Journal of Peptide Science
J. Peptide Sci. **8**: 118–124 (2002)
DOI: 10.1002/psc.374

The Production of Anti-Hexapeptide Antibodies which Recognize the S7, L6 and L13 Ribosomal Proteins of *Escherichia coli*

ARISTOMENIS METAXAS,[a] SOCRATES TZARTOS[b] and MARIA LIAKOPOULOU-KYRIAKIDES[a]*

[a] Department of Chemical Engineering, Section of Chemistry, Aristotle University of Thessaloniki, Greece
[b] Hellenic Pasteur Institute, Athens, Greece

Received 23 November 2001
Accepted 26 November 2001

Abstract: Here we report the synthesis of the *N*-terminal hexapeptide H-Pro-Arg-Arg-Arg-Val-Ile-OH of the *E. coli* ribosomal protein S7, the *C*-terminal hexapeptide H-Lys-Glu-Ala-Lys-Lys-Lys-OH of L6 and the *C*-terminal hexapeptide H-Pro-Gln-Val-Leu-Asp-Ile-OH of L13. All peptides were prepared by SPPS following the Fmoc-strategy, using DIC/HOBt and/or HBTU as coupling reagents and 2-chlorotrityl chloride resin as the solid support. The carrier linked synthetic peptides were injected into rabbits and elicited an anti-peptide response. These anti-hexapeptide antibodies were found to recognize the corresponding peptides and proteins.

Keywords: synthetic surface peptides; ribosomal proteins; *Escherichia coli*; ovalbumin; anti-hexapeptide antibodies; ELISA; immunoblotting

INTRODUCTION

Synthetic peptides are used extensively for the induction of antibodies specific for the corresponding primary amino acid sequences in intact proteins [1–4]. Therefore, antibodies for relevant surface-exposed sequences of ribosomal proteins from *E. coli* are valuable probes for studying the fine structure at the surface of the ribosome and its functions [5–10].

The S7 ribosomal protein of the small subunit of the *E. coli* ribosome consists of 177 amino acids and interacts specifically with 16S RNA [11]. Furthermore, the *N*-terminal decapeptide is located close to or at the peptidyltransferase centre [12]. L6 is an ubiquitous protein and has been found in prokaryotes, archaebacteria and eukaryotes with a highly conserved primary structure. In *E. coli* the *C*-terminal domain of L6 protein is associated with 23S ribosomal RNA and it also influences the acyl-tRNA binding site [13]. Protein L13 seems to have contact points with widely separated regions of the 23S RNA within the subunit. Studies on the 50S assembly *in vitro* revealed that the L13 protein is essential for the formation of the RI^*_{50} intermediate particle. Omission of L13 prevents the formation of this intermediate and of the 50S particle [14].

Here we report the synthesis, for the first time, of three hexapeptides corresponding respectively to the *N*-terminal sequence of the S7 and the *C*-terminal sequences of the L6 and L13 ribosomal proteins of *E. coli*. Conjugates of the peptides with ovalbumin were prepared and used to induce anti-peptide antibodies.

MATERIALS AND METHODS

Materials

All amino acid derivatives and 2-chlorotrityl-chloride resin were purchased from the Chemical and

* Correspondence to: Dr M. Liakopoulou-kyriakides, Department of Chemical Engineering, Section of Chemistry, Aristotle University of Thessaloniki, Thessaloniki 54006, Greece.
Contract/grant sponsor: Institution of National Scholarships, Greece.

Biochemical Laboratories of Patras (Greece). All other reagents and the solvents were purchased from Sigma Chemical Co (Germany) and used without further purification.

Synthesis of H-Pro-Arg-Arg-Arg-Val-Ile-OH (I)

Fmoc-Ile-O-resin. 2-chlorotrityl chloride resin (0.5 g, 0.8 mmol active chloride) was swelled in 5 ml DCM. Fmoc-Ile-OH (177 mg, 0.5 mmol) and DIEA (0.35 ml, 2 mmol) were added and the reaction mixture was stirred for 25 min at room temperature. Subsequently, MeOH : DIEA 9 : 1 (1 ml) was added in order to destroy the excess of active chloride on the resin and stirring was continued for a further 15 min. The resin was filtered, washed with 2 × DCM : MeOH : DIEA (92 : 5: 3), 3 × DCM, 2 × isopropanol and 2 × diethyl ether and dried *in vacuo* for 24 h. The yield was 75%.

H-Ile-O-resin. Fmoc-Ile-O-resin (0.64 g) was treated with 6 ml of 20% piperidine in DMF for 25 min at room temperature. The resin was filtered, washed with 3 × DMF, 2 × isopropanol, 3 × DMF, 2 × isopropanol, 1 × diethyl ether and dried *in vacuo* for 24 h.

Fmoc-Val-Ile-O-resin. Fmoc-Val-OH (339 mg, 1 mmol, 3-fold excess of the resin substitution) and HOBt (135 mg, 1 mmol) were dissolved in 1 ml DMF : DCM 1 : 1 and chilled at 0 °C. DIC, (155 µl, 1 mmol) was added and the resulting solution was stirred for 15 min at 0 °C and for a further 10 min at room temperature. The precipitated DIU was filtered, washed with DMF and the combined filtrates containing Fmoc-Val-benzotriazolylester were transferred to the solid phase reactor containing the corresponding deprotected peptide-resin. NMM (110 µl, 1 mmol) was added and the reaction mixture was stirred for 24 h at room temperature. After coupling, the resin was washed with 3 × DMF, 2 × isopropanol, 3 × DMF, 2 × isopropanol, 1 × diethyl ether and dried *in vacuo* for 24 h. The yield of the reaction was estimated with Kaiser's test and found to be ~99%.

H-Pro-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Val-Ile-O-resin. Following the above procedure, Fmoc-Arg-(Pbf)-OH and Fmoc-Pro-OH were added and the final hexapeptide, obtained as Fmoc-Pro-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Val-Ile-O-resin was treated with piperidine, as mentioned above, for the removal of the *N*-terminal protecting group.

H-Pro-Arg-Arg-Arg-Val-Ile-OH. The partially protected peptide-resin ester was cooled in an ice bath and treated with a cool mixture of TFA : phenol : thioanisole : H_2O : EDT 82.5 : 5 : 5 : 5 : 2.5 (10 ml) for 2 h at room temperature. The resin was separated by filtration and washed with DCM (2 × 10 ml). The combined filtrates were concentrated *in vacuo* to a volume of 1–2 ml and the residual solution was diluted with a ten-fold excess of H_2O. Scavengers were extracted with an equivalent amount of cold Et_2O and the peptide was obtained as a solid material by lyophilization.

H-Lys-Glu-Ala-Lys-Lys-Lys-OH (II)

This was synthesized following steps similar to the above procedure and obtained as Fmoc-Lys(Boc)-Glu(Bu^t)-Ala-Lys(Boc)-Lys(Boc)-Lys(Boc)-resin. It was deprotected with TFA : H_2O 95 : 5 and precipitated by addition of cold Et_2O.

H-Pro-Gln-Val-Leu-Asp-Ile-OH (III)

This was obtained as Fmoc-Pro-Gln(Trt)-Val-Leu-Asp-(Bu^t)-Ile-O-resin. It was deprotected using the mixture TFA : H_2O : EDT 95 : 2.5 : 2.5 and isolated as described above for peptide I.

The three hexapeptides were also synthesized using HBTU as the coupling reagent and the yields were comparable.

HPLC Purification

The fully deprotected peptides were purified by RP-HPLC on a C18 column, under isocratic conditions with H_2O/acetonitrile 70 : 30, containing 0.1% TFA as eluent, flow rate 1 ml/min, at room temperature. The UV detector was set at λ 210 nm.

N-terminal Sequencing and Mass Spectrometry

Peptides I, II and III were analysed by amino acid hydrolysis with 6N HCl at 110 °C, *N*-terminal sequencing and nanoelectrospray tandem mass spectrometry. *N*-terminal sequence analysis was performed in a PE Biosystems sequencer (Procise 494; Foster City, CA, USA).

For mass spectrometric analysis, the peptides were desalted and concentrated on a pulled capillary, containing approximately 100 nl of POROS R2 reverse phase material (Perseptive Biosystems, Framingham, MA), and eluted with 1 µl of 60% methanol in 5% formic acid directly into the nanoelectrospray needle. Electrospray mass spectra were

acquired on an API 365 triple quadropole mass spectrometer (PE-Sciex, Torondo, Canada) equipped with a nanoES ion source. Q1 scans were performed with a 0.2 Da mass step. For operation in the MS/MS mode, Q1 was set to transmit a mass window of 2 Da and spectra were accumulated with 0.2 Da mass steps. Resolution was set so that the fragment masses could be assigned with an accuracy better than 1 Da.

Carrier-linked Peptides

Peptide I with ovalbumin (method A). One ml of citraconic anhydride solution in H_2O (3.6 mg) was added dropwise to 1 ml of peptide I solution in H_2O (3.6 mg/ml) and the mixture was incubated at room temperature for 1 h. The pH was adjusted to 8.5 with NaOH, 20 mg of 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) was added and the mixture was stirred for 5 min. Subsequently, 5 mg of ovalbumin was added and the reaction mixture was incubated at room temperature for 4 h. The reaction was terminated by adding sodium acetate buffer, pH 4.2, to a final concentration of 100 mM and stirring for 1 h. The conjugate was separated from the non-reacted peptide by dialysis, first against sodium acetate buffer and then against PBS (four changes overnight).

Peptides II and III with ovalbumin (method B). Peptide II (10 mg) was dissolved in PBS (100 µl) and 33 µl of this solution was added to 5 mg ovalbumin in 2 ml of PBS. An equal volume (2 ml) of 0.2% glutaraldehyde in PBS was added dropwise and the mixture was incubated at room temperature for 1 h. The reaction was stopped by adding glycine 1 M in PBS (pH 7.2) to a final concentration of 100 mM and stirring for 1 h. The conjugate was separated from the non-reacted peptide by dialysis against PBS (four changes overnight).

The same procedure was followed for the conjugation of peptide III with ovalbumin, but with a higher concentration of glutaraldehyde (2%).

Immunization Procedure

Six-month old New Zealand rabbits received subcutaneous injections of 1 ml peptide-ovalbumin conjugate emulsified with 1 ml Freund's complete adjuvant. After 1, 2 and 3 months, the rabbits were boosted with an equal quantity of conjugate emulsified with 1 ml Freund's incomplete adjuvant. Blood samples were obtained 6 days after each boost. The blood was centrifuged and the serum was stored at −20 °C.

Purification of the Antisera

Each antiserum was centrifuged at 3000 rpm for 30 min for the removal of debris. Subsequently, while the antibody solution was being stirred in an ice bath, an equal volume of saturated ammonium sulphate solution was added and stirring was continued for 1 h at 0 °C. The solution was centrifuged at 3000 rpm for 30 min, the supernatant was discarded and the pellet was resuspended in half of the starting volume of the antiserum with PBS. Finally, the solution was dialysed against PBS (three changes overnight) and centrifuged at 3000 rpm for 15 min.

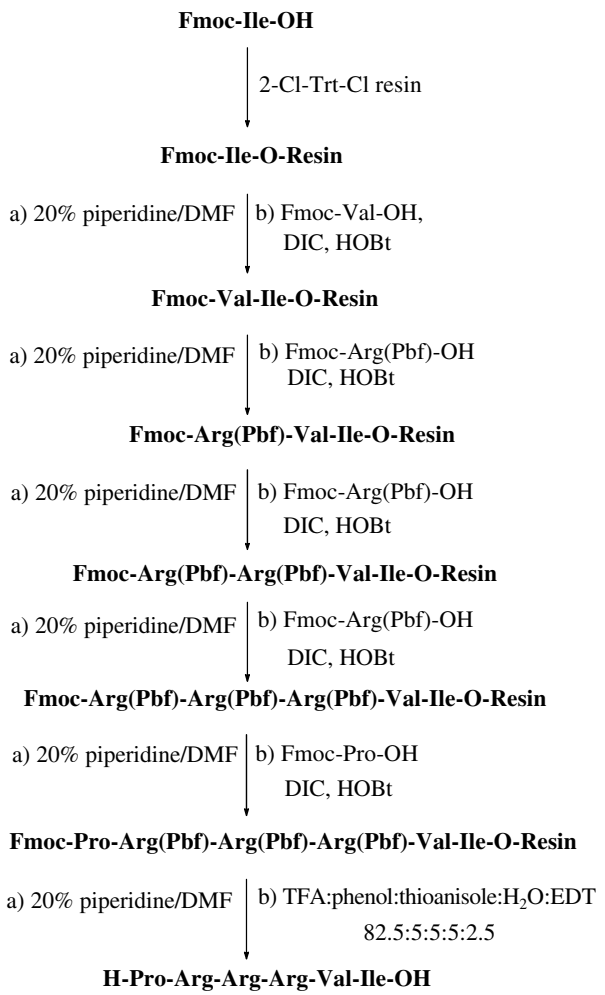
ELISA Assessment of Peptide Binding

Wells of microtitre plates (MaxiSorp F96, Nunc) were coated, by overnight incubation at 4 °C, with 1 µg peptide per well in 100 µl of 50 mM sodium carbonate buffer, pH 9.6. The coated plates were washed with PBS (3 × 10 ml), and then the wells were saturated with 200 µl of PBS-2.5% BSA for 1 h at room temperature. After PBS washes (3 × 10 ml), antiserum diluted (1/100 and 1/1000) in 100 µl of PBS-0.2% BSA was added to the wells and incubated for 3 h at room temperature. After further washes with PBS-0.05% Tween (1 × 10 ml) and PBS (1 × 10 ml), 100 µl of goat anti-rabbit IgG conjugated with horseradish peroxidase, diluted (1/1500) in PBS-0.2% BSA was added and incubated for 1.5 h at room temperature. The wells were washed with PBS-0.05% Tween (3 × 10 ml) and with PBS (3 × 10 ml), then the bound sera were measured by adding the peroxidase substrate, containing 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and H_2O_2. The colour developed approximately after 20 min and was measured at 405 nm using a microtitre plate reader.

Immunoblotting

Ribosomal proteins from the large and the small subunits of *E. coli* were separated by SDS-PAGE electrophoresis on 15% acrylamide/bisacrylamide (29 : 1) gel, under reducing conditions, electrotransferred to a nitrocellulose membrane and subjected to immunoblotting.

The membrane was incubated initially in 10 ml PBS-5% milk for 1.5 h and washed with phosphate-buffered saline (PBS-0.05%) Tween 20 (polyoxyethylene(20)sorbitan monolaurate) (2 × 10 ml). Subsequently, antiserum was added, diluted (1 : 1000) in 10 ml PBS-5% milk and incubated for 2 h at room temperature. After washes with PBS-0.05% Tween (2 × 10 ml), goat anti-rabbit IgG, labelled with alkaline phosphatase was added, diluted (1 : 2000) in 10 ml PBS-5% milk-0.05% Tween and incubated for 2 h at room temperature. The membrane was washed with PBS-0.05% Tween (2 × 10 ml) and with PBS (1 × 10 ml) and finally the bound proteins were detected (stained) adding the alkaline phosphatase substrate, containing 66 µl NBT (nitroblue tetrazolium) and 33 µl BCIP (5-Bromo-4-chloro-3-indonyl phosphate) in 10 ml of the alkaline phosphatase buffer.



Scheme 1 Synthesis of hexapeptide I.

RESULTS

Scheme 1 shows diagrammatically the synthesis of peptide I, using DIC/HOBt as coupling reagents. Similar synthetic routes were used for peptides II and III. The overall yield of synthesis for peptides I, II and III was found to be 29%, 38% and 35%, respectively. In the case of HBTU, the yields were slightly higher, 32%, 40% and 43%, for peptides I, II and III, respectively. Anchoring of the *C*-terminal amino acids to the resin gave a 75% yield for Fmoc-Ile-OH (peptides I and III) and 70% for Fmoc-Lys(Boc)-OH (peptide II).

The fully deprotected peptides were analysed by amino acid hydrolysis, *N*-terminal sequencing and HPLC. Amino acid analysis yielded a satisfactory correspondence between experimental and expected values for all peptides. Figure 1 shows the HPLC separation of peptide III as obtained after final deprotection and isolation. Peak 6 (with a retention time of 3.27 min) corresponds to peptide III, as judged by amino acid analysis and electrospray mass spectra. Peptides I and II were eluted, under the same experimental conditions with retention times of 3.45 and 2.18 min, respectively. Mass spectra of the purified products gave $(M + H)^+$ at 796 corresponding to peptide I, $(M + H)^+$ at 731 for II and 685 for III.

The study of the anti-peptide antisera against the synthetic peptides and the corresponding ribosomal proteins was carried out by ELISA and immunoblotting, respectively. ELISA tests showed that the anti-peptide sera for peptides I and II recognize the corresponding peptides and there is also cross-reaction between antisera and peptides.

Figure 2 shows the immunoblots of the ribosomal proteins of *E. coli* with anti-peptide sera I, II and III. According to this figure, analysed with the computer program GelPro Analyzer, antiserum I recognizes ribosomal proteins with molecular weights ~20 kDa and 17.5 kDa from the small subunit of the *E. coli* ribosome, which presumably correspond to S7 and S5, respectively. Antiserum II seems to recognize the ribosomal protein L6 (18.7 kDa), while antiserum III recognizes L13 (16 kDa).

DISCUSSION

In this report, we describe the synthesis of the *N*-terminal hexapeptide H-Pro-Arg-Arg-Arg-Val-Ile-OH of the *E. coli* ribosomal protein S7, the *C*-terminal hexapeptide H-Lys-Glu-Ala-Lys-Lys-Lys-OH of L6 and the *C*-terminal hexapeptide H-Pro-Gln-Val-Leu-Asp-Ile-OH of L13, the production of

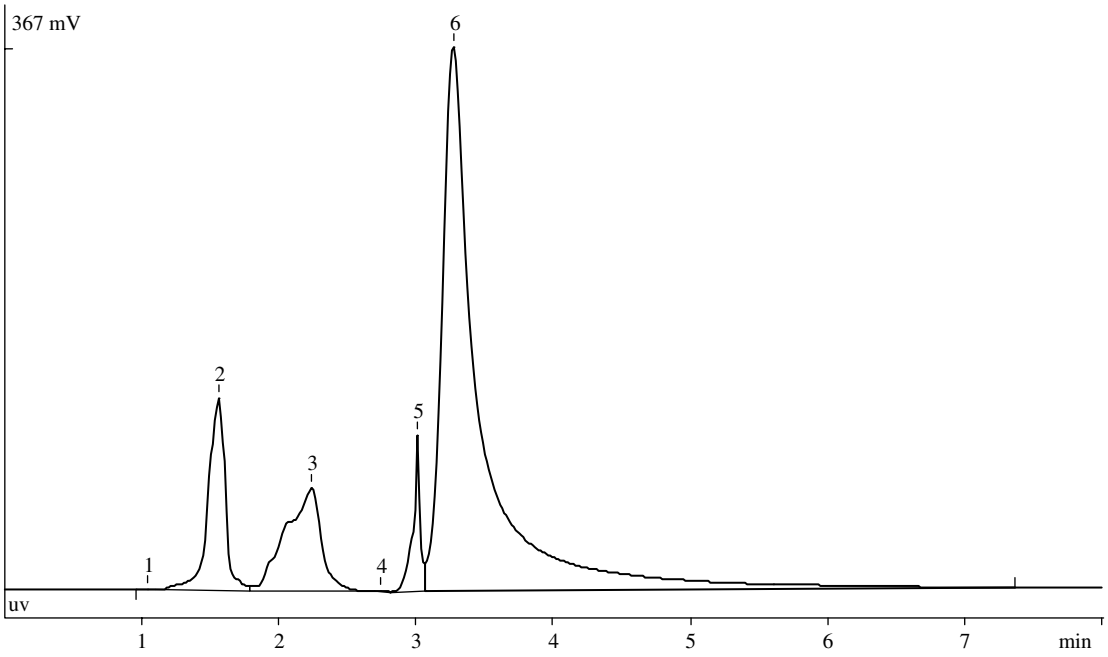


Figure 1 HPLC analysis of peptide III. Conditions as described in Materials and Methods.

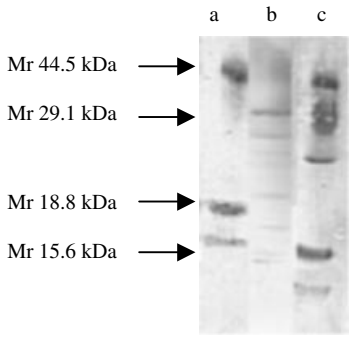


Figure 2 Immunoblotting of ribosomal proteins of *E. coli* with antisera I, II and III. Immunoblotting of 30S ribosomal subunit with antiserum I (lane a); immunoblotting of 50S ribosomal subunit with antiserum II (lane b); immunoblotting of 50S ribosomal subunit with antiserum III (lane c).

antisera elicited from the above peptides and their study against the *E. coli* ribosomal proteins.

Potential antigenic determinants of proteins can be predicted combining the results from calculation of hydrophilicity [15,16], turn structure predictions and flexible regions of proteins [17]. We chose small regions from the terminal sequences because they fulfil the above mentioned criteria of known antigenic sites. In addition, we selected hydrophilic regions because they have a high probability for surface exposure, whereas hydrophobic regions are not soluble. The *N*- and *C*-terminals of the protein chains are less constrained for rigidity than the internal regions and are considered to be flexible. Proteins containing sequences in which α-helices can be interrupted are known as possible antigenic sites. The peptides were synthesized by the solid phase method using 2-chlorotrityl-chloride resin [18] and Fmoc [19] as the *N*-terminal protecting group of the amino acids. For protection of the guanidine group of arginine, 2,2,5,7,8-pentamethylchroman-6-sulphonyl group (Pmc) [20] and the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl group (Pbf) [21] were used in two different experiments. It was found that the Pbf-group is the preferable protecting group because deprotection resulted in fewer impurities. For the synthesis of these peptides, DIC/HOBt [22] and HBTU [23] were used as coupling reagents. Both are excellent coupling reagents providing satisfactory yields. The yield of each coupling reaction was estimated with Kaiser's test. Problems occurred when the incorporated amino acid was Fmoc-Arg(Pbf)-OH or Fmoc-Arg(Pmc)-OH, Fmoc-Ile-OH and Fmoc-Pro-OH and the reaction step had to be repeated in these cases.

For the final deprotection of peptides, the selection of the deprotection mixtures was based on the composition of the particular peptides. For peptide I that contained three residues of protected arginine,

ethanodithiol and phenol were used as scavengers and thioanisole for the acceleration of the reaction. In the case of peptide II, with the presence of Boc- and tBu- protecting groups, TFA:H_2O was used, whereas for peptide III ethanodithiol was used as the scavenger for the Trt-protecting group.

Since HPLC analysis of the fully deprotected peptides showed that the peptides were over ~80% pure, they were used for conjugation with ovalbumin without further purification. It is known that haptens, generally, are not immunogenic, so they have to be conjugated with a protein-carrier. Such proteins, including keyhole limpet hemocyanine, bovine serum albumin, ovalbumin etc, provide the class II-T cell receptor binding sites, and thus contain regions which are helper T-cell epitopes and are detected by T-cells during the immuno-response processing. In the case of ovalbumin, the region I_{323}–R_{339} is the possible epitope [24]. For the conjugation of peptide I to ovalbumin, EDC was used, while the free imino-group and the guanidine groups of arginine residues were protected with citraconic anhydride. The conjugation of peptides II and III to ovalbumin was done using glutaraldehyde. In the case of peptide II, a very low concentration of glutaraldehyde was used, and the pH of the reaction was adjusted to below the pK of the amino group to avoid the participation of the ε-amino groups of lysine residues in the conjugation process.

The study of the anti-peptide antisera against the synthetic peptides carried out by ELISA tests, showed that anti-peptide sera raised against peptides I and II recognize the corresponding peptides and there is also cross-reaction between antisera and peptides. Anti-peptide serum III was unable to bind to peptide III (which was less hydrophilic than the other two peptides), possibly due to unsatisfactory immobilization of the peptide in the microplates.

Further, the immunoblotting experiments showed that the anti-peptide antibodies directed to these synthetic hexapeptides recognize the corresponding S7, L6 and L13 ribosomal proteins of *E. coli*.

In conclusion, the results presented here establish the generality that synthetic oligopeptides from various domains of proteins with a hydrophilic character are useful tools for the production of antibodies reactive with the corresponding proteins.

Acknowledgements

The authors are indebted to Dr. M. Foundoulakis (Hoffmann Le Roche, Switzerland) for the mass spectra and *N*-terminal sequencing analysis and Professor D. A. Kyriakidis and his group (Biochemistry Laboratory, Department of Chemistry, Aristotle University of Thessaloniki, Greece) for helpful discussions and some experiments. This work was partly supported by the Institution of National Scholarships, Greece.

REFERENCES

1. Lerner RA. Chemically synthesized peptides predicted from nucleotide sequences of the hepatitis B virus genome elicit antibodies reactive with the native envelope protein of Dane particles. *Cell* 1981; **23**: 309–310.
2. Lerner RA. Tapping the immunological repertoire to produce antibodies of predetermined specificity. *Nature* 1982; **299**: 592–596.
3. Sutcliffe JC, Shinnick TM, Green N, Liu FT, Niman HL, Lerner RA. Chemical synthesis of a polypeptide predicted from nucleotide sequence allows detection of a new retroviral gene product. *Nature* 1980; **237**: 801–805.
4. Sutcliffe JG, Shinnick TM, Green N, Lerner RA. Antibodies that react with predetermined sites on proteins. *Science* 1983; **219**: 660–666.
5. Herfurth E, Wittmann-Liebold B. Determination of peptide regions exposed at the surface of the bacterial ribosome with antibodies against synthetic peptides. *Biol. Chem. Hoppe-Seyler* 1995; **376**: 81–90.
6. Walleczek J, Albrecht-Ehrlich R, Stoffler G, Stoffler-Meilicke M. Three-dimensional localization of the NH_2- and carboxyl-terminal domain of ribosomal protein S1 on the surface of the 30S subunit from *Escherichia coli*. *J. Biol. Chem.* 1990; **265**: 11338–11344.
7. McKuscie H, Sommer A, Tewari DS, Traut RP, Glitz DG. Localization of two epitopes of protein L7/L12 to both the body and stalk of the large ribosomal subunit. *J. Biol. Chem.* 1986; **261**: 6924–6932.
8. McKuscie H, Nag B, Etchison JR, Traut RP, Glitz DG. Differential localization of two epitopes of *Escherichia coli* ribosomal protein L2 on the large ribosomal subunit by immunoelectron microscopy using monoclonal antibodies. *J. Biol. Chem.* 1991; **266**: 1898–1902.
9. Nag B, Tewari D, Sommer A, McKuskie Olson H, Glitz D, Traut R. Probing ribosome function and the location of *Escherichia coli* ribosomal protein L5 with a monoclonal antibody. *J. Biol. Chem.* 1987; **262**: 9681–9687.
10. Nag B, Akella SS, Cann PA, Tewari DS, Glitz DG, Traut RR. Monoclonal antibodies to *Escherichia coli* ribosomal proteins L9 and L10. *J. Biol. Chem.* 1991; **266**: 22129–22135.

11. Reinbolt J, Tritsch D. The primary structure of ribosomal protein S7 from *E. coli* strains K and B. *FEBS Lett.* 1978; **91**: 297–301.
12. Bischof O, Kruft V, Wittmann-Liebold B. Analysis of the pyromycin binding site in the 70S ribosome of *Escherichia coli* at the peptide level. *J. Biol. Chem.* 1994; **269**: 18315–18319.
13. Golden B, Rramakrishnan V, White SW. Ribosomal protein L6: structural evidence of gene duplication from a primitive RNA binding protein. *EMBO J.* 1978; **12**: 4901–4909.
14. Mende L. The primary structure of protein L13 from the large subunit of Escherichia coli ribosomes. *FEBS Lett.* 1978; **96**: 313–316.
15. Hopp TP, Woods KR. Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA* 1981; **78**: 3824–3828.
16. Hopp TP, Woods KR. A computer program for predicting protein antigenic determinants. *Mol. Immunol.* 1983; **20**: 483–489.
17. Westhof E, Altschuh D, Moras D, Bloomer AC, Mondragon A, Klug A. Van Regenmortel MHV. Correlation between segmental mobility and the location of antigenic determinants in proteins. *Nature* 1984; **311**: 123–126.
18. Barlos K, Chatzi O, Gatos D, Stavropoulos G. 2-Chlorotrityl chloride resin. *Int. J. Pept. Prot. Res.* 1991; **37**: 513–520.
19. Carpino LA, Han GY. The 9-fluorenylmethoxycarbonyl function, a new base-sensitive amino-protecting group. *J. Am. Chem. Soc.* 1970; **92**: 5748–5749.
20. Ramage R, Green J, Blake AJ. An acid labile arginine derivative for peptide synthesis: N^G-2,2,5,7,8-pentamethylchroman-6-sulphonyl-L-arginine. *Tetrahedron Lett.* 1991; **32**: 6353–6370.
21. Carpino LA, Shroff H, Triolo SA, Sayed E, Mansour E, Wenschuh H, Albericio F. The 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl group (Pbf) as arginine side chain protectant. *Tetrahedron Lett.* 1993; **34**: 7829–7832.
22. Liakopoulou-Kyriakides M, Metaxas A, Sarigiannis Y, Magafa V, Stavropoulos G, Laidou I, Thanassoulopoulos CC. Analogues of Ser-Val-Gly-Glu (AS-I) toxin pathogenic to sunflower. Synthesis and biological activities. *J. Phytopathol.* 1998; **146**: 279–282.
23. Dourtoglou V, Gross B, Lambropoulou V, Zioudrou C. O-benzotriazolyl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate as coupling reagent for the synthesis of peptides of biological interest. *Synthesis* 1984; 572–574.
24. Shimonkevitz R, Colon S, Kappler JW, Marrack P, Grey HM. Antigen recognition by H-2-restricted T cells. A tryptic ovalbumin peptide that substitutes for processed antigen. *J. Immunol.* 1984; **133**: 2067–2074.

