

## **A new synthesis of the tripeptide Gly-His-Lys with antimicrobial activity**

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**Summary.** A new solid phase synthesis of the growth-modulating tripeptide Gly-His-Lys is described. 2-Chlorotrityl chloride resin and 9-fluorenylmethoxycarbonyl-(Fmoc), 4-methyltrityl-(Mtt) protecting groups were used. The synthetic tripeptide was tested for its activity against bacteria, yeast and fungi. The *in vitro* effect of the tripeptide on DNA, RNA and protein synthesis was studied as well.

**Keywords:** Amino acids – Tripeptide GHK - Synthesis - Antimicrobial activity

### **Introduction**

Gly-His-Lysine (GHK) is a tripeptide found in normal human plasma (Pickart et al., 1973; Schlesinger et al., 1977). Synthetic GHK was found to accelerate growth in cultured cells and tissues e.g. hepatoma cells (Pickart et al., 1973), neurons and glial cells (Sensenbrenner et al., 1975) macrophages (Joseph et al., 1978), mast cells (Mazingue et al., 1978), fibroblasts (Slotta et al., 1975) and lymphocytes (Dessaint et al., 1977).

Isolation and purification of the growth-modulating peptide GHK from natural sources (e.g. serum) has not been very successful due to co-isolation of copper and iron with the peptide (Pickart et al., 1979). The solid phase method of Stewart and Young (1959) modified by Gutte and Merrifield (1971) has been used so far for the synthesis of GHK.

Here, we report another solid phase synthesis of the tripeptide GHK and its *in vitro* antimicrobial activity against bacteria, fungi and yeast. Moreover we describe the effect of GHK on DNA, RNA and protein synthesis of bacterial cells.

### **Materials and methods**

2-Chlorotrityl chloride resin, Fmoc-Lys(Mtt)-OH, Fmoc-Gly-OH and Fmoc-His(Mtt)-OH were purchased from Chemical and Biopharmaceutical Labs, Technological Park

(Patra, Greece). All other reagents and solvents used were obtained from Sigma Chem. Co (Germany). TLC was performed on silica gel F<sub>254</sub> plates (d, 0.1 mm, Merck, Germany). Amino acid analysis was done on a Beckman model C200 Analyzer. The acid hydrolysis of the final tripeptide was carried out at 110°C under a nitrogen vacuum atmosphere for 22 h. W-1 single column and AA15 for basic amino acids was used. HPLC analysis was performed on a Lichrosphere RP-C8 column, 5 m. Flow speed 0.6 ml/min, A220 nm, paper speed 2 mm/min. Solvent 70:30 methanol/water. Optical density measurements were taken on a Hitachi U-1100 spectrophotometer. High resolution mass spectra were recorded on a TS-250 VG spectrometer. Radioactivity was measured in a Packard liquid Scintillation Analyzer (Tri-Carb 1500TR).

### *Chemistry*

Acid labile 2-chlorotrityl chloride resin was used for the solid phase synthesis of the tripeptide GHK. Fmoc-Lys(Mtt)-OH was esterified to the resin with a procedure similar to that reported for other amino acids (Barlos et al., 1991). Incorporation of the second amino acid Fmoc-His(Mtt)-OH was done with DCCI/HOBt at 0°C in the presence of diisopropylamine (DIPA).

The same procedure was followed for the addition of the third amino acid Fmoc-Gly-OH. Quantitative determination of free amino groups during solid-phase synthesis of the tripeptide was performed by the ninhydrin reaction (Sarin et al., 1981).

Base labile Fmoc-blocking group, in all steps of the synthesis, was removed with piperidine 25% in dichloromethane. Side chain functional protecting group (Mtt) was removed at the end of the synthesis with trifluoroacetic acid (TFA) 50% in dichloromethane, with simultaneous cleavage of the tripeptide from the resin. The tripeptide TFA salt was precipitated by ether and recrystallized from methanol/ether.

Tripeptide free base (GHK), was obtained after treatment of the correspondent TFA salt with 0.1 N KOH and extraction with ethyl acetate. It was further purified by HPLC on a Lichrosphere RP-C8 column and identified by amino acid analysis and FAB mass spectrum.

### *Synthesis*

#### Attachment of Fmoc-Lys(Mtt)-OH to the resin

2-Chlorotrityl chloride resin (1 g) was swelled in 10 ml DMF for 3 min. Then a solution of 0.6 g (1 mmol) of N,N'-diisopropylethylamine (DIPA) in 5 ml DMF was added to the resin and the mixture was stirred for 30 min at room temperature.

The reaction was completed by the addition of 20 ml of methanol/trifluoromethanol (2:1) and immediate filtration. Subsequently the Fmoc-Lys(Mtt)-resin was washed (3 × DMF, 3 × 2-propanol, 2 × methanol, and 3 × ether) and dried in vacuo at room temperature (1.35 g).

#### Fmoc-Gly-His(Mtt)-Lys(Mtt)-resin

After the attachment of lysine to the resin, the desired tripeptide was synthesized by addition of Fmoc-His(Mtt)-OH and Fmoc-Gly-OH respectively. Coupling was performed with DCCI/HOBt, at 0°C in the presence of DIPA. The yields were respectively 67% for the dipeptide and 48% for the tripeptide.

#### Cleavage of the peptide from the resin

Removal of 4-methyltrityl groups and subsequent cleavage of the tripeptide from the resin was achieved by treatment of the tripeptide-resin (1.2 gr) with 5 ml 50% TFA in dichloromethane for 5 min followed by immediate filtration. The resin was washed with methanol (3 × 20 ml) and the filtrate after evaporation precipitated by ether. It was recrystallized from methanol/ether to afford a white solid material.

Tripeptide GHK, was obtained after treatment of the correspondent tripeptide TFA salt with equimolar amount (plus 10% excess) 0.1N KOH and extraction with ethyl acetate. After evaporation of organic layer, the remaining oily material was purified by HPLC. Fractions corresponding to GHK were collected, dried in vacuo and kept at 4°C. Amino acid analysis gave G, H, K at a 1:0.97:1.02 ratio respectively. Its molecular formula  $C_{14}H_{24}O_4N_6$  was determined by high resolution MS ( $m/z$  340).

#### Antimicrobial activity measurements

Antibacterial activity was evaluated by means of the Minimal Inhibitory Concentration (MIC), using the method of progressive double dilution in liquid media (Manousakis et al., 1987; Stokes 1975). Bacterial strains were obtained on Luria Bertani broth and agar (LB broth/agar). Tests were carried out using Mueller Hinton broth (BBL), LB broth and Minimal Salts broth. Inocula were about  $10^7$  cells/ml. The tripeptide was dissolved in distilled water (10mg/ml) and kept at 4°C. Further dilution in the medium was done in the range of 6–200  $\mu$ g/ml. Measurements were taken after 7, 10 and 24 h of incubation at 37°C.

MIC method was also used for measurements of antimicrobial activity of GHK against yeast and fungi. The cultures were obtained on Sabouraud Dextrose agar and tests were carried out on Sabouraud broth. Inocula were about  $10^6$  cells for yeast and  $10^6$  spores for fungi. Data were recorded after 48h for yeast and 4 days for fungi. Incubation was carried out at 28°C (Porretta et al., 1993).

#### Determination of the rate of DNA, RNA and protein synthesis

Bacterial cultures, after 3 1/2 h of incubation with various concentrations of GHK, were labeled with (methyl- $^3$ H)-thymidine, ( $^3$ H)-uridine and DL-(4,5- $^3$ H) leucine, 1 $\mu$ Ci/ml for 35 minutes. Radioactivity was measured by acid precipitation method as it was described by Ausubel et al. (1987).

### Results and discussion

The percentage of lysine attached to the resin was determined under various solvents. The results are shown at Table 1. 4-Methyltrityl group, which has been used for the protection of the side chain carboxamide of asparagine and glutamine (Sax et al., 1992) proved to be a good protective group for  $\epsilon$ -amino group of lysine and imino group of imidazole ring of histidine as well.

Coupling reactions reported here gave the highest yield on the basis of many experimentations under different conditions. Quantitative cleavage of the tripeptide from the resin was achieved at the time of 5 min whereas shorter

**Table 1.** Resin loading by Fmoc-Lys(Mtt)-OH

| Solvent | % Esterification of<br>Fmoc-Lys(Mtt)-OH |
|---------|-----------------------------------------|
| DCM     | 77                                      |
| DMF     | 84                                      |
| THF     | 69                                      |
| Dioxane | 38                                      |

**Table 2.** Antibacterial screening data, 10h of incubation ( $OD_{600}$ ), with 50  $\mu$ g/ml of GHK

|       |    | <i>B. cereus</i> | <i>S. aureus</i> | <i>X. campestris</i> | <i>E. coli</i> |
|-------|----|------------------|------------------|----------------------|----------------|
| GHK   |    | 1.180            | 0.870            | NM                   | 0.590          |
| "     | LB | 1.178            | 1.132            | NM                   | 0.570          |
| "     | MH | 0.385            | 0.612            | NM                   | 0.063          |
| "     | MS | 1.243            | 0.950            | 0.396                | 0.640          |
| Blank |    | 1.125            | 1.253            | 0.447                | 0.632          |
| "     | LB | 0.400            | 0.516            | 0.246                | 0.321          |
| "     | MH |                  |                  |                      |                |
| "     | MS |                  |                  |                      |                |

Average values of 3 measurements are presented.  
*NM* no measurements.

**Table 3.** Antibacterial screening data, 24h of incubation ( $OD_{600}$ ) with 50  $\mu$ g/ml of GHK

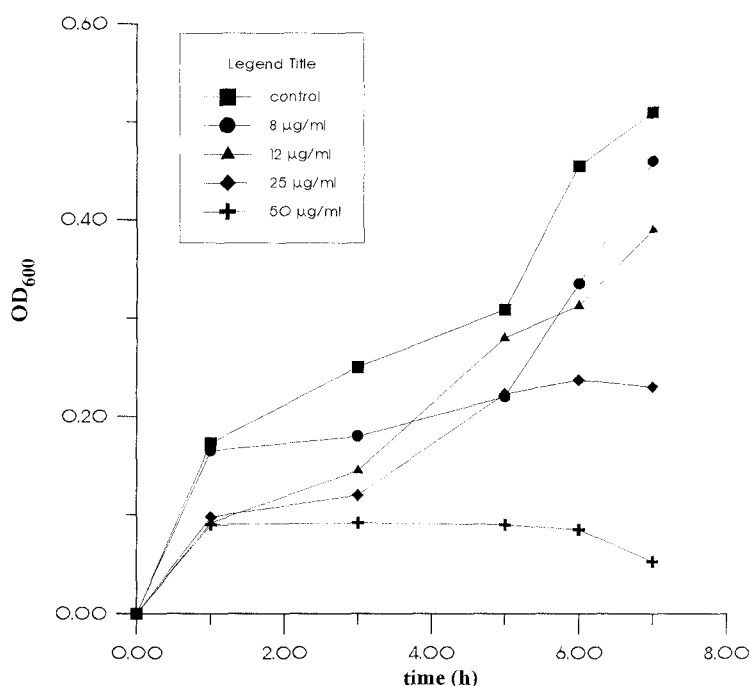
|       |    | <i>B. cereus</i> | <i>S. aureus</i> | <i>X. campestris</i> | <i>E. coli</i> |
|-------|----|------------------|------------------|----------------------|----------------|
| GHK   |    | 1.626            | 1.618            | 0.940                | 1.348          |
| "     | LB | 1.520            | 1.520            | 0.640                | 1.066          |
| "     | MH | 0.895            | 1.008            | 0.570                | 0.338          |
| "     | MS | 1.243            | 0.950            | 0.983                | 1.324          |
| Blank |    | 1.125            | 1.253            | 0.447                | 1.203          |
| "     | LB | 0.930            | 0.516            | 0.530                | 0.980          |
| "     | MH |                  |                  |                      |                |
| "     | MS |                  |                  |                      |                |

Average values of 3 measurements are presented.

or longer splitting times led to either incomplete cleavage or to a lot of by-products as it can be judged by TLC in different solvent systems.

In conclusion, in the new synthesis of the tripeptide GHK that we report here, the side chain protection of lysine and imidazole with 4-methyltrityl (Mtt) group represents advantages in comparison with the older groups used (e.g. carbobenzoxy, used for side chain of lysine) in terms of ease of removing them, yields and lack of side products in the deprotection step and cleavage of the tripeptide from the resin.

Antibacterial screening data on Gram-positive bacteria *Bacillus cereus* ATCC 11778 and *Staphylococcus aureus* and Gram-negative bacteria



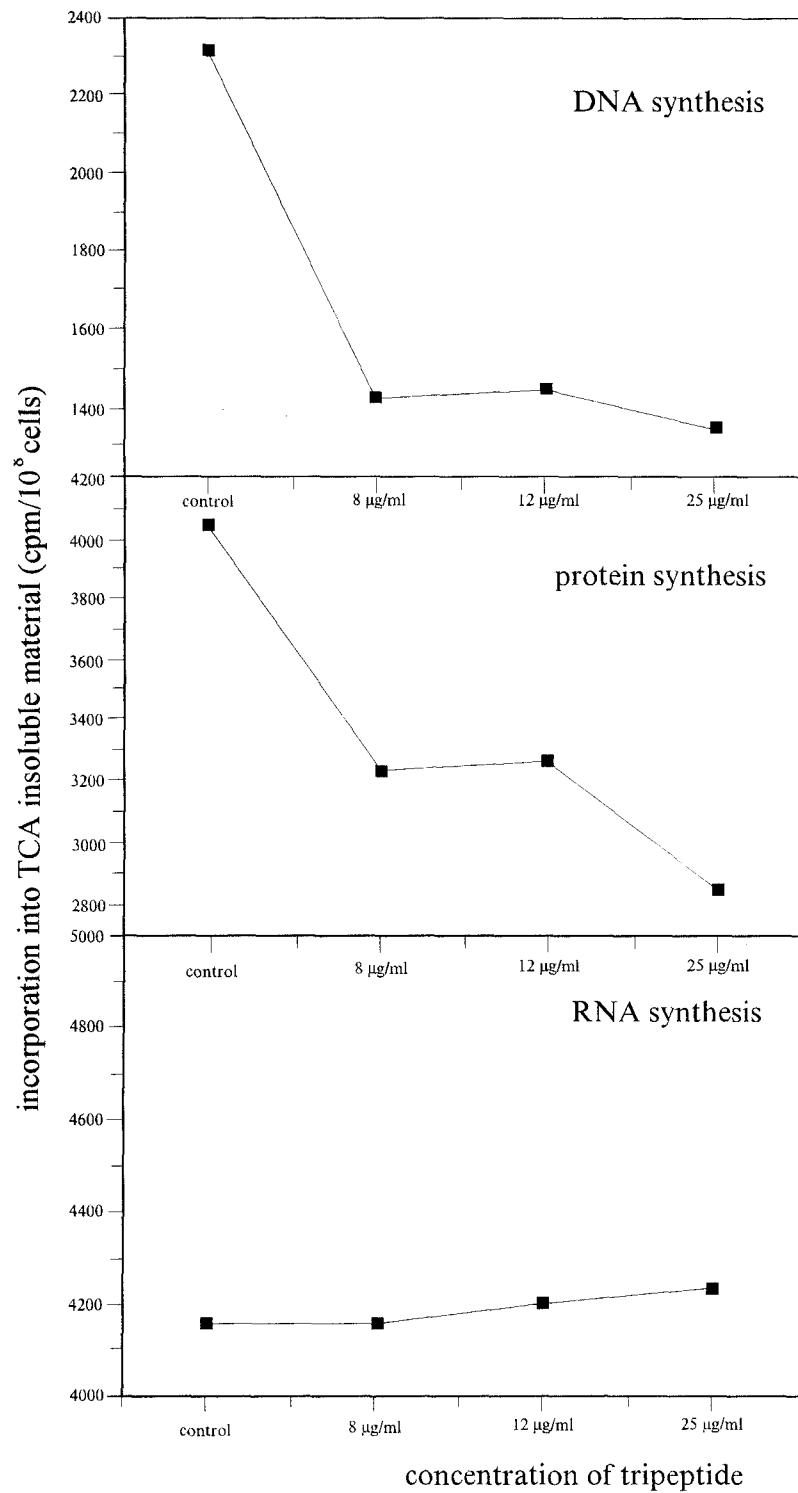
**Fig. 1.** Time and concentration dependent effect of GHK on growth of *E. coli* in MS broth. Equal samples of *E. coli* cultures (20ml each) were placed in 250ml flasks supplemented with 8, 12, 25 and 50 µg/ml of GHK. Control culture was also used

*Xanthomonas campestris* ATCC13951 and *Escherichia coli* DH5a obtained after 10h of incubation, indicated that the tripeptide GHK at a concentration of 50 µg/ml inhibits the growth of *E. coli* only in minimal salts broth which contains 0.1% yeast extract (Table 2). No growth inhibitory effect is observed with *B. cereus*, *S. aureus* and *X. campestris* at the same time of incubation. Figure 1 shows the effect of GHK at different concentrations on the rate of growth of *E. coli*. The minimal inhibitory concentration (MIC) was found to be 50 µg/ml. Interesting enough, the inhibitory effect of tripeptide on *E. coli* cultures appears at low protein concentration in the medium. Based on the data of Table 3 (24h incubation) GHK rather stimulates the growth of *B. cereus* and *S. aureus* and the effect is similar to that observed in neurons and glial cells.

Furthermore, it was found that after 24h incubation, the tripeptide at the same concentration of 50 µg/ml, retards the rate of growth of *E. coli*.

Antifungal (on *Aspergillus spp*, *Microsporum canis*, *Trichophyton mentagrophytes*) and yeast (on *Candida albicans*, *Rhodotolura spp*) screening (data not shown) indicated that GHK does not inhibit the growth of yeast and fungi even at high concentrations 100-200 µg/ml.

Pulse-labeling experiments of *E. coli* cultures with radio-labeled thymidine (for DNA synthesis) uridine (for RNA synthesis) and leucine (for protein synthesis) showed that GHK inhibits both DNA and protein synthesis and does not affect RNA synthesis (Fig. 2).



**Fig. 2.** Concentration dependent effect of GHK on the rate of DNA, RNA and protein synthesis

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