SYNTHETIC SEGMENTS OF INFLUENZA VIRUS HAEMAGGLUTININS*

Jan JEŽEK[a], Ivo BLÁHA[a], Milan ZAORAL[a], Eva HAMŠÍKOVÁ[b], Hana ZÁVADOVÁ[b] and Vladimír VONKA[b]

[a] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6 and*
[b] *Department of Experimental Virology, Institute of Sera and Vaccines, 101 03 Prague 10*

Received April 28, 1988
Accepted July 20, 1988

Ala^{97}HA-1(91–108) (*I*), Met^{97}HA-1 (91–108) (*II*), HA-2 (1–13) (*III*), HA-1 (185–200) (*IV*) segments of influenza virus *Victoria*/75 (H3N2) haemagglutinins were prepared by solid phase peptide synthesis and conjugated to TT and BSA, respectively. The conjugates were tested for immunogenicity in rabbits.

Significant results were obtained by immunizing experimental animals with an analog of HA-1 (91–108)** peptide segment[1,2] of haemagglutinin (see, e.g., ref.[3–8]) of influenza virus type *Victoria*/75, Ala^{97}-HA-1(91–108) peptide (*I*), conjugated to a high-molecular-weight carrier. The animals raised antibodies able to protect them against further challenge with the influenza virus[9,1]. The results suggested a chance of obtaining a synthetic antiinfluenza vaccine. They seemed so much interesting that we decided to verify and complement them – the more so, as they were obtained with synthetic peptide material the chemical characterization of which did not fully satisfy the usual standards. In addition to peptide *I* we prepared its Met^{97} analog, peptide *II*, HA-2(1–13) peptide (*III*) and HA-1(185–200) peptide (*IV*). Peptide *II* is chemically more closely related to the naturally occurring sequence than the Ala^{97} analog. Peptide *III* takes part in the interaction of the virus with the cell membrane[10]. Peptide *IV* comprizes the antigenic determinant B of virus A/*Victoria*/75 H3N2 (ref.[11,12]).

Peptides *I*–*IV* were prepared by solid-phase methodology[13] using the Merrifield

* Presented in part at the International Symposium on Synthetic Immunomodulators and Vaccines, Třeboň 1985 (see ref.[16]).

** Recommended abbreviations and symbols are used (Eur. J. Biochem. *138*, 9 (1984)). Further abbreviations: HA, haemagglutinin; TT, tetanus toxoid; HPLC, high performance liquid chromatography; CDI, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride; MDP, muramyldipeptide (N-acetylmuramyl-L-alanyl-D-isoglutamine); BSA, bovine serum albumin; SPRIA, solid phase radioimmunoassay; HOBt, N-hydroxybenzotriazole; DMF, N,N-dimethylformamide.

peptide resin as support, symmetrical anhydrides[14] for elongation of the peptide chain and liquid HF[15] for splitting off the peptide from the support and for removal of the protecting groups. The products were purified by reversed-phase HPLC. Peptide *III* was poorly soluble and was purified by precipitation from hot aqueous acetic acid (60%). Poor solubility also prevented its use for immunization.

The conjugation[16-18] of *I* and *II* with TT yielded, in our hands, only insoluble, immunologically inactive, materials. A control experiment showed this to be due to the attachment of peptides *I* and *II* to TT and not by the reaction of TT with CDI. When the conjugation of *I* and *II* with TT was carried out in the presence of MDP, approx. 35% of a water-soluble material was obtained. Peptides *I* and *II* were also coupled to TT with glutardialdehyde. Peptide *IV* was conjugated with BSA (CDI) (see Table I). The conjugates of *I* and *II* with TT and MDP and of *IV* with BSA were used for immunization of rabbits.

A detailed survey of the work done with peptides *I*–*IV* and of the results obtained was presented elsewhere[18]. Only those of the results will be mentioned here which are essential for the subsequent discussion.

Some of the results obtained were in agreement with the published data, e.g. the formation of antibodies (all the immunized experimental animals developed antibodies reacting with *I* and *II* and with HA) and the specificity of the antibodies

TABLE I
Conjugation of peptides *I* and *IV* with carriers

Component	Experiment No.			
	1	2	3	4
Carrier (μmol)	TT (1)	TT (1)	TT (1)	BSA (0·6)
Peptide (μmol)	*I* (100)	*I* (100)	*I* (100)	*IV* (15·1)
CDI (μmol)	500	200	200	30·2
MDP (μmol)	0	100	100	0
Soluble conjugate (mg)	0	60	60	52
Insoluble conjugate (mg)	156	104	110	5·4
Peptide content[a] in the conjugate (soluble/insoluble)	0/29	63/11	38/6	3·3/13·9
Content[b] of MDP in the conjugate (soluble/insoluble)	0/0	6/3	20/—	0/0

[a] Mol of peptide/mol of carrier, determined by comparison of amino acid analyses of the carrier and the conjugate; [b] mol of MDP/mol of carrier, determined indirectly as the difference between the experimental value for Glu (or Ala) and the theoretical value corresponding to the given substitution of the carrier with the peptide.

(only homotypic reaction with HA, cross-reactivity with the whole virion). In contrast, we were not able to confirm the key finding, that the antibodies formed against peptide *I*–TT conjugates possessed protective activity[1,9], i.e., that they were able to protect the experimental animal against further challenge with the virus. The available data do not allow an exact explanation of this disappointing discrepancy. However, the present, and a number of similar results produce an impression that the work on "synthetic vaccines" was based on oversimplified conceptions of the underlying idea of antigenic determinants. And moreover, that a greater attention has to be paid to identification, characterisation and purity of the components employed, especially peptides.

EXPERIMENTAL

Chloromethylated styrene–divinylbenzene copolymer (1% divinylbenzene, 0·97 mmol Cl/g, Institute of Chemical Process Fundamentals, Prague) was used as carrier. Carboxyterminal amino acid was linked using the potassium fluoride method[19] and its content was determined by amino acid analysis, elemental analysis and picric test[20]. The following protecting groups were used: N^{α}-Boc, N^{ω}-Z, OH (Ser, Thr) and COOH (Asp, Glu) — Bzl, OH (Tyr) — Z. The scheme of the synthesis was that according to Zaoral and coworkers[21], with the use of a three-fold excess of symmetric anhydrides in dichloromethane. Asn and Gln were condensed via active esters with HOBt in DMF (3 eq). The completeness of the reaction was checked by the ninhydrine test[22]. After the completion of the synthesis the Boc group was split off and neutralization carried out. The splitting off of the protecting groups and the peptides from the carrier was carried out with liquid hydrogen fluoride, with addition of 10% of anisole (0°C, 45 min); in peptide containing Met 5% 2-mercaptopyrimidine was added. Hydrogen fluoride was evaporated at 0°C in a stream of nitrogen. TT was from the Institute of Sera and Vaccines. Dialysis was carried out in a Serva-Visking tube (for substances with a molecular weight lower than 10^4). Preparative separation of gram amounts of compounds was carried out by HPLC using a Jobin–Yvon column of 500 × 80 mm dimension and packed with Separon SI C 18 (mobile phase 0·1% TFA/methanol, detection with UV light 230 nm). Preparative separation of smaller amounts of compounds (50—100 mg) was carried out on a Spectra Physics Instrument with a 250 × 9 mm column, packed with Partisil ODS 2 (mobile phase 0·1% TFA/methanol). The analytical control of the fractions obtained was carried out on a Spectra-Physics instrument with a 250 × 4·6 mm column packed with Spherisorb ODS and the same mobile phase. Samples for amino acid analysis were hydrolysed at 110°C for 20—48 h in 6M-HCl. The amino acid analyses were carried out on a Beckman–Spinco 120 B instrument. UV spectra were measured on a Varian–Cary 219 spectrometer.

H-Ser-Lys-Ala-Phe-Ser-Asn-Ala-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Ser-Leu-OH (*I*)

From 6·88 g (4 mmol) of Boc-Leu-O-(R), 16·8 g of peptide–resin was obtained (after completion of the synthesis, splitting off the Boc group and neutralization). The increase in weight was 9·92 g (89·7%). A part of the peptide–resin (4·4 g) was stirred at 0°C with 8 ml of anisole, 4 g of 2-mercaptopyridine and 65 ml of liquid HF for 45 min. After evaporation of HF in a stream of N_2, the resin was washed with three 60 ml portions of ethyl acetate (0°C) and the peptide was extracted with four 40 ml portions of 20% acetic acid. After lyophilization, 1·9 g of product

was obtained. The lyophilisate was dissolved in 10 ml of acetic acid, diluted with 60 ml of water and the pH was adjusted to 3·9 with acetic acid. The compound was desalted on a column of Amberlite IRC-50 (124 ml, column 2 × 40 cm). The salts were eluted with 500 ml of 0·25% acetic acid and the product with 400 ml of 50% acetic acid. After lyophilization, 1·1 g of product was obtained. For $C_{93}H_{130}N_{20}O_{30}$ (2 008) calculated: 13·95% N; found 9·76% N. The content of the peptide in the lyophilisate was 70%, yield 36·6% (based on the first amino acid attached to the resin and corrected for lyophilisate peptide content).

The crude peptide material from several synthetic runs (1·5 g) was dissolved in 100 ml of 23% acetic acid and placed onto a HPLC column (500 × 80 mm) preequilibrated with 5 l of 0·1% TFA in 30% MeOH. Elution was carried out in a stepwise manner, with 5 l 30% MeOH, 10 l 40% MeOH, 10 l 45% MeOH, 5 l 50% MeOH and 5 l 60% MeOH. Flow rate 100 ml/min, UV detection at 230 nm, fractions of 500 ml each. Fractions 51—57 (3·5 l) were pooled, pH adjusted with 4M-NaOH to 6, and concentrated in vacuo to 500 ml. pH was adjusted with NaOH to 8 and, after 2—3 min, to 3·9 with acetic acid. The solution was desalted on a column of Amberlite IRC-50 (100 ml, column 1·5 × 57 cm). After lyophilization, 440 mg of the product was obtained. The product contained 12·3% of N, i.e. the content of peptide in the sample was 87%. Yield 13·3% (based on the first amino acid attached to the resin and corrected for the sample peptide content). For $C_{93}H_{130}N_{20}O_{30}.CH_3COOH.6\ H_2O$ (2 176) calculated: 52·43% C, 6·76% H, 12·87% N; found: 52·69% C, 6·75% H, 12·74% N. (The sample for elemental analysis was dried at 100°C/10 Pa over P_2O_5 for 8 h). $[\alpha]_D^{22}$ −86·5° (*c* 0·5, 1M-AcOH). Edman's degradation was in accordance with the assumed sequence. UV (1% TFA), λ_{max} (ε): 223·6 nm (29 100), 278 nm (3 730). Amino acid analysis (hydrolysis 20 h, 110°C, 6M-HCl): Lys 0·91(1), Asp 3·23(3), Ser 2·92(3), Pro 1·98(2), Ala 3·15(3), Val 1·02(1), Tyr 2·75(3), Phe 0·97(1).

H-Ser-Lys-Ala-Phe-Ser-Asn-Met-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Ser-Leu-OH (*II*)

The synthesis was carried out in a similar manner as in the preceding case. From 3·44 g (2 mmol) of Boc-Leu-O-(R), 8·9 g (97%) of peptide-resin was obtained. From 6·88 g of peptide resin 2·22 g of product was obtained after splitting off with liquid HF, washing with three 160 ml portions of ethyl acetate (0°C), extracting the product with four 80 ml portions of 20% aqueous acetic acid, adjusting to pH 8 (NaOH, 1 h), acidification to pH 4 (AcOH), desalting on 200 ml Amberlite IRC-50 and lyophilization. The crude product was purified by HPLC using a stepwise gradient elution with 30% MeOH (50 min), 40% MeOH (40 min), 50% MeOH (95 min), 60% MeOH (50 min), 70% MeOH (50 min), at a 100 ml/min flow rate. Detection was carried out with UV (230 nm). From 1·82 g of the crude product, 575 mg of HPLC pure peptide was obtained. Amino acid analysis: Asp 3·16(3), Ser 2·73(3), Pro 2·10(2), Ala 2·08(2), Val 1·02(1), Met 0·90(1), Leu 1·10(1), Tyr 2·94(3), Phe 0·98(1), Lys 1·01(1). For $C_{95}H_{134}N_{20}O_{30}S.3\ CF_3COOH.1/2\ CH_3COOH.3\ H_2O$ (2 494·5) calculated: 49·11% C, 5·86% H, 11·23% N, 1·29% S, 6·85% F; found: 49·03% C, 5·81% H, 11·09% N, 1·59% S, 6·62% F. The sample for elemental analysis was dried at 100°C/10 Pa over P_2O_5 for 10 h. A part of the material (150 mg) was dissolved in 10 ml of water, pH of the solution was adjusted to 8 (NaOH) and then immediately to 3·9 with AcOH. The solution was desalted on a column of Amberlite IRC-50 (30 ml). After lyophilization 129 mg of desalted product was obtained. For $C_{95}H_{134}N_{20}O_{30}S.3\ CH_3COOH.5\ H_2O$ (2 338·5) calculated: 51·87% C, 6·72% H, 11·98% N, 1·37% S; found: 52·04% C, 6·66% H, 11·50% N, 1·36% S. The content of the peptide in the sample was 85% (based on the N content). The overall yield of HPLC pure peptide was 13·8%, $[\alpha]_D^{22}$ −78·5° (*c* 0·6, 1M-AcOH). The results of Edman's degradation of the product and its chymotryptic cleavage were in agreement with the assumed primary structure. UV (5% AcOH) λ_{max} (ε): 275 nm (2 630).

H-Gly-Ile-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile-Glu-Asn-Gly-OH (*III*)

From 5·7 g (4 mmol) of Boc-Gly-O-(R), 9·8 g (94%) of peptide–resin was obtained. A part of the peptide–resin (4·5 g) was cleaved in 80 ml of liquid HF and 10 ml of anisole at 0°C for 45 min. After evaporation of HF in the stream of N_2 and washing of the resin with 60 ml of ethyl acetate (3×, 0°C), the peptidic material was extracted with twelve 50 ml portions of 50% AcOH (60°C). The combined extracts were concentrated in vacuo, water was added, and the mixture was lyophilized. Yield, 1·8 g (77·4%, uncorrected), HPLC purity 82·3% (215 nm). The material was dissolved in 210 ml of 60% AcOH, warmed to 60°C and the insoluble material was filtered off. The filtrates were diluted with 150 ml of water and allowed to stand in a water-ice bath for one hour. The product which had separated was filtered off and washed four times with 20 ml portions of cold water. Yield, 1·07 g (40%, based on the content of the peptide in the lyophilisate), HPLC purity 91% (215 nm), m.p. 295–302°C. For $C_{59}H_{88}N_{14}O_{17}$.HF.2 CH_3COOH.1 H_2O (1 424) calculated: 53·16% C, 7·01% H, 13·77% N; found: 53·24% C, 6·82% H, 13·53% N. The sample for elemental analysis was dried at 22°C/10 Pa over P_2O_5 for 8 h. Amino acid analysis (hydrolysis 20 h, 6M-HCl, 110°C): Asp 1·01(1), Glu 1·06(1), Gly 4·01(4), Ala 2·07(2), Ile 2·84(3), Phe 1·99(2).

H-Pro-Ser-Thr-Asp-Lys-Glu-Glu-Thr-Asp-Leu-Tyr-Val-Gln-Ala-Ser-Gly-OH (*IV*)

From 2 g (1 mmol) of Boc-Leu-O-(R), 4·21 g (95%) of peptide–resin was obtained. The splitting off of the peptide from the carrier was carried out with 50 ml of liquid hydrogen fluoride with addition of 6 ml of anisole (0°C, 1 h). After evaporation of the hydrogen fluoride in a stream of N_2 the reaction mixture was washed with ether, the peptide dissolved in 200 ml of 20% acetic acid and lyophilized. Yield 1·63 g (94%) of crude peptide. This was purified by HPLC in the system 0·05% of aqueous TFA/methanol, with a gradient of 1% MeOH/min, beginning with 30% MeOH. The main product was isolated, concentrated in vacuo and lyophilized. Yield 747 mg (43%) of the product. For $C_{73}H_{114}N_{18}O_{31}$.4 H_2O (1 812) calculated: 48·39% C, 6·79% H, 13·91% N; found: 48·09% C, 6·91% H, 13·90% N. Amino acid analysis: Pro 1·02(1), Ser 1·89(2), Thr 1·94(2), Asp 2·01(2), Lys 1·05(1), Glu 3·09(3), Leu 1·01(1), Tyr 0·93(1), Val 0·99(1), Ala 1·01(1), Gly 1·01(1).

Conjugation of TT With Peptide *I* and MDP*

A) A solution of 150 mg (1 μmol) of TT in 3·75 ml of cooled (0°C) physiological solution was added to a turbid solution of 51 mg (100 μmol) of MDP.H_2O and 200·8 mg (100 μmol) of peptide *I* in 30 ml of physiological solution at 0°C and under stirring. A cooled physiological solution (6 ml) containing 60 mg (200 μmol) of CDI was then added to the mixture and the reaction was allowed to proceed under stirring for 24 h at 0°C and another 24 h at room temperature. At the end the reaction mixture contained a precipitate. The mixture was dialysed in a physiological solution (4 × 5 l) for 48 h and the content of the dialysation tube was centrifuged at 3 000 rpm for 10 min. The supernatant was pipetted off and the precipitate recentrifuged with 15 ml of physiological solution. The combined supernatants afforded — after lyophilization and subtracting the weight of sodium chloride — 60 mg of conjugate. The weight of the precipitate after lyophilization was 104 mg.

* Conjugation of peptide *II* with TT was carried out in an analogous way. Analogous results were obtained.

B) Owing to the poor solubility of peptide *I* in physiological solution the experiment was repeated after the dissolution of peptide *I* by 30 min stirring at 20°C and 5 min at 40°C. A weakly opalescent solution was formed. Further conditions were the same as sub *A*). After 24 h the reaction mixture was milky white, without a visible precipitate, but after 48 h a precipitate was formed. After centrifugation and lyophilization 60 mg of a soluble and 110 mg of an insoluble conjugate were obtained. The molar ratios of the starting compounds, the yields of conjugates and the substitutions of the carriers with the peptide are given in Table I.

Conjugation of BSA with peptide *IV*

BSA (39·6 mg, 0·6 μmol) was added to a solution of 26·3 mg (15·1 μmol) of peptide *IV* in 5 ml of physiological solution. The weakly turbid solution was cooled at 0°C and 5·8 mg (30·2 μmol) of CDI in 5 ml of physiological solution were added under stirring. After 3 h standing at 0°C and 1 h at 5°C the opalescent solution was dialysed for 36 h against physiological solution at room temperature, with four exchanges. During dialysis a part of the conjugate separated in the form of a precipitate. The reaction mixture was centrifuged and 10 ml of supernatant were obtained. The precipitate was washed twice with 5 ml of physiological solution and centrifuged. The combined supernatants (20 ml) were lyophilized and the weight of sodium chloride was subtracted to give 52 mg of soluble conjugate. The precipitate was lyophilized with 2·5 ml of physiological solution to give 5·4 mg of insoluble conjugate (after subtraction of the sodium chloride weight). The molar ratios of the starting compounds, the yields of conjugates and the substitutions of the carrier with the peptide are given in Table I.

The authors are indebted to Dr M. Ryba for HPLC *analyses, to Dr J. Horáček for elemental analyses, to Mr J. Zbrožek for amino acid analyses and to Mrs Z. Ledvinová for optical rotation measurements.*

REFERENCES

1. Müller G. M., Shapira M., Arnon R.: Proc. Natl. Acad. Sci. U.S.A. *79*, 569 (1982).
2. Jackson D. C., Dopheide T. A., Russel R. J., White D. O., Ward C. W.: Virology *93*, 458 (1979).
3. Shapira M., Arnon R. in: *Options for the Control of Influenza, Proc. Viratek-UCLA Symp. Keystone, Colorado 1985* (A. P. Kendal and P. A. Patriarca, Eds), p. 391. Alan R. Liss, Inc., New York 1986.
4. Arnon R., Shapira M. in: *Modern Approaches to Vaccines. Molecular and Chemical Basis of Virus Virulence and Immunogenicity* (R. M. Chanock and R. A. Lerner, Eds), p. 109. Cold Spring Harbor Lab., Cold Spring Harbor, New York 1984.
5. Arnon R. in: *Advances in Immunopharmacology, Proc. 3rd Int. Conf. Immunopharm., Florence 1985* (L. Chedid, J. W. Hadden, F. Spreafico, P. Dukor and D. Willoughby, Eds), p. 347. Pergamon Press, London, 1985.
6. Arnon R. in: *Immunochemistry of Viruses. The Basis for Serodiagnoses and Vaccines* (M. H. V. van Regenmortel and A. R. Neurath, Eds), p. 139. Elsevier, Amsterdam, 1985.
7. Arnon R., Jacob Ch. O., Shapira M. in: *Prog. Immunol., Int. Congr. Immunol., 5th, 1983* (Y. Yamamura and T. Tada, Eds), p. 1327. Academic, Tokyo, 1984.
8. Arnon R., Sela M.: Ann. Inst. Pasteur/Immunol. *136D*, 271 (1985).
9. Shapira M., Jolivet M., Arnon R.: Int. J. Immunopharmacol. *7*, 719 (1985).
10. Skehel J. J., Bayley P. M., Brown E. B., Martin F. R., Waterfield M. D., White J. M., Wilson J. A., Wiley D. C.: Proc. Natl. Acad. Sci. U.S.A. *79*, 968 (1982).

11. Ward F. W.: Curr. Top. Microbiol. *94/95*, 1 (1981).
12. Wiley D. C., Wilson I. A., Skehel J. J.: Nature *289*, 373 (1981).
13. Barany G., Merrifield R. B. in: *The Peptides* (E. Gross and J. Meienhofer, Eds), Vol. 2, p. 1. Academic Press, New York 1980.
14. Hagenmeier H., Frank H.: Hoppe-Seylers Z. Physiol. Chem. *353*, 1973 (1972).
15. Sakakibara S., Shimonishi Y., Kishida Y., Okada M., Sugihara H.: Bull. Chem. Soc. Jpn. *40*, 2164 (1967).
16. Bláha I., Ježek J., Zaoral M., Hamšíková E., Závadová H., Vonka V. in: *Synthetic Immunomodultors and Vaccines* (M. Zaoral, Z. Havlas, O. Mikeš and Ž. Procházka, Eds), p. 203. Inst. Org. Chem. Biochem., Czechoslov. Acad. Sci., Prague 1986.
17. Hamšíková E., Závadová H., Zaoral M., Ježek J., Vonka V.: ibid. p. 208.
18. Hamšíková E., Závadová H., Zaoral M., Ježek J., Bláha I., Vonka V.: J. Gen. Virol. *68*, 2249 (1987).
19. Horiki K., Igano K., Ynouye K.: Chem. Lett. *1978*, 165.
20. Hancock W. S., Battersby J. E., Harding D. R. K.: Anal. Biochem. *69*, 497 (1975).
21. Zaoral M., Yamashiro D., Hammonds R. G. jr, Li C. H.: Int. J. Peptide Protein Res. *17*, 292 (1981).
22. Kaiser E., Colescott R. L., Bossinger C. D., Cook P. I.: Anal. Biochem. *34*, 595 (1970).

Translated by Ž. Procházka.