Synthesis Of Three Analogues Of Trypsin-Modulating Oostatic Factor (TMOF) And Screening Of Their Insecticidal Properties Towards Cabbage Cluster Caterpillar

Rani Maharani, Ari Hardianto, Safri Ishmayana, Eka Fitri Yanti, Devia Irma Melati, Daniel Sihotang, Lindung Tri Puspasari, Danar Dono

Abstract: Three analogues of TMOF, [PP (H-Pro-Pro-OH), PPPPPP (H-Pro-Pro-Pro-Pro-Pro-Pro-OH), and APPPPPP (H-Pro-Pro-Pro-Pro-Pro-Pro-OH)], were successfully synthesised and screened for their insecticidal properties against cabbage cluster caterpillar (Crocidolomia pavonana). All compounds were prepared through Fmoc-based solid-phase peptide synthesis (SPPS) method. The synthesis employed chlorotrityl resin as solid support. A combination of N,N-diisopropylcarbodiimide (DIC) and ethyl 2-cyano-2-(hydroxyimino)acetate (oxyma) was chosen as coupling reagent in the synthesis. The peptide was cleaved by using TFA cocktail. All peptide crudes were purified by using reversed-phase flash chromatography with octadecyl silane (ODS) as stationary phase. All purified peptides were characterized by using TOF-ESMS and their purity was analysed using thin layer chromatography and analytical RP-HPLC. The synthesised TMOF analogues, together with TMOF, were biologically tested towards the (C. pavonana) at 1000 ppm. The results indicated that TMOF and the analogues were less effective for the cabbage cluster caterpillar, compared to previously reported activity of TMOF towards Aedes aegypty.

Index Terms: Trypsin-modulating oostatic factor, TMOF, Aedes aegypty, Crocidolomia pavonana, solid-phase peptide synthesis, 2-chlorotrityl chloride resin, DIC/Oxyma.

1 INTRODUCTION

Studies on trypsin-modulating oostatic factor (TMOF) (Fig. 1), a decapeptide (YDPAPPPPPP) isolated from ovaries of the female Aedes aegypty, have been reported by some researchers and has become our research interest [1, 2]. TMOF with its insecticidal activity towards the mosquito larvae was found to inhibit the trypsin- or chemotrypsin-like enzyme biosynthesis in the mosquito [3]. Borovsky stated that TMOF is potential as a future biorationale larviside [2]. TMOF is considered to be environmentally friendly and effective. It is also deemed that TMOF can replace current resistance insecticides. Some analogues of TMOF have been synthesised and screened for their insecticidal properties against A. aegypty [2]. The result showed that some analogues have a better activities against A. aegypty compared to the TMOF.

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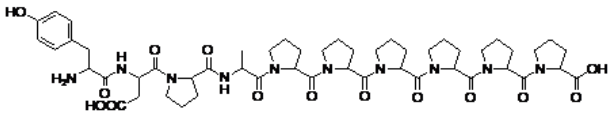

Fig. 1. *Structure of TMOF (YDPAPPPPPP).*

In our current studies we are interested to explore insecticidal activity of TMOF and its analogues against another insect, C. pavonana. The lepidopteran C. pavonana is known as cabbage cluster caterpillar in agriculture field. It is becoming enemies for farmers including farmers in Indonesia. Currently, we are interested in studying TMOF and analogues for their biological properties against C. pavonana. Since some Lepidoptera such as Heliothis virescens and Plutella xylostella have been found to use trypsin to digest food,[3] the study is aimed to test if TMOF and analogues could also control trypsin biosynthesis in C. pavonana. The chemical synthesis of TMOF has been reported by our research group[4] and in the present paper, we are going to describe the synthesis of the analogues and also to report the result of biological assays of the synthesisied products in a comparison with the insecticidal activity of TMOF against C. pavonana. Solid-phase peptide synthesis method was chosen over solution-phase peptide synthesis. TMOF analogues were synthesised by using the same strategy of the synthesis of TMOF [4]. They were prepared on chlorotrityl resin. This selection was based on the fact that the resin does not have a tendency towards diketopiperazine formation particularly during the attachment of the first two residues on resin. This is particularly the case when proline is present as the C terminus of the peptide. Chlorotrityl resin has also been shown to be able to avoid extensive racemization during the attachment of the first amino acid particularly in Fmoc-based peptide synthesis [5]. The synthesis was based on Fmoc strategy and employed DIC/oxyma as coupling reagent. DIC (N,N′-diisopropylcarbodiimide) is one of carbodiimide reagents

designed to prevent the formation of N-acylurea [6]. The use of DIC is preferred to that of dicyclohexylcarbodiimide (DCC) because the urea generated by the former is soluble in DMF [7]. The reagent is mostly employed with a combination with some additives. Oxyma (ethyl 2-cyano-2-(hydroxy-imino)acetate) as the coupling addive is chosen since it shows superiority over famous additive HOBt (N-hydroxybenzotriazole) in avoiding racemisation. Oxyma can also give coupling efficiency for the carbodiimide approach [8].

2 Methodology

2.1 Chemicals

Commercial-grade reagents and solvents were used without purification unless needed. All Fmoc amino acids were purchased from Peptide Solutions Ltd. and Merck company.

2.2 Procedures

All of the procedures were based on protocols described in Maharani et al. (2015) [4]

Resin loading

To 2-chlorotrityl resin (1.0-1.6 mmol) was added dry dichloromethane (5 mL). The resin was shaken for 5 min and then filtered. A solution of Fmoc-L-proline (0.50 mmol) that was previously treated with dry dichloromethane (4 mL) and N,N-diisopropylethylamine (DIPEA) (1.25 mmol) was added onto resin. The mixture was shaken for 30 min, filtered, and then washed with dimethylformamide (DMF) (2 x 2 min). A mixture of dichloromethane:methanol:DIPEA (80:15:5/5 mL) was added and the mixture was shaken for another 10 min. The latter step was undertaken in two cycles. The resin was then filtered and washed successively with 3 x 2.5 mL DMF and 3 x 2.5 mL dichloromethane. The resin was dried in vacuo for 30 min by a stream of air to obtain dry Fmoc-propyl resin.

Fmoc deprotection

Fmoc-peptidyl resin was shaken with 25% dry piperidine in dry DMF (10 mL) for 30 min. The resin was then filtered and washed with DMF and dichloromethane successively. The reaction was monitored by thin layer chromatography where the successful Fmoc removal was shown with the absence of the compound spot on the thin layer chromatography (TLC) plate under 254 nm Ultra Violet (UV) lamp.

DIC/Oxyma-mediated coupling

Fmoc-amino acid (4 eq.) and oxyma (4 eq.) were weighed in a dry vial and dissolved in a small amount of DMF. DIC (4 eq.) was then added until the solution turns yellow. The solution was added onto dry peptidyl resin and shaken for 30 min. Coupling effectivity was tested by chloranil test. A positive result is shown by dark blue to green beads and a negative result is shown by colourless to yellowish beads. If the coupling was done, the peptidyl resin was washed with DMF and dichloromethane successively.

Resin cleavage

To the peptidyl-trichlorotrityl resin was added a cleavage cocktail of trifluoroacetic acid (TFA)dichloromethane (2:8/10 mL). The yellow resin turned bright red. The resin was then shaken for 1 h and then filtered. The resin was washed subsequently with further cleavage cocktail (10 mL x 2) and dry dichloromethane (10 mL x 2). The combined solution was evaporated and the resulting residue was dissolved in acetonitrile:water (1:1, 10 mL). The crude solution (1 mg/1mL in 50% acetonitrile in water) was subjected to analytical Reversed Phase High Performance Liquid Chromatography (RP-HPLC) and monitored at 240 nm. The purification was undertaken on open column chromatography using octadesylsilane-coated silica and the purity of the peptide was tested by thin layer chromatography. The purity of the product will be checked by analytical RP-HPLC and characterized by mass spectrometry.

Chloranil test general procedure[9]

The stock solutions are kept in a fridge for a maximum of one month.

Solution 1: 2% acetaldehyde in dimethylformamide

Solution 2: 2% chloranil in dimethylformamide

A few beads of resin are placed in a small test tube and 2-5 drops of a solution of 2% acetaldehyde in dimethylformamide and 2% chloranil in dimethylformamide are added. The mixture is mixed for a short period and left at room temperature for 5 min and the color of the beads is observed. A positive result is shown by dark blue to green beads and a negative result is shown by colourless to yellowish beads.

Thin layer chromatography (TLC)

A few beads of resin are placed in a small test tube and 5 drops of TFA:dichloromethane (2:8) was added. The mixture was shaken for 5 min. The reaction mixture was spotted on the TLC plate and eluted by eluent of chloroform/methanol/acetic acid (90:8:2). The plate was then observed under 254 nm UV lamp.

Reversed-phase flash column chromatography

Peptide crude were applied on flash column that has been packed with octadecyl-functionalized silica as stationary phase. The gradient eluent or mobile phase of methanol:water was employed with the increasing methanol volume and the addition of 1% TFA. Every fraction was spotted on the TLC plate and fractions with similar Rf values were combined and characterized with ESI-MS.

Bioassay

Bioassay consisted of two treatments with two repetitive protocols. The first treatment was applied for control and another treatment was for the samples. For the control treatment, the larvae were fed by leaves having been smeared by solvent only. Two pieces of letucce leaves with 4x4 cm size were placed in petri dish (diameter 9 cm) after being smeared by 200 μl of 1000 ppm TMOF in methanol. After the solvent evaporated, ten larvae (first instar) of C. pavonana were placed into each petri dish. Larvae were fed with leaves for 48 hours. Everyday larvae was fed with leaves with no new treatment until achieving fourth- instar larvae. Observation carried out everyday since 48 hours after treatment until larvae get into fourth instar. The number of C. povanana larvae which are died were counted. The larve mortality of C. pavonana is counted by using equation (1).

$$P = \frac{a}{b} \times 100\% \quad \text{.....(1)}$$

P = Mortality (%)
a = Number of C. povanana larvae that are died
b = Number of C. povanana larvae that are tested

3 Results and Discussion

Synthesis of three linear peptides (Fig. 2), PP (H-Pro-Pro-OH), PPPPPP (H-Pro-Pro-Pro-Pro-Pro-Pro-OH), and APPPPPP (H-Pro-Pro-Pro-Pro-Pro-Pro-OH) were carried out by using a protocol applied for synthesis of TMOF [4].

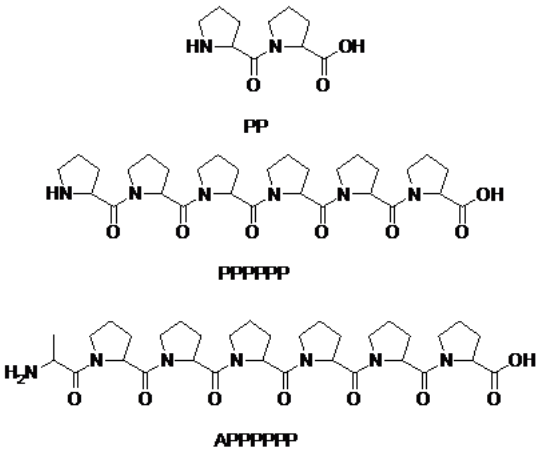

Fig. 2. *Structures of PP, PPPPPP, and APPPPPP.*

All synthesis in three different tubes were initiated by attaching the first amino acid, Fmoc-L-proline, onto the 2-chlorotrityl resin (Fig. 3). This step was undertaken in dichloromethane in the presence of a base, DIPEA. The mixture was shaken for 30 minutes to facilitate complete attachment. The resin was found to have an amino acid loading of 0.52 mmol/ 1.00 g resin.

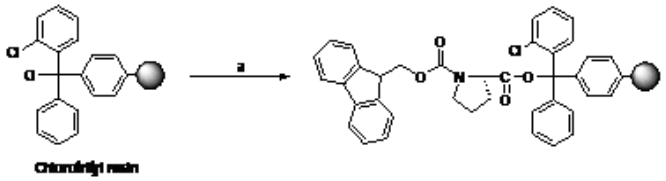

Fig 3. *Attachment of the first amino acid, (a) Fmoc-L-Pro, CH_2Cl_2, DIPEA.*

To avoid truncated products, methanol was then added in order to cap any of the remaining chloro groups on the resin (Fig. 4). This capping would ensure that there was only the amino group of the proline available for the next coupling.

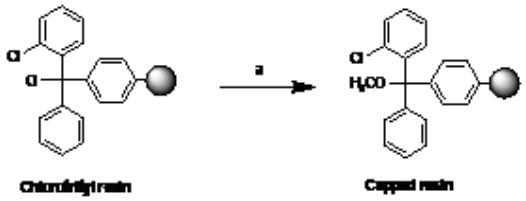

Fig. 4. *Capping the resin, (a) Dichloromethane:methanol:DIPEA (80:15:5).*

The next step involved removal of the Fmoc protecting group which took advantage of the basic conditions of 25% piperidine in DMF for 30 minutes (Fig. 5). After washing the resin with DMF and dichloromethane, the relatively dry resin was tested before it was ready for coupling with a second amino acid. A few beads of the dry resin were tested with a chloranil test with a positive result suggesting the presence of a secondary amino acid due to complete removal of the Fmoc protecting group. The few beads of resin were also tested by spotting the cleaved peptide on silica gel F254 plate of thin layer chromatography (TLC) (chloroform:methanol:acetic acid/90:8:2). The absence of fluorescencing spot under 254 nm UV lamp indicating that the Fmoc group has been completely removed.

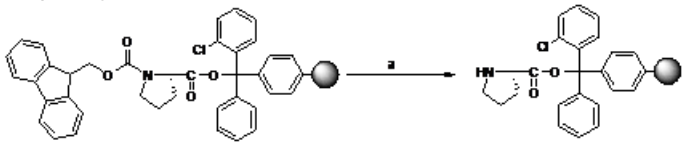

Fig. 5. *Fmoc removal, (a) 25% piperidine in DMF, 30 minutes.*

Coupling of the second amino acid, Fmoc-L-proline, was carried out using a combination of DIC and oxyma as coupling reagent (Fig. 6). The coupling system was found to give a complete attachment of the second residue, Fmoc-L-proline, after 24 hours coupling. The chloranil test was applied to test the coupling effectiveness. The negative test of the chloranil test showing that the coupling was successful. The next step was Fmoc deprotection using 25% piperidine in DMF to give the dipeptidyl resin that was ready for the next coupling (Fig. 6). The successful deprotection was again tested by TLC as before, where the absence of the Fmoc group was shown by the absence of the spot on the TLC plate.

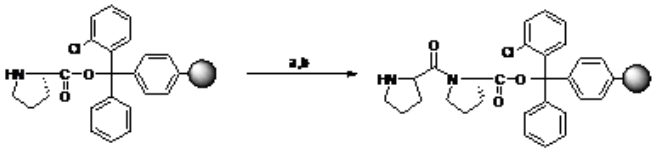

Fig. 6. *Second residue attachment, (a) Fmoc-L-proline, DIC/oxyma, DMF (b) 25% piperidine in DMF.*

The following treatment for first PP-containing tube is to cleave the dipeptide from the resin that will be described in the next paragraph. For another three tubes, repetitive protocol (coupling and Fmoc deprotection) were carried out until all of the desired sequences obtained. As found in Maharani et al. (2015). [4], it was found that the Fmoc removal step was more difficult when four residues were attached on the resin (for PPPPPP and APPPPPP). A single deblocking cycle (1 x 30 min) was not sufficient when 25% piperidine in DMF was employed. The presence of desired peptidyl resin of all TMOF analogues were confirmed by ESI-MS. The ESI-MS data suggested that the peptides were ready to be cleaved from the resin. The cleavage conditions employed for removal of all peptides from the resin were TFA:dichloromethane (2:8) (Fig. 7). The cocktail was added to the unprotected decapeptidyl resin and shaken for 1 hour resulting in the cleaved decapeptide. This was observed by the yellow beads turning red, indicating that the decapeptide had been cleaved from the resin. The resin was washed with dichloromethane several times with the cleavage cocktail and the washings were

combined. The solvent was then removed by rotary evaporation at room temperature. This step was undertaken several times in order to remove any trace of TFA.

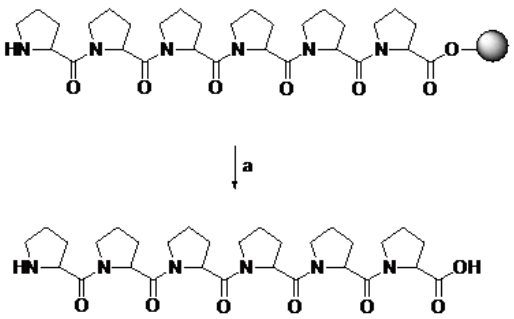

Fig. 7*. Resin cleavage of PPPPPP, (a) TFA:dichloromethane = 2:8) over 1 hour.*

All peptide crudes were purified by reversed-phase flash column chromatography and were characterized by ESI-MS. Compound PP, PPPPPP and APPPPPP were obtained as white solid with 12%, 5.4%, and 14% yields, respectively. At the end of the synthesis, ESI-MS analysis provided the molecular mass ion of m/z 213.2631, 601.5361 and 672.9623, indicating the $[M+H]^+$ ions of the respective PP, PPPPPP, and APPPPPP, respectively. The results of biological assay of the three TMOF analogues towards C. pavonana is presented on Table 1.

Table 1. *Percentage of mortality of TMOF and its three analogues towards C. pavonana*

Entry	Percentage of mortality (%)
TMOF	20
PP	23.33
PPPPPP	30
APPPPPP	23.33

The biological assay data were obtained after 200 μl of 1000 ppm of TMOF was smeared onto the cabbage leaves, prepared for ten larvae. The mortality of the larvae after seven days is presented on Table 1. Unexpectedly, the PPPPPP analogue showed the highest biological activity, while previous report indicate that PPPPPP has only 17% activity of TMOF when assayed against A. aegypty. The present work found that TMOF gave 20% mortality, while PPPPPP gave 30% mortality, which mean the analogue is about 10% better. This result indicates that TMOF activity is most likely species specific, which mean it has different effect on different species. However, further investigation need to be performed to prove this hypothesis. These results also indicated that TMOF and the analogues that we synthesized were ineffective to control the growth of the cabbage cluster caterpillar (C. Pavonana) and the screening for the new biorational is needed. However, TMOF and the three analogues may also be tested again other pests, particularly pests relying on trypsin biosynthesis as TMOF works on the inhibition of trypsin biosynthesis.

4 Conclusions

Three analogues of TMOF were successfully synthesised by solid-phase peptide synthesis with DIC/oxyma as coupling reagent. All synthesis were carried out on chlorotrityl resin with Fmoc strategy with 12%, 5.4% and 14% yields for respective PP, PPPPPP, and APPPPPP. The peptide crude were purified by reverse-phase flash column chromatography and characterized by TOF-ESMS. TMOF and the analogues were ineffective to overcome the cabbage pest C. pavonana and the screening for the new biorational is needed.

Acknowledgement

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