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SYNTHESIS AND EVALUATION OF SOME PEPTIDE CHAINS USING THE LIQUID PHASE METHOD AS BIOLOGICALLY ACTIVE SUBSTANCES

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ABSTRACT

Some peptide chains different in lengthes and sequences were synthesized using polyethylene glycol as polymeric support and t-butyl oxy carbonyl group as N-terminal group. p-Bromoethyl benzoyl chloride was used as anchoring group. 18 crown ether-6 was employed to catalyse the esterification of the first amino acid to the anchoring group.The yield of the esterification was improved to 95.5% in case of Butyl oxy carbonyl (BOC) Glycine and 79.2% in case of BOC Tyrosine. The strategy of the liquid phase method was employed.

The antimicrobial activity of the pure synthesized peptides were tested after cleavage of the polymeric support agoinst two Gram + va bacteria, two Gram-ve bacteria, two filamentous fungi and yeast.

INTRODUCTION

The liquid phase method of peptide synthesis using soluble polyethylene glycol was introduced by Bayer et al.[1,2].

Since then, several biological active peptides have been synthesized using this method. It offers many advantages over classical and solid phase methods of peptide synthesis. The use of an insoluble support as COOH-terminal protecting group, as realized in the solid-phase synthesis by Merrifield[3], enables easy separation of excess reagents from the polymer-

bound peptide but suffers from inherent deficiencies induced by the polymeric, heterogeneous matrix. The use of soluble polymeric supports was devised to eliminate some of the short-comings of solid-phase synthesis while retaining the enormous advantages of a polymer-mediated synthesis. From the great number of functionalized linear polymers available, polyethylene glycol (PEG) proved to be most compatible with respect to the physical and chemical properties necessary for "liquid-phase" strategy[4,5]. The liquid-phase method, as developed by Bayer et al[1]is therefore primarily based upon the use of PEG, which can be regarded as solubilizing polymeric protecting group, as the COOH-terminal "liquid support". Viewed from the chemical methodology, liquid-phase synthesis is identical to classical solution methods, however, the presence of a solubilizing macromolecular protecting group affords (i) higher solubility of peptides during stepwise synthesis and (ii) facilitated separation of low- molecular- weight compounds from the polymeric peptide ester.

The present work represents the synthesis of five peptides chains using liquid phase method, aiming to study their antimicrobial activity towards two Gram + ve bacteria, two Gram - ve bacteria, two filamentous fungi and yeast.

RESULTS AND DISCUSSION

The following peptide chains were synthesized using the liquid phase method:

1. H-Glu-Ala-Leu-Val- Lys-Gly-OH.
2. H-Leu-Tyr-Leu-Val-Cys-Gly-OH.
3. H-Lys-Val-Tyr-OH.
4. H-Gly-Ala-Leu-Tyr-Leu-Cys-Gly-OH.
5. H-Gly-Ala-Leu-Tyr-Cys-Gly-OH.

These peptides are co-valently bound to monofunctional polyethylene glycol monomethyl ether (PEGM) of molecular weight 5000. The esterification of the polymer with first amino acid was catalysed by 18 crown-6[6,7]

in DMF at 60 °C, which was found to be the best condition for quantitative esterification. The incorporation of the first amino acid with p-bromomethyl benzoyl polyethylene glycol monomethyl ether in this reaction was resulted in 95.5% yield in case of BOC Gly and 79.2% yield in case of BOC Tyr. Without using of this catalyst the yield of esterification of these two amino acids were 79% nad 68% respectively. This avoid any undesirable side reactions.

All the synthesized peptides were chromatographically separated and purified. Analytical controls were carried out after each step of the synthesis, purity were indicated by amino acid analysis and thin-layer chromatography. All the amino acids were BOC-protected at o -amino group.

The BOC-group was removed by treatment of MPEGA-peptide for 30 min with trifluoroacetic acid-dichloromethane (1 : 1) using (10 ml) of the deprotecting agent per (1.0 g) MPEGA- peptide. The volume of th solution was then reduced by flash evaporation to an oil and the polyethylene glycol-peptide was precipitated by the addition of anhydrous ether under vigorous stirring. The mixture was stirred over 15-30 min at 30°C, the precipitate was filtered, washed with ether, and dried under vacuum. The coupling reactions were carried out by symmetrical anhydride method[8] applying excess anhydride component. To this end, the BOC-protected amino acid derivative was dissolved in a minimum amount of dichloromethane and the solution was cooled to 0 °C, equivalent of dicyclohexylcarbodiimide (DOC) in a 2 M stock solution of dichloromethane was added and the mixture was allowed to stand 30 min at 0 °C. The precipitated dicyclohexylurea was removed by filtering the anhydride solution directly into a flask containing the deprotected aminocomponent in dichloromethane.

The extent of coupling was mentioned first by qualitative UV test on thin-layer plates. Quantitative ninhydrin tests[9] were carried out after isolation of protected poly ethylene glycol- peptide by precipitation. The purity was tested after each step by thin-layer chromatography.

The amino acid analysis of the synthesized peptide:

1. H-Glu-Ala-Leu-Val-Lys-Gly-OH

	Glu	Ala	Val	Lys	Gly	Leu
calculated:	1	1	1	1	1	1
found:	0.89	0.97	1	1.02	1	1

2. H-Leu-Tyr-Leu-Val-Cys-Gly-OH

	Leu	Tyr	Val	Cys	Gly
Calculated:	2	1	1	1	1
found:	2	0.78	1	0.91	1

3. H-Lys-Val-Tyr-OH

	Lys	Val	Tyr
Calculated:	1	1	1
found:	1	1	0.8

4. H-Gly-Ala-Leu-Tyr-Leu-Cys-Gly-OH

	Gly	Ala	Leu	Cys	Tyr
calculated:	2	1	2	1	1
found:	1.9	1	1.98	1	0.77

5. H-Gly-Ala-Leu-Tyr-Cys-Gly-OH

	Gly	Ala	Tyr	Cys	Leu
calculated:	2	1	1	1	1
found:	1.91	1	0.78	0.91	1

Biological Activity:

The antimicrobial properties of five pure synthesized peptides were tested after cleavage of the polymeric support against two Gram +ve bacteria (Bacillus subtilis and Staphylococcus aureus), two Gram-ve bacteria (Escherichia coli and Pseudomonas aeruginosa), yeast (Candide albicans) and two filamentous fungi (Aspergillus niger and Penicillium chrysogenum). Results in the present investigation indicated that no inhibition zones caused on the growth of all tested microorganisms by the water soluble pure peptides at all applied concentrations (500, 1000, 5000 and 10000 ppm). By increasing the incubation period the tested microorganisms were grown on the water soluble synthesized peptides. It can be concluded from these results that the five tested synthesized peptides have no activity against the tested bacteria and fungi, and the tested microorganisms may use these peptides as nutrient substances.

EXPERIMENTAL

1. Amino- acid Analysis: (By using Beckmann Unichrom Analyser)

0.1 (uM) of the peptide was hydrolysed by 3-4 ml 6 N HCl in evacuated sealed tube for 24hr at 110 °C. Then the solvent was removed in vacuum to give a residue analyzed according to Moore method[10].

2. Thin-Layer Chromatography (TLC)[11,12] :

Thin-layer chromatography is useful for estimating the purity both of starting materials for liquid-phase peptide synthesis. TLC plates may be spread using Brinkman Silica Gel G or Silica Gel H, both give essentially the same Rf's.

Thin-layer plates used were Merk Silica Gel 60 with and without fluoresence indicator. The plates were developed by Ninhydrin solution (300 mg Ninhydrin dissolved in 100 ml n- butanol and 3 ml Acetic acid[13,14]

Solvent system used is 1- butanol: acetic acid: water (30: 10: 10)

3. Synthesis:

3.1 Synthesis of BOC-amino-acid[15] :

A suspension of (0.05 mole) of the amino acid and (0.055 mole) of BOC azide[16] in (10 ml) of water and (10 ml) of dioxane, was placed in a vessel of a pH-stat(autotitrator). The reservoir of the pH-stat was filled with (4N) NaOH. The pH control of the instrument was advanced to the point where continued uptake of base indicates that the reaction is proceeding at a reasonable rate. A few amino acids will react at pH 8.5, and most at pH 9.8, a few require pH 10.2 for a reasonable rate of reaction. The reaction is usually completed in a few hours, although the reaction with certain amino acids is quite sluggish and may requires more than 24 hr. The end of the reaction is indicated by cessation of base uptake. Extraction of the solution 3 times with ether to remove unreacted azide, then chilling the aqueous phase in ice, the reaction mixture was then acidified to pH 2 with HCl in a pH-stat, then extracted 3 times with ethyl acetate. The ethyl acetate extract was washed 3 times with small portions of water (saturated NaCl solution for water-soluble derivatives), dry over $MgSO_4$, and evapourate under reduced pressure.

3.2 Purification of MPEG:

MPEG was dissolved in a least amount of distilled methylene chloride and reprecipitated by dropwise addition of dry ether under cooling and vigorous stirring. The precipitate was filtered, washed with dry ether without suction. The process was repeated several times till pure crystalline substance was obtained and dry under vacuo for 2 hr.

TLC; Rf= 0, solvent system 1- butanol (30), acetic acid (10), water (10).

3.3. Synthesis of p-bromomethyl benzoyl chloride[17] :

3.3.1. Synthesis of p-bromomethyl benzoic acid:

Benzoyl peroxide (0.2 g) and N-bromosuccinimide (17.8 g; 100 m.mol) (recrystallized from hot water) were added to a suspension of p-toluic acid (13.6g; 100 m.mol) (recrystallized from $CHCl_3$- MeOH) in dry benzene (100 ml). The mixture was heated under reflux for 24 hr. Removal of the solvent in vacuo gave a white residue which was suspended in (100 ml) of boiling water, collected by filtration and washed with boiling water (4 x 100 ml). The crude product was dried and recrystallized from hot MeOH to give pure acid (17.5 g, 81.4%): mp. 225-227°C (Lit. 224 - 226°C)[18] Anal calcd. for $C_8H_7BrO_2$: C, 44.68, H, 3.28; Br, 37.15 found : C, 44.50; H, 3.18; Br, 37.02.

3.3.2. Synthesis of the acid chloride:

(10.75 g; 0.05 M) of p-bromomethyl benzoic acid was refluxed with (0.075m) thionyl chloride for 6 hr, the excess of the thionyl chloride was distilled over a water bath. The product was crystallized from Pet. ether (60 - 80%) to give the pure acid chloride (10.05 g. -90%) mp 57°C (Lit. 56°C)[19].

4. Esterification of p-bromomethyl benzoyl chloride with MPEG:

(50 g; 10mM) MPEG and (7g, 30 mM) p-bromomethyl benzoyl chloride were dissolved in (500 ml) dry toluene and refluxed in a three necked flask in nitrogen atmosphere for 24 hr. The excess of toluene was distilled under vacuo till the total volume becomes (60 ml) after cooling, the product was crystallizd by dropwise addition of dry ether during vigorous stirring, then filtered, redissolved in methylene chloride and reprecipitated by dry ether. The last step was repeated twice till pure product was obtained.

TLC; Rf = O, solvent system l-butanol (30), acetic acid (10), water (10).

5. Synthesis of BOC amino acid potassium salt[20] :

(1.0 g) of a BOC amino acid was dissolved in a mixture of ethanol (6 ml), H_2O (4 ml) and 1.0 equivalent of a (1.0 N) KOH solution, the solvent

was removed by azeotropic distillation in the presence of toluene and dried in vacuo over P_2O_5, the resultant hygroscopic white salt was used without further treatment.

5. Coupling reaction of potassium salts of BOC amino acids with p-bromomethyl benzoyl polyethylene glycol monomethyl ether (MPEGA) catalyzed by 18- crown-6:

6.1. Coupling reaction of p-bromomethyl benzoyl polyethylene glycol monomethyl ether with different BOC amino acid potassium salts:

A mixture of (1 mM) BOC amino acid potassium salt, (2.61 g; 0.5 mM) MPEGA and (0.279; 1 mM) 18-crown-6 were dissolved in DMF and refluxed for 48 hr at 60°C. The reaction mixture then, dried under vacuo and the residue was dissolved in CH_2Cl_2 and reprecipitated by dry ether with cooling and vigorous stirring. Then, the product was filtered, dried and redissolved several times in CH_2Cl_2 and reprecipitated by dry ether till chromatographically pure product was obtained. The yield of incorporations are about 100%.

For cleavage of BOC group, 12 g of MPEGA- bound peptide dissolved in (120 ml) TFA CH_2Cl_2 (1:1) and the solution was stirred for 30 min at RT. Then, the reaction mixture was distilled under vacuum, the produced oil was dissolved in CH_2Cl_2 and the product was precipitated by dropwise addition of dry ether under cooling and vigorous stirring. The pure product was filtered and dried under vacua, yield 91.7%.

TLC: Rf = O, solvent l-butanol (30), acetic acid (10), water (10).

5.2. Cupling reaction of Gly with the second amino acid:

(2 mM) MPEGA- Gly was dissolved in 20 ml dichloromethane, in another flask, (10 mM) of the second amino acid was dissolved in (10 ml) dichloromethane and cooled to 0°C, 0.48 equivalent (5 mM) of dicyclohexylcarbodiimide dissolved in dichloromethane was added and the mixture was allowed to stand for 30 min at 0°C while stirring. The precipitate of dicyclohexyl urea was removed by filtrating the anhydride solu-

tion directly into the flask containing the deprotected amino component in dichloromethane with slow stirring. The above solution was neutralized by N-methyl morpholine till pH=7, then the solution was concentrated to about (10 ml) and stirred over night at RT. The product was precipitated by dropwise addition of dry ether under vigorous stirring while cooling, then the product was recrystallized twice till pure crystals were obtained. The coupling was controlled by quantitative ninhydrine test.

TLC; Rf = 0, solvent system l-butanol (30) acetic acid (10), water (10).

other amino acids were coupled by the above described method.

7. Investigation of antimicrobial activity:

The antimicrobial activity for each of the five water soluble synthesized peptides at different applied concentrations (500, 1000, 5000 and 10000 ppm) were investigated against 2 Gram +ve bacteria (Bacillus subtilis and Staphylococcus aureus), 2 Gram-ve bacteria (Escherichia coli and Pseudomonas aeruginosa), two filamentous fungi (Aspergillus niger and Penicillium chrysogenum) and yeast (Candide albicans).

The bacterial tested organisms were cultivated on nutrient agar medium, where as yeast and moulds were cultivated on malt extract agar and Czapek's Dox agar media, respectively according to Harrigan and McCane[21].

The antimicrobial activity of the studied peptides were assayed by measuring the inhibition zones of microbial growth caused by known volume of the water soluble peptide. The method was essentially as foolows:

Petri-dishes containing base layer of (2%) agar in water and an upper layer of the specific culture medium seeded with the tested microorganism. Hold glass microfibre filters discs (0.6 cm) with saturated known volume (0.1 ml) of each different concentration of water soluble peptide put on the surface of the medium. Then, the plates were incubated at 37°C for 2 days for bacteria, and at 28°C for 4 days for yeast and fungi, and the diameter of inhibition zones were measured.

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