

Stabilization of Angiotensin-(1-7) by key substitution with a Cyclic Non-natural Amino Acid

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Abstract

Angiotensin-(1-7) [Ang-(1-7)], a heptapeptide hormone of the renin-angiotensin-aldosterone system (RAAS), is a promising candidate as a treatment for cancer that reflects its anti-proliferative and anti-angiogenic properties. However, the peptide's therapeutic potential is limited by the short half-life and low bioavailability resulting from rapid enzymatic metabolism by peptidases including angiotensin-converting enzyme (ACE) and dipeptidyl peptidase 3 (DPP 3). We report the facile assembly of three novel Ang-(1-7) analogues by solid-phase peptide synthesis which incorporates the cyclic non-natural δ -amino acid ACCA. The analogues containing the ACCA substitution at the site of ACE cleavage exhibit complete resistance to human ACE, while substitution at the DPP3 cleavage site provided stability against DPP 3 hydrolysis. Furthermore, the analogues retain the anti-proliferative properties of Ang-(1-7) against the 4T1 and HT-1080 cancer cell lines. These results suggest that ACCA-substituted Ang-(1-7) analogues which show resistance against proteolytic degradation by peptidases known to hydrolyze the native heptapeptide may be novel therapeutics in the treatment of cancer.

1. Introduction

Angiotensin-(1-7) [Ang-(1-7)] is a heptapeptide hormone of the renin-angiotensin-aldosterone system (RAAS) that mediates its biological effects by activating the G-protein coupled receptor *mas* (Santos 2003). Ang-(1-7) is generated by enzymatic cleavage of the decapeptide Angiotensin I by endopeptidases including neprilysin (NEP) and thimet oligopeptidase and from breakdown of the octapeptide Angiotensin II by carboxypeptidases including angiotensin-converting enzyme 2 (ACE2) and propyl carboxypeptidase (PCP) (Chappell 2016).

Ang-(1-7) was identified as a promising therapeutic for the treatment of cancer based on the anti-proliferative and anti-angiogenic properties of the peptide in models of lung, breast and prostate cancer (Menon 2007; Soto-Pantoja 2009; Cook 2010; Krishnan 2013a, b; Gallagher 2014). In a mouse model of lung cancer, Ang-(1-7) reduced the growth of human A549 xenograft tumors that was associated with a decrease in cyclooxygenase 2 (COX-2) mRNA and protein (Menon 2007). Attenuation of tumor growth by Ang-(1-7) was also associated with reduced vessel density in the tumors of mice treated with the heptapeptide hormone as compared to saline-treated animals (Soto-Pantoja 2009). The reduction in angiogenesis was associated with a decrease in the proangiogenic protein vascular endothelial growth factor-A (VEGF-A), suggesting that Ang-(1-7) reduces VEGF-A to inhibit angiogenesis (Soto-Pantoja 2009). Ang-(1-7) reduced cell migration and invasion *in vitro* in A549 human lung cancer cells in association with a decrease in matrix metalloproteinases MMP-2 and MMP-9 expression and activity, suggesting that the heptapeptide hormone may inhibit metastasis (Ni 2012). In two different models of metastatic prostate cancer in the bone, Ang-(1-7) prevented the formation of metastatic tumors, demonstrating the inhibition of metastasis (Krishnan 2013a). Furthermore Ang-(1-7) regulates the tumor microenvironment by inhibiting the proliferation of cancer-associated fibroblasts (CAF) and fibrosis by reducing the mitogen-activated protein kinases (MAPK) ERK1/2 through increasing the MAPK phosphatase DUSP1 in orthotopic breast tumors (Cook 2010). Collectively, these results demonstrate that Ang-(1-7) inhibits proliferation, inflammation, angiogenesis, fibrosis and metastasis to reduce tumor growth.

Published clinical trials further demonstrate the therapeutic potential of Ang-(1-7) for the treatment of cancer (Petty 2009; Petty 2012; Pham 2013; Rodgers 2006). With limited toxicity and pleiotropic effects on cancer cells and the tumor microenvironment, Ang-(1-7) shows

promise as a first-in-class anti-cancer agent (Gallagher 2014). However, a problem commonly associated with peptide therapeutics is their short half-life and limited bioavailability due to rapid degradation by endopeptidases and exopeptidases. Ang-(1-7) is degraded in the circulation by angiotensin-converting enzyme (ACE) resulting in a short half-life of 29 minutes (Rodgers 2006). ACE hydrolyzes Ang-(1-7) to the pentapeptide Ang-(1-5) and the dipeptide His⁶-Pro⁷ (**Fig. 1**) (Chappell 1998) and ACE inhibitor treatment increases the half-life of Ang-(1-7) in the circulation (Chappell, 2016). Dipeptidyl peptidase 3 (DPP 3) was recently identified as an enzyme which degrades Ang-(1-7) in the brain, kidney and human HK-2 renal epithelial cells, and its activity was enhanced in fetal programming (Cruz-Diaz 2016; Marshall 2014a, b). In contrast to ACE, DPP 3 cleaves the first two residues from the N-terminus of Ang-(1-7) to yield Ang-(3-7) which is then rapidly hydrolyzed to Ang-(5-7) (**Fig. 1**).

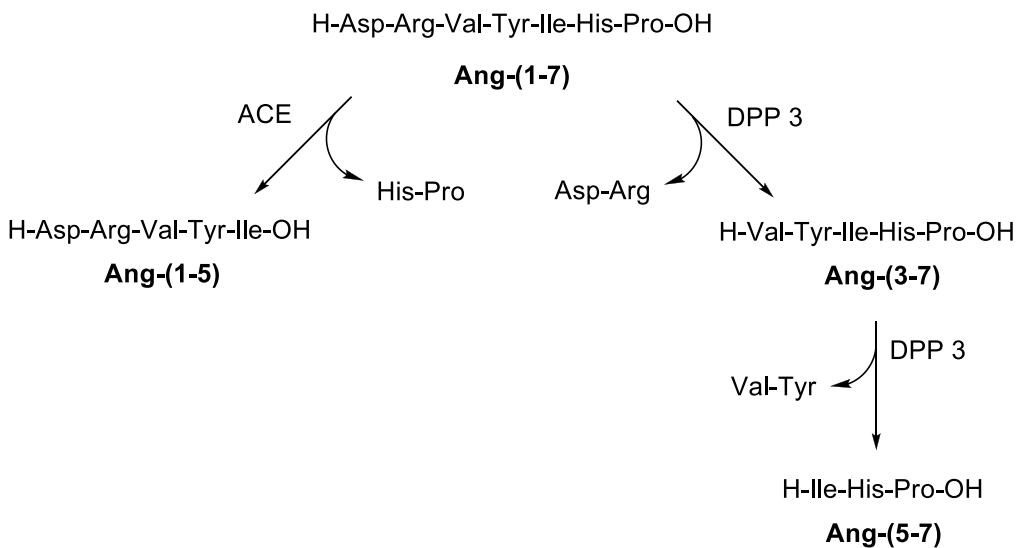


Fig. 1. Proteolytic cleavage of Angiotensin-(1-7) by angiotensin converting enzyme (ACE) and dipeptidyl peptidase 3 (DPP 3).

Previous attempts to stabilize the heptapeptide hormone for clinical application include encapsulation of the native peptide in the cyclic oligosaccharide cyclodextrin (CyD) [Ang-(1-7)-CyD] (Lula 2007, Fraga-Silva 2011) and in a liposomal delivery system (Silva-Barcellos 2001). Furthermore, a cyclic analogue of Ang-(1-7) was previously shown to be resistant to purified human ACE (Kluskens 2009).

Here we report the synthesis and biological evaluation of three novel stabilized Ang-(1-7) analogues: ACCA1 **1**, ACCA2 **2** and ACCA3 **3**. The increase stability to enzymatic degradation was achieved by incorporation of the non-natural δ -amino acid ACCA **4** which was previously employed successfully in dipeptide coupling (O'Reilly 2013). Non-natural amino acids in general and those exhibiting cyclic moieties in particular are valuable building blocks for peptidomimetic synthesis as their incorporation into the sequence of a peptide drug candidate often results in increased stability as recognition by proteolytic enzymes is prevented. Furthermore, rigidity inferred to the usually very flexible peptide structure can lead to enhanced selectivity and efficiency. ACCA **4** was substituted for the native amino acid residues Ile⁵ and His⁶ to give compounds **1** and **2** respectively (**Fig. 2**). Compound **3** (**Fig. 2**), in which ACCA replaced the Val³ residue, was also synthesized. The first two peptide analogues target directly the site of hydrolysis by ACE, while the third leaves the ACE hydrolysis site intact and introduces rigidity earlier in the sequence to compare affinity and activity. Compound **3** was also considered to be a potential stable compound as ACCA was substituted at the initial cleavage of site for DPP 3.

The stability of the analogues was assessed against both human ACE and DPP 3. The biological efficacy of the compounds was investigated in cell proliferation assays testing their inhibitory effect on cell growth of 4T1 breast cancer and HT-1080 fibrosarcoma cells.

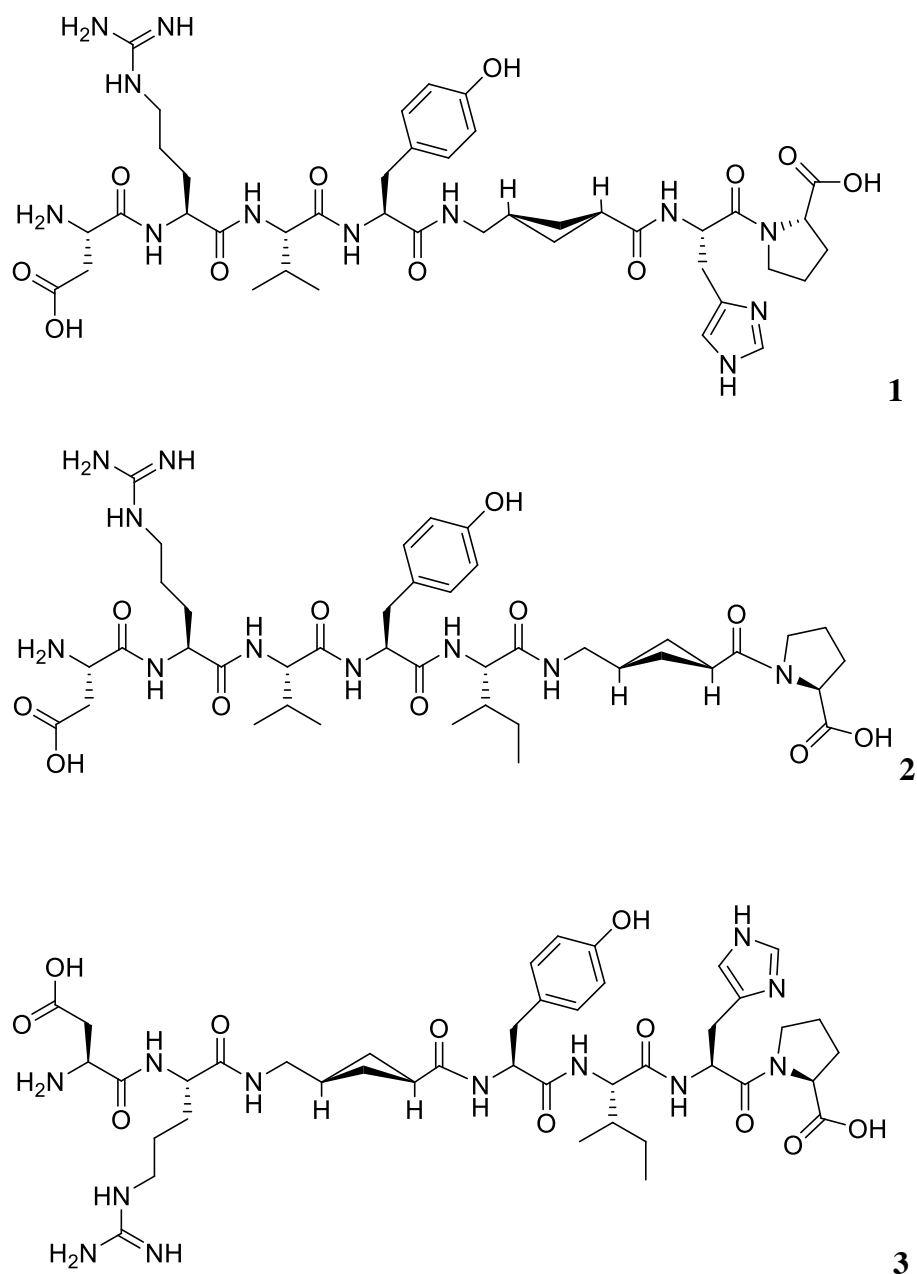
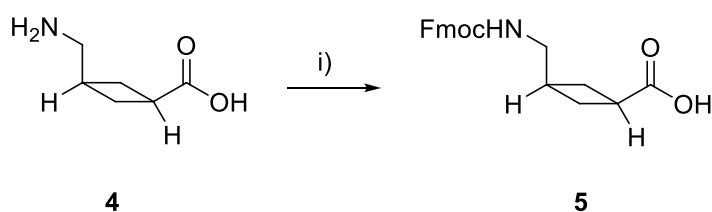


Fig. 2. Structures of the Ang-(1-7) analogues ACCA1 **1**, ACCA2 **2** and ACCA3 **3**.

2. Results and discussion

2.1 Chemistry

The synthesis of ACCA **4** was carried out as previously described (O'Reilly 2013) except for the reduction of the heterocycle double bond which was revised and achieved by transfer hydrogenation (Paryzek 2003). Fmoc protection of ACCA facilitated its use in solid-phase peptide synthesis (**Scheme 1**). The X-ray crystal structure of **5** visualizes the cyclobutane moiety of the non-natural amino acid and the *cis* orientation of the amino and carboxylic acid group (**Fig. 3**).



Scheme 1. Synthesis of Fmoc-ACCA 5. i) Fmoc-OSu (1.5 eq.), 10% (w/v) aq. Na₂CO₃, dioxane, 0 °C - r.t., 1 h 45 min, 95%.

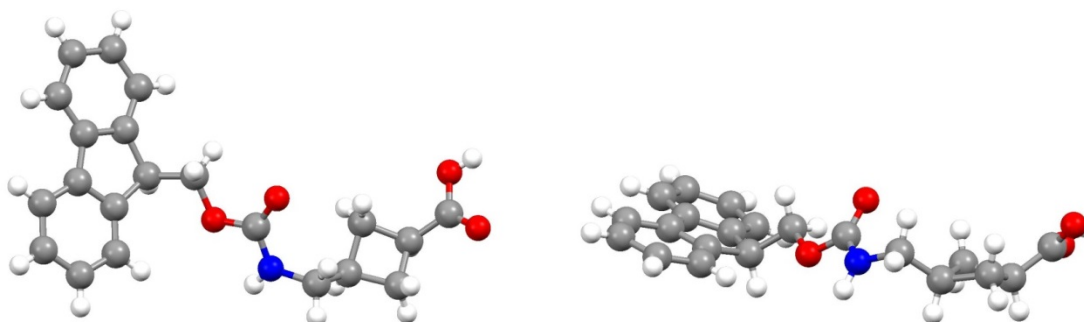


Fig. 3. Two orientations of the X-ray crystal structure of Fmoc-ACCA **5** (CCDC 1514790).

The peptides incorporating ACCA **4** were prepared employing a standard Fmoc/*t*-Bu solid-phase peptide synthesis protocol using an automated peptide synthesizer as described in the experimental procedure. Formation of the peptides was confirmed by MALDI-TOF MS analysis and purification by RP-HPLC generated peptides **1**, **2** and **3** with yields of 64%, 46% and 63% yield with >99%, >99% and 91% purity, respectively.

2.2 Proteolytic stability

Figure 4 shows the HPLC-UV profiles of the Ang-(1-7) standard and the purified ACCA peptides alone (left panels, Figure 4, A, C, E and G) and following a 60 minute incubation with human ACE at 37°C (right panels). As we previously reported (Chappell 1998), ACE catalyzed the metabolism of Ang-(1-7) to the pentapeptide Ang-(1-5) (**Fig. 4, B**). Despite its smaller molecular weight, Ang-(1-5) (20 minutes) exhibits a greater retention time than Ang-(1-7) (18.3 minutes) under the current chromatographic conditions. In comparison to Ang-(1-7), the ACCA1 and ACCA2 analogues show complete resistance to ACE metabolism (**Fig. 4, D and F** respectively), while the ACCA3 analogue was metabolized by ACE. Note that intact ACCA3 eluted at 18 minutes (**Fig. 4, G**) and the ACE-dependent metabolite ACCA3-Ang-(1-5) eluted later at 19.2 minutes (**Fig. 4, H**).

We then established the extent of metabolism of Ang-(1-7) and the ACCA peptides by human DPP 3 (**Fig. 5**). As previously reported (Cruz-Diaz 2016), DPP 3 ultimately metabolizes Ang-(1-7) (**Fig. 5, A**) to the tripeptide Ile⁵-His⁶-Pro⁷ or Ang-(5-7) that elutes at 8 minutes (**Fig. 5, B**). This profile reflects the initial metabolism of Ang-(1-7) to the dipeptide Asp¹-Arg² and Ang-(3-7) followed by the more rapid hydrolysis of Ang-(3-7) to Val³-Tyr⁴ and Ang-(5-7) which

accounts for the major peak of Ang-(5-7) in the chromatograph. **Figure 5, D** demonstrates that ACCA1 (**Fig. 5, C**) is metabolized by DPP 3 to ACCA1-(3-7) [6.2 minutes]. It is possible that the 2.8 minute peak is ACCA1-(5-7); however, the ACCA substitution of Ile⁵ may prevent the hydrolysis of ACCA1-(3-7) by DPP 3 and the 2.8 minute peak may not represent a metabolite of ACCA1. In comparison, ACCA2 (**Fig. 5, E**) is metabolized by DPP 3 to the tripeptide ACCA2-(5-7) (**Fig. 5, F**). In this case, substitution of His⁶ with the ACCA residue may allow for more complete metabolism of the ACCA2-(3-7) peptide following the initial hydrolysis of ACCA2 (see pathway). In contrast, the ACCA3 analogue (**Fig. 5, G**), where the Val³ residue is replaced, is resistant to the initial hydrolysis step by DPP 3 (**Fig. 5, H**).

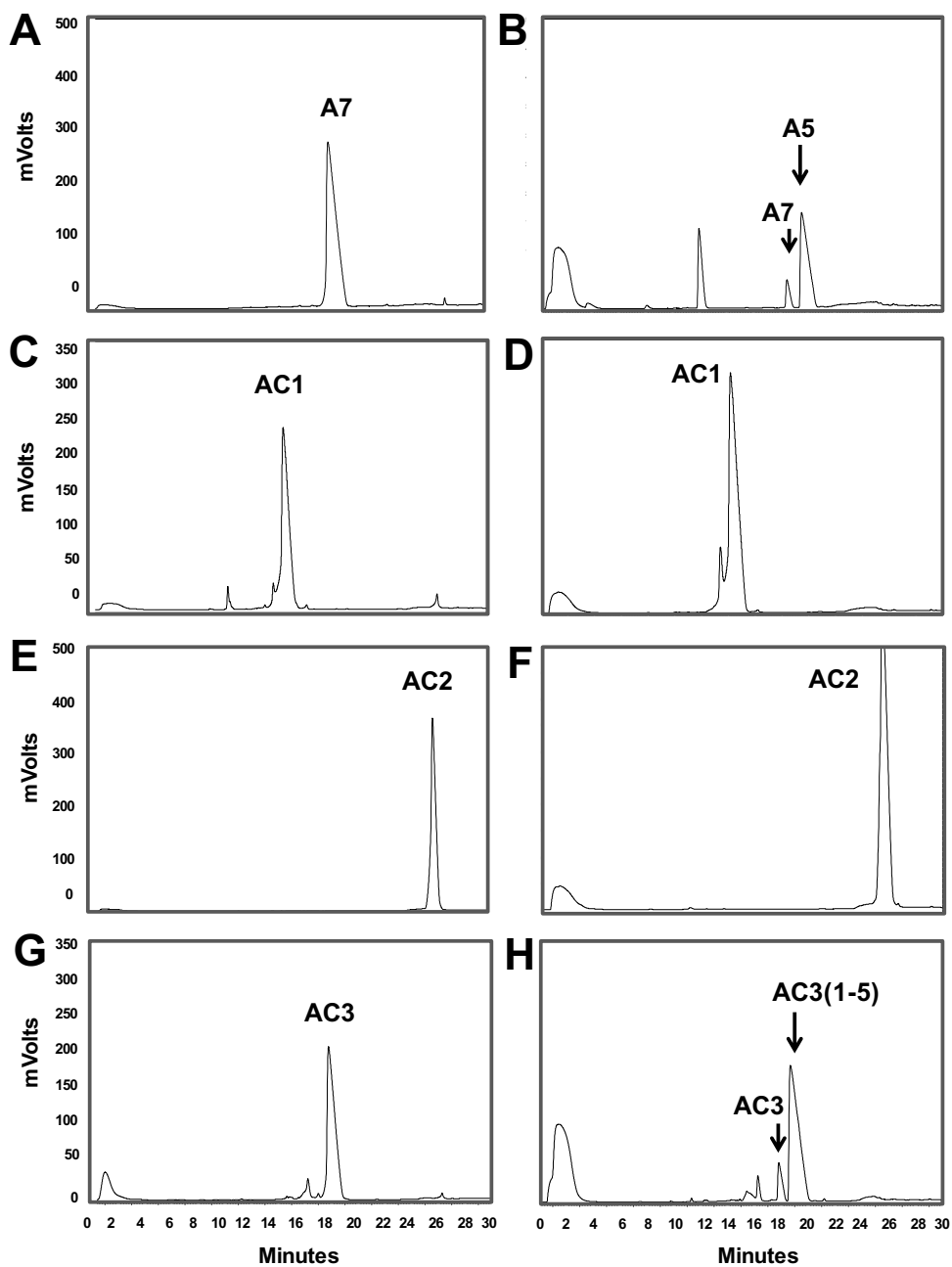


Fig. 4. HPLC-UV profile of the ACCA peptides and following exposure to ACE. A: Ang-(1-7) (A7) standard. **B:** ACE metabolizes Ang-(1-7) to Ang-(1-5) (A5). **C:** ACCA1 (AC1) standard. **D:** ACCA1 is not metabolized by ACE. **E:** ACCA2 (AC2) standard. **F:** ACCA2 is not metabolized by ACE. **G:** ACCA3 (AC3) standard. **H:** ACCA3 (AC3) is metabolized by ACE to ACCA3-(1-5) [AC3(1-5)].

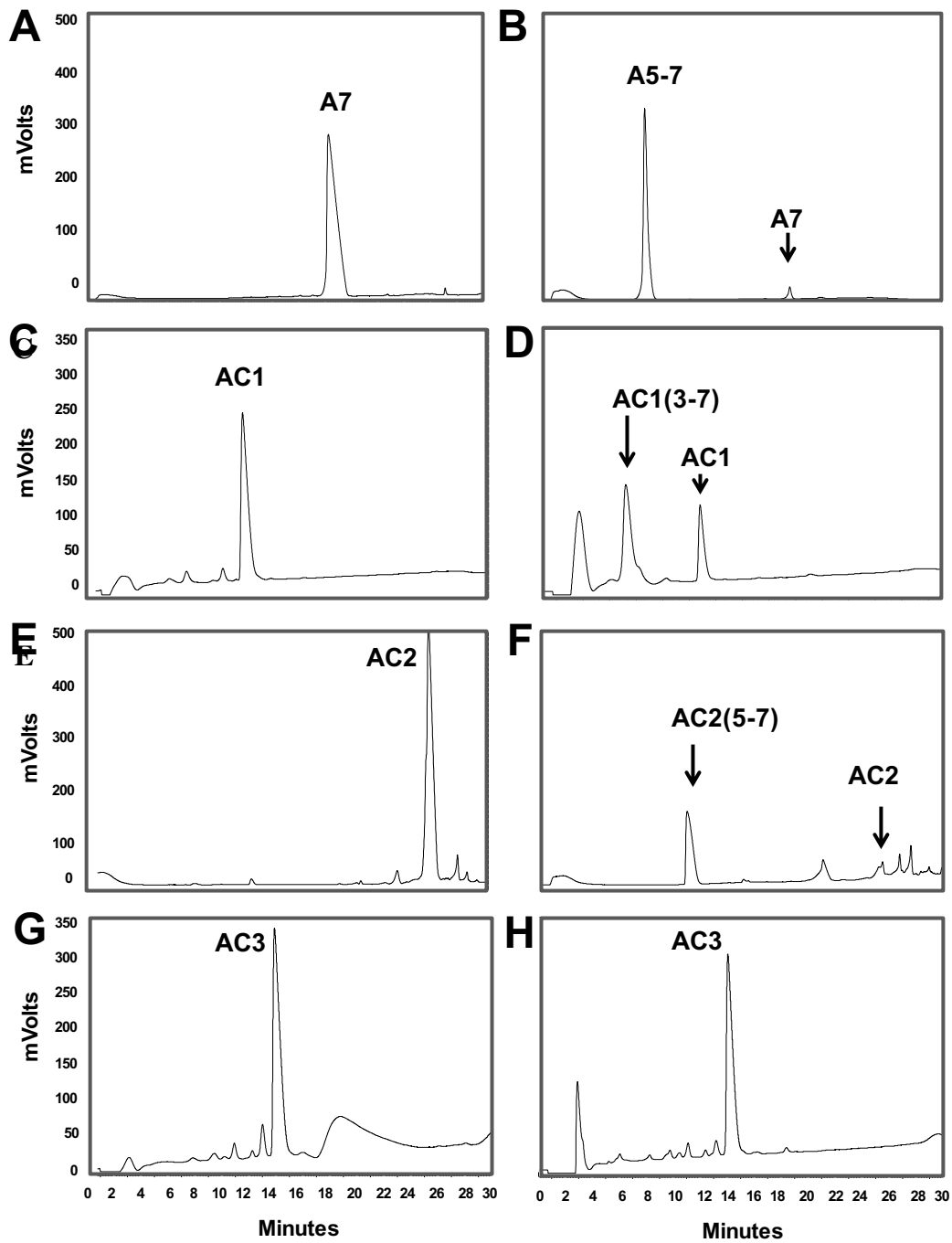


Fig. 5. HPLC-UV profile of the ACCA peptides and following exposure to DPP 3. **A:** Ang-(1-7) (A7) standard. **B:** DPP 3 metabolizes Ang-(1-7) to Ang-(3-7) and ultimately to Ang-(5-7) (A5-7). **C:** ACCA1 (AC1) standard. **D:** ACCA1 is metabolized by DPP 3 to likely ACCA1-(3-7) [AC1(3-7)], eluting at 6.2 minutes. **E:** ACCA2 (AC2) standard. **F:** ACCA2 is likely degraded

fully to ACCA2-(5-7) [AC2(5-7)]. **G:** ACCA3 (AC3) standard. **H:** ACCA3 is more stable to DPP 3 hydrolysis in comparison to Ang-(1-7) and the other analogues.

2.3 Cell Growth

Murine 4T1 breast cancer cells and human HT-1080 fibrosarcoma cells were incubated with one of the three ACCA analogues or Ang-(1-7), to preliminary assess whether the modified peptides inhibited cell proliferation similar to the parent compound Ang-(1-7). 4T1 cells closely mimic human triple negative breast cancer cells and HT-1080 cells are human sarcoma cells. Ang-(1-7) [100 nM] served as the control and was added daily, as it is rapidly degraded. ACCA1, ACCA2 or ACCA3 were added as a single aliquot on day 0, at either a concentration of 100 nM or 1 μ M, and the total number of cells per well was counted after 3 or 4 days. The Ang-(1-7)-ACCA analogues did not require further additions for the duration of the study. ACCA, and a range of ACCA dipeptides, did not show any toxicity on C6 glioma cell lines up to 200 μ M, significantly higher than the concentrations used in this study (Pes 2013). As shown in **Figure 6**, 100 nM Ang-(1-7) reduced proliferation of the mouse triple negative breast cancer cells by 34% compared to the untreated control cells. ACCA1 significantly reduced proliferation at the single treatment dose of 1 μ M, but a 10-fold lower dose was not effective. In contrast, ACCA2 and ACCA3 significantly reduced proliferation of the 4T1 cells at both single doses 100 nM and 1 μ M. The growth of HT-1080 sarcoma cells was reduced 44% by 100 nM Ang-(1-7) that was again added daily as compared to the control (**Fig. 7**). Although ACCA2 did not reduce the growth of HT-1080 cells, both ACCA1 and ACCA3 reduced proliferation at single doses of 1 μ M.

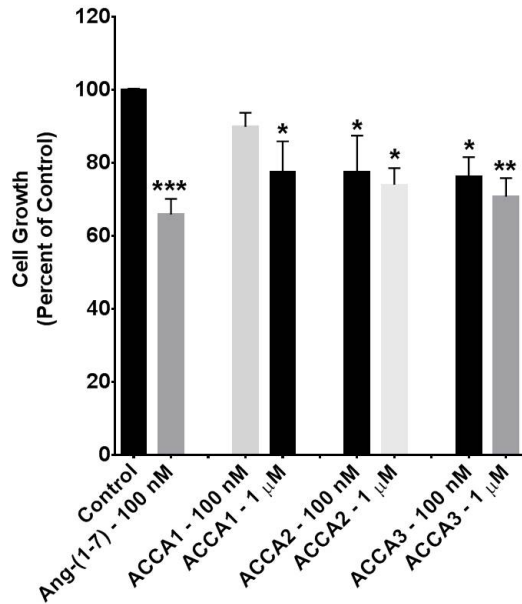


Fig. 6. Effect of Ang-(1-7) or each of the Ang-(1-7)-ACCA analogues on the proliferation of 4T1 murine triple negative breast cancer cells. Ang-(1-7) [100 nM] was added daily to rapidly growing cells while each of the ACCA analogues was added once to rapidly growth cells at Day 0. The total cell number per well was counted after 3 to 4 days. n = 4-5 in triplicate; * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$.

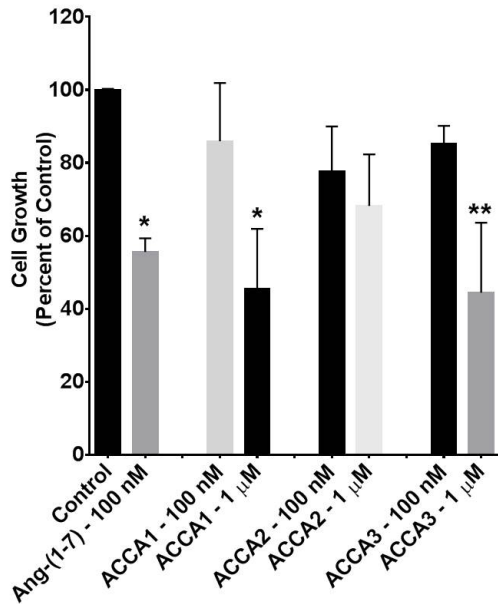


Fig. 7. Effect of Ang-(1-7) or each of the Ang-(1-7)-ACCA analogues on the proliferation of HT-1080 human sarcoma cells. Ang-(1-7) [100 nM] was added daily to rapidly growing cells while each of the ACCA analogues was added once to rapidly growth cells at Day 0. The total cell number per well was counted after 3 to 4 days. n = 4-5 in triplicate; * denotes $p < 0.05$ and ** denotes $p < 0.01$.

3. Conclusion

The present study is the first to explore key substitutions with a non-natural amino acid as a strategy for stabilizing Ang-(1-7) against degradation by both ACE and DPP 3. ACCA is a non-functionalized amino acid which offers structural rigidity without introducing additional reactivity. The advantage of this approach is the ease of synthesis of the desired targets that can be accomplished by standard solid-phase protocols, as well as the possibility that the introduction of additional structural modifications will further optimize the stability and activity of the peptide.

Our study of the three ACCA substitutions at various positions within Ang-(1-7) structure indicated differential sensitivities of the three analogues to hydrolysis by ACE and DPP 3. When ACCA is substituted for the two native amino acid residues at the Ile⁵-His⁶ site of ACE hydrolysis it confers to the peptide significant resistance to ACE enzymatic degradation, but only limited or no protection from DPP 3 hydrolysis which is consistent with the catalytic preference of the aminopeptidase. However, the opposite effect was observed when ACCA replaced Val³; the peptide was hydrolyzed by ACE to a similar extent as Ang-(1-7) but showed resistance to degradation by DPP 3. The ACCA substitution at Ile⁵ may allow for the initial hydrolysis to ACCA1-(3-7), but could potentially prevent the subsequent hydrolysis step to generate ACCA1-(5-7) by the aminopeptidase. Additional studies are necessary to fully elucidate the hydrolysis profile of the ACCA1 **1** analogue by DPP 3.

Preliminary tests on two cancer cell lines which respond to the anti-proliferative effects of Ang-(1-7) (4T1 and HT-1080) were also carried out on the synthesized analogues. All three Ang-(1-

7)-ACCA analogues significantly reduced cell growth of the 4T1 breast cancer cells by approximately 25% at the single dose of 1 μ M, that was comparable to the extent of inhibition with 100 nM Ang-(1-7) (repeated daily dosage) after 4 days. In contrast, ACCA1 **1** and ACCA3 **3** were significantly more active than ACCA2 **2** with HT-1080 cells (growth reduction by 40-50% and ~20%, respectively), also comparable to Ang-(1-7). Metabolism of Ang-(1-7) or the Ang-(1-7)-ACCA analogues in either cell line is not currently known and it is possible that ACCA2 is more susceptible to degradation by other peptidases expressed in the HT-1080 cells. While this study is preliminary, it offers significant opportunities to further explore these types of modification to the Ang-(1-7) peptide structure. Future studies will focus on expanding the cell lines tested, and degradation pathways for Ang-(1-7) and the Ang-(1-7)-ACCA analogues will be performed. Nevertheless, our results clearly demonstrate that the ACCA-substituted Ang-(1-7) analogues retain the anti-proliferative properties of the native peptide while exhibiting enhanced enzymatic stability. The successful synthesis of these peptidase-resistant and bioactive analogues suggests that ACCA-substituted Ang-(1-7) may reduce tumor growth as well as have the therapeutic potential for use in other cardiovascular pathologies.

4. Experimental section

Non-natural amino acid synthesis. Fmoc-OSu was purchased from Iris Biotech. Li⁺ pellets were purchased from Fluka. Ammonia was purchased from BOC gases. All other reagents and solvents were purchased from Sigma-Aldrich and used without further purification. Anhydrous CH₂Cl₂ and THF were obtained from a Puresolv-ENTM solvent purification system. When necessary, reactions were performed under inert atmosphere of nitrogen, using oven dried

glassware. Oxygen-free nitrogen was obtained from BOC gases and used without further drying. NMR spectra were recorded on Varian VNMRS 300, 400 and 500 MHz instruments at 25 °C. Tetramethylsilane was used as an internal standard. The coupling constants (J) are in hertz (Hz) and the chemical shifts (δ) are in parts per million (ppm). Signals were assigned with the aid of COSY, HSQC and HMBC spectra. High resolution mass spectra were recorded using electron spray ionization (ESI) on a Waters Micromass LCT (MeOH/H₂O, 60:40) TOF instrument with a cone voltage of 35 V and a capillary voltage of 3000 V and are reported in the form m/z (intensity relative to base = 100). Infrared spectra were recorded on a Bruker ALPHA Platinum ATR spectrometer and melting points were determined on a StuartTM SMP10 melting point apparatus. Crystal data were collected using a Rigaku Oxford Diffraction SuperNova A diffractometer fitted with an Atlas detector. Evaporation *in vacuo* refers to the removal of solvent on a Büchi rotary evaporator with an integrated vacuum pump. Compounds were purified by flash chromatography using Davisil[®] Chromatographic Silica Media (40 - 63 μ m) or ion-exchange chromatography using a DOWEX[®] 50WX8 resin (100 - 200 mesh, Sigma-Aldrich). Thin-layer chromatography (TLC) was carried out on aluminum sheets pre-coated with silica gel 60 F₂₅₄ (Merck) and compounds were visualized by UV or by treatment with potassium permanganate stain (0.75 g KMnO₄, 5 g K₂CO₃ and 0.625 mL 10% (w/v) NaOH in 100 mL H₂O) or ninhydrin stain (1.5 g ninhydrin and 3 mL AcOH in 100 mL *n*-butanol) followed by heating.

***N*-(9-Fluorenylmethoxycarbonyl)-*cis*-3-(aminomethyl) cyclobutane carboxylic acid (5).** ACCA **4** (101 mg, 0.78 mmol, 1.0 eq.) was dissolved in 10% (w/v) aq. Na₂CO₃ (2.1 mL, 1.95 mmol, 2.5 eq.), 1,4-dioxane (5 mL) was added and the reaction was cooled to 0°C. Fmoc-OSu

(395 mg, 1.17 mmol, 1.5 eq. in 1,4-dioxane (2.5 mL) was added and the mixture was stirred for 45 min at 0 °C and for 1 h at room temperature. The reaction mixture was then washed with Et₂O (30 mL). The aqueous layer was acidified with 1 M HCl and extracted with EtOAc (4 x 25 mL). The combined organic layers were washed with brine, dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude material was purified by silica gel column chromatography (cyclohexane/EtOAc, from 95:5 to 40:60) and crystallized from acetone and petroleum sprits (b.p. 40 - 60 °C) to give the product (260 mg, 95%) as a white solid: R_f = 0.36 (cyclohexane/EtOAc, 1:5); m.p. 134 -135 °C; ¹H NMR (500 MHz, (CD₃)₂CO) δ = 7.71 - 7.70 (m, 2H, Ar), 7.54 - 7.53 (m, 2H, Ar), 7.27 - 7.16 (m, 4H, Ar), 6.41 (s, 1H, NH), 4.18 (d, *J* = 7.2 Hz, 2H, C=OOCH₂), 4.08 - 4.05 (m, 1 H, C=OOCH₂CH), 3.05 - 3.02 (m, 2H, NCH₂), 2.91 - 2.83 (m, 1H, CHC=OOH), 2.38 - 2.29 (m, 1H, NCH₂CH), 2.15 - 2.09 (m, 2H, C=OCH(CHH)₂), 1.88 - 1.82 (m, 2H, C=OCH(CHH)₂); ¹³C NMR (126 MHz, CD₃OD) δ = 175.4, 156.4, 144.3 , 141.2 , 127.6 , 127.0 , 125.2 , 119.9 , 65.9, 47.2, 45.7 33.5, 31.2, 28.5; ν_{max} (neat) = 1709 cm⁻¹, 1690 cm⁻¹; HRMS (ES) *m/z* = 374.1377 [M+Na]⁺ C₂₁H₂₁NO₄Na requires 374.1368.

Peptide synthesis, purification and characterization. SPPS was carried out on a 0.1 mmol scale. Amino acids were purchased Fmoc protected (Iris Biotech) with Trt as side-chain protecting group for His, Pbf for Arg and *t*-Bu for Tyr and Asp. The polystyrene resin (1% DVB crosslinks, particle size: 100 - 100 mesh, loading: 0.52 mmol/g) was purchased with a 2-chlorotriyl (2CT) linker preloaded with the proline residue (Iris Biotech). HATU (Iris Biotech) was employed as the coupling reagent and DIPEA (Sigma-Aldrich) as the base. All other reagents and solvents were purchased from Sigma-Aldrich and used without further purification. Peptide synthesis was carried out using an automated peptide synthesizer (Applied Biosystems

433A) in the Royal College of Surgeons in Ireland (RCSI). Single coupling cycles (except for the amino acid coupling to the resin-bound proline, double coupling cycle) using a 10-fold excess of Fmoc protected amino acid to resin substitution were carried out employing HATU/DIPEA coupling chemistry in NMP (Thermo Fisher Scientific). Fmoc cleavage was monitored with a PerkinElmer Series 200 inline spectrophotometer and the UV absorbance was detected at 301 nm. Peptide cleavage from the resin and side-chain deprotection was achieved by gentle stirring in a mixture of 87.5% TFA, 5% H₂O, 5% thioanisole and 2.5% triisopropylsilane for 2.5 h at room temperature. The volume of the TFA and scavenger mixture was reduced by half under a stream of nitrogen and the peptide was precipitated by the addition of cold ether. Precipitation from the full volume of the cleavage cocktail had been attempted but was found not to be possible. The suspension was centrifuged for 5 min at 8000 rpm, the supernatant was decanted and the peptides were re-suspended in ether. This step was repeated three times. The peptides were then air dried, dissolved in double distilled water and lyophilized to give the crude material as white solids. Chromatographic purification was performed on a Shimadzu Prominence HPLC using an Aeris Peptide column (3.6 μm XB-C18 4.6 mm x 150 mm; Phenomenex, Torrance CA). 0.1% phosphoric acid (Sigma, Chicago IL) in Milli-Q water (Millipore Systems, Billerica MA) was employed as mobile phase A and 80% acetonitrile/0.1% phosphoric acid as mobile phase B with a linear gradient of 0-15% B in 20 min, 15% to 35% B in 10 min and 35% isocratic for 5 min at a flow rate of 1 mL/min at room temperature. The UV absorbance was detected at 220 nm with an inline spectrophotometer. The peptide peaks were collected and completely evaporated in a vacuum centrifuge. MALDI TOF MS spectra were acquired in Trinity College Dublin using a Waters MALDI Q-TOF Premier. The instrument was operated in positive or negative mode as required. Laser energy was provided by a N₂ laser operating at 337 nm. Samples were mixed

with a CHCA (α -cyano-4-hydroxycinnamic acid) matrix. 1 μ L of the matrix/sample solutions were loaded onto a MALDI plate and allowed to air dry. The instrument was calibrated using PEG. The internal lock mass used was [Glu] Fibrinopeptide B. MassLynx 4.1 software was used to carry out the analysis.

ACCA1 (**1**) H-Asp-Arg-Val-Tyr-ACCA-His-Pro-OH: white solid, yield = 64%, >99% purity, R_t = 14.5 min; MALDI-TOF MS (m/z) = 897.4580 [M+H]⁺ C₄₁H₆₁N₁₂O₁₁ requires 897.4583.

ACCA2 (**2**) H-Asp-Arg-Val-Tyr-Ile-ACCA-Pro-OH: white solid, yield = 46%, >99% purity, R_t = 25.8 min; MALDI-TOF MS (m/z) = 873.4827 [M+H]⁺ C₄₁H₆₅N₁₀O₁₁ requires 873.4834.

ACCA3 (**3**) H-Asp-Arg-ACCA-Tyr-Ile-His-Pro-OH: white solid, yield = 63%, 91% purity, R_t = 18.11 min; MALDI-TOF MS (m/z) = 911.4763 [M+H]⁺ C₄₂H₆₃N₁₂O₁₁ requires 911.4739.

Proteolytic stability. The stability of the three purified ACCA analogues was assessed against human ACE and DPP 3. The angiotensin standards Ang-(1-7) and Ang-(1-5) were obtained from Bachem (Torrance, CA). The purified analogues or Ang-(1-7) (100 nmol) were incubated with human ACE (1.0 μ g, R&D Systems, Minneapolis MN) or human DPP 3 (0.1 μ g, R&D Systems) for 60 minutes at 37 °C in 0.1 mL of the HEPES assay buffer (25 mM HEPES, 125 mM NaCl, 10 μ M ZnCl₂, pH 7.5) and the assays were terminated with 1.0% phosphoric acid. The purity of each compound and the extent of peptide metabolism by ACE or DPP 3 were assessed with the Shimaduz Prominence HPLC system equipped with a narrow-bore Aeris Peptide XB-C18 (2.0 \times

100 mm, Phenomenex) using identical gradient conditions described above and the UV profile was monitored at 220 nm.

Cell culture. Murine breast cancer cells (4T1; ATCC CRL-2539) were derived from a spontaneously-arising tumor in the mammary tissue of a BALB-c mouse. Human fibrosarcoma cells (HT-1080; ATCC CCL-121) were derived from a fibrosarcoma from a 35 year-old male. 4T1 cells were grown in RPMI media with 10% fetal bovine serum (FBS), 10 nmol/L HEPES, 100 µg/mL penicillin and 100 U/mL streptomycin while HT-1080 cells were grown in EMEM with 10% FBS, 10 nmol/L HEPES, 100 µg/mL penicillin and 100 U/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Cell growth. 4T1 cells or HT-1080 cells were seeded into 24-well cluster plates and allowed to grow overnight, in 10% FBS. After 24 h, the FBS was reduced to 1% and the rapidly proliferating cells were incubated with 100 nM Ang-(1-7) as previously described (Cook 2010; Gallagher 2004; Krishnan 2013b), added daily, or either 100 nM or 1 µM ACCA1, ACCA2 or ACCA3 for 3 or 4 days. The total number of cells per well was counted using a Nexelcom Cellometer and compared to the control.

Statistical analysis. The data was analyzed using GraphPad Prism 5 software and results are displayed as the mean ± standard error of the mean (SEM) of at least three independent experiments (n = 3) measured in triplicate, unless otherwise stated. Statistical analysis was by one-way ANOVA followed by Dunnett's multiple comparison test. Values of p < 0.05 were considered significant; * denotes p < 0.05, ** denotes p < 0.01 and *** denotes p < 0.001.

Compliance with Ethical Standards

The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

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Abbreviations

ACCA, *cis*-3-(aminomethyl)cyclobutanecarboxylic acid; ACE, Angiotensin-converting enzyme; HATU, *N*-[(Dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; HRMS, High-resolution mass spectrometry; 2CT resin, 2-Chlorotriyl resin; NMP, *N*-methylpyrrolidone; DIPEA, *N,N*-Diisopropylethylamine; DPP 3, Dipeptidyl peptidase 3; Fmoc, 9-fluorenylmethoxycarbonyl; Fmoc-OSu, *N*-(9-Fluorenylmethoxycarbonyloxy)succinimide; Pbf,

2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl; *t*-Bu, *t*-butyl; Trt, trityl; TFA, trifluoroacetic acid, SPPS, solid-phase peptide synthesis

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