Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Synthesis of Antioxidant Peptide SCAP1 (Leu-Ala-Asn-Ala-Lys)

I. R. Sabana[1], M. Naufal,[1] I. Wiani[1], A. Zainuddin[1], A. T. Hidayat[1, 2], D. Harneti[1], Nurlelasari[1], J. Al-Anshori[1], U. Supratman[1,2], R. Maharani[1, 2*]

[1]*Department of Chemistry Universitas Padjadjaran, Jl. Raya Bandung-Sumedang Km 21 Jatinangor 45363, Sumedang, West Java, Indonesia.*
[2]*Central Laboratory Universitas Padjadjaran, Jl. Raya BandungSumedang Km 21 Jatinangor 45363, Sumedang, West Java, Indonesia.*

SCAP1 is a pentapeptide isolated from the hydrolysate of oyster (*Saccostrea cucullata*), which has antioxidant and anticancer properties. This study aims to find a novel strategy to obtain antioxidant peptide SCAP1 (Leu-Ala-Asn-Ala-Lys) through chemical synthesis. This study reports the first synthesis of SCAP1 through the solid phase peptide synthesis (SPPS) method with Fmoc strategy on 2-chlorotrityl chloride resin. A HATU-HOAt coupling reagent was employed in all amide bond formations and 95% TFA in water was employed to release the desired peptide from the solid support. The crude peptide was purified using successive preparative-RP-MPLC and RP-HPLC to obtain the pure peptide. The synthesized peptide was confirmed by mass spectrometry, showing a correct molecular ion peak at m/z 516.3143 (M+H)+, referring to the desired SCAP1. 1H-NMR spectra of the peptide revealed protons signals consistent to the structure of SCAP1. During the synthesis, the coupling between Ala and Asn(Trt) was not straightforward, which led to a low yield of the final product. The use of Trt-based Asn was not a suitable option, as it could cause aggregation during the synthesis, eventually leading to a poor yield of the final product. SCAP1 was obtained as a white solid with a percent yield of 8.28%.

Keywords: Antioxidant peptide, pentapeptide, SCAP1, Solid-phase peptide synthesis.

Introduction

Bioactive peptides are peptides with hormones- or drugs- like activities that modulate physiological functions through binding interactions to specific receptors on target cells [1]. This group of compounds is worth further exploration due to their future possibility as new drugs candidate. Moreover, the resistance of commercially available drugs that leads to diseases becoming tolerant to pharmaceutical treatment, has made even it more attractive [2].

Bioactive peptides can easily be found in animals and plants either naturally or buried in their proteins [3,4]. Glutathione (γ-Glu-Cys-Gly), carnosine (β-alanyl-L-histidine), anserine (β-alanyl-L-1-methylhistidine), and ophidine (β -alanyl- L -3-methylhistidine) are bioactive peptides found in muscle tissue [4]. Antioxidant dipeptides, Tyr-Leu and Phe-Tyr, were obtained from hydrolysates of

seed protein [5], and antioxidant Pro-Ala-Gly-Tyr was obtained from hydrolysates of amur sturgeon skin gelatin [6]. Recently, SCAP1(Leu-Ala-Asn-Ala-Lys) was isolated from hydrolysate of oyster (Saccostrea cucullata) protein [7], which shows a good antioxidant activity with the percentage of DPPH radical binding activity of 83.79±0.53% and triggers the HT-29 colon cancer cell death. Structurally, SCAP1 consists of one leucine, two alanines, one asparagine, and one lysine, where all of the residues are L-configured. Currently, there is no report on the exploration of the SCAP1, including on the chemical access into the peptide.

A chemical synthesis is one of method that can access the peptide, including solid-phase peptide synthesis (SPPS) method. Reports regarding the success of SPPS in the preparation of the peptides have been described in many papers. Bradykinin, a linear nonapeptide, was successfully

synthesized in the early years of the discovery of the SPPS method [8]. Until recently, the synthesis of bradykinin analogues was still carried out using the SPPS method [9, 10]. Two peptides, SAGNPN and GLAGA, were successfully synthesized by Escudero et al. in 2013 [11]. Other peptides, RPNYTDA, TSQLLSDQ, TRTGDPFF and NFHPQ reported by Yan et al., were also successfully prepared by SPPS in 2015 [12]. The latest research was reported by Maharani et al., in 2018, synthesizing antioxidant tetrapeptide PAGY and its analogues through Fmoc/t-Bu SPPS strategy with excellent yields [13]. With the same method used in Maharani et al. (2018), SCAP1 would be synthesized and discussed here. This report is the first synthesis of SCAP1 with solid-phase peptide synthesis (SPPS) method.

Experimental

Chemicals and instrumentation

The chemical used in this research were 2-chlorotrityl chloride resin, dichloromethane, dimethylformamide (DMF), N,N-diisopropyletilamine (DIPEA), Fmoc-Ala-OH, Fmoc-Asn(trt)-OH, Fmoc-Lys(boc)-OH, Fmoc-Leu-OH, 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU), 1-hydroxy-7-azabenzotriazole (HOAt), trifluoroacetic acid (TFA), acetaldehyde, p-chloranyl, methanol, and piperidine. All materials used are proanalyst. Amino acids and resin were purchased from GL-Biochem, Shanghai, China.

The instrumentations used for synthesis were SPPS tubes, freeze dryer, rotary evaporator, shaking rotator, reverse phase medium pressure liquid chromatography (RP-MPLC) (BuchiC-620 Sepacore C18 4g column), analytical reverse-phase high performance liquid chromatography (RP-HPLC) (Waters 2998 Photodiode Array Detector, LiChrospher 100, column C-18 5 mm), preparative reverse-phase high performance liquid chromatography (RP-HPLC preparative) (Agilent SD1, Photo Diode Array (PDA) detector, C-18 column 250 x 21.2 mm), mass spectrometer (Waters HR-TOF-MS Lockspray), UV-Vis spectrophotometer (TECAN Infinite pro 200), and nuclear magnetic resonance (NMR) spectroscopy (JEOL JNM-ECZ600R 600 MHz).

Research methodology

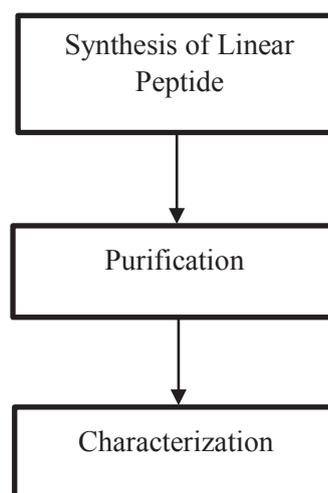To achieve the target of synthesis, a research methodology in Fig. 1 was applied.

Fig. 1. Research methodology flowchart.

Procedures

C-terminal amino acid binding to resin 2-chlorotrityl chloride resin (0.45 mmol, 0.4 g) was added with 10 mL dichloromethane, and then the mixture was shaken for 15 minutes. After a simple filtration, a cocktail of first amino acid, Fmoc-Leu-OH, (0,6 mmol, 212,046 mg) in dichloromethane (10 mL) and DIPEA (2.3 mmol, 385 µL) was added and shaken for 24 h at room temperature. Resin was then filtered and washed with a solvent.

To determine the capacity of loading resin, 1 mg of resin was added with 0.3 mL piperidine in DMF 25% and set aside for 1 hour. After 1 h, 2700 µL piperidine in DMF 25% was added and the absorbance was measured using UV spectrometer in 290 nm wavelength.

A mixture of methanol: dichloromethane: DIPEA (15:80:5) was added to the resin and shaken for 10 minutes. After that, the resin was filtered and washed with solvent. This procedure was performed twice.

In the next step, Fmoc deprotection was carried out. A 4 mL solution of 20% piperidine in DMF was added to the resin-peptidyl-Fmoc and the mixture was shaken for 30 minutes. Resin was then filtered and washed with solvent.

Amino acid coupling

A cocktail of amino acid and coupling reagent [Fmoc-AA2-OH (2 eq.), HATU (2 eq.), HOAt (2 eq.), DIPEA (8 eq.) in 4 mL dichloromethane: DMF (1:1)] was added to resin in SPPS tube. A mixture was then shaken for 24 h. Resin was

filtered and washed with washing solvents. Reaction was monitored with chloranil test. After that, Fmoc deprotection was carried out using 20% piperidine and monitored by chloranil test. A series of amino acid coupling and Fmoc deprotection was performed until pentapeptide was constructed on the resin.

Resin cleavage

Trifluoroacetic acid in water (95%, 10 mL) was added to an SPPS tube, and shaken for 10 minutes. Filtrate was collected, and this reaction was performed twice. The combined filtrate was then evaporated using a rotary evaporator, and crude was characterized using a mass spectrometer and analytical RP-HPLC. Purification of this compound was undertaken with successive preparative RP-MPLC and preparative RP-HPLC. The purified fraction was then characterized by the mass spectrometer and 1H-NMR.

Chloranyl test

A small amount of resin was placed in an eppendorf tube, and 2-5 drops of 2% acetaldehyde in DMF solution were added. After that, 2-5 drop of second solution, 2% p-chloranil in DMF, was added and shaken. After 5 minutes, a colour change in the resin was observed.

(2S,5S,8S,11S,14S)-14-amino-8-(2-amino-2-oxoethyl)-2-(4-aminobutyl)-5,11,16-trimethyl-4,7,10,13-tetraoxo-3,6,9,12-tetraazaheptadecan-1-oic acid (7)

Yield 9 mg (8.28%), white solid. Mass spectrum: m/z 516.3143 [M + H]+. C22H41N7O7.

Calculated: 515.3146. 1H NMR in CD3OD (600 MHz): See Table 1.

Results and Discussion

The synthesis of SCAP1 was undertaken on 2-chlorotrytil chloride resin with Fmoc-protecting group strategy (Scheme 1).

Scheme 1. Synthetic route of SCAP1. (a) (1) Fmoc-Lys(Boc)-OH, DIPEA, dichloromethane, 24 h,rt, (2) methanol: dichloromethane: DIPEA (15:80:5), 10 min, rt; (b) 20% piperidine in DMF; (c) Fmoc-Ala-OH, HOAt, HATU, DIPEA, dichloromethane:DMF (1:1), 4 h, rt; (d) Fmoc-Asn(Trt)-OH, HOAt, HATU, DIPEA, dichloromethane:DMF (1:1), 4 h, rt; (e) Fmoc-Leu-OH, HOAt, HATU, DIPEA, dichloromethane:DMF (1:1), 4 h, rt; (f) Trifluoroacetic acid 95% in water.

Initially, the 2-chlorotrytil chloride resin was swollen in dichloromethane to prepare it for the attachment of the first amino acid, Fmoc-Lys(Trt)-OH, the condition of basic DIPEA in dichloromethane, carried out for 24 h. Loading resin value was then calculated to obtain 0.52 mmol/g resin, which was categorized to be suitable, since previous studies mentioned that a suitable loading resin value should not give more than 0.6 mmol/g resin [14]. The next step involved resin capping, undertaken through the addition of methanol on the resin to close the active sites of the resin that do not form an ester bond with the first amino acid [15].
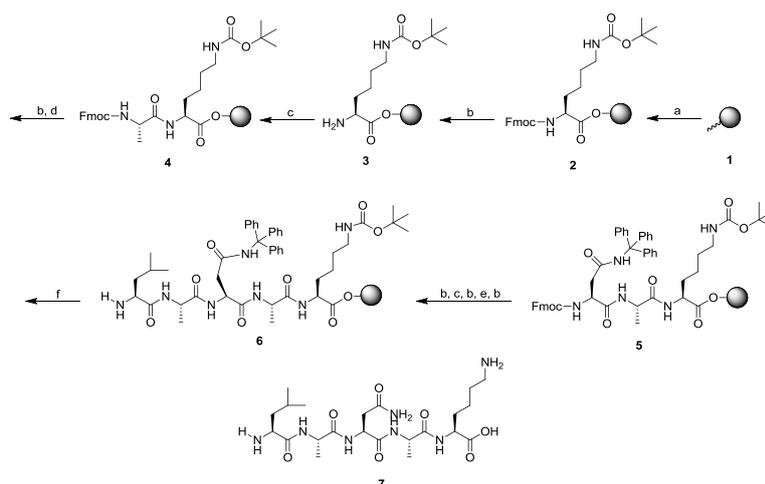
Scheme 1. Synthetic route of SCAP1. (a) (1) Fmoc-Lys(Boc)-OH, DIPEA, dichloromethane, 24 h,rt, (2) methanol: dichloromethane: DIPEA (15:80:5), 10 min, rt; (b) 20% piperidine in DMF; (c) Fmoc-Ala-OH, HOAt, HATU, DIPEA, dichloromethane:DMF (1:1), 4 h, rt; (d) Fmoc-Asn(Trt)-OH, HOAt, HATU, DIPEA, dichloromethane:DMF (1:1), 4 h, rt; (e) Fmoc-Leu-OH, HOAt, HATU, DIPEA, dichloromethane:DMF (1:1), 4 h, rt; (f) Trifluoroacetic acid 95% in water.

To provide the Nα active site of amino acid to be coupled with the second amino acid, deprotection of the Nα amino acid protecting group using a 20% basic piperidine in DMF was conducted for 30 minutes, to obtain 3. The success of this stage was analyzed through a qualitative chloranil test on a few beads of the resin. The positive results of the test show a dark green resin, indicating that the Fmoc group has been released from the amino acid on the resin.

The next step was to couple the second amino acid, Fmoc-Ala-OH, onto resin-Lys(Trt)-NH2. A coupling solution of HATU, HOAt, DIPEA in DMF: dichloromethane (1: 1) was added to the SPPS tube and the mixture was shaken for 24 hours. An excess amount of the second amino acid was used to multiply collisions between the amino acids. After 24 hours, the resin was filtered, washed, dried, and a few beads of the dried resin were taken for the chloranil test, which showed that some beads gave green color, describing that there remains some of the first amino acid that did not bound completely to the second amino acid. Consequently, re-coupling was required using the same coupling solution within 24 hours. The chloranil test was re-applied, and eventually, a positive result was obtained when all of the resins turned yellow. From that, it could be assumed that all the first amino acid on the resin was bound to the second amino acid, giving dipeptidyl resin 4.

After the dipeptide was successfully attached on the resin, the next step was a Fmoc deprotection to give resin-Lys(Trt)-Ala-NH2 that was ready to be coupled with the third amino acid. A coupling with the third amino acid, Fmoc-Asn(Trt)-OH took advantage of the same HATU/HOAt reagent. Unfortunately, a triple-coupling protocol was required until the tripeptidyl resin was obtained.

A series of a Fmoc deprotection and a coupling of the following residues were repeated until the desired peptidyl resin 6 were gained. During the subsequent coupling reaction, the attachment of the fourth amino acid (Fmoc-Ala-OH) required a two-times coupling until the tetrapeptide was completely formed on the resin. It seems likely that couplings between Fmoc-Ala-OH and Fmoc-Asn (Trt)-OH are not straightforward, since the side chain of Asn is known to aggregate due to its capability to form a hydrogen bond with its own peptide backbone [16]. Unfortunately, a combination of Asn with Trt protecting group was found to effectively promote the aggregation than the free Asn itself. In addition to that, it was

found that a high proportion of Ala and Asn in the peptide is vulnerable to aggregation [7].

After the pentapeptidyl resin 6 was gained, the pentapeptide was released from resin using 95% TFA in water. The high concentration of TFA was employed because the solution not only was used to release the pentapeptide, but also all the protective groups of the side chains of Asn and Lys. Water was added in the cleavage cocktail as a scavenger for carbocations released from the protective groups. The reaction was marked by a resin color turning blackish. From this synthesis, we obtained 364.3 mg of yellowish crude.

To determine whether the peptide was successfully synthesized, an analysis of the crude using mass spectrometer was carried out. The presence of molecular ion peak [M+H]+ 516.3143 corresponded to the calculated m/z 515.3146 of SCAP1, showing that SCAP1 has been successfully synthesized. Furthermore, an analytical RP-HPLC was performed to determine the purity of the crude. The chromatogram showed two peaks at the retention times of 1.34 and 25.77 minutes, which showed that the pentapeptide was impure, necessitating further purification, using semi preparative RP-MPLC (10–90% acetonitrile in water over 60 min, flow rate 3 mL/min, monitored at 210 nm). The two peaks had been successfully separated into two fractions, Fractions 1 and 2. HR-TOF-ESMS analysis showed that the desired peptide was contained in Fraction 1. Further, to check the purity of this fraction, analytical RP-HPLC (10–90% acetonitrile in 1% TFA in water over 30 min, flow rate 1 mL/min, wavelength at 210 nm) was re-applied. Unfortunately, the chromatogram of this fraction showed overlapping peaks, indicating that the pentapeptide was still impure. Hence, re-purification was undertaken using preparative RP-HPLC (methanol:water 1.2:8.8, 35 minute, 6.25 mL/min). Fractions from the second purification were confirmed by analytical RP-HPLC (10–90% acetonitrile in 1% TFA in water over 30 min, flow rate 1 mL/min, wavelength at 210 nm) and there was a fraction with only one peak at 2.80 minutes, indicating that the pentapeptide was pure.

To ensure that the peak was the expected pentapeptide peak, characterization using mass spectrometer was carried out. The results showed the presence of a molecular ion peak [M + H]+ at m/z 516.3143, according to the calculated m/z pentapeptide 515.3146.

Further, a characterization using 1H-NMR analysis was carried out. The spectrum disclosed chemical shifts of the expected protons of SCAP1, indicating that the NMR data was consistent with the structure of SCAP1 (Table 1). NMR data of SCAP1 were compared with NMR data obtained from peptides, which have similar sequence and residues, reported by Pusterla & Bode in 2015 [18], Elipe et al. in 2001 [19], and Marcelo et al. in 2012 [20]. Structural assignment of SCAP1 using 1H-NMR showed proton signals of peptide side chain that were distributed between δH 0.77 and 2.82 ppm. The methylene signals of Lys were found at δH 1.22 – 1.27 ppm (2H, m), 1.45 – 1.53 ppm (2H, m), 1.53-1.72 ppm (2H, m), and 2.78 – 2.82 ppm (2H, m). The methyl proton signals of two Ala were found at δH 1.20 ppm (6H) as a triplet signal, explained as two overlapping doublet signals. The methylene signals of two Asn were shown at δH 2.56-2.64 ppm (2H, m). For the side chain of Leu, two methyl signals were found at δH 0.77 ppm (6H) as two overlapping doublets, while methine and methylene signals were seen at δH 1.53 – 1.60 (1H, m) and 1.45 – 1.53 ppm (2H, m), overlapping with proton signals of Lys side chain. Proton α for Leu was revealed at δH 3.82 ppm (1H, t, J = 7.2 Hz), while protons α for one Lys, two Ala and one Asn were disclosed at δH 4.05 – 4.09 (1H, m), 4.10-4.15 (1H, m), 4.15-4.20 (1H, m) and 4.42-4.45 ppm (1H, m), respectively.

TABLE 1. ^{1}H-NMR spectral data of SCAP1 in CD$_3$OD.

Residue	δ$_H$ (ppm) ($\sum$H, m, J)
Leu	
Hδ	0.77 (6H, dd, J = 10.3, 6.2 Hz)
Hβ	1.53-1.60 (2H, m)
Hγ	1.45-1.53 (2H, m)
Hα	3.82 (1H, t, J = 7.2 Hz)
Ala	
Hβ	1.20 (3H, t, J = 6 Hz)
Hα	4.10-4.15 (1H, m)
Asn	
Hβ	2.64 (dd, J = 12.0, 5.4 Hz, 1H),
	2.56 (dd, J = 12.0, 6.0 Hz, 1H)
Hα	4.42-4.45 (1H, dd, J = 3.6, 6.9 Hz)
Ala	
Hβ	1.20 (3H, t, J = 6 Hz)
Hα	4.15-4.20 (1H, m)

SCAP1 was successfully synthesized as a white solid (9 mg) with a percent yield of 8.28%. The small number of peptides that had been synthesized can be attributed to several factors, including the difficult coupling between Asn and Ala, the potential aggregation during the synthesis step and the loss of peptide during the purification step.

Conclusion

In this study, SCAP1 was successfully synthesized using solid-phase peptide synthesis with Fmoc strategy, resulting in the final peptide with a 8.28% yield. A HATU-HOAt coupling reagent was employed in all amide bond formations, while basic piperidine in DMF was employed to remove the Fmoc group to give free amino group during the synthesis; and 95% TFA in water was employed to release the desired peptide from the solid support. A subsequent preparative-RP-MPLC and RP-HPLC on the crude peptide was applied to get the purified peptide. Both purifications were run on C18 column with eluent of acetonitrile:water. The purifications were monitored under wavelength of 210 nm. The synthesized peptide was characterized by high resolution mass spectroscopy (HR-TOF-ESMS) and 1H-NMR to confirm that SCAP1 has been obtained. The low yield of the synthesized peptide was due to the difficult coupling between Asn and Ala, necessitating 2-3 times coupling protocol. In addition to this, the low yield of the product was caused by a potential aggregation during the synthesis, primarily due the Trt-protected Asn employed in the synthesis, as well as the presence of Asn and Ala in sequence of the peptide. Moreover, a double-step purification could also contribute to the loss of the peptide.

Acknowledgement

References

1. Fitzgerald, R. J., Murray, B. A. Bioactive peptides and lactic fermentations. *Society of Dairy Technology*, **59**(2), 118-125 (2006).

2. Housman, G., Byler, S., Heerboth, S., Lapinska, K., Longacre, M., Snyder, N., Sarkar, S. Drug resistance in cancer: an overview. *Cancers*, **6**, 1769-1792 (2014).

3. Castro, R. J. S. D., Sato, H. H. Biologically active peptides: processes for their generation,

purification and identification and applications as natural additives in the food and pharmaceutical industries. *Food Research International*, **74**, 185-198 (2015).

4. Samaranayaka, A. G. P., Chan, E. C. Y. L. Food-derived peptidic antioxidants: a review of their production, assessment, and potential applications. *Journal of Functional Foods*, **3**, 229-254 (2011).

5. Yang, J., Hu, L., Cai, T., Chen, Q., Ma, Q., Yang, J., Meng, C., Hong, J. Purification and identification of two novel antioxidant peptides from perilla (*Perilla frutescens* L. Britton) seed protein hydrolysates. *Public Library of Science One,* 1-15 (2018).

6. Nikoo, M., Benjakul, S., Ehsani, A., Li, J., Wu, F., Yang, N., Xu, Baocai, X., Jin, Z., Xu, X. Antioxidant and cryoprotective effects of a tetrapeptide isolated from Amur sturgeon skin gelatin. *Journal of Functional Foods*, **7**, 609-620 (2014).

7. Umayaparvathi, S., Meenakshi, S., Vimalraj, V., Arumugam, M., Civagami, G., Balasubramanian, T. Antioxidant activity and anticancer effect of bioactive peptide from enzymatic hydrolysate of oyster (*Saccostrea cucullata*). *Biomedicine & Preventive Nutrition*, **4**, 343-353 (2014).

8. Merrifield, R. B. The synthesis of bovine insulin by the solid phase method1. *J. Am. Chem. Soc.* **86**(2), 304-305 (1964).

9. Zewail, M. A., Khalil, E. M., Mohamed, S. M., Sharada, H. M. Synthesis of some analogs of bradykinin hormone using modified solid phase peptide synthesis and microwave technique (Part 1). *Egypt. J. Chem.* **53**(2), 267-277 (2010).

10. Zewail, M. A., Naglah, A. M., Osman, S. M. Solid phase peptide synthesis of some analogs of bradykinin hormone using microwave energy application (part II). *Egypt. J. Chem.* **56**(2), 141-153 (2013).

11. Escudero, E., Mora, L., Fraser, P. D., Aristoy, M. C. Toldra, F. Identification of novel antioxidant peptides generated in Spanish dry-cured ham. *Food Chemistry*, **138**, 1282-1288 (2013).

12. Yan, Q. J., Huang, L. H., Sun, Q., Jiang, Z. Q., Wu, X. Isolation, identification and synthesis of four novel antioxidant peptides from rice residue protein hydrolyzed by multiple proteases. *Food Chemistry*, **179**, 290-295 (2015).

13. Maharani, R., Sumiarsa, D., Zainuddin, A., Ammatillah, N., Hidayat, A. T., Harneti, D., Nurlelasari, Supratman, U. Synthesis of tetrapeptides and screening of their antioxidant properties. *Current Bioactive Compounds*, **14**, 1-6 (2018).

14. Barlos, K., Chatzi, O., Gatos, D., Stavropoulos, G. 2-Chlorotrityl chloride resin. *Int. J. Peptide Protein Res.* **37**, 513-520 (1991).

15. Chan, W. C., White, P. D. "*Fmoc Solid Phase Peptide Synthesis*", Oxford University Press, New York, (2000).

16. Hyde, C., Johnson, T., Owen, D., Quibell, M., Sheppard, R. C. Some difficult sequences made easy: A study of interchain association in solid-phase peptide synthesis. *International Journal Of Peptide & Protein Research,* 431-440 (1994).

17. Rahman, S. A., El-Kafrawy, A., Hattaba, A., Anwer, M. F. Optimization of solid-phase synthesis of difficult peptide sequences via comparison between different improved approaches. *Amino Acids*, **33**, 531-536 (2007).

18. Pusterla, I., Bode, J. W. An oxazetidine amino acid for chemical protein synthesis by rapid, serine-forming ligations. *Nature Chemistry,* **7**, 668-672 (2015).

19. Elipe, M. V. S., Bednarek, M. A., Gao, Y. D. ^{1}H NMR structural analysis of human ghrelin and its six truncated analogs. *Biopolymers,* **59**, 489-501, (2001).

20. Marcelo, F., Cañada, F. J., Barbero, J. J. α-N-Linked glycopeptides: conformational analysis and bioactivity as lectin ligands. *Org. Biomol. Chem.* **10**, 5916-5923 (2012).