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Construction of combinatorial "Library" of cyclopeptides and screening for biological activities

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ABSTRACT Two combinatorial libraries of hexa- and heptacyclopeptides were synthesised by the split and mixed method. These libraries were tested for antibacterial activity against the bacterial strains *Escherichia coli* 25922, *Escherichia coli* 35213, *Staphyloccus aureus* 24213 and *Staphyloccus aureus* 29213. Results indicated that these cyclopeptides demonstrated no activities against all four bacterial strains tested. Preliminary screening of the libraries against dengue virus protease however indicated some inhibitory activities from compounds in both libraries.

ABSTRAK Dua "combinatorial libraries" mengandungi heksa- dan heptasiklopeptida telah disintesis menggunakan kaedah "split dan mixed". Librari ini diskrin untuk keaktifan antibakteria terhadap strain bakteria *Escherichia coli* 25922, *Escherichia coli* 35213, *Staphyloccus aureus* 24213 and *Staphyloccus aureus* 29213. Keputusan menunjukkan bahawa siklopeptida dalam library yang disintesis tidak menunjukkan sebarang keaktifan biologi. Akan tetapi, pengskrinan awal library siklopeptida ini menunjukkan kesan penahanan terhadap protease virus denggi.

(Combinatorial library, solid-phase synthesis, cyclopeptide, antibacterial activity, dengue virus protease inhibition)

INTRODUCTION

Classical methods for obtaining biologically active compounds are through screening a wide range of chemical compounds either obtained from natural products (herb) or synthetic compounds. This would involve extraction, isolation, purification and synthesis of the biologically active compound for further lead generation. At best, a chemist might be expected to synthesise or purify approximately 100 compounds each year.

With the advancement in technology such as in combinatorial synthesis, chemists can now synthesise more than 10,000 compounds within a week. Usually, these compounds are simultaneously produced in a set, commonly called combinatorial libraries, through a reaction of one set of compounds with a second set of compounds. These libraries are then used to screen for biological activities for lead generation.

Combinatorial libraries of peptides have offered state-of-the-art tools to the pharmaceutical industries where the compounds in these libraries are exploited to identify inhibitors and agonists of new target molecules [1]. The same libraries could also be used to screen for potential new lead molecules for the design of drugs to address specific diseases [2,3].

Cyclic peptides are amongst the important classes of compounds used as therapeutic agents. For example, cyclic peptides such as bacitracin and vancomycin have shown activities as antibiotics [4]. The use of cyclic peptides in drug development is enhanced by their conformational restraints imposed by their cyclised nature enabling their control of spatial orientation as well as making them more resistant towards enzymatic degradation [5]. Thus, this increases their potential in pharmaceutical applications.

Unfortunately, many pathogens have very effectively demonstrated that virtually all of the modern anti- infectives can be circumvented by various resistance mechanisms. Thus, antibiotic resistance has emerged as a serious concern and pharmaceutical companies continue to search for a broad-spectrum drug that could kill them quickly and safely without falling prey to bacterial resistance.

Some researchers are returning to old ideas, once considered as possibilities but ignored when classic screening reliably yielded new compounds. Our research aim, like many others, however, is to search for the possibilities for new antibiotics created from novel approaches, particularly cyclic peptides. In this project, we proposed to synthesise two sets of combinatorial libraries of cyclohexapeptides and of cycloheptapeptides to be screened for anti-microbial activities. In turn, we hope that this will lead to the generation of a new lead towards anti-microbial active compounds.

MATERIALS AND METHOD

Synthesis of the cyclopeptide library

The cyclopeptides were synthesised by the solid phase method. The Merrifield resins were used as the solid support for the syntheses. Two libraries of cyclopeptides were generated combinatorially via the split and mixed method. The first library consists of the combination of the amino acids alanine (A), leucine (L), valine (V) and phenylalanine (F) to make cyclohexapeptides while the second library consists
of cycloheptapeptides with the amino acids alanine (A), leucine (L), valine (V), glycine (G) and phenylalanine (F).

Screening of anti-bacterial activities.

Test discs containing 10 μg of Gentamicin – form Oxoid with the product serial number CN10 was used as a control in all the antibacterial activity testing. The bacterial broth or Mueller-Hinton broth was purchased from Oxoid with the product serial number CM405, while the bacterial agar or Mueller-Hinton agar was obtained from Oxoid with the product serial number CM337. Pure bacteria *Escherichia coli* strain 25922, *Escherichia coli* strain 35213, *Staphyloccus aureus* strain 24213 and *Staphyloccus aureus* strain 29213 were pre-prepared into a bacterial broth and were used for the testing.

After the cleavage from the solid phase and cyclization reaction, the cyclopeptides in the first library and second library were dissolved in methanol. 50 μl of the solubilized cyclopeptides at concentration around 50 mg/ml were then dispensed onto the three paper discs containing 10 μg of Gentamicin on the first paper disc, cyclopeptides on the second, and methanol on the third (used as solvent control). Three paper discs were then placed onto the surface of the bacteria cultured agar and the plates were incubated at 37 °C for the *E. coli*, and 28 °C for the *S. aureus*. The diameter of the zone of inhibition (mm) was measured in mm after 48 hours. The same method was carried out for the second library of cyclopeptides.

Screening of dengue virus protease inhibition using fluorogenic peptides as substrate

For cis-cleavage assay, fluorogenic peptide substrate containing two basic residues at P1 and P2 were used in this assay. Assays were carried out using a Shimadzu RF-5301PC spectrofluorometer. The standard reaction mixtures (100μl) contained 200mM Tris-HCl, pH 8.5, and 200μM fluorogenic peptide substrate. After enzyme addition, the reaction mixture was incubated at 37°C for 30 min and terminated by addition of 1.0ml of 125mM $ZnSO_4$. The precipitate was removed by centrifugation for 1 min in a microcentrifuge (13,000 rpm), and the rate of product (7-amino-4-methyl-coumarin) released into the supernatant was determined fluorometrically (λ (excitation)=385nm, λ (emission)=465nm). The substrate used was Boc-Gly-Arg-Arg-4-methylcoumaryl-7-amide (MCA). Stock solutions (100nm) were prepared by dissolving peptides in dimethyl sulfoxide that are then diluted in water to 1mM working stock before use. Substances with potential inhibitory activity will then be screened for their effect on this protease activity by measuring fluorometrically the rate of product (7-amino-4-methyl coumarin) released into the supernatant solution.

RESULTS AND DISCUSSION

Synthesis of the cyclopeptide library

The strategy chosen for the preparation of cyclic peptides mirrors the method used by Sila and Mutter [6]. The linear precursor peptide was synthesised on the resin aminomethylpolystyrene crosslinked with 1 % divinylbenzene (DVB). The first amino acid was then attached to the resin solid support through 4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid (HMBA) which acts as the linker between the solid support and the peptide chain to be synthesised.

The libraries of cyclic peptides were then generated using the Furka's split-and-mix method [7] as shown by the example in Figure 1. It is envisaged that cyclization of the linear peptides

will invoke duplicity in the type of cyclic peptides generated if not controlled. Hence, to overcome such problems, the first amino acid linked to the solid support was chosen as a fixed amino acid. For this purpose, leucine was arbitrarily chosen as the first amino acid to be linked. Cyclization of the peptides was carried out only after the peptides were cleaved from the resin. A total of 243 cyclohexapeptides (Figure 2) and 729 cycloheptapeptides (Figure 3) were generated following the cyclization step in the first and second library, respectively.

Screening for anti-bacterial activities.

Four types of bacteria have been cultured for the screening of anti-bacterial activities. These four types of bacteria were the *Escherichia coli* strain 25922, the *Escherichia coli* strain 35213, the *Staphyloccus aureus* strain 24213 and the *Staphyloccus aureus* strain 29213. The pictures in Figure 4 show the results of the anti-bacterial activities of the first library of hexacyclopeptides (Figure 4a) and of the second library of cycloheptapetides (Figure 4b). The paper disc on the left corner of the petri-plate that shows the anti-bacterial positive result was the antimicrobial agent CN10. The paper disc containing the sample of the cyclopeptide from the first synthesized library is on the right side of the plate and shows a negative result. The paper disc at the bottom of the plate shows negative result for the methanol used as the solvent control.

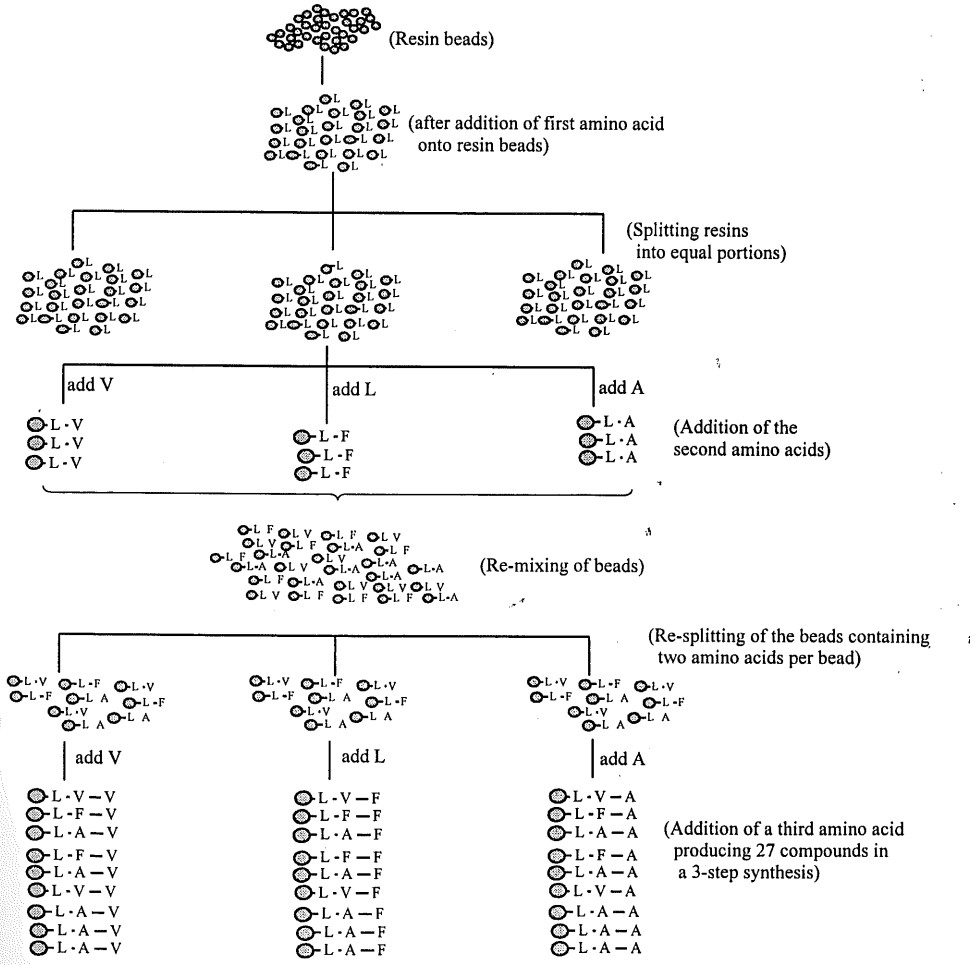


Figure 1 The split-and-mix method for generating a combinatorial library of peptides.

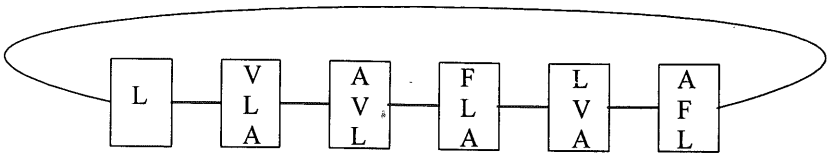


Figure 2. Amino acid units in the combinatorial library of cyclohexapeptides

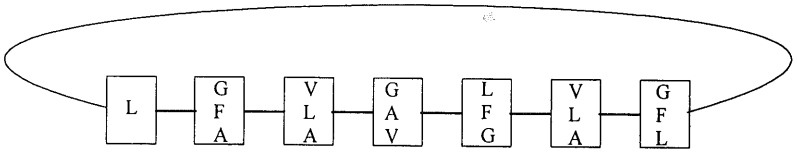
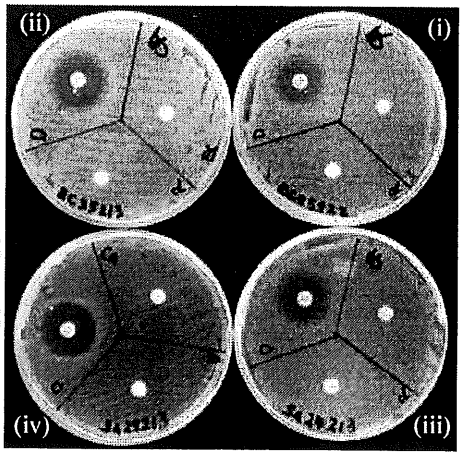
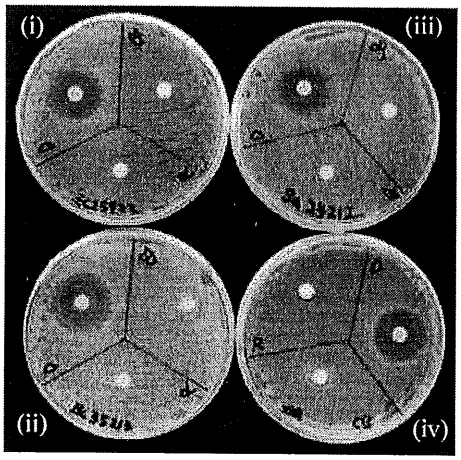


Figure 3. Amino acid units in the combinatorial library of cycloheptapeptides



(Figure 4a)



(Figure 4b)

Figure 4. The anti-bacterial screen results of the four bacterial strains for the cyclopeptides synthesised; i.e., (i) with *Escherichia coli* strain 25922, (ii) with the *Escherichia coli* strain 35213, (iii) with *Staphyloccus aureus* strain 24213 and (iv) with *Staphyloccus aureus* strain 29213. The paper disc on the left corner shows a positive result with the antimicrobial agent CN10. The disc on the right corner is the screening with the cyclopeptide library while the disc at the bottom of the plate is a solvent control. Fig. 4a shows the results with the library of cyclohexapeptide while Fig. 4b shows the results with the library of cycloheptapeptide synthesised. No anti-bacterial activities were observed with both the libraries of cyclopeptides screened.

The results of the antibacterial screenings are tabulated in (Table 1). As expected, no antibacterial activities were observed with both libraries synthesised. This could be due to several factors. The type of amino acids used when generating the library of the cyclic peptides may influence the activity of the cyclic peptides. Other cyclic peptides that have shown activities such as bacitracin have amino acids such as histidine and aspartic acid in the cyclic chain. Furthermore, known antibiotics such as vancomycin and other antibodies consist of large number of amino acids in the molecule. It is therefore also be possible that the size of the cyclic peptides in the library generated is not big enough to induce any activities.

Table 1. Anti-bacterial effects of the cyclopeptides in first and second library.

Sample Dissolved In Paper Disc	Concentration	Solvent	Antibacterial Activity Against			
			E. coli 25922	*E. coli* 35213	*S. aureus* 24213	*S. aureus* 29213
Cyclopeptide of First Library	50 mg/ml	Methanol	Negative	Negative	Negative	Negative
Cyclopeptide of Second Library	50 mg/ml	Methanol	Negative	Negative	Negative	Negative
Control 1 - Solvent Methanol Only	-	Methanol	Negative	Negative	Negative	Negative
Control 2 -Antibacterial Agent (CN10)	10 µg of Gentamicin	Distilled Water	Positive	Positive	Positive	Positive

Screening for dengue virus protease activity.
Dengue virus type 2 (DEN 2), a member of Flaviviridae, has a single-stranded RNA genome of positive polarity. This genome encodes for a single polyprotein precursor arranged in the order of C-prM-E-NS1 -NS2A-NS2B-NS3-NS4A-NS4B-NS5 [8]. Analysis of polyprotein processing of the virus established that NS3 protease, when complexed with the viral activator protein NS2B, catalyses the cleavages at NS2A - NS2B, NS2B-NS3, NS3-NS4A and NS4B-NS5 sites. This, in turn, activates the polyprotein processing and causes viral replication. These sites have in common Lys-Arg, Arg-Arg, Arg-Lys and occasionally Glu-Arg at P1 and P2 positions followed by a short chain amino acid Gly, Ala or ser at the P1' position (see Ref .9 for nomenclature).

The libraries of cyclic peptides generated were also screened for inhibition activities against dengue virus protease which would block the polyprotein processing and hence viral replication. Two different concentrations of cyclopeptides were used in this experiment. Preliminary screening of the libraries against dengue virus protease indicated obvious inhibitory activities from the cyclopeptides in both libraries. Both cyclic peptide libraries reduces the enzyme cleavage of the fluorogenic peptide substrate AMC (Boc-Gly-Arg-Arg-4-methylcoumaryl-7-amide{MCA}). However, from the results in Table 2, it is shown that the library containing hexacyclopeptides shows greater proteases inhibitory effect than the library of cycloheptapeptides. It was also observed that increasing the concentration of cyclohexapeptide library does not increase the inhibition but increasing the concentration of cycloheptapeptide library seems to increase the inhibition by cycloheptapeptide by 10%. The result obtained, however, is only a preliminary one. Work is still in progress to deconvulate the library and identify the active cyclic peptide, which could then be used as a template for lead generation.

Table 2. Percentage inhibition on dengue NS2B/3 protease activity in the presence of various concentrations of cyclopeptides.

Concentration of library of cyclopeptides used	Percentage inhibition
20 μL^{-1} of Cyclohexapeptide Library	80
30 μL^{-1} of Cyclohexapeptide Library	78
20 μL^{-1} of Cycloheptapeptide Library	30
30 μL^{-1} of Cycloheptapeptide Library	42

CONCLUSION

Two sets of libraries of cyclopeptides were successfully synthesized. It is essential that the first amino acid used in the solid-phase synthesis of the peptide before cyclization be fixed in order to avoid duplicity of compound produced upon cyclisation.

Screening of the cyclohexapeptides consisting of amino acids building blocks; leucine, alanine, valine and cycloheptapeptides build with leucine, alanine, glycine and phenylalanine showed that these cyclopeptides were not active towards the bacterial strains *Escherichia coli* 25922, *Escherichia coli* 35213, *Staphyloccus aureus* 24213 and *Staphyloccus aureus* 29213.

Preliminary screening of cyclopeptide libraries showed some activity for dengue virus protease inhibition. Results seemed to indicate that cyclohexapeptides have better inhibitory activity on the dengue protease than cycloheptapeptides.

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