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The synthetic molecules YK51 and YK73 attenuate replication of dengue virus serotype 2

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Abstract. Dengue virus infection has been posing alarming economic and social burden on affected nations. It is estimated that 50-100 million dengue infections occur annually with over 2.5 billion people at risk for endemic transmission. In the effort to develop effective antiviral agents, we previously reported potential antiviral activities from selected array of natural products and compounds against dengue virus serotype 2 (DV2). In this study, we report the synthesis of two efficacious novel compounds, YK51 and YK73, and their activities against DV2 replication. Both compounds were chemically synthesised from nicotinic acid using a modified method for the synthesis of dihydropyridine. The products were tested with cell-based assays against DV2 followed by a serine protease assay. As a result, both YK51 and YK73 exhibited intriguing antiviral properties with EC_{50} of 3.2 and 2.4 µM, respectively. In addition, YK51 and YK73 were found to attenuate the synthesis of intracellular viral RNA and protect the switching of non-classic mechanism of protein translation. These compounds demonstrated inhibitory properties toward the activity of DV2 serine protease in a dose dependent manner. These findings demonstrate that both YK51 and YK73 serve as DV2 serine protease inhibitors that abrogate viral RNA synthesis and translation. Further investigation on these compounds to corroborate its therapeutic properties towards dengue is warranted.

INTRODUCTION

Dengue virus (DV) is an RNA virus belonging to the *Flaviviridae* family. There are four distinct serotypes (DV1 to DV4) that circulate the tropical and subtropical regions of the world accounting to over hundreds of millions of annual infections, with approximately 75% of cases being endemic to Asia-Pacific (Murray *et al*., 2013). All DV serotypes share similar genome organisation and replication strategies. Its genome size of 11 kb forms a single open reading frame that is translated into a ~370 kDa precursor polyprotein containing the structural proteins (C, prM, and E) and non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Henchal & Putnak, 1990). DV is predominantly transmitted in a cycle that involves humans and mosquito vectors, *Aedes aegypti* and *Aedes albopictus* (Guzman *et al*., 2010). Despite the newly lounged tetravalent dengue vaccines Dengvaxia® (Sanofi Pasteur) in the market, the coverage period of the vaccine is yet to be identified. To back up the potential drawbacks from the current vaccine candidate as experienced with the vaccine against Japanese Encephalitis virus, another member of the *Flaviviridae* family (WHO, 2016), continuous efforts in developing efficacious antiviral agent against dengue diseases remain imperative.

In recent years, many investigators have looked at compounds from plants and their derivatives to develop new antiviral drugs (Kumar & Pandey, 2013; Kiat *et al*., 2006). In terms of DV replication in a host system, to

our best knowledge, NS2B-NS3pro has emerged as the most potential antiviral target due to its pivotal role in viral replication (Baharuddin *et al.*, 2014). Upon translation, the viral precursor polyprotein is subsequently co- and post-translationally cleaved by proteases of the host and the virus. Together with the cofactor NS2B, NS3pro catalyses the *cis*-cleavage of NS2A-NS2B and NS2B-NS3, as well as *trans*-cleavage of NS3-NS4 and NS4B-NS5 junctions. This releases the individual proteins necessary for further viral RNA synthesis, assembly and maturation (Henchal & Putnak, 1990; Kautner *et al.*, 1997; Yamschcikov & Compans, 1995; Liu *et al.*, 2002).

Our previous study demonstrated the potential inhibition of DV2 NS2B-NS3pro by several natural products isolated from Asian fingerroot *Boesenbergia rotunda* (Kiat *et al.*, 2006). These bioactive compounds have led to the design and synthesis of new compounds with different chemical space that were shown to possess antiviral effects against DV propagation (Muhamad *et al.*, 2010). This study reports the synthesis of two piperidinyl compounds (YK51 and YK73) and characterisation of their *in vitro* activities as antiviral agents.

MATERIALS AND METHODS

Synthesis of YK51 and YK73

YK51 and YK73 were synthesised from nicotinic acid via a method derived from dihydropyridine synthesis as reported by Hilgeroth *et al.* (2002). Both compounds were later subjected to NMR and the purity of each compounds were greater than 95%. The synthesis began with esterification of nicotinic acid **1**, in the presence of sulfuric acid in ethanol under reflux condition for 4 hours (Sambrook *et al.*, 2005). The ester **2** was subsequently converted to 1,4-dihydropyridine **3** by reacting nicotinate ester **2** with phenyl chloroformate, followed by treatment with phenyl magnesium chloride in the presence of CuI as catalyst (Hilgeroth *et al.*, 2002). The 1,4-dihydropyridine **3** with phenylcarbamate was then converted to a BOC-protected dihydropyridine **4** by stirring with t-BuOK in THF at -42°C (Comins 1983). The dihydropyridine **4** readily underwent 1,4-addition reaction with cuprate prepared from copper (I) cyanide to give 31% yield of adduct **5**. The subsequent step was the reduction reaction of the compound **5** with hydrogen gas and 10% palladium on activated carbon to produce YK51 with 99% yield (Scheme 1).

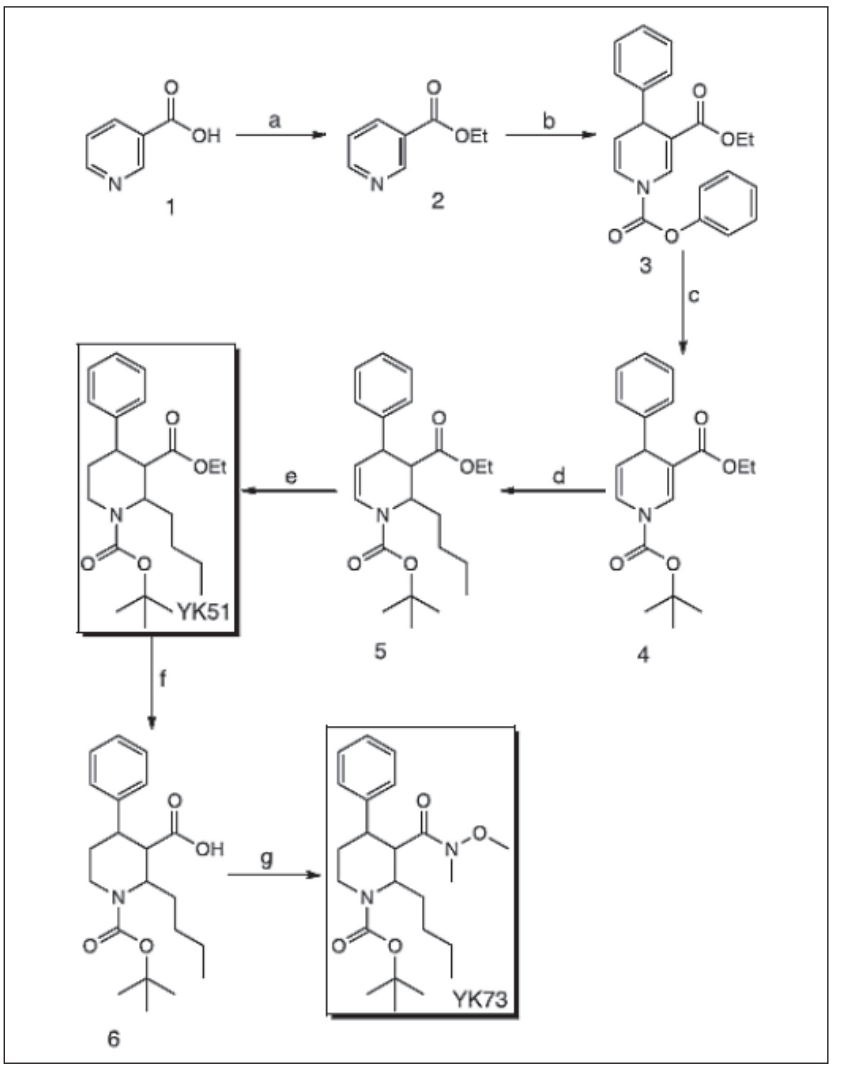
YK73 was synthesised in continuation from YK51 via two additional steps of reactions. YK51 was hydrolysed with potassium hydroxide in ethanol solution under reflux condition to make carboxylic acid **6** in quantitative yield. It was then coupled with Weinreb amine via amide coupling reaction to furnish YK73 in 49% yield (Scheme 1). Detailed synthesis condition is appended as supplementary (Figure 1S).

Cell culture

HepG2, Vero and C6/36 cells were obtained from American Type Culture Collection (ATCC, USA). The HepG2 and Vero cell lines were maintained by regular sub-cultivation in 1X growth media of Dulbecco's modified Eagle medium, DMEM (Gibco, USA), supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 U/ml of penicillin, 100 U/ml of streptomycin and 20 mM of HEPES buffer at 37°C in a humidified incubator in the presence of 5% CO_2. The C6/36 cell line was maintained by regular sub-cultivation in a 1X Leibowitz's growth medium L-15 (Gibco, USA), supplemented with 10% FBS, 100 U/ml of penicillin, 100 U/ml of streptomycin and 10% tryptose phosphate broth (Sigma, USA) at room temperature.

Virus stocks

Dengue virus serotype 2 (DV2) New Guinea C strain was a kind gift from the Department of Medical Microbiology, Faculty of Medicine, University of Malaya. The virus stock was prepared by promoting infection on preformed monolayer of C6/36 cells, in a standard 75 cm^2 tissue culture flask, in three (3) ml of Leibovitz's L-15 medium containing 2% FBS, 100 U/ml of penicillin, 100 U/ml of streptomycin and 10% tryptose phosphate



Scheme 1. Synthesis of YK51 and YK73. Reagents, conditions and percent yield: a: EtOH, H_2SO_4, reflux, 4h, then NH_3(aq), 82%; b: PhOCOCl, -10°C, 30min, then PhMgCl, THF, r.t., 2h, 62%; c: t-BuOK, THF, -42°C, 30 min, 72%; d: $Bu_2(CuCN)Li_2$, THF, -78°C, 31%, 6h; e: 10% Pd/C, H_2, MeOH, r.t, 24h, 99%; f: KOH, EtOH, reflux, 3h, 100%; g: NHMe(OMe).HCl, DMAP, PyBrOP, DCM, r.t., 19h, 49%.

broth. The flask were left for 2 hours for viral adsorption. Subsequently, to encourage viral propagation, cell culture medium was added to 10 ml and incubated at room temperature for 6 to 7 days or until the cytophatic effect (CPE) was confirmed. Virus was harvested by one repetition of freeze-thaw cycle, centrifuged at 1,500X g for 5 minutes, collected in small aliquots and stored at -80°C until further use. Titers of the virus stocks were determined as previously described (Payne *et al.*, 2006).

DV2 infection

Infection was performed as mentioned elsewhere (Gan *et al.*, 2015). Briefly, medium from the overnight culture was removed and the preformed monolayer of HepG2 cells were washed with phosphate buffered saline (PBS) prior to the addition of 100 µl of DV2 containing media with a multiplicity of infection (moi) factor of one. The plate was left for 2 hours with occasional rocking to assist viral adsorption. Subsequently, infected cells were washed with PBS and fresh media

with 2% FBS or specified otherwise was added and maintained for further 48 hours in the humidified incubator.

Virus yield reduction

HepG2 cells were infected with DV2 and incubated for two (2) hours. Then, the DV2 containing medium was removed and the cells were washed with PBS. Two-fold serial dilution of the antiviral compounds was prepared with the media starting from 766.9 nM to 12.7 µM. The culture media containing varying concentration of antiviral compound including a set of control without any antiviral compound was supplemented into respective wells. After 48 hours of treatment, the medium containing the newly released virus was collected and stored at -80°C until used. Titres of virus released in the presence of antiviral at varying concentrations were determined by plaque assay using the Vero cells. The percentage of virus yield was then calculated using the following formula:

% of virus yield = [(viral titre with compound) / (viral titre without compound)] X 100

Cell viability assay

The cell viability assay was carried out on the synthesised compounds to determine the potential cytotoxicity of the concentrations used in this study. The compounds were added into the fresh media with 2% FBS and made to the final concentration specified before supplementing it to the preformed monolayer of HepG2 cells in 96-well plates. Cells supplemented with only fresh media with 2% FBS were concurrently performed and referred as control for each plate. The tetrazolium solution (Promega, USA) was added into each well at end-point of 72 hours post-incubation and colour changes were monitored and quantified using spectrophotometer. Reading values from the control were normalised to 100% and percentage of cytotoxicity was estimated from subtracting the percentage cell viability in treatment from 100%.

Reverse transcription-PCR

Cellular total RNA was isolated with the RNAeasy system (Qiagen, CA), and one (1) µg was used to generate cDNA using High Capacity RNA-to-cDNA Kit reverse transcription system (Applied Biosystem, USA) in accordance with the manufacturer's instructions. The cDNA was then used as a template for PCR amplification of the gene products DV2 NS2B/NS3 (Gan *et al.*, 2015) and GAPDH (Razali *et al.*, 2010). Amplification of the DV2 NS2B/NS3 was performed over 35 cycles at 50°C using eight (8) µl of cDNA as the starting material while the GAPDH was amplified with 30 cycles at 56°C using two (2) µl of cDNA as starting material. Products from these independent PCR reactions were resolved by 2% agarose gel electrophoresis, visualised by ethidium bromide staining and the pictures were captured by gel documentation system (Bio-Rad, CA).

Solid phase sandwich ELISA

Whole cell proteins were isolated with Cell Lysis Buffer #9803 (Cell Signalling Technology, USA). Detection of the phosphorylation level of 4E-BP1 was determined with PathScan® Phospho-4E-BP1 (Thr37/Thr46) Sandwich ELISA system (Cell Signalling Technology, USA) in accordance with the manufacturer's instructions.

DV2 NS2B/NS3 protease assay

The study was performed in 96-well plate with Cary Eclipse Varian fluorescent spectrophotometer system (Agilent Technology, USA) as previously described (Yusof *et al.*, 2000; Heh *et al.*, 2013). The 200 µL of standard reaction mixtures contained the final concentration of 200 mM Tris-HCl (pH 8.5), and were optimised at two (2) µM of dengue protease CF40.gly(T).NS3pro (Heh *et al.*, 2013) and 100 µM of fluorogenic peptide substrate (Boc-Gly-Arg-Arg-MCA) (Peptide Institute Inc., Minoh-Shi, OK, Japan). Substrate cleavage was observed and optimised by monitoring the emission at 440 nm upon excitation at 350 nm. The assays were carried out by mixing protease with or without the presence of inhibitor (YK51 or YK73) in varying concentrations. The inhibitors were initially prepared in dimethylsulfoxide (DMSO) and assayed at five different concentrations, ranging

from 64 µM to one (1) mM for YK51 and 62 to 989 µM for YK73. Reaction mixtures without the addition of dengue protease CF40.gly(T).NS3pro were referred as blanks to correct the error obtained in the measurement of the fluorescence intensity due to the release of 7-Amino-4-methylcoumarin (AMC) by self-hydrolysis of the peptide substrate. Mixtures without inhibitors were tested with the addition of DMSO of the same amount to eliminate solvent effect. The reaction mixtures were incubated at 37°C for 30 min before the addition of the final fluorogenic peptide substrate. The mixtures were then further incubated at 37°C for another 30 min before measurements were taken.

Statistical analyses

Unless specified, all experiments were conducted in three biological and technical replicates. Results are expressed as mean ± standard error of the mean (SEM). Comparisons between groups were made using Student's t-test using statistical computer package SPSS version 17.0 software for Windows (IBM, USA). Half maximal inhibitory concentration (IC_{50}) and half maximal effective concentration (EC_{50}) were calculated using Graph Pad Prism version 5 software for Windows (Graph Pad Software Inc., USA).

RESULTS

YK51 and YK73 inhibit DV2 propagation in a dose-dependent manner

The antiviral activity of the synthesised compounds was evaluated by viral yield reduction assay. Viral yields from three independent replicates with the same concentration of supplemented compound were pooled. Serial dilution of factor 10 was then prepared for each pooled sample prior to viral titration. Results demonstrated that both antiviral compound YK51 and YK73 exerted anti-DV2 propagation in a dose-dependent manner (Figure 1). At the concentration of ~12.5 µM, reduction in plaque formation was drastically apparent in the presence of both compounds when compared to the virus yield from untreated controls. Treatment with 12.7 µM of YK51 demonstrated a greater potential with 95.5% inhibition while treatment with 12.4 µM of YK73 resulted in 82.7% inhibition. Nevertheless, treatment with YK73 exerted a better inhibition potential at lower concentrations compared to those treated with YK51. At a concentration of three (3) µM, treatment with YK73 marked almost 70% reduction of virus yield and treatment with YK51 marked around 46% reduction of virus yield. The half maximal inhibitory concentration (EC_{50}) for both compounds, YK51 and YK73, were 3.2 and 2.4 µM, respectively.

YK51 and YK73 show no toxicity at concentration used

The synthesised compounds were subjected to MTS assay to demonstrate that the reduction in nascent viral release was due to the bioactivity of the respective compounds and not due to the inability of effective viral propagation in dying cells. In this assay, culture media supplemented at varying concentrations of synthesised compound ranging from 766.9 µM to 12.7 mM. Concentrations of compounds of less than this showed no antiviral activity, hence were not tested. The results show no toxicity for both independent compounds at all concentrations of treatment with similar cell viability to that of untreated cell sets (data not shown).

YK51 and YK73 inhibit the switching of intracellular translational mechanism post-DV2 infection

It has previously been delineated that DV replicates through a cap-dependent translation during the first six (6) hours of infection and switched to cap-independent translation, as seen by the dephosphorylation of p70S6K and 4E-BP1 (Villas-Boas *et al.*, 2009). In order to determine whether the synthetic compounds (YK51 and YK73) would have any effects on the switching of translation mechanism by DV2 infection, the phosphorylation level of 4E-BP1 was evaluated at 48 hours post DV2 infection. To abrogate the DV2 propagation effectively, 12.5 µM of both compounds were used in the treatment for this assay. Since cellular proteins were extracted after 48 hours post

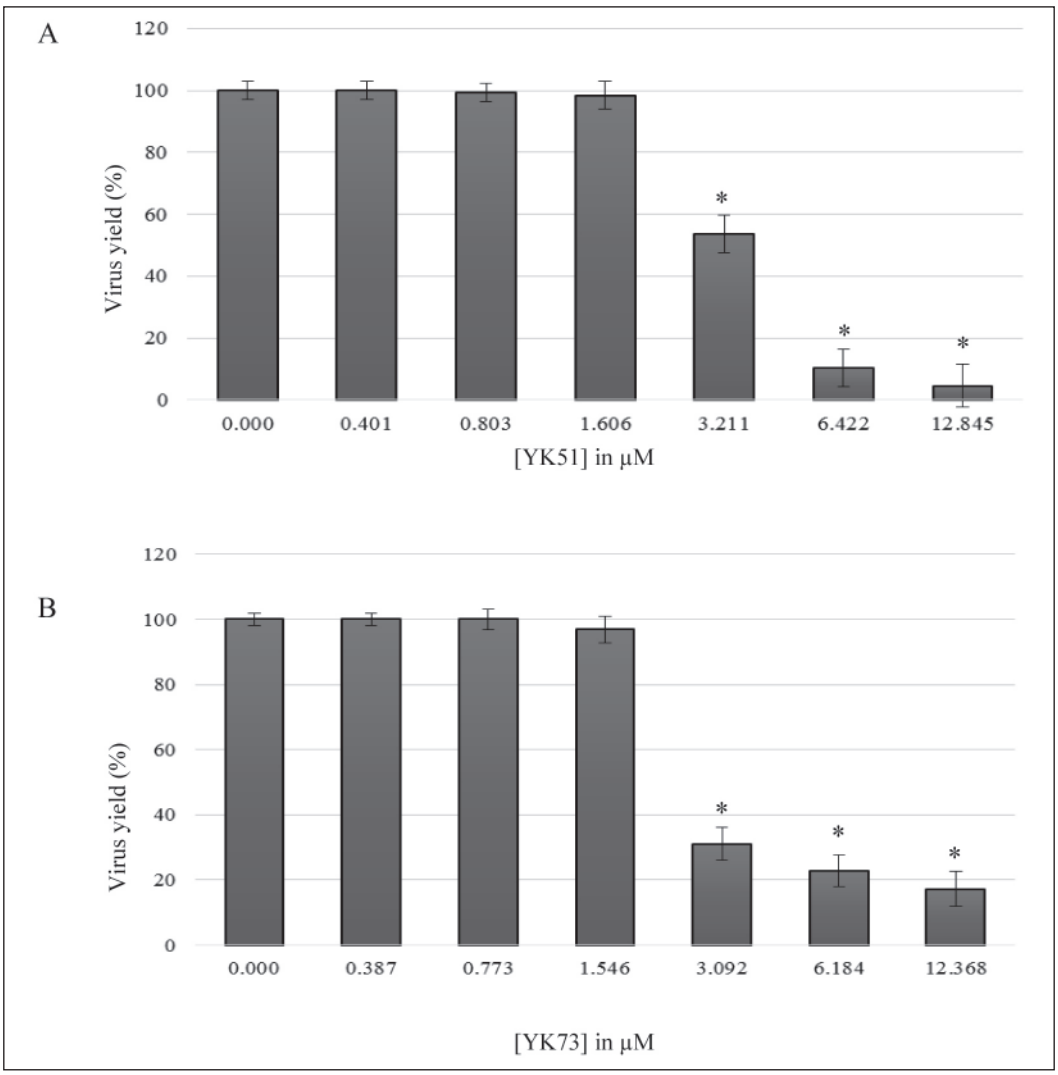


Figure 1. Antiviral activity of A) YK51 and B) YK73 against DV2 infection. Virus yield reduction assay and plaque assay were used to evaluate the *in vitro* antiviral activity of the compounds. Pooled samples from four independent assays were used for titration. Results are presented in percentage virus yield with negative control normalised to 100% and percentage of inhibition was calculated from subtracting the percentage of virus yield in treatment from 100%. Error bars are expressed as standard deviation, comparison was made between antiviral compounds and statistical significance ($p<0.05$) is indicated by asterisk.

viral infection, as expected, infection with DV2 resulted in about 34% reduction of the phosphorylated 4E-BP1 compared to the mock infected cells (Figure 2). The observed phosphorylation level corresponds to the switching of the cellular translational pattern from the cap-dependent into cap-independent translational mechanism (Pause *et al.*, 1994). In contrast, the switching of translational pattern was not observed in those compound-treated DV2 infections. This was shown by the high levels (greater than 50%) of phosphorylated 4E-BP1 protein detected in cells treated with both compounds compared to those without treatments. This observation demonstrated the preventive effects of these YK51 and YK73 synthetic compounds against the switching of translation mechanism by DV during its replication, thus affecting viral propagation.

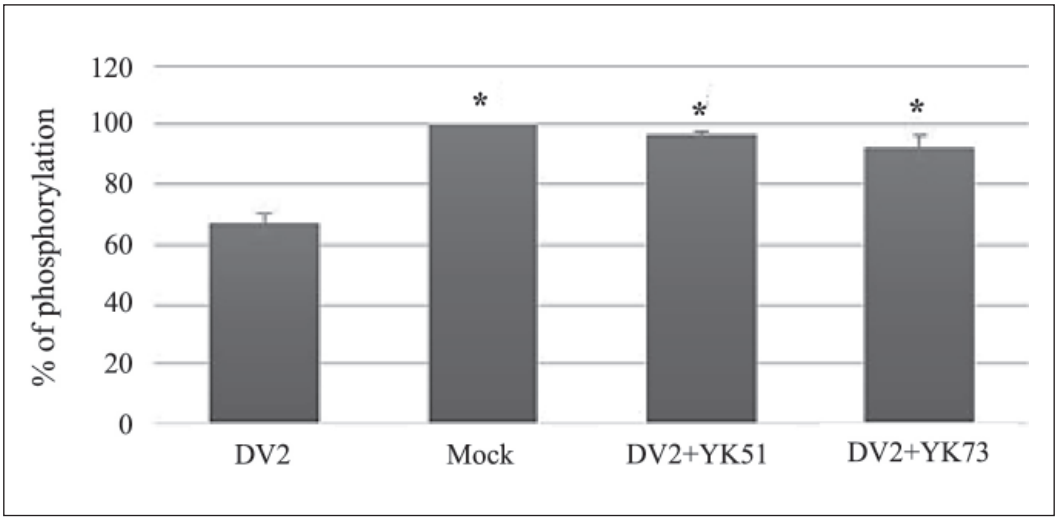


Figure 2. The synthetic compounds, YK51 and YK73, retained the phosphorylation level of 4E-BP1 in HepG2 infected with DV2. Whole cell protein was extracted for relative quantification of 4E-BP1 phosphorylation level. Results are averages of four independent replicates (n=4). Error bars are expressed as SEM and statistical significance ($p<0.05$) is indicated by an asterisk above the bar.

YK51 and YK73 inhibit *in vitro* replication of DV2

To determine whether the synthetic compounds (YK51 and YK73) affect replication of the viral genome, DV2 viral RNA transcripts in HepG2 cells was quantified via RT-PCR. It is noteworthy that treatment with both synthetic compounds resulted in a dose-dependent reduction of the amplified DV2 gene compared to infected cells without treatment (Figure 3). To further evident that the presence of gene amplified via RT-PCR originated from DV, a mock experiment was carried out and the observation revealed the absence of DV2 gene product. The GAPDH gene of the host was co-amplified in the same PCR mixture as a reference gene and the bands produced for mock infected and DV-infected cells were of similar intensities. It was interesting to note that only treatment with YK51 reduced viral replication by half in accordance to its respective EC_{50} value (Figure 4B) whereas that of YK73 did not.

YK51 and YK73 inhibit *in vitro* DV2 serine protease activity

To investigate whether the synthetic compounds render the inhibition of DV2 serine protease, the NS2B-NS3pro was cloned and expressed as previously described (Yusof *et al.*, 2000). The results from the serine protease assays indicated that both the compounds inhibit the catalytic activity of the enzyme at a concentration-dependent manner (Figure 4). YK73 demonstrated a better inhibition profile with an IC_{50} of 213.7 µM than YK51 with an IC_{50} of 257.4 µM. Remarkably, both compounds are in fact better inhibitors when compared to a known inhibitor for DV2 protease, pinostrobin with an IC_{50} of 614.2 µM (Kiat *et al.*, 2006; Heh *et al.*, 2013). These therefore demonstrated that the synthetic compounds, YK51 and YK73, have great potentials as DV2 serine protease inhibitors.

DISCUSSION

Our previous study has presented the antiviral potentials of several piperidinyl derivatives against DV2 infection (Muhamad *et al.*, 2010). These derivatives were designed based on the cyclohexenyl chalcone, i.e. 4-hydroxypanduratin A, obtained from Asian fingerroot *Boesenbergia rotunda* that exhibited remarkable inhibitory effect against DV2 serine protease activity (Kiat *et al.*, 2006). Screening of the synthetic

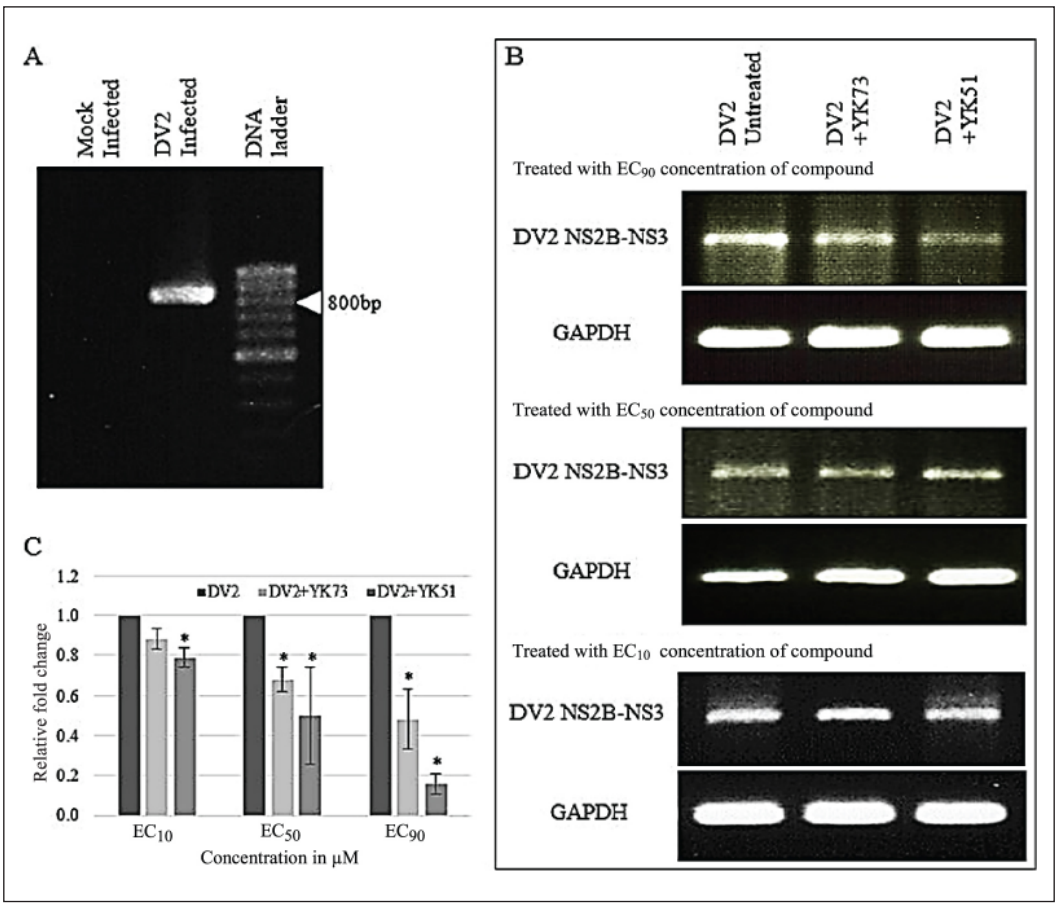


Figure 3. The synthetic compounds, YK51 and YK73, inhibit DV2 replication in HepG2. A) Total cellular RNA was extracted from mock infected and DV2 infected cells. The cDNA was then subjected to PCR for DV2 specific gene, NS2B-NS3. A gel representative shows the absence of PCR product from mock infected cells (lane 1), a distinct band of ~820 bp PCR product from DV2 infected cells (lane 2) and the 100 bp DNA ladder (lane 3). B) Total cellular RNA was extracted, followed by semi-quantitative PCR for the genes indicated. The compilation of gels are from one representative experiment out of the many performed showing bands from untreated DV2 infected cells (lane 1), DV2 infected cells treated with YK73 (lane 2) and DV2 infected cells treated with YK51 (lane 3). C) Average band intensities of three (n=3) different experiments described above. Error bars are expressed as SEM and statistical significance ($p<0.05$) is indicated by an asterisk above the bar.

compounds revealed two outstanding candidates, namely the YK51 and YK73, which required as low as 5 µM of independent compound to render ±90% inhibition of DV2 infection at median tissue culture infective dose (Gan *et al.*, 2017). These results therefore warranted further investigation to corroborate the potentials of these compounds as potent antivirals against dengue.

The present study revealed the synthetic pathway and demonstrates the ability of YK51 and YK73 to reduce the nascent virus production in DV2 infected cells with EC_{50} of 3.2 and 2.4 µM, respectively. Administration of the compounds did not show any cytotoxic effect to the cells at all tested concentrations. In fact, the YK51 and YK73 have higher maximum non-toxic doses (MNTD) of 102.8 and 61.8 µM, respectively, (Muhamad *et al.*,

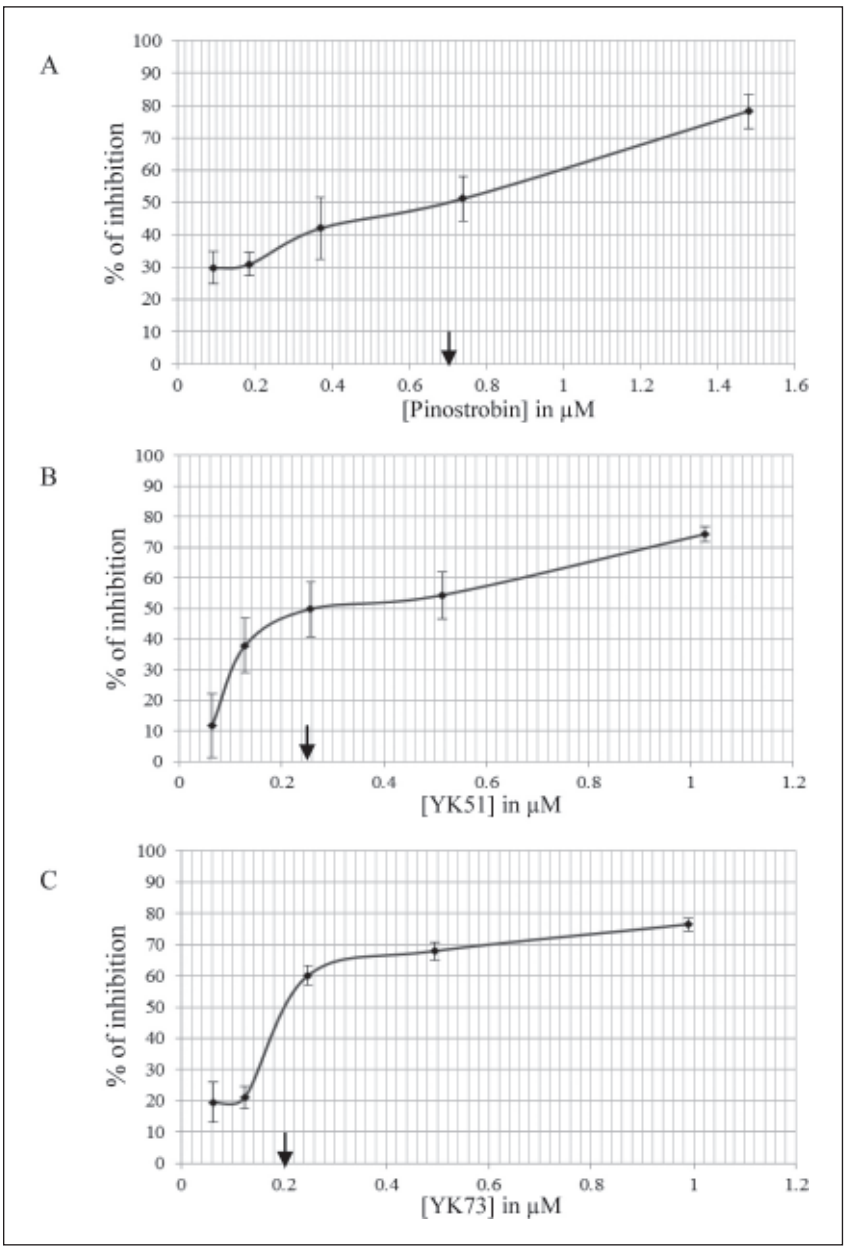


Figure 4. The synthetic compounds A) pinostrobin (positive control), B) YK51 and C) YK73 inhibit DV2 NS2B-NS3pro proteolytic activity. The activity curves were generated from average values of three replicates (n=3). Error bars are expressed as standard deviation. The activity is expressed in percentage and derived from the percentage of odd fluorogenic intensity value of enzyme activity left after inhibition over the fluorogenic intensity value of enzyme activity (negative control). Arrow at x-axis indicates the IC_{50} concentration.

2010) compared to the lead 4-hydroxypanduratin A with an MNTD of 45.9 µM (unpublished data). This suggests that the new compounds are better in terms on cellular cytotoxicity. Furthermore, the DV2 serine protease bioassay validated the conserved interaction between the synthetic compounds, YK51 and YK73 with serine

protease; similar to that of the prototype structure 4-hydroxypanduratin A. It is worth mentioning that studies have shown strong correlation between the amount of DV present in the blood during the viremic phase and the severity of the disease (Vaughn *et al.*, 2000). Therefore, the effective reduction of viral release upon treatment even at low concentrations of YK51 or YK73 may be able to offer protection from severe dengue, both in primary and secondary infections. Moreover, acute reduction of nascent virions in antivirus-treated infected individuals could also subsequently reduce viral transmission to mosquitoes, especially during a dengue outbreak.

Treatments with YK51 and YK73 following DV2 infection were shown to exhibit protective effects to the translational commitment of the host cells. Unlike other viruses, flaviviruses are RNA viruses that sequester the host protein machinery by inactivating the translation factor, such as 4E-BP1, in a cap-dependent protein synthesis pathway (Villas-Boas *et al.*, 2009). Despite the resemblance of DV RNA to that of typical host mRNA, DV was shown to be capable of alternating between a classical cap-dependent translation and a non-classical mechanism that appears not to require a functional cap (Edgil *et al.*, 2006). In line with that, DV infected cells demonstrated an inhibition of classical protein synthesis pathway at 24 hours onwards (Villas-Boas *et al.*, 2009). The switch in protein translation preference from a cap-dependent to cap-independent mechanism during dengue infection would halt the protein translation process of the host cell but favours the translation of viral polyprotein. Results of our current study suggest that treatments with YK51 and YK73 are able to prevent the switch of translational mechanism induced by DV infection. It is noteworthy that NS3pro of the flavivirus member has been described to interplay with host proteins to orchestrate the translation-replication switch of the virus (Villas-Boas *et al.*, 2009). This indicates that YK51 and YK73 may also exhibit their antiviral effects by impairing the function of DV2 NS2B-NS3pro during virus replication. Such inhibitory mechanism was previously reported for pinostrobin which inhibits DV2 NS2B-NS3pro (Kiat *et al.*, 2006; Heh *et al.*, 2013).

Further investigation on the antiviral property of the compounds also revealed the *in vitro* attenuation of viral RNA amplification. This demonstrates that both compounds have significant effects on viral RNA synthesis and consequently on the translation of viral proteins or vice versa. The reduction of viral transcript shown in this study by both YK51 and YK73 may also suggest some degree of anti-helicase and/or anti-RNA dependent RNA polymerase activities. This is because both helicase and RNA-dependent RNA polymerase are also encoded by DV NS3 and NS3-NS5, respectively (Benarroch *et al.*, 2004). Moreover, the inverse relationship between the phosphorylation state of 4E-BP1 and the DV RNA copy number seen in this study strongly suggests that YK51 and YK73 inhibit the intracellular replication of the virus. Despite the 'weak' interaction shown by both compounds in DV2 NS2B-NS3pro assay, the *in vitro* viral inhibition of these novel compounds was remarkable. This indicates that the mode of action for both YK51 and YK73 is not exclusively acting on the virus serine protease alone but also extends to other mechanisms of viral inactivation. Investigation on these mechanisms is underway.

In conclusion, the synthesised compounds, YK51 and YK73, exert antiviral activities against DV2 replication. The current study therefore, provides evidence that both YK51 and YK73 interfere with intracellular viral RNA amplification and inhibit the induction of cap-independent translation mechanism by DV2 during viral replication. These subsequently affect the production of nascent virus. However, further study is warranted for an in-depth investigation to elucidate the mode of action of these studied compounds that contribute to their antiviral activities against DV2 replication.

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REFERENCES

Baharuddin, A., Hassan, A.A., Sheng, G.C., Nasir, S.B., Othman, S., Yusof, R., Othman, R. & Rahman, N.A. (2014). Current approaches in antiviral drug discovery against the flaviviridae family. *Current Pharmaceutical Design* **20**(21): p. 3428-3444.

Benarroch, D., Egloff, M., Mulard, L., Guerreiro, C., Romette, J. & Canard, B. (2004). A structural basis for the inhibition of the NS5 dengue virus mRNA 2'-O-methyltransferase domain by ribavirin 5'-triphosphate. *Journal of Biological Chemistry* **279**(34): p. 35638-43.

Comins, D.L. (1983). Alpha-metalation of 1-(tert-butoxycarbonyl)-1,4-dihydropyridines. *Tetrahedron Letters* **24**(28): p. 2807-2810.

Edgil, D., C. Polacek, C. & Harris, E. (2006). Dengue virus utilizes a novel strategy for translation initiation when cap-dependent translation is inhibited. *Journal of Virology* **80**(6): p. 2976-2986.

Gan, C.S., Lim, P.J., Mohamad Razif, M.F., Yusof, R. & Othman, S. (2017). Subversion of immunoproteasome subunit expression in dengue virus serotype 2-infected HepG2 cells. *Revista da Sociedade Brasileira de Medicina Tropical* **50**(1): doi: 10.1590/0037-8682-0207-2016.

Gan, C.S., Yusof, R. & Othman, S. (2015). Different serotypes of dengue viruses differently regulate the expression of the host cell antigen processing machinery. *Acta Tropica* **149**: p. 8-14.

Guzman, M.G., Halstead, S.B., Artsob, H., Buchy, P., Farrar, J., Gubler, D.J., Hunsperger, E., Kroeger, A., Margolis, H.S., Martínez, E., Nathan, M.B., Pelegrino, J.L., Simmons, C., Yoksan, S. & Peeling, R.W. (2010) Dengue: a continuing global threat. *Nature Reviews Microbiology*, p. S7-S16.

Heh, C.H., Othman, R., Buckle, M.J., Sharifuddin, Y., Yusof, R. & Rahman, N.A. (2013). Rational discovery of dengue type 2 non-competitive inhibitors. *Chemical Biology & Drug Design* **82**(1): p. 1-11.

Henchal, E.A. & Putnak, J.R. (1990). The dengue viruses. *Clinical Microbiology Reviews* **3**(4): p. 376-396.

Hilgeroth, A., Heinemann, F.W. & Baumeister, U. (2002). First rotameric anti dimers and 3,9-diazatetraasteranes from unsymmetrically substituted N-acyl- and N-acyloxy-4-aryl-1,4dihydropyridines. *Heterocycles* **57**(6): p. 1003-1016.

Kautner, I., Robinson, M.J. & Kuhnle, U. (1997). Dengue virus infection: epidemiology, pathogenesis, clinical presentation, diagnosis, and prevention. *Journal of Pediatrics* **131**(4): p. 516-24.

Kiat, T.S., Pippen, R., Yusof, R., Ibrahim, H., Khalid, N. & Rahman, N.A. (2006). Inhibitory activity of cyclohexenyl chalcone derivatives and flavonoids of fingerroot, Boesenbergia rotunda (L.), towards dengue-2 virus NS3 protease. *Bioorganic & Medicinal Chemistry Letters* **16**(12): p. 3337-3340.

Kumar, S. & Pandey, A.K. (2013). Chemistry and biological activities of flavonoids: an overview. *Scientific World Journal*, http://dx.doi.org/10.1155/2013/162750.

Liu, W.J., Sedlak, P.L., Kondratieva, N. & Khromykh, A.A. (2002). Complementation analysis of the flavivirus Kunjin NS3 and NS5 proteins defines the minimal regions essential for formation of a replication complex and shows a requirement of NS3 in cis for virus assembly. *Journal of Virology* **76**(21): p. 10766-10775.

Muhamad, M., Lee, Y.K., Rahman, N.A. & Yusof, R. (2101). Antiviral actions of flavanoid-derived compounds on dengue virus type-2. *International Journal of Biological Sciences* **6**(3): p. 294-302.

Murray, N.E., Quam, M.B. & Wilder-Smith, A. (2013). Epidemiology of dengue: past, present and future prospects. *Clinical Epidemiology* **5**: p. 299-309.

Pause, A., Belsham, G.J., Gingras, A.C., Donzé, O., Lin, T.A., Lawrence, J.C. Jr. & Sonenberg, N. (1994). Insulin-dependent stimulation of protein-synthesis by phosphorylation of a regulator of 5'-cap function. *Nature* **371**(6500): p. 762-767.

Payne, A.F., Binduga-Gajewska, I., Kauffman, E.B. & Kramer, L.D. (2006). Quantitation of flaviviruses by fluorescent focus assay. *Journal of Virological Methods* **134**(1-2): p. 183-189.

Ray, U. & Das, S. (2011). Interplay between NS3 protease and human La protein regulates translation-replication switch of Hepatitis C virus. *Scientific Reports*, **1**: doi:10.1038/srep00001.

Razali, N., Aziz, A.A. & Junit, S.M. (2010). Gene expression profiles in human HepG2 cells treated with extracts of the Tamarindus indica fruit pulp. *Genes and Nutrition*, **5**(4): p. 331-341.

Sambrook, M.R., Beer, P.D., Wisner, J.A., Paul, R.L., Cowley, A.R., Szemes, F. & Drew, M.G. (2005). Anion-templated assembly of pseudorotaxanes: Importance of anion template, strength of ion-pair thread association, and macrocycle ring size. *Journal of the American Chemical Society* **127**(7): p. 2292-2302.

Vaughn, D.W., Green, S., Kalayanarooj, S., Innism, B.L., Nimmannitya, S., Suntayakorn, S., Endy, T.P., Raengsakulrach, B., Rothman, A.L., Ennis, F.A. & Nisalak, A. (2000). Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *Journal of Infections Disease* **181**(1): p. 2-9.

Villas-Boas, C.S.A., Conceição1, T.M., Ramírez, J., Santoro1, A.B.M. & Da Poian, A.T. (2009). Dengue virus-induced regulation of the host cell translational machinery. *Brazilian Journal of Medical and Biological Research* **42**(11): p. 1020-1026.

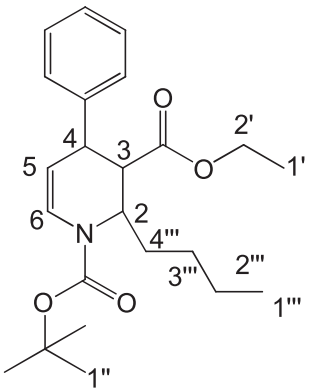
World Health Organization (2016). Japanese encephalitis. http://www.who.int/biologicals/areas/vaccines/jap_encephalitis/en/ (assessed 24.06.2016).

Yamshchikov, V.F. & Compans, R.W. (1995). Formation of the flavivirus envelope – role of the viral NN2B-NS3 protease. *Journal of Virology* **69**(4): p. 1995-2003.

Yusof, R., Clum, S., Wetzel, M., Murthy, H.M. & Padmanabhan, R. (2000). Purified NS2B/NS3 serine protease of dengue virus type 2 exhibits cofactor NS2B dependence for cleavage of substrates with dibasic amino acids *in vitro*. *Journal of Biological Chemistry* **275**(14): p. 9963-9969.

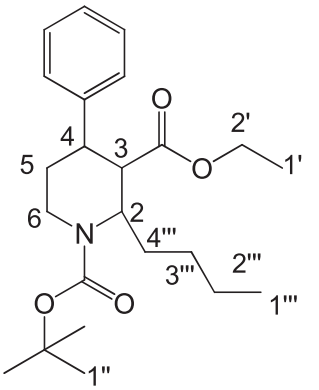
SUPPLEMENTARY DATA

Synthesis of ethyl (1-tert-butoxycarbonyl-2-butyl-4-phenyl)-3,4-dihydro pyridinyl-3-carboxylate (5)



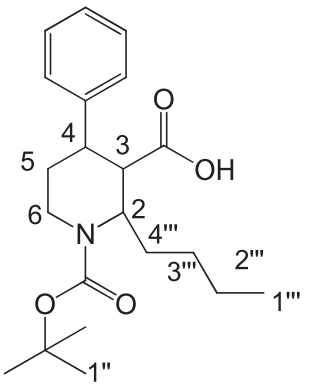
In anhydrous condition, 2 equivalents of n-BuLi (1.6 M in hexanes, 12.2 ml) was added dropwise into a cooled (-40°C to -50°C) CuCN (0.875 g, 9.77 mmol) in 100 ml THF and stirred for 30 minutes under cold bath. The resulting 2 immiscible layers, where cuprate was formed at the lower layer as pale yellow coloured solution was then cooled to -78°C and the resulting solution (86.7 ml) was cannulated into the substrate **4** (2.79g, 8.47 mmol) at this temperature and stirred for 6 hours. The reaction was quenched with NH_4Cl saturated solution, extracted with Et_2O, followed by washing with more NH_4Cl, then water and brine, finally dried with Na_2SO_4 anhydrous. Further purification was performed using flash column chromatography using petroleum ether and EtOAc as eluting solvent to afford 31% yield of adduct. ^{1}H NMR (400MHz, $CDCl_3$): 0.81 (m, 3H, H1'"), 1.05 (t, 3H, *J*=7.2Hz, H1'), 1.28 (m, 6H, H2'", H3'", H4"'), 1.46 (s, 9H, H1"), 2.81 (dd, 1H, *J*=62.7Hz, 3.8Hz, H3), 3.73 (brd, 1H, *J*=11.6Hz, H4), 3.94 (m, 2H, H2'), 4.55 (brd, 1H, *J*=10.8Hz, H2), 4.72 (brd, 1H, *J*=8.4Hz, H5), 6.76 (d, 1H, *J*=7.6Hz, H6), 7.14 (m, 5H, H Phenyl). ^{13}C NMR (100MHz, $CDCl_3$): 13.95, 22.55, 27.96, 28.21, 37.98, 50.65, 51.14, 51.58, 52.94, 60.51, 81.04, 109.41, 122.92, 123.16, 126.63, 128.30, 142.96, 152.20, 171.72. LRMS (EI): m/z 387 (M^+, 1), 214 (65), 156 (100), 69 (77), 41 (91). HRMS (EI) calculated for $C_{23}H_{33}O_4N$ (M^+): 387.2410, found 387.2403.

Synthesis of ethyl (1-tert-butoxycarbonyl-2-butyl-4-phenyl)-piperidinyl-3-carboxylate (YK51)



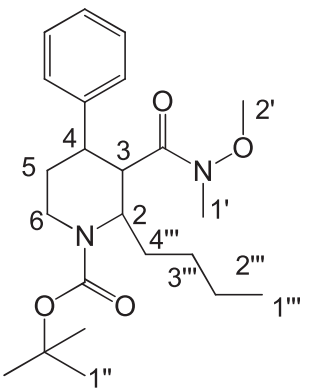
The reaction flask was charged with substrate **5** (0.107g, 0.277 mmol) and Pd/C catalyst (0.029g, 0.0277 mmol Pd). 10 ml of MeOH was added and then the mixture was purged with nitrogen gas to remove oxygen. The reaction began when it was then purged with hydrogen and stirred under hydrogen at room temperature. The completion of reaction was monitored by TLC. After completion of reaction, the reaction mixture was again purged with nitrogen and the catalyst was then filtered off. The product was obtained at 99% yield after the solvent was evaporated off. 1H NMR (400MHz, $CDCl_3$): 0.82 (m, 3H, H1'"), 0.96 (m, 3H, H1'), 1.17 (m, 6H, H2'", H3'", H4"'), 1.44 (s, 9H, H1"), 1.71 (m, 1H, H5), 1.81 (m, 1H, H5), 2.81 (m, 1H, H6), 2.94 (dt, 1H, *J*=12.4Hz, 4.8Hz, H3), 3.11 (m, 1H, H4), 3.88 (m, 2H, H2'), 3.95 (brd, 1H, *J*=13.2Hz, H6), 4.62 (dd, 1H, *J*=79.1Hz, 10.0Hz, H2), 7.15 (m, 5H, H Phenyl). ^{13}C NMR (100MHz, $CDCl_3$): 13.84, 22.45, 25.6, 27.70, 28.40, 33.63, 37.19, 38.36, 42.76, 50.62, 51.99, 57.65, 79.73, 126.68, 127.31, 128.32, 144.13, 154.82, 209.88. LRMS (EI): m/z 389 (M^+, 5), 276 (80), 232 (100), 128 (21), 84 (17), 57 (57). HRMS (EI) calculated for $C_{23}H_{35}O_4N$ (M^+): 389.2566, found 389.2559.

Synthesis of (1-tert-butoxycarbonyl-2-butyl-4-phenyl)-piperidinyl-3-carboxylic acid (6)



The substrate **YK51** was dissolved in little amount of THF, then 2M KOH solution in EtOH 95% was added. The mixture was then refluxed for 3 hours. The mixture was acidified with 2M HCl and extracted with EtOAc. The combined extracted product was then washed with water brine then dried with Na_2SO_4 anhydrous, followed by evaporation of the solvent to get the product **6** in quantitative yield. ^{1}H NMR (400MHz, $CDCl_3$): 0.82 (m, 3H, H1'"), 1.20 (m, 6H, H2'", H3'", H4'"), 1.41 (s, 9H, H1"), 1.66 (brd, 1H, *J*=13.2Hz, H5), 1.77 (m, 1H, H5), 2.72 (m, 1H, H6), 2.95 (dt, 1H, *J*=12.2Hz, 4.8Hz, H3), 3.04 (m, 1H, H4), 3.95 (dd, 1H, *J*=64.3Hz, 11.2Hz, H6), 4.60 (dd, 1H, *J*=71.9Hz, 13.2Hz, H2), 7.09 (m, 5H, H Phenyl). ^{13}C NMR (100MHz, $CDCl_3$): 14.00, 22.33, 26.11, 27.80, 28.40, 29.67, 33.73, 37.91, 50.73, 52.35, 80.15, 126.51, 127.23, 128.40, 143.79, 154.83, 176.51. LRMS (EI): m/z 361 (M^+, 5), 304 (22), 260 (29), 204 (38), 57 (100). HRMS (EI) calculated for $C_{21}H_{35}O_4N$ (M+): 361.2253, found: 361.2242.

Synthesis of 1-tert-butoxycarbonyl-2-butyl-3-(methoxy(methyl) carbamoyl)-4-phenylpiperidine (YK73)



The substrate **6** was dissolved in CH_2Cl_2. DMAP and PyBrOP were then added and stirred for 1 hour. NHMe(OMe).HCl was then added and strirred for 19 hours at room temperature. The reaction mixture was quenched by adding water, extracted by CH_2Cl_2 and the organic layer was washed with brine. Purification of the crude through column chromatography (hex:EtOAc= 3:17) afforded 49% of the product. ^{1}H NMR (400MHz, $CDCl_3$): 0.81 (m, 3H, H1'"), 1.22 (m, 4H, H2'", H3'"), 1.42 (s, 9H, H1"), 1.58 (m, 2H, H4'"), 1.80 (m, 2H, H5), 2.84 (m, 1H, H6), 2.92 (s, 3H, H1'), 3.30 (m, 2H, H3, H4), 3.62 (s, 3H, H2'), 3.99 (dd, 2H, *J*=72.3Hz, 13.6Hz, H6), 4.60 (brd, 1H, *J*=43.16Hz, H2), 7.13 (m, 5H, H Phenyl). ^{13}C NMR (100MHz, $CDCl_3$): 13.84, 22.10, 25.61, 27.64, 28.25, 31.84, 33.76, 36.93, 38.40, 47.22, 50.91, 61.49, 79.23, 125.99, 127.09, 128.03, 144.08, 154.51, 172.18. LRMS (EI): m/z 404 (M^+, 1), 303 (24), 273 (40), 247 (43), 57 (100). HRMS (EI) calculated for $C_{23}H_{36}O_4N_2$ (M^+): 404.2675, found 404.2682.