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Synthesis, Spectroscopic and Investigation on Potential of *p*-Nitroanilide Derivatives as Chromogenic Substrates for Detection of Endotoxin

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The development of *p*-nitroanilide derivatives as chromogenic substrates has become a subject of interest due to their prominent use in detecting endotoxin chromogenically. In this study, two new *p*-nitroanilide compounds namely *tert*-butyl(1-((2-((4-nitrophenyl)amino)-2-oxoethyl)amino)-1-oxopropan-2-yl)carbamate (**P1**) and *tert*-butyl(1-((4-(methylthio)-1- ((4-nitrophenyl)amino)-1-oxobutan-2-yl)amino)-1-oxopropan-2-yl)carbamate (**P2**) were obtained in good yield and characterized using combination of common spectroscopic techniques such as Fourier transform infrared, ultraviolet-visible, ^{1}H and ^{13}C nuclear magnetic resonances. The synthesized compounds were subjected to biological study to investigate their potential to detect endotoxin. From this approach, it was revealed that compound **P1** gave positive response towards endotoxin by rapidly changed into cloudy solution. However, compound **P2**, which has limited solubility in the analysis gave negative responses where the solution of **P2** remained unchange.

Keywords: *p*-Nitroanilide, Endotoxin chromogenically, Amino acids and Peptides.

INTRODUCTION

Chromogenic substrate used to measure endotoxin and examine the activities of enzyme by resulting rapid changes of colour or rapid precipitation during the analysis [1-6]. The rapid responses of chromogenic substrates towards enzyme activity have driven to their major use in pharmaceutical [7,8] and food industries as quality control [9]. Chromogenic substrates are commonly derived from *p*-nitroaniline, which was combined with amino acids or peptides [10-15]. In the structure of chromogenic substrate, the C-terminal from the amino acids is functioned as the enzyme cleavage sites for the nitroanilide, before this molecule is being hydrolyzed into *p*-nitroaniline by the enzyme-substrate activity (Fig. 1) [16]. Incorporation of *p*-nitroaniline in chromogenic substrates are important as this molecule can readily separate by the enzyme, gives strong colour of changes towards bacterial activity and soluble in water [17]. According to few reports, the synthesis of *p*-nitroanilide is troublesome and quite complicated due to the poor nucleophilicity of *p*-nitroaniline [18-21]. However, according to Oyamada *et al.* [22], these problems can be solved by using a strong activating agent such as phosphorus trichloride. Other types of activating agent that can be used are pivaloyl chloride [23] and thionyl chloride [24]. The use of phosphorus oxychloride as activating agent is limited for certain protecting groups such as carboxybenzyl and phthaloyl only [2,25]. The other approach that can be used to optimum the reaction is by using protecting amino acids such *t*-butoxylcarbonyl (Boc) amino acids or peptides. This will allow the *in situ* intermediate to react with the carboxylic acids of the amino acids instead of amine.

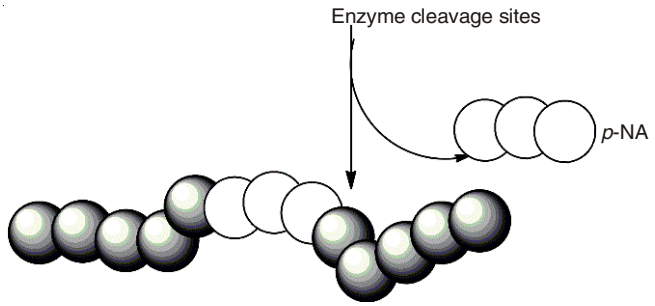


Fig. 1. Illustration of *p*-nitroanilide cleavage by enzyme-substrate activities

In this study, two new *p*-nitroanilide compounds were synthesized by using modification on the methods described in the literature. The synthesized compounds were tested against endotoxin (in this case is *E. coli*) and further analyzed using enzyme-linked immunosorbent assay (ELISA). The function of *Escherichia coli* is to catalyze the proenzyme to enzyme in limulus amebocyte lysate (LAL) in this study is

derived from horseshoe crab's blood [26]. Endotoxin is the cell wall component of gram-negative bacteria (in this case is *E. coli*) and made up by lipopolysaccharide (LPS) that owns a broad variety of biological fields [27,28].

EXPERIMENTAL

All chemicals or reagents used were purchased from Merck and Sigma Aldrich and used as received without further purification. Melting points were measured using BÜCHI Melting Point B-545. The infrared spectra were recorded on a Fourier transform-infrared spectrometer, Perkin Elmer spectrum 100 in the range of 4000-400 cm^{-1} using potassium bromide (KBr) pellets. For UV-visible analysis, all compounds were recorded by using spectrophotometer Shimadzu UV-1601PC in 1 cm^3 cuvette in methanolic solution for absorbance analysis. NMR spectra of ^1H and ^{13}C NMR were recorded using Bruker Avance III 400 spectrometer with deuterated chloroform ($CDCl_3$) and deuterated dimethyl sulfoxide (DMSO) as the solvents and chemical shift values were given in parts per million (ppm) relative to solvent resonances as internal standard. Thin layer chromatography was carried out on silica-gel plates (Merk, Kieselgel 60 F_{254}) in the following solvent systems; **A**: $CHCl_3$:CH_3OH = 9:1, **B**: DCM:CH_3OH = 9:1.

Syntheses of simple peptides with *p*-nitroaniline: Phosphorus trichloride (0.1 mmol, 0.087 mL) and *p*-nitroaniline (0.2 mmol, 0.27 g) were mixed pyridine (10 mL). The mixture was stirred at 25 °C for 24 h under nitrogen atmosphere to afford the corresponding intermediate phospho-azo compound. Then a solution of *N*-Boc-ala-gly-OH (0.1 mmol, 0.5 g) in pyridine (20 mL) was added to the above solution of intermediate phospho-azo compound by a syringe. The reaction was stirred at 40 °C for 5 days under a nitrogen atmosphere. After the reaction was completed, pyridine solvent was removed by rotavap and triturated with ethyl acetate and water. The precipitate was filtered off. The organic layer was separated by using solvent 5 % citric acid solution (20 mL, 3 times), water, 5 % of sodium hydrogen carbonate (20 mL, 3 times), water and saline (20 mL, 3 times) and dried over with sodium sulfate. The crude product was separated by using column chromatography and the product was rinsed with cold chloroform, dried and recrystallized with ether to give **P1** as an 'off-white' powder (0.1942 g, 63 %). *N*-Boc-ala-gly was replaced with *N*-Boc-ala-met to give **P2** as pale yellow powder (0.264 g, 60.0 %).

Biological assay: Screening of endotoxin was carried out using enzyme-linked immunosorbent assay (ELISA) recorded by using Biodex reader. Compounds **P1** and **P2** (0.15 mol-0.25 mol) were diluted with 4 mL tris HCl buffer solution (pH10) and 10 % of DMSO (1 mL) before being sonicated for 0.5 h. The forming precipitate was filtered and the filtrate was reserved for analysis. 50 µL solutions of each substrate was pipetted into 96 well plate along with 50 µL (0.03 EU- 0.05 EU) of *E. coli* and 50 µL of limulus amebocyte lysate reagent. For standard, 50 µL of commercial chromogenic substrate was pipetted into 96 micro well plates combined with 50 µL of 0.03-0.05 EU of *E. coli* and 50 µL of limulus amebocyte lysate reagent. The assay was prepared in 4 duplicates. The 96 well plate, then were placed in ELISA recorded by Biodex and analyzed at 405 nm over 1 h.

Spectral data

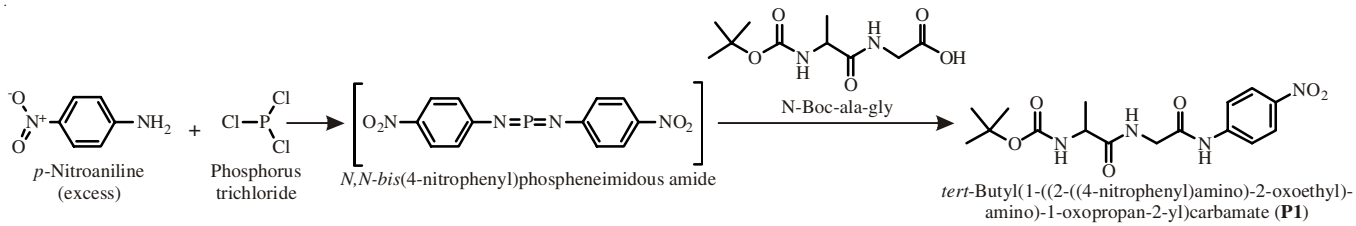
***tert*-Butyl(1-((2-((4-nitrophenyl)amino)-2-oxoethyl)-amino)-1-oxopropan-2-yl)carbamate (P1) :** ^1H NMR (700 MHz; DMSO-d_6) δ = 1.2 (3H, d, *J* = 7.7 Hz CH_3) 1.3 (9H, s, CH_3), 3.9 (2H, d, *J* = 5.6 Hz, CH_2), 4.0 (1H, m, *J* = 7.0 Hz), 7.8 (2H, d, *J* = 9.1 Hz, C aromatic), 8.2 (2H, d, *J* = 9.1 Hz, C aromatic) 6.7, 7.0 and 10.4 (1H, s, NH). ^{13}C NMR (700 MHz; DMSO-d_6) δ = 18.45, 28.6, 43.36, 50.26, 78.7, 119.26, 125.51, 155.8, 169.21, 173.85. Selected IR band (KBr disk, cm^{-1}): 3429 (b), N-H str.; 2981 (m), C-H str.; 1645 (s), C=O str.; 1384 (s), N-O str. (sym); 1523 (m), C-N str. mp = 197.2-206.4 °C. Compound obtained as off-white powder (0.1942 g, 63 %).

***tert*-Butyl(1-((4-(methylthio)-1-((4-nitrophenyl)amino)-1-oxobutan-2-yl)amino)-1-oxopropan-2-yl)carbamate (P2):** ^1H NMR (700 MHz; D_2O) δ = 1.2 (2H, m, *J* = 5 Hz CH_2), 1.5 (3H, d, *J* = 4 Hz, CH_3), 2.6 (9H, s, $3CH_3$), 3.0 (3H, s, CH_3), 3.1 (2H, d, *J* = 4 Hz, CH_2), 4.1 (1H, m, *J* = 5.4 Hz, CH), 4.5 (1H, m, *J* = 5.2 Hz, CH), 6.3, 7.2, 8.7 (1H, s, NH) 8.0 (2H, m, *J* = 4.4 Hz, H aromatic), 8.5 (2H, m, *J* = 5.1 Hz, H aromatic). ^{13}C NMR (700 MHz; D_2O) δ = 8.35, 16.46, 23.87, 23.93, 36.65, 46.78, 48.85, 127.51, 141.13, 147.33, 170.92 and 173.98. Selected IR band (KBr disk, cm^{-1}): 3412 (b), N-H str.; 3067 (m), C-H str.; 1636 (s), C=O str.; 1531 (s), N-O str. (sym); 1055 (m), C-O str.; 676 (s), C-S str. mp = 115.4-127.9 °C. Compound obtained as pale yellow powder (0.264 g, 60.0 %).

RESULTS AND DISCUSSION

Synthesis of *p*-nitroanilide derivatives: Compounds *tert*-butyl(1-((2-((4-nitrophenyl)amino)-2-oxoethyl)amino)-1-oxopropan-2-yl)carbamate **(P1)** and *tert*-butyl (1-((4-(methylthio)-1-((4-nitro-phenyl)amino)-1-oxobutan-2-yl)-amino)-1-oxopropan-2-yl)carbamate (**P2)** were prepared by reacting protective peptides, namely *N*-Boc-ala-gly and *N*-Boc-ala-met with *p*-nitroaniline under inert condition. In this approach, phosphorus trichloride was used as an activating agent where excess amount of *p*-nitroaniline was used to ensure the coupling between the intermediate (*N,N-bis*(4-nitrophenyl)-phospheneimidous amide) with the peptide is occurred. The reaction scheme between the *p*-nitroaniline, phosphorus trichloride and *N*-Boc-ala-gly is shown in **Scheme-I** to show the formation of **P1**.

Compounds **P1** and **P2** were collected and characterized by elemental analysis, FTIR, UV-visible and NMR spectroscopy techniques. The FTIR spectra showed several important vibrational modes for ν(N-H), ν(C-H) of aromatic groups and alkyl group, ν(C=O) amide, ν(NO_2), ν(C-N) and ν(C-S) that were assigned for 3481-3361, 3367-3294, 1631-1603, 1531-1516, 1320-1315 and 1269-1257 cm^{-1}, respectively. As stated by Stuart [29] the ν(N-H) usually appeared at the range of approximately 3400-3300 cm^{-1}. However, in the IR spectra of compounds **P1** and **P2**, the peaks for ν(N-H) were indicated at higher frequency. This might be due to the presence of intermolecular hydrogen bonds [30] and mesomeric (phi polarization) effect contributed by the *p*-nitroaniline ring [31,32]. The N-H peak for compound **P2** was indicated at 3481 cm^{-1} as symmetric stretch [33] at higher frequency compared to compounds **P1**. This is affected by the presence of sulfur atom in the molecule that shifted the N-H into higher frequency.



Scheme-I: Synthesis of **P1**

The ν(C=O) for compound **P2** has the lowest frequency because the alkyl substituted group such as methione and alanine were employed. The only C-S band for **P2** was found at 695 cm^{-1}, the common range reported for this functional group [34]. In the UV spectra, the absorption band for phenyl ring was indicated in the range of 221-222 nm (ε = 3900-8100 L mol^{-1} cm^{-1}) and was assigned to π-π* transition [35]. A broad band in the range 312-331 nm corresponded to NO_2 and C=O and was represented as π-π* electron transition. The absorption for NO_2 was indicated at region 312-314 nm. The absorption was varied due to the different types of amino acids used as the alkyl substituents. The shift to the shorter wavelength is called hypsochromic shift [36]. According to Takada *et al*. [37] the addition of alkyl group to the conjugated molecule, such as nitrophenyl contributes to hypsochromic shift, which was affected by the inductive effect that occurred between the alkyl groups, C=O and NH [38]. The compilation of the FTIR and UV data is shown in Table-1. In the ^{1}H NMR spectra for compounds **P1** and **P2**, the amide and amine protons were indicated at similar range from δ_H 6.3-10.4 ppm, respectively. The signals for methyl protons which sourced from *tert*-butyl functional group, were indicated at region δ_H 1.2-1.5 ppm as a singlet [39].

TABLE-1
FTIR AND UV DATA FOR COMPOUNDS **P1** AND **P2**

Compd.	IR stretching (cm^{-1})	Chromophore	λ_{max} (nm)	Transition
P1	ν(N-H) 3361s ν(C-H) 3294w ν(C-H) 2981m ν(C=O) 1631s ν(NO_2) 1516s ν(NO_2) 1315s	Phenyl ring C=O NO_2	222 312 312	π-π* π-π* π-π*
P2	ν(N-H) 3481m ν(C-H) 3367w ν(C=O) 1603s ν(NO_2) 1531m ν(NO_2) 1320s ν(C-S) 695w	Phenyl ring C=O NO_2	221 314 314	π-π* π-π* π-π*

Compounds **P1** and **P2** also showed two set resonances of phenyl proton as a pseudo-doublet pattern because of the effect from *para*-disubstituted phenyl ring. However, the methyl protons in compound **P2** appeared further downfield at δ_H 2.6 ppm. As well as the phenyl protons (δ_H 8.0-8.6 ppm) due to the presence of sulfur in the compound. The presence of an electronegative atom such as sulfur tends to shift the ^{1}H NMR signals of nearby proton slightly downfield.

In the ^{13}C NMR, methyl carbons of *tert*-butyl (protecting group) were observed in the ranges δ_c 23.87-28.67 ppm, respectively. These data are similar to the studies reported by Cellier *et al*. [5] and Sun *et al*. [40] in which methyl carbon for protecting group of *tert*-butyl-amino acid was reported at region δ_c 28.4 ppm and δ_c 28.31 ppm. The most deshielded region was assigned for carbonyl (-C=O) with the chemical shift at ranges δ_c 173.85-173.98 ppm due to the electronegativity effects [41]. Oxygen atom was considerably more electronegative in comparison with other atoms, hence causing a larger chemical shift. In contrast to **P1**, the signal for compound **P2** appeared at upfield region of δ_c 52 ppm, which is quite similar with the compound studied by Xu *et al*. [42] (Table-2).

TABLE-2
^{13}C NMR DATA FOR COMPOUNDS **P1** AND **P2**

Compound	Moieties	Chemical shift (δ_H, ppm)
P1	C, (CH_3)	18.45
	C, (3×CH_3)	28.67
	C, (CH_2)	43.36
	C, (CH_2)	50.26
	C, (C-O)	78.70
	C, (aromatic)	112.84, 119.26, 125.51, 142.74 and 145.35
	C, (C=O)	155.80
	C, (C=O)	169.21
	C, (C=O	173.85
P2	C, (CS)	8.35
	C, (CH_3)	16.48
	C, (3×CH_3)	23.87
	C, (CH_2)	36.65
	C, (CH_2)	36.71
	C, (CH)	46.76
	C, (CH)	48.85
	C, (C-O)	52.00
	C, (aromatic)	127.51 and 141.13
	C, (C=O)	147.33
	C, (C=O)	170.92
	C, (C=O)	173.98

Bioassay analysis: In this analysis, endotoxin was combined with limulus amebocyte lysate extracted from American Horseshoe Crab, *Limulus polyphemus*. The principle of screening test is that, *E. coli* will induce initial rate of enzyme activation. Once it was activated, the splitting of *p*-nitroaniline from amino acid or peptide linkage occurred. The absorbance reading for nitro (NO_2) was detected at 405 nm [19]. The bioassay analysis was run with a blank prepared by mixing a buffer with limulus amebocyte lysate. Then, a standard consisting of commercial chromogenic substrate (pyrochrom), limulus amebocyte lysate and *E. coli* was prepared. The solution of compounds **P1** and **P2** at concentration of 0.03-0.05 M were also prepared by dissolving 0.15-0.25 mmol in 1 mL DMSO and 4 mL tris-HCl buffer (pH 10). In the preparation of the solution, the mixtures were sonicated in 0.5 h to increase

the solubility in the buffer solution. The solution was left for 10 min at room temperature before the supernatant was separated and pipetted into the 96 well plates. Similar to the standard, the *E. coli* and limulus amebocyte lysate were added into the supernatant, which has been pipetted earlier into the 96 well plates. When all the samples are ready for analysis, the samples were analyzed by ELISA for 1 h. After the analysis was completed, the 96 well plate was taken out from the incubator. Interestingly, **P1** showed colour changes from clear to cloudy solution except for compound **P2** that remained unchanged. This screening result shows that P1 has positive result and gave signals in term of precipitation as reported by Stumacher *et al.* [43]. Further investigation was investigated using ELISA. The assay solution of **P1** and **P2** in 0.03 Eu/mL were prepared in the similar manner described earlier. From the ELISA analysis, it can be concluded that compound **P1** display chromogenic substrate activity by hydrolyzing the enzyme (from horseshoe crabs) (Fig. 2).

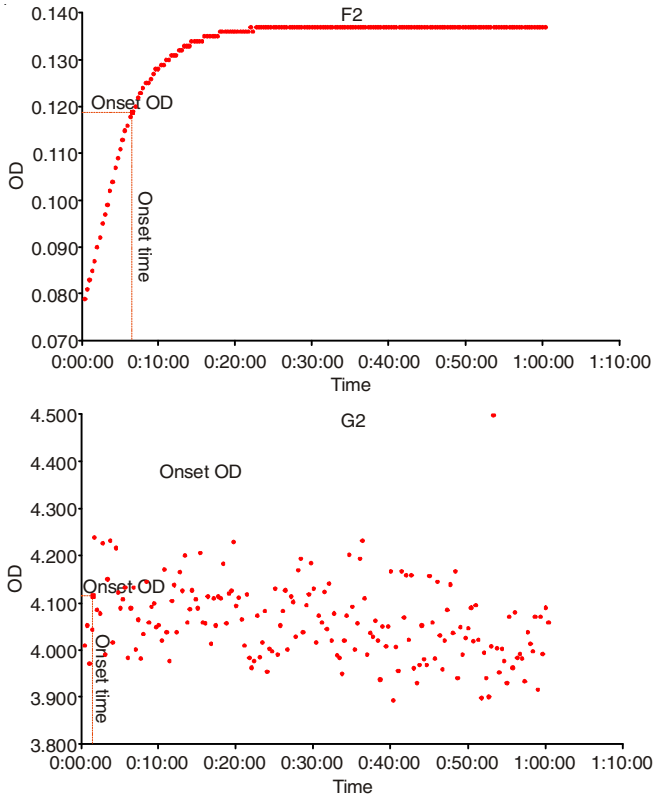


Fig. 2. Comparison between the results of **P1** and **P2** from ELISA

In contrast, compound **P2** was unable to react with *E. coli* and enzyme. This unsuccessful reaction might be attributed to the presence of sulfur atom in the compound (methionine) that disturbed the hydrolysis process. This finding was supported by Kwon *et al*. [19] which reported that some residue amino acid such as methionine, tyrosine, cysteine and tryptophan were sensitive and not really efficiency with *p*-nitroaniline cleavage. Enzymes are very specific and only work with certain substrate. Del Mar *et al*. [44] reported that some of chromogenic substrates such as suc-ala-ala-pro-gly-*p*NA have less efficient towards enzyme elastase because the hydrolysis results showed that the choromogenic substrate was unable to cleave peptide linkage.

Conclusion

In conclusion, two novel compounds, *p*-nitroanilides derivatives (**P1 and P2**) were successfully synthesized and characterized by using Fourier transform infrared, elemental analysis (CHNS), ultraviolet-visible, ^{1}H and ^{13}C nuclear magnetic resonances (NMR). The frequency of FTIR for C=O in these compounds are varied, depends on the lengths and molecular weights of the substituted alkyl group. In the UV-visible spectra, the absorption for NO_2 was indicated at region 312-375 nm. Compounds **P1** and **P2** were subjected for biological assay analysis using enzyme-linked immunosorbent assay (ELISA) to investigate the potential of these compounds to act as chromogenic substrates for bacterial endotoxin such as *E. coli* in particular. This study utilized proteolytic enzyme activity sourced from limulus amoebocyte lysate (LAL). The results show that compound **P1** able to detect endotoxin until concentration at 0.03 EU/mL by apparently change colours from clear to cloudy solution in the presence of *E. coli*. However, compound **P2** has limited solubility and unable to cleavage the peptide in the hydrolysis process thus enable its potential as chromogenic substrate for endotoxin.

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