SUMMARY

Sorbents for the immobilization of lipases have been synthesized from Silokhrom and Porokhrom and also from the biospecific sorbent Liposorb, based on microcrystalline cellulose. Immobilized forms of the lipase from the fungus Oospora lactis have been obtained, and some of their properties have been studied.

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SYNTHESIS OF C-TERMINAL PEPTIDE FRAGMENTS OF THE HEAVY CHAIN OF HEMAGGLUTININ OF INFLUENZA VIRUS OF SUBTYPES H1 AND H3

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It is proposed to use the chromogenic 2-[4-phenylazo)benzylsulfonyl]ethyl group, which can be eliminated by organic bases in aprotic solvents, for the protection of a C-terminal carboxy group in the synthesis of peptides. The synthesis of a number of 10–16-membered peptides corresponding to C-terminal fragments of the heavy chain of the hemagglutination of influenza virus of subtypes H1 and H3 has been performed with the use of this group.

The main protective antigen of the influenza virus, hemagglutinin (HA), is a highly variable membrane protein consisting of two polypeptide chains – heavy (HA_1) and light (HA_2) – linked by disulfide bonds. According to their antigenic affinities, HAs are divided into several serosubtypes of which subtypes H1 and H3 are of the greatest importance for man. Within the serosubtypes, also, an extremely high variability of the amino acid sequence is observed, but the HA of each subtype has several extended conservative regions having undergone practically no amino acid substitutions in the process of the drift evolution of the subtype. One of the most interesting regions is the C-end of HA_1. It has been shown in a number of papers [1-3] that antibodies obtained against synthetic C-terminal fragments of HA_1 of subtype H3 also interact specifically with the HAs and viruses of different strains of subtype H3 immobilized on a solid support, but the biological properties of such antibodies have not been studied.

With the aim of a detailed investigation of their immunogenic properties, we have synthetized several peptides, (Ia-c) and (IIa-c), consisting of C-terminal fragments of HA_1 of subtypes H1 and H3 having different lengths – from 10 to 16 amino acid residues (Table 1).

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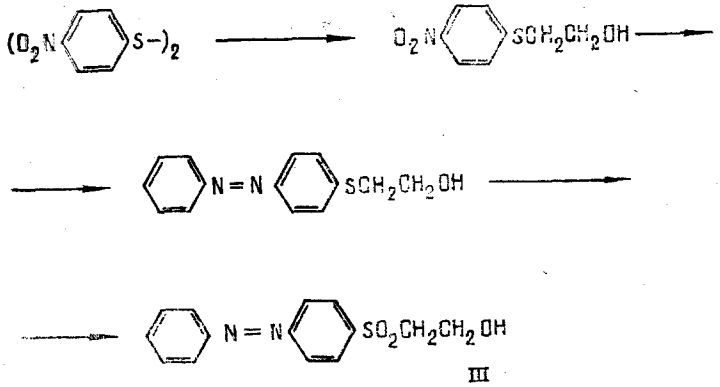
TABLE 1. Synthetic C-terminal Fragments of Influenza Virus HA_1

Compound	Subtype of HA	No. of the amino acids in the HA	Amino acid sequence
Ia	H1	320—328	Cys(Acm)-Leu-Arg-Asn-Ile-Pro-Ser-Ile-Gln-Ser
Ib	H1	317—328	Cys(Acm)-Val-Thr-Gly-Leu-Arg-Asn-Ile-Pro-Ser-Ile-Gln-Ser
Ic	H1	314—328	Cys(Acm)-Leu-Arg-Met-Val-Thr-Gly-Leu-Arg-Asn-Ile-Pro-Ser-Ile-Gln-Ser
IIa	H3	320—328	Cys(Asm)-Met-Arg-Asn-Val-Pro-Glu-Lys-Gln-Thr
IIb	H3	317—328	Cys(Acm)-Ala-Thr-Gly-Met-Arg-Asn-Val-Pro-Glu-Lys--Gln-Thr
IIc	H3	314—328	Cys(Acm)-Leu-Lys-Leu-Ala-Thr-Gly-Met-Arg-Asn-Val--Pro-Glu-Lys-Gln-Thr

N-Terminal cysteine residues, which are absent from the natural structure of HA_1, were introduced for the subsequent conjugation of the peptides with a protein carrier.

To synthesized the peptides we made use of tactics which we have described previously [4], based on the use as the constant C-protection of a chromogenic group removable selectively by bases through a β-elimination mechanism. In the present syntheses, in place of the known 2-[4-(phenylazo)benzylsulfonyl]ethyl(Pse) protective group, we have used for the first time the 2[4-(phenylazo)phenylsulfonyl]ethyl (Ppse) group, which is very similar in properties to the Pse group. The elimination of a methylene unit between the phenyl ring and the sulfonyl group in the Ppse protection increased the rate of its splitting out by strong organic bases — 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) or piperidine — in aprotic solvents by a factor of approximately 5.

For the temporary protection of the N-amino group we used the tert-butyloxycarbonyl (Boc) group, and for the protection of the side chains of trifunctional amino acids we used a set of groups eliminated simultaneously by acidolysis after the end of the synthesis: the benzyl (Bzl) group for Thr, Ser, and Glu; the benzyloxycarbonyl group (Z) for Lys; and the N^G-mesitylenesulfonyl group for Arg. The cysteine residues were protected by the selectively eliminable S-acetyamidomethyl (Acm) group.



III

Scheme of the synthesis of 2-[4-(phenylazo)phenylsulfonyl]ethanol (III).

The 2-[4-(phenylazo)phenylsulfonyl]ethanol (III) required for the introduction of the Ppse protection was synthesized in good overall yield from the readily available 4,4'-dinitrodiphenyl disulfide by the scheme shown. The Ppse esters of the Boc-amino acids (IV-VI) and (XIII) were obtained by the method described previously for the synthesis for the Ppse esters [4] and were characterized by physicochemical methods (Table 2).

The protected decapeptides (IXa) and (XIIa) (Table 3), and also peptide blocks for the assembly of longer peptides were synthesized by the stepwise growth of the peptide chain using activated esters of Boc-amino acids, beginning with the Ppse ester of the C-terminal amino acid. After each acylation cycle, the Boc-protection was eliminated with trifluoroacetic acid (TFAA). The method of synthesis in this case was identical with that developed previously for the synthesis of peptides using Ppse protection [4, 5].

The Ppse group possesses all the useful properties of the Pse group, facilitating the isolation and purification of the peptides synthesized — the Ppse esters of protected peptides

TABLE 2. Ppse Esters of Protected Amino Acids

Compound	Yield, %	mp, °C	R_f in systm. 1	R_f in systm. 2	$[\alpha]_D^{20}$ (c 0,5; DMFA)	FAB mass spectrum ($M+H^+$)
IV. Boc-Gly-OPpse	85	100—101	0,63	0,73	—	448
V. Boc-Ser(Bzl)-OPpse	90	106—107	0,64	0,88	24,0	538
VI. Boc-Thr(Bzl)-OPpse	90	45*	0,69	0,89	52,0	582
XIII. Boc-Phe-OPpse	88	91—92	0,65	0,84	19,2	538

*Amorphous compound.

TABLE 3. Ppse Esters of Protected Peptides Synthesized

Structure of the peptides	Yield, %	Yield on the elimination of Ppse, %	Deblocking product, yield, %
VII. Boc-Leu-Arg (Mts)- Asn-Ile-Pro-Ser (Bzl)-Ile-Gln-Ser (Bzl)-OPpse	56*	—	—
VIIIa. Boc-Cys (Acm)-Val-Thr (Bzl)-Gly-OPpse	66*	94	—
VIIIb. Boc-Cys (Acm) - Leu - Arg (Mts)- Met (O)-Val-Thr (Bzl)-Gly-OPpse	49*	81	—
IXa. Boc-Cys (Acm)- Leu- Arg (Mts)- Asn- Ile-Pro- Ser (Bzl)-Ile-Gln-Ser (Bzl)-OPpse	52*	89	Ia (44)
IXb. Boc-Cys (Acm)- Val-Thr (Bzl)-Gly-Leu-Arg (Mts)-Asn-Ile-ProSer (Bzl)-Ile-Gln-Ser (Bzl)-OPpse	73**	87	Ib (45)
IXc. Boc -Cys (Acm) - Leu -Arg (Mts) -Met(O)-Val-Thr (Bzl) - Gly - Leu - Arg (Mts)-Asn-Ile-Pro-Ser(Bzl)-Ile-Gln-Ser (Bzl)-OPpse	97**	86	Ic (31)
X. Boc-Met (O)- Arg (Mts)- Asn-Val-Pro-Glu (OBzl)-Lys (Z)-Gln-Thr (Bzl) OPpse	54*	—	—
XIa. Boc-Cys (Acm)-Ala-Thr (Bzl)-Gly-OPpse	80*	66	—
XIb. Boc - Cys (Acm) - Leu - Lys (Z)-Leu-Ala-Thr (Bzl)-Gly-OPpse	65*	94	—
XII. Boc- Cys (Acm) - Met (O)- Arg (Mts)-Asn-Val-Pro-Glu (OBzl)-Lys (Z)-Gln-Thr (Bzl)-OPpse	48*	87	IIa (42)
XII. Boc - Cys (Acm) - Ala-Thr (Bzl) - Gly-Met (O)-Arg (Mts) - Asn-Val - Pro - Glu (OBzl)-Lys (Z)-Gln-Thr (Bzl)-OPpse	88**	91	IIb (34)
XII. Boc - Cys (Acm) - Leu - Lys (Z) -Leu-Ala-Thr-(Bzl - Gly-Met (O)-Arg (Mts) -Asn-Val- Pro-Glu (OBzl)-Lys (Z)-Gln-Thr (Bzl)-OPpse	97**	90	IIc (36)

*Yield calculated on the Ppse ester of the C-terminal amino acid.
†Yield at the stage of the condensation of two blocks.

are readily soluble in dimethylformamide (DMFA), dimethyl sulfoxide (DMSO), and TFAA, and are practically insoluble in ether and isopropanol. Because of a shift of the optical absorption maxima into the UV and the long-wave region of the visible spectrum, the Ppse group has a deeper red coloration than the Pse group, which increases the sensitivity and reliability of the thin-layer chromatographic analysis of the purity of the peptides synthesized.

The protected peptides (IXb, c) and (XIIb, c) were obtained by block condensation using (4 + 9) and (7 + 9) schemes. The Ppse protection was eliminated from the peptide blocks (VIIIa, b) and (XIa, b) by the brief action of a small excess of DBU in DMFA or DMSO, and peptides with free carboxy groups were obtained in high yields and were then condensed with the tosylates of nonapeptides (VII) or (X), respectively, under the action of dicyclohexylcarbodimmide (DCC) and 1-hydroxybenzotriazole in a DMFA–DMSO mixture. The protected trideca- and hexadecapeptides (IXb, c) and (XIIb, c) were purified by repreciptation from DMFA in ethyl acetate.

After the elimination of the Ppse and Boc protective groups, the peptides were deblocked with a mixture of 1 M trifluoromethanesulfonic acid and thioanisole in TFAA and at 0°C [6]. Methionine sulfoxide residues were reduced by treating the deblocked peptides with large excesses of dithiothreitol [7]. Peptides (Ia-c) and (IIa-c) were purified by a unitary scheme — gel chromatography on Sephadex G-15 in 1 M acetic acid, and then preparative reversed-phase chromatography on LiChrosorb RP-18. All the stages of deblocking and purification were monitored by analytical high-performance liquid chromatography (HPLC). According to the results of HPLC, the purity of the peptides synthesized was not less than 95%, and

TABLE 4. Analysis of the Amino Acid Compositions of the Synthetic Peptides

Amino acid	Peptide					
	Ia	Ib	Ic	IIa	IIb	IIc
Asx	1,00 (1)	1,09 (1)	1,00 (1)	0,98 (1)	1,01 (1)	1,00 (1)
Thr	— —	0,91 (1)	0,87 (1)	0,97 (1)	2,15 (2)	2,07 (2)
Ser	1,87 (2)	2,04 (2)	1,89 (2)	— —	— —	— —
Glx	1,03 (1)	1,10 (1)	1,07 (1)	1,87 (2)	1,95 (2)	1,93 (2)
Pro	1,10 (1)	1,15 (1)	1,09 (1)	1,20 (1)	1,10 (1)	1,16 (1)
Gly	— —	1,06 (1)	1,01 (1)	— —	1,00 (1)	1,00 (1)
Ala	— —	— —	— —	— —	1,00 (1)	1,00 (1)
Val	— —	0,91 (1)	2,04 (2)	1,00 (1)	1,02 (1)	1,00 (1)
Leu	1,06 (1)	1,09 (1)	2,00 (2)	— —	— —	1,90 (2)
Ile	1,90 (2)	2,10 (2)	2,00 (2)	— —	— —	— —
Met	— —	— —	0,90 (1)	0,99 (1)	0,91 (1)	0,89 (1)
Lys	— —	— —	— —	1,00 (1)	1,05 (1)	2,07 (2)
Arg	0,93 (1)	1,03 (1)	2,03 (2)	0,03 (1)	1,02 (1)	1,00 (1)

*The expected values are given in parentheses; Cys was not determined.

their amino acid compositions corresponded to those expected. The yields of the peptides of the deblocking and purification stages are given in Table 3.

The Acm groups were laminated from the N-terminal cysteine residues by the action of mercury(II) acetate, and the peptides were conjugated with bovine serum albumin with the aid of the N-hydroxysuccinimide ester of 3-(2-pyridyldithio)propionic acid by a procedure described previously [4]. The degree of conjugation, evaluated from the liberation of 2-thiopyridone, amounted to from 15 to 33 mmole of peptide per 1 mmole of protein. The antigenic and immunogenic properties of the peptides and conjugates synthesized are being investigated.

EXPERIMENTAL

L-Amino acids produced by Merck and Fluka, and 4-dimethylaminopyridine, di-tert-butyl pyrocarbonate, dithiothreitol, trifluoromethanesulfonic acid, and DCC from Fluka were used. Information on the preparation of the protected amino acids and their activated esters is given in [4].

Thin-layer chromatography was conducted on Merck Kieselgel 60 F_{254} plates in the following solvent systems: 1) chloroform–ethanol–acetic acid (95:5:3); and 2) benzene–acetone–acetic acid (100:50:2). The spots were revealed on the plates in visible and UV light, and also after treatment with the ninhydrin or the chlorine/benzidine reagent. Reversed-phase HPLC was performed on a LKB chromatograph in a gradient of from 5 to 90% of acetonitrile in water containing 0.1% of TFAA. For analytical HPLC, 4.6 × 125 mm and 3.2 × 250 mm columns with the support LiChrosorb RP-18 (5 and 10 μm) (Merck) were used, and for prepartive HPLC a 10 × 250 mm column containing LiChrosorb RP-18 (10 μm), at rates of flow of 1 and 4-5 ml/min, respectively. The wavelength of the UV detector was 220 nm.

The amino acid compositions of the peptides was analyzed after hydrolysis in 6 N HCl (20 and 45 min, 155°C, in sealed ampuls) on a Biotronik 7000 analyzer. Mass spectra were recorded on a MS 7070 HS instrument (VG Analytical) under regimes of electron impact and of bombardment with accelerated argon atoms (FAB). Optical rotations were measured on a DIP-360 polarimeter (JASCO).

2-(4-Nitrophenylthio)ethanol. With stirring, 3.3 g (44 mmole) of sodium tetrahydroborate was added in portions over 30 min to 12.3 g of 4,4'-dinitrodiphenyl disulfide (40 mmole) in 200 ml of ethanol. Stirring was continued for another 30 min, and then 4.56 g (80 mmole) of ground KOH in 16 ml (240 mmole) of 2-chloroethanol was added. The reaction mixture was heated at 80°C under reflux until the red color changed to yellow. After cooling in an ice bath, the precipitate that deposited was filtered off and was washed with water and cold ethanol. An additional amount of product was obtained by evaporating and diluting the aqueous mother solutions. The combined precipitates were recrystallized from benzene. Yield 11.5 g (72%), mp 59-60°C (according to the literature: mp 60-62°C [8]); R_f 0.60 (ethylacetate); 0.55 (system 1).

2-(4-Aminophenylthio)ethanol. A mixture of 150 ml of ethanol, 9.95 g (50 mmole) of 2-(4-nitrophenylthio)ethanol, and 59.0 g (250 mmole) of stannous chloride dihydrate was boiled for 30 min. The hot mixture was poured with stirring onto ice, solid NaOH was added to give a strongly alkaline reaction, and the product was extracted with ethyl acetate (3 × 100 ml). The organic phase was washed with water to neutrality, dried with anhydrous Na_2SO_4, and evaporated in vacuum. The residue was triturated with pentane, with cooling, and the resulting crystalline product was filtered off and washed with pentane. Yield 6.7 g (79%), mp 36-37°C, R_f 0.45 (ethyl acetate).

2-[4-(phenylazo)phenylthio]ethanol. A solution of 6.7 g (40 mmole) of 2-(4-aminophenylthio)ethanol and 4.8 g (45 mmole) of nitrobenzene in 40 ml of glacial acetic acid was kept in an atmosphere of argon at 50°C for 30 min. Then the mixture was poured, with stirring, onto ice, and the dark precipitate that formed was collected and was washed with water on the filter. The crude product was dissolved in 150 ml of chloroform, and the solution was washed with 5% $NaHCO_3$ solution and with water and was dried with anhydrous Na_2SO_4. Then it was filtered through a layer of dry silica gel, and the sorbent was washed with chloroform. Evaporation of the combined filtrates yielded 7.6 g (74%) of crystalline product having mp 91-92°C, R_f 0.66 (ethyl acetate), 0.54 (system 1). UV spectrum $\lambda_{max}^{C_2H_5OH}$ 360 (lg ε 4.40).

2-[4-(Phenylazo)phenylsulfonyl-ethanol] (III). With stirring, 6.2 ml of 30% hydrogen peroxide was added dropwise to a mixture of 6.27 g of 2-[4-(phenylazo)phenylthio]ethanol (24.3 mmole), 2 ml of 0.3 M aqueous ammonium molybdate solution, and 60 ml of acetone, whereupon the temperature of the mixture rose and it became homogeneous. The reaction mixture was kept at 40°C for an hour and was then cooled in an ice bath, and the precipitate that deposited was filtered off. An additional amount of product was obtained after diluting the filtrate with water. The combined precipitates were carefully washed with water, dried, and recrystallized from ethanol. Yield 6.41 g (91%), mp 175-177°C, R_f 0.67 (ethyl acetate), 0.40 (system 1). UV spectrum $\lambda_{max}^{C_2H_5OH}$ 360, 454 (lg ε 4.38; 2.76).

The synthesis of the Ppse Esters and Boc-protected amino acids was carried out by a published method for synthesizing Ppse esters [4], using for 1 equivalent of the alcohol (III) 1.1-1.3 equivalents of the Boc-amino acid, 0.1 equivalent of 4-dimethylaminopyridine, and 1.1-1.2 equivalents of DCC. The yields and physicochemical properties of the products are given in Table 2.

Elimination of the Ppse-Protection. The Ppse ester of the completed protected peptide was dissolved in DMFA, DMSO, or a mixture of them (5 ml/g of substance), and 1.2-1.5 equivalents of DBU were added. The mixture was stirred at room temperature for 3-5 min and was diluted with 10-fold volume of a mixture of ether and ethyl acetate (9:1). The resulting precipitate was filtered off, washed with ether, and twice reprecitated from glacial acetic acid into ether.

Peptides with free carboxy groups were obtained in chromatographically pure form, according to HPLC, and were used further without additional purification. The yields of the peptides after elimination of the Ppse protection are given in Table 3.

Synthesis of Peptides by the Block Condensation Method. Preparation of the Hexadecapeptide (IXc). The Boc-protection was eliminated from 2.37 g of the nonapeptide (VII) by dissolving it in 30 ml of TFAA (20 min, 0°C), adding 0.5 g of p-toluenesulfonic acid monohydrate to the solution, and distilling off the TFAA in vacuum. The residue was re-evaporated in chloroform and DMFA and was triturated with ether. The precipitate was filtered off, washed with ether, and dried in vacuum over P_2O_5. This gave 2.41 g (98%) of the nonapeptide tosylate. A solution of 0.46 g (0.25 mmole) of this tosylate, 0.403 g (0.325 mmole) of Boc-Cys(Acm)-Leu-Arg(Mts)-Met(O)-Val-Thr(Bzl)-Gly-OH, and 0.2 g (1.5 mmole) of 1-hydroxybenzotriazole in 3 ml of DMFA–DMSO (1:1) was cooled to 0°C, and to it were added 0.1 g (0.49 mmole) of DCC and, after 45 min, 0.055 ml (0.5 mmole) of N-methylmorpholine. The mixture was kept at 0°C for 4 h, and then at room temperature for 15 h. The dicyclohexylurea that had deposited was filtered off and was washed with DMFA (2 × 3 ml), and, with stirring the combined filtrates were diluted with 50 ml of ether–ethyl acetate (9:1). The resulting precipitate was filtered off and was washed with ether and with ethyl acetate and was reprecipitated from 5 ml of DMFA in 50 ml of ethyl acetate. The yield of peptide (IXc) was 0.69 g (96%).

Peptides (IXb) and (XIIb, c) were obtained similarly starting from the tosylates obtained from peptides (VII) and (X) and the appropriate carboxylic components. The yields at the stage of condensation of the blocks are given in Table 3.

Deblocking and Purification of the Synthetic Peptides. The Ppse group group was eliminated from 180-230 mg of a protected peptide as described above, and then the N-terminal Boc group was eliminated by dissolving the material in 2 ml of TFAA (20 min, 0°C).

The solution was poured into 20 ml of cooled ether, and the resulting precipitate was filtered off and was washed with ether and dried in vacuum. The partially deblocked peptide was dissolved in 4 ml of a 1 M solution of trifluoromethanesulfonic acid–thioanisole in TFAA containing 0.5 ml of m-cresol that had been cooled to 0°C, and the solution was kept in an ice bath for 2 h. Then it was rapidly added dropwise to 20 ml of dry ether cooled to −20°C, and the precipitate was filtered off, washed with ether, and dried in vacuum. It was reprecipitated with ether from 2 ml of glacial acetic acid.

When a methionine sulfoxide residue was present in the peptide, the precipitate obtained was dissolved in 5 ml of 0.1 M ammonium acetate buffer (pH 5), 50-100 equiv. of dithiothreitol was added, and the mixture was kept at 40°C for 16 h.

A solution of a peptide in 3-5 ml of 20% acetic acid or in buffer after reduction was chromatographed on a 25 × 800 mm column of Sephadex G-15 in 1 M acetic acid. The fractions containing the peptides were combined and evaporated and the residue was dissolved in water containing 0.1% of TFAA and was chromatographed on a preparative column of LiChrosorb RB-18 in a gradient of from 5 to 90% of acetonitrile in water containing 0.1% of TFAA. The duration of the gradient was 2 h. The fractions containing the pure desired peptide were combined and lyophilized.

The yields of the peptides at the deblocking and purification stages, calculated on the initial completely protected peptides, are given in Table 3, and the results of amino acid analysis in Table 4.

SUMMARY

For the protection of a C-terminal carboxy group in the synthesis of peptides it is proposed to use the chromogenic 2-[4-(phenylazo)phenylsulfonyl]ethyl group, which can be eliminated by organic bases in aprotic solvents. The synthesis of a number of 10–16-membered peptides corresponding to the C-terminal fragments of the heavy chain of the hemagglutinin of influenza virus of subtypes H1 and H3 has been carried out with the use of this group.

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