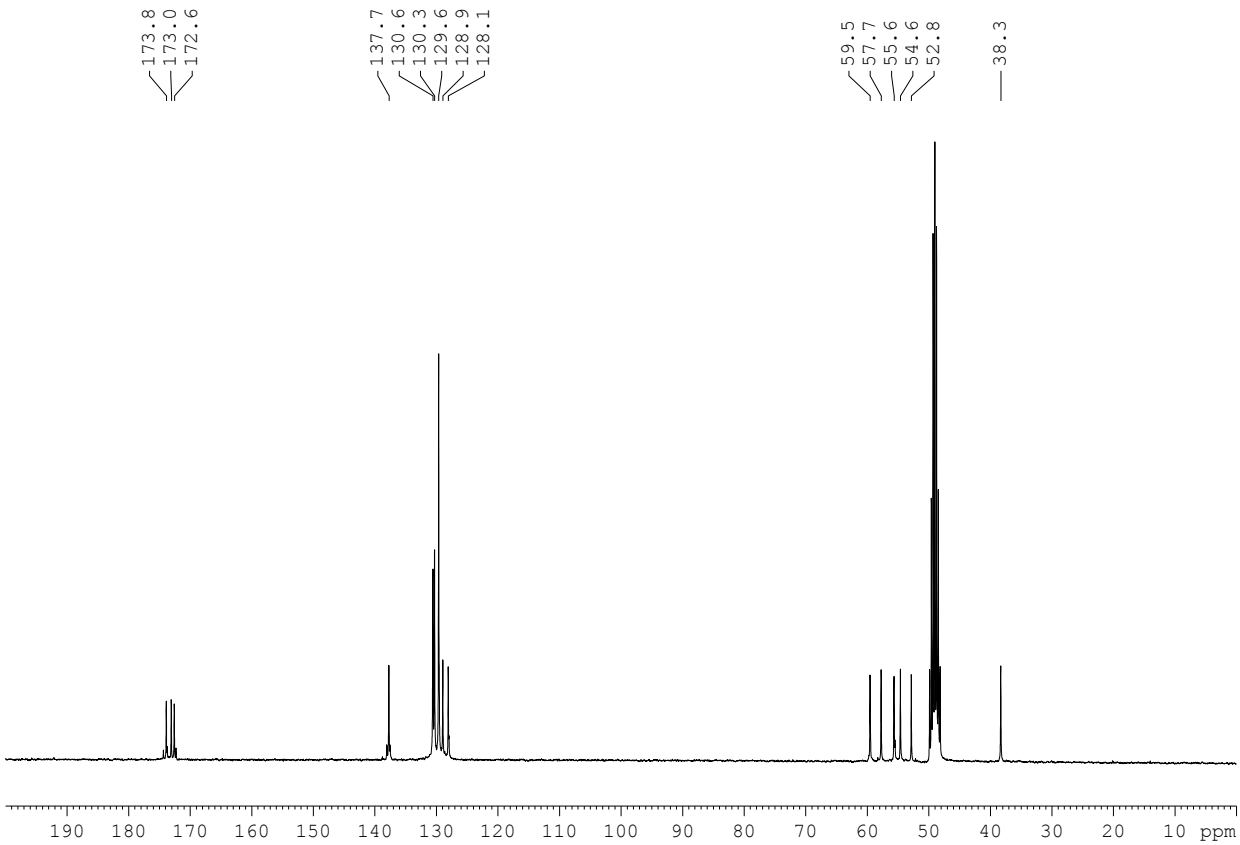


*((((S)-2-(1H-Imidazol-4-yl)-1-methylcarbamoyl-ethylcarbamoyl)-methyl)-(3-phenyl-propyl)-amino)-acetic acid.2TFA (**5**)*

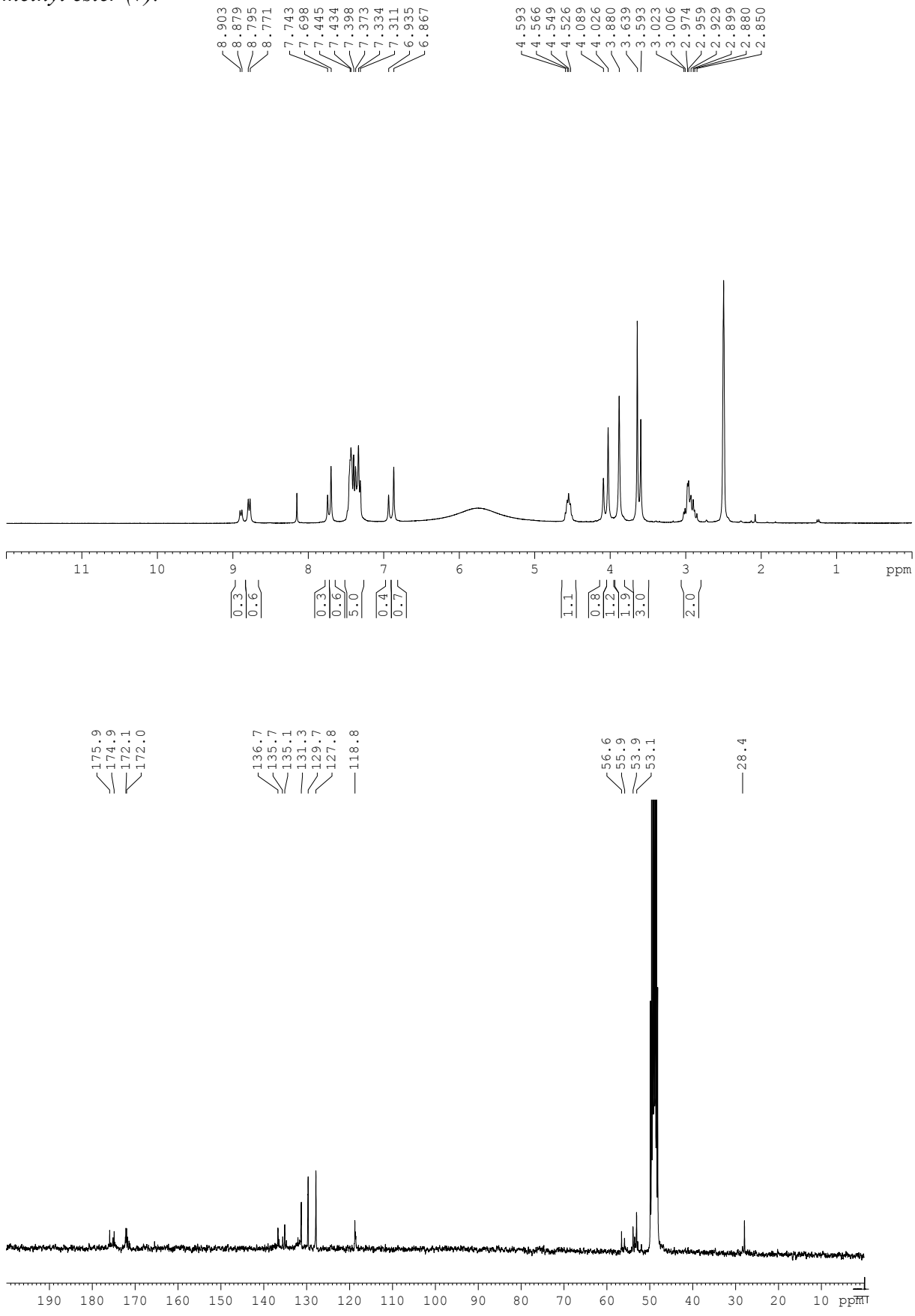


*(S)-2-(2-(Benzyl-carboxymethyl-amino)-acetylamino)-3-phenyl-propionic acid methyl ester.*
***(6)***



*(S)-2-(2-(Benzoyl-carboxymethyl-amino)-acetylamino)-3-(1H-imidazol-4-yl)-propionic acid methyl ester (7).*



*((((S)-2-(1H-Imidazol-4-yl)-1-methylcarbamoyl-ethyl-carbamoyl)-methyl)-(3-phenyl-propyl)-amino)-acetic acid methyl ester. 2TFA* **(8)** *(BDM43124*