Solid Phase Synthesis of Potential Antigenic Peptides and New Lipopeptides of Hepatitis B Virus

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Peptides belonging to the envelope protein of HBV [Tyr^{148}]*S*(139–148) and pre*S*(120–145) have been synthesized using the continuous-flow fluoren-9-ylmethoxycarbonyl (Fmoc)-polyamide solid phase methodology. Furthermore, the synthesis of a new series of hydrophobic derivatives involving N$^{\alpha}$-acylation of both peptides with stearic and cholanic acid as well as the introduction of the synthetic B-cell and macrophage activator Pam_3-Cys-Ser-Ser is also described. Benzotriazol-1-yloxytris-(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) and benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) proved to be convenient reagents to promote the coupling of these lipid moieties to peptides attached to Kieselguhr-supported polyacrylamide resins. Some synthetic aspects concerning reaction conditions and the use of different scavengers at the cleavage stage are discussed. Finally, a cyclic derivative of the *S* peptide was obtained through a disulphide bond formation.

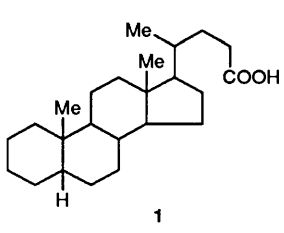
Hepatitis B virus (HBV) is endemic throughout much of the world. There are 280 million people who carry persistent HBV infection and in certain areas more than 10% of the population are chronic carriers. In humans, HBV infection involves a wide spectrum of clinical states ranging from the healthy carrier state to acute or chronic hepatitis and liver cirrhosis. Furthermore, a relationship between hepatocellular carcinoma and chronic HBV infection has already been established.[1]

The human HBV, a member of the Hepadnaviridae family, is composed of a genomic, partially double-stranded, DNA-containing core that is surrounded by a lipid envelope bearing multiple copies of the hepatitis B surface antigen, HBsAg.[2,3] The gen of the surface antigen encodes three different polypeptides: p25 or small protein which bears 226 amino acids of the *S* protein, gp33 or middle protein (*S* region + 55 amino acids of the pre*S2* region) and p39 or large protein (*S* region + pre*S2* region + 119 amino acids of the pre*S1* region).

Current hepatitis B vaccines are obtained either from plasma of human carriers or by recombinant DNA technology.[1,4] Both vaccines are composed of the surface antigen. However, their main drawback is their high cost of production whereas the countries that need them the most can afford them the least. Therefore, chemically synthesized peptides appear as a promising approach towards the production of synthetic immunizing antigens for human vaccination against hepatitis B. Moreover, chemical synthesis precludes exposure of vaccines to viral nucleic acids and avoids contamination by cellular proteins.

Many groups using synthetic peptides of the *S* region have identified putative antibody binding sites mainly in the hydrophillic domain, between residues 139–147 where the *a* epitope common to all HBV serotypes has been located. In addition, antibodies elicited with peptides of the *S* region have been found reactive with the natural envelope protein of HBV particles.[5,6] On the other hand, truncated and conjugated versions of pre*S* sequences have been found to elicit protective antibodies in chimpanzees.[7,8] Furthermore, Neurath and colleagues have demonstrated that the synthetic peptide preS(120–145) binds human antibodies elicited by HBV infection.[9] In fact, the sequence 132–145 is the dominant antibody recognition site within pre*S2* region.[10]

However, synthetic peptides are known to induce considerably weaker immune responses than those elicited by intact proteins or intact viruses.[11] These weak responses may be overcome by the use of immunoadjuvants such as liposomes.[12] Liposomes are lipid vesicles that elicit potent immune responses against the antigen entrapped or linked to them. No toxicity and lack of unwanted side effects as well as absence of immunogenicity by themselves are other interesting characteristics shown by liposomes. In this report, the synthesis of two peptides belonging to the sequence of the envelope proteins [Tyr^{148}]*S*(139–148)† and pre*S*(120–145) is described. Moreover, in an attempt to increase both entrapment yields of the peptides into liposomes and therefore immunogenicity, several derivatives involving *N*$^{\alpha}$-acylation of the parent peptides with lipid moieties have also been prepared. These modifications include *N*-stearoyl[13] and *N*-cholanoyl peptides {cholanic acid [17-β-(1-methyl-3-carboxypropyl)etiocholane] **1** is a natural compound structurally similar to cholesterol, a component of biological membranes that stabilize liposomes} and the synthesis of a conjugate with built-in adjuvancy, Pam_3-Cys-Ser-Ser {Pam_3-Cys, *S*-[2(*R*,*S*)-2,3-bis(palmitoyloxy)propyl]-*N*-palmitoyl-(*R*)-cysteine, **2**}, which is a B-cell and macrophage activator present in the bacterial walls of Escherichia coli.[14] Finally, a conformationally restricted cyclic analogue of the *S* peptide has been obtained through a disulphide bond formation.[15]



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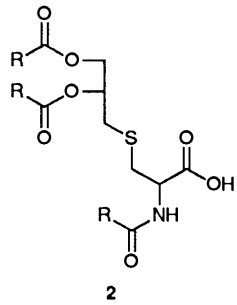

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R = Me[CH_2]$_{14}$

† Abbreviations used for amino acids follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in the *J. Biol. Chem.*, 1972, **247**, 977. All amino acids are of the L-configuration unless stated otherwise.

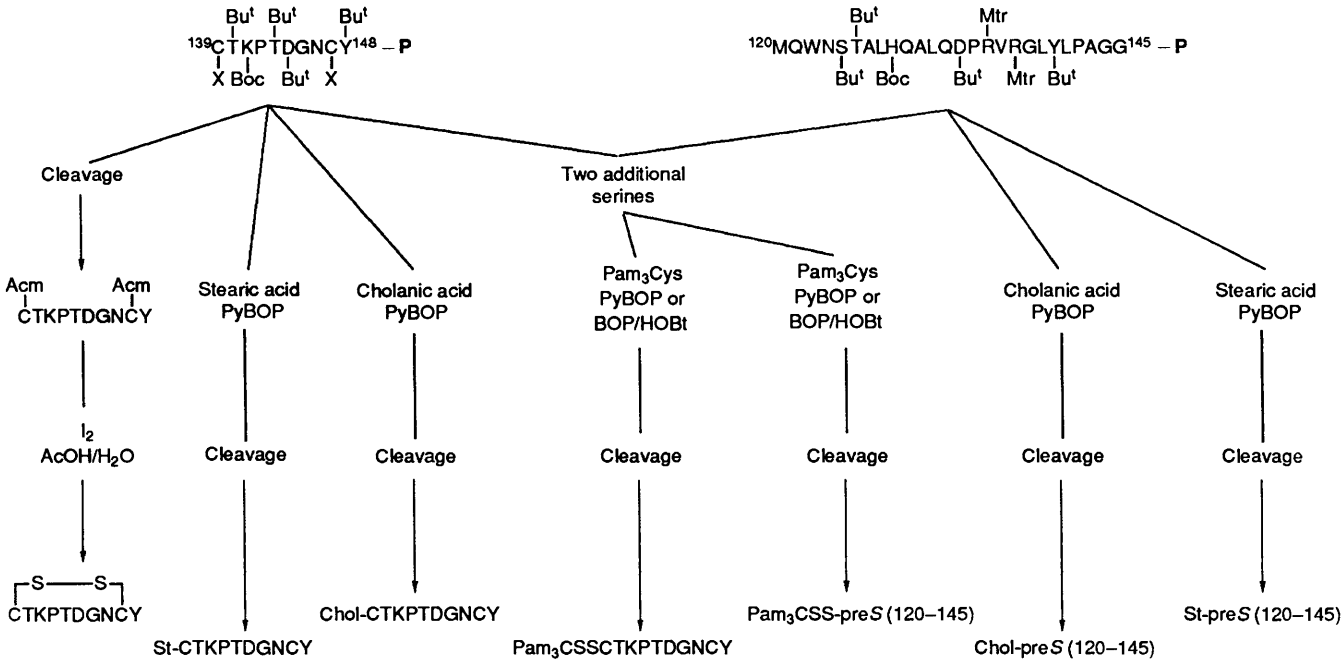

Fig. 1 Scheme of the products synthesized and reactions described. The two protected peptide resins are shown at the top of the page (amino acids are presented in single-letter code, **P** stands for Kieselguhr-supported polyamide resin). Stearic and cholanic acid are coupled directly to both protected peptide resins (X = Trt for the *S* peptide) followed by cleavage to yield the corresponding stearoyl (St) or cholanoyl (Chol) peptides. Similarly, Pam_3-Cys is coupled after addition of two serine residues. On the other hand, the protected decapeptide *S* resin (X = Acm) is cleaved from the resin to afford the Acm protected peptide. The disulphide bond is formed in solution with iodine.

Results and Discussion

The synthesis of the two peptides belonging to the natural sequences of the envelope proteins of HBV (adw serotype) [Tyr^{148}]*S*(139–148) and pre*S*(120–145) has been accomplished by the continuous-flow Fmoc-polyamide solid-phase method which has been successfully employed for the synthesis of acid labile peptides[16,17] as in this case (sequences Asn-Ser, Asp-Gly or Asp-Pro).

The assembly was carried out in a stepwise manner using largely standard procedures (Fig. 1).[18] A functionalised poly(dimethylacrylamide) resin supported in macroporous Kieselguhr was used. The first amino acid of each sequence was esterified to the resin bound linkage (4-hydroxymethylphenoxyacetic acid) using pentafluorophenyl esters of the Fmoc-amino acids and 4-dimethylaminopyridine (DMAP) as the catalyst. The reaction conditions were similar to those previously found to minimise racemization.[19] This esterification step was carried out twice for the attachment of Fmoc-Gly and three times for Fmoc-Tyr(Bu^t) in order to achieve anchoring yields >95% (98% and >99%, respectively). Thus, the residual benzyl hydroxy groups (2% or less) were left uncapped as they do not usually interfere in subsequent amino acid couplings.

The following amino acids were assembled straightforwardly in a continuous-flow synthesizer. A Fmoc/Bu^t scheme of protection was used. Side chains of Lys, Thr, Asp, Ser, His and Tyr were blocked with t-butyl esters, ethers or urethane functions (t-butyloxycarbonyl, t-Boc). On the other hand, guanidine function of Arg was protected with 4-methoxy-2,3,6-trimethylbenzenesulphonyl (Mtr) groups while Cys was protected either with triphenylmethyl (Trt) groups to provide the linear [Tyr^{148}]*S*(139–148) peptide or with acetamidomethyl (Acm) for a later disulphide bond formation. Four-fold molar excesses of activated Fmoc-amino acids were used throughout the synthesis. Most amino acids were coupled as their pentafluorophenyl esters in the presence of an equivalent of 1-hydroxybenzotriazole (HOBt) except Arg, Ser and Thr which were activated as 3-hydroxy-2,3-dihydro-4-oxobenzotriazine (DHBT) esters. Acylation times of 60 min were programmed in the synthesizer (recirculation mode) in order to ensure complete couplings. Samples of peptide–resin were removed during the course of the synthesis and subsequent Kaiser's tests were carried out. Continuous UV monitoring of the column effluent at 304 nm gave typical acylation and deprotection traces. Only a progressive broadening of the deprotection profiles was observed between the fifth and thirteenth residues, sharpening smoothly from this last one onwards. This phenomenon has been attributed to a slow chemical cleavage of the protecting group and slow release of the fluorene derivative from the resin matrix into the solvent.[18] Both effects result from internal aggregation of the growing peptide chains (intermolecular β-sheet secondary structures[20,21]) which introduces additional cross-linking and usually provides qualitative diagnostics for a sluggish acylation in the following cycle. In spite of that, no incomplete couplings were observed in the synthesis of pre*S*(120–145) and [Tyr^{148}]*S*(139–148).

The pre*S*(120–145) peptide was synthesized in a single column and no physical problems were detected in the reaction column during the assembly. However, at the end of the synthesis a dense packing of the resin was observed and after filtering and drying it, the production of a significant amount of resin fines was confirmed. The chemical integrity of the peptide resin was not affected at all but when the peptide–resin was again introduced into the column in order to enlarge the sequence, blockage of the 100 μm filter at the top and bottom of the column increased back pressure of the pump and DMF leaked from the valve fittings.[17] This unexpected production of fines may have been due to a lack of space in the column that did not allow the resin to swell as the peptide was growing. The problem was overcome by sifting the peptide–resin to remove fines.

The *S* peptide was assembled in two column mode since two different groups were chosen to protect cysteine: Acm and Trt. Thus, in a single run of 11 cycles—7 common cycles corresponding to the inner sequence Thr^{140}-Lys-Pro-Thr-Asp-Gly-Asn^{146} and 4 independent cycles, 2 for each peptide resin corresponding to both cysteine residues—the protected peptide–

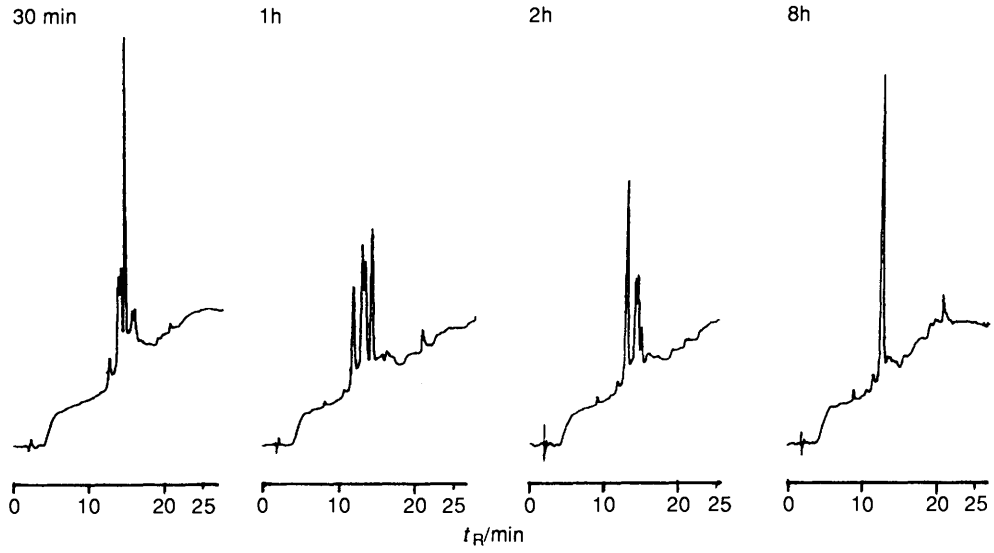

Fig. 2 Monitoring by HPLC of the cleavage reaction of pre*S*(120–145) peptide. Elution conditions: A = H_2O–0.1% TFA; B = ACN–0.1% TFA; gradient from 20% B to 80% B in 20 min + 80% B to 100% B in 10 min; flow 1.2 ml min^{-1}; detection at 215 nm.

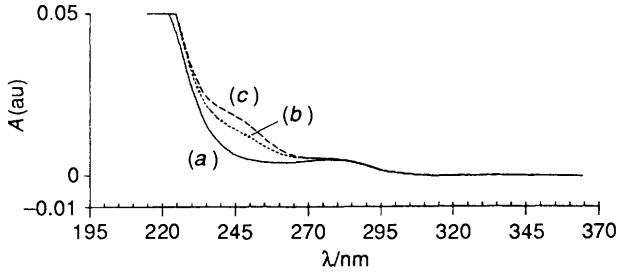

Fig. 3 Diode array UV overlay spectra of the sample at 1 h: (*a*) pre*S*(120–145); (*b*) partially Mtr protected pre*S*(120–145); (*c*) partially Mtr protected (presumably double protected) pre*S*(120–145). The absorption band of Trp can be also observed at 276 nm.

resins [Cys(Acm)^{139}, Cys(Acm)^{147}, Tyr^{148}]*S*(139–148) and [Cys(Trt)^{139}, Cys(Trt)^{147}, Tyr^{148}]*S*(139–148) were obtained.

The incorporation of two additional serines to both peptide–resins was carried out in the synthesizer with two-column mode following standard procedures. However, longer recirculation times and finally, a double coupling was needed in both serine cycles for the pre*S* peptide owing to a weak positive Kaiser's test and because a loss in intensity of the test was observed when a blank of Fmoc-free Ser-pre*S* or Ser-Ser-pre*S* peptide resins was assayed.[18]

As regards acylation with lipid moieties, stearic and cholanic acid were successfully coupled to both peptide resins with Castro's new reagent PyBOP.[22] Addition of 2 equiv. of the fatty acid with 2 equiv. of PyBOP and almost 6 equiv. of *N*,*N*-diisopropylethylamine (DIEA) afforded the lipopeptide resin in less than 30 min as monitored by the ninhydrin test. On the other hand, coupling of Pam_3Cys, a more hindered amino acid, required a longer reaction time[14b] (*ca.* 20 h). Indeed, when only 1.3 equiv. of Pam_3Cys were used, the coupling was incomplete after 24 h and a second acylation was needed to give a negative Kaiser's test. Alternatively, the coupling method BOP–HOBt[23] was also used and, in our hands, similar results were achieved.

Cleavage of the peptide and lipopeptide resins was carried out with trifluoroacetic acid and convenient scavengers. To ensure optimum cleavage conditions (choice of scavengers, length of reaction time) preliminary small scale experiments were carried out. Peptide and lipopeptides of the *S* protein were successfully cleaved in 1.5–2 h with trifluoroacetic acid (TFA)–ethanedithiol (95:5). A single major peak was obtained by HPLC as shown in Figs. 4 and 6.

On the other hand, in the cleavage of pre*S* peptides, which contain two Mtr protected arginines, one tryptophan and one methionine, a mixture of scavengers was necessary so as to quench the cleaved carbocations and to prevent back addition of the detached peptide to the resin.[24] Anisole and ethanedithiol have been found effective scavengers for peptides containing Mtr protected arginines and tryptophan. Furthermore, thioanisole is known to catalyze the removal of Mtr protecting groups from arginine. Thus, a mixture of TFA–thioanisole–ethanedithiol–anisole (90:5:3:2) was chosen for the cleavage. The Mtr protecting group is cleaved slowly by TFA. Therefore, a careful monitoring of the cleavage reaction is needed to establish the optimum reaction time to remove Mtr and minimise the possibility of unwanted side reactions, such as Mtr addition, thioketal formation in the indole ring of tryptophan[25] or those coming from acid labile Asp-Pro or Asn-Ser sequences. In Figs. 2 and 3, an example of cleavage for the pre*S*(120–145) peptide is shown. The other three lipopeptides offered similar profiles (Fig. 4), although at longer reaction times. UV spectra afforded by the diode array detector was extremely useful to ascertain whether the Mtr group has been removed or not (see Fig. 3).

The length of reaction times were very much dependent on the lipid moiety. Thus, 8 h were needed to completely deprotect pre*S*(120–145), 28 h for the stearoyl-pre*S*(120–145) and 35 h for the cholanoyl-pre*S*(120–145). With respect to the Pam_3Cys-Ser-Ser-pre*S*(120–145), additional care should be taken as there is circumstantial evidence that thioanisole can modify cysteine residues.[26] Therefore, two pilot experiments were carried out, one with the aforementioned cleavage mixture and another without thioanisole but with a higher proportion of acid: TFA–anisole–ethanedithiol (94:5:1). Both cleavage reactions were monitored, and showed similar HPLC profiles although in this case, a much slower rate was observed and heating to 45 °C was implemented to speed Mtr removal[25] (30 h at room temperature and 16 h at 45 °C).

Finally, the cyclization of the *S* decapeptide *via* a disulphide bond was attempted (Figs. 5 and 6). The Acm-protected cysteine [Tyr^{148}]*S*(139–148) peptide resin was treated with TFA–water (95:5) for 1.5 h. This time, cleaved t-butyl ions were quenched effectively by water since Acm groups still remained, protecting both cysteine groups. The peptide was

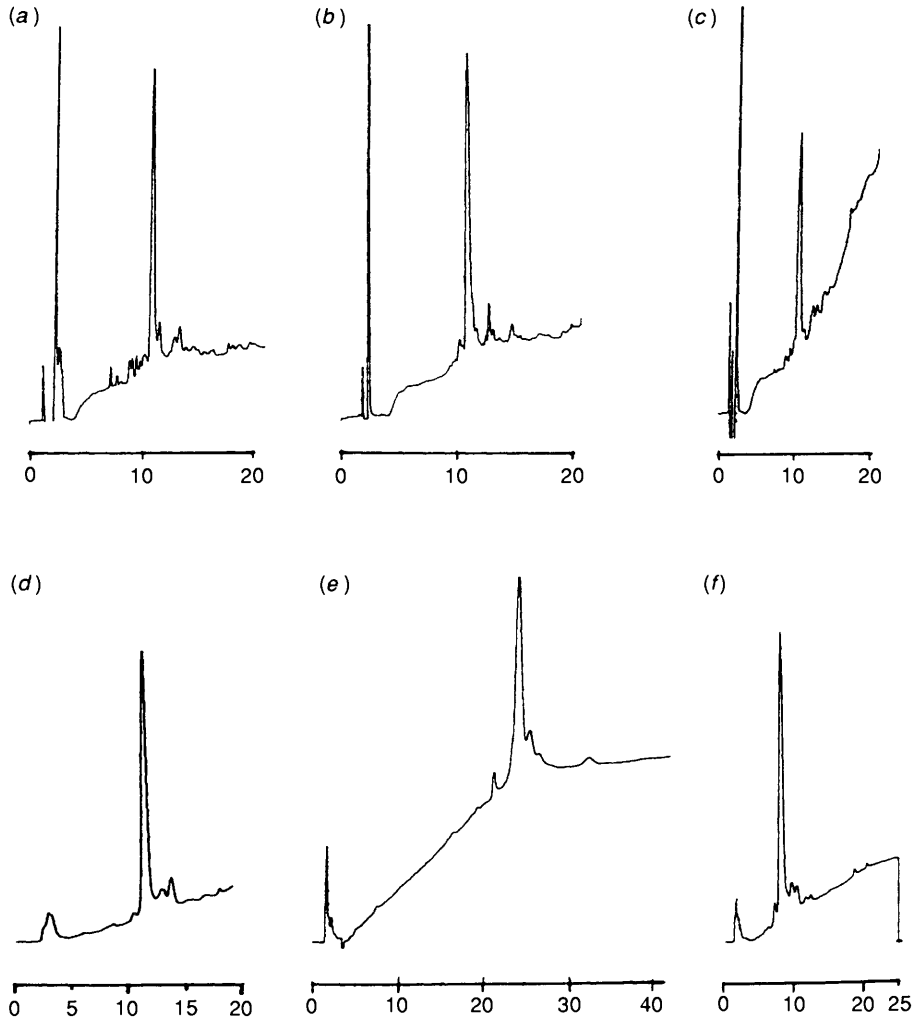

Fig. 4 HPLC elution profiles of some crude lipopeptides: (*a*) stearoyl-pre*S*(120–145); (*b*) Pam_3CSS-pre*S*(120–145); (*c*) cholanoyl-pre*S*(120–145); (*d*) stearoyl-[Tyr^{148}]*S*(139–148); (*e*) Pam_3CSS-[Tyr^{148}]*S*(139–148); (*f*) cholanoyl-[Tyr^{148}]*S*(139–148). Elution conditions: for samples (*a*) to (*c*) the same as Fig. 2; for samples (*d*) to (*f*): A = H_2O–THF (8:2) with 0.1% TFA; B = THF–H_2O (8:2) with 0.1% TFA; gradient from 50% B to 100% B in 20 min + 100% B for 10 min; flow 1.2 ml min^{-1}; detection at 230 nm (1 au).

Table 1 Characterisation of *S* peptides by amino acid analysis (theoretical values in parentheses) and FAB-MS

	CTKPTDGNCY	cyclic CTKPTDGNCY	St-CTKPTDGNCY	Chol-CTKPTDGNCY	Pam_3 CSSCTKPTDGNCY
Asx	2.10(2)	2.01(2)	2.03(2)	1.96(2)	2.07(2)
Ser	—	—	—	—	1.91(2)
Gly	1.08(1)	1.03(1)	1.12(1)	1.10(1)	1.07(1)
Thr	2.03(2)	1.94(2)	1.85(2)	1.92(2)	1.87(2)
Pro	0.92(1)	1.05(1)	1.03(1)	1.01(1)	1.04(1)
Tyr	0.89(1)	0.97(1)	0.91(1)	0.94(1)	0.93(1)
Lys	1.01(1)	1.06(1)	1.05(1)	1.07(1)	1.09(1)
m/z (FAB)	1102 $(M + H)^+$	1099.4 $(M)^+$ 1121.4 $(M + Na)^+$	1367.5 $(M + H)^+$ 1389.5 $(M + Na)^+$	1443.5 $(M)^+$ 1466.6 $(M + Na)^+$	2169 $(M + H)^+$

isolated in the usual manner and disulphide bond formation was achieved by treating the peptide in a dilute solution (0.01 mmol in 40 ml) with a moderate excess of iodine (10 x) in a highly acidic medium [AcOH–water (8:2)] which avoided over oxidation to cysteic acid.[27,28] Moreover, iodine was easily and rapidly removed by extraction with carbon tetrachloride, thus avoiding the use of reductors such as thiosulphite or ascorbic acid which contaminates the crude peptide and can promote unwanted side reactions.

Analytical HPLC of each crude peptide was performed in reversed phase (Spherisorb ODS-2) with acetonitrile-0.1% TFA–H_2O/0.1% TFA. Elution with THF/0.1% TFA–H_2O[9]/0.1% TFA was needed for the more lipophilic *S* peptides. Purification of the peptides was also carried out when needed by medium pressure liquid chromatography on a Lobar System in isocratic conditions or by semipreparative HPLC.[30] All the peptides were characterized by amino acid analysis (each sample gave correct amino acid composition although neither Cys nor Trp were determined) and fast atom bombardment mass spectroscopy.

Table 2 Characterisation of pre*S* peptides by amino acid analysis (theoretical values in parentheses) and FAB-MS spectrometry

	pre*S*(120–145)	St-pre*S*(120–145)	Chol-pre*S*(120–145)	Pam_3CSS-pre*S*(120–145)
Asx	2.17(2)	1.95(2)	2.01(2)	2.11(2)
Gln	2.91(2)	2.96(3)	3.05(3)	2.89(3)
Ser	0.95(1)	1.02(1)	0.93(1)	3.12(3)
Gly	3.09(3)	3.10(3)	2.97(3)	2.95(3)
His	1.00(1)	1.03(1)	0.96(1)	0.99(1)
Arg	2.15(2)	2.03(2)	2.11(2)	2.07(2)
Thr	1.13(1)	1.01(1)	0.96(1)	1.00(1)
Ala	3.05(3)	3.01(3)	2.95(3)	3.08(3)
Pro	2.07(2)	1.95(2)	1.98(2)	1.97(2)
Tyr	1.00(1)	1.01(1)	1.06(1)	0.94(1)
Val	0.87(1)	1.05(1)	1.09(1)	0.98(1)
Met	0.84(1)	0.86(1)	0.93(1)	0.95(1)
Leu	3.78(4)	4.02(4)	3.97(4)	3.91(4)
m/z (FAB)	2880 $(M + H)^+$	3147 $(M + H)^+$	3223 $(M + H)^+$	
	1440 $(M + H)^{2+}$	1256 (Y_{12}'')		
	1256 (Y_{12}'')		1256 (Y_{12}'')	1256 (Y_{12}'')*

* In this sample only the fragmentation Y_{12}'' corresponding to the amide bond Asp^{133}-Pro^{134} was detected.

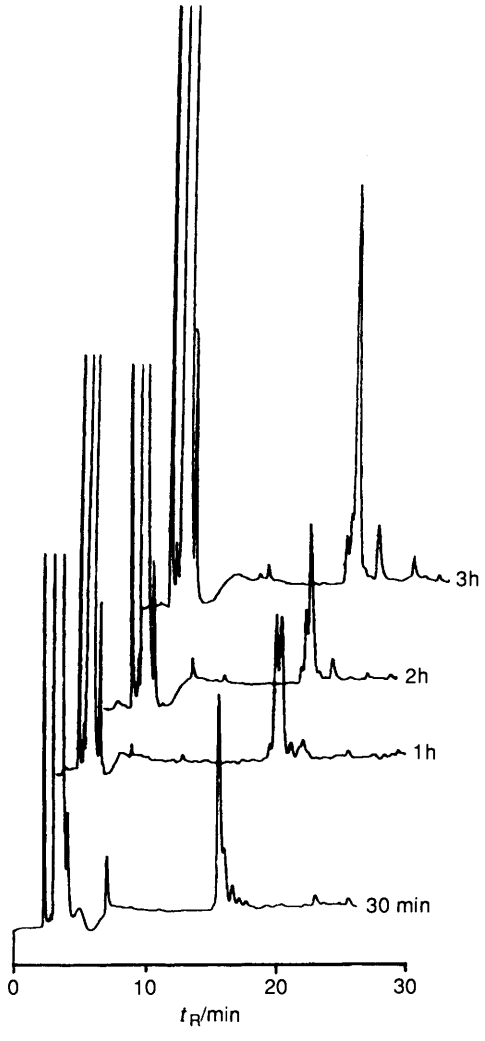

Fig. 5 Progress of the disulphide bond formation in *S* peptide monitored by HPLC. Elution conditions: A = H_2O–0.1% TFA; B = acetonitrile–0.1% TFA; convex gradient from 8% B to 20% B in 30 min. Flow 0.9 ml min^{-1}; detection at 215 nm.

The immunogenicity of these peptides and lipopeptides and their usefulness as potential components of a vaccine is currently being evaluated and will be reported elsewhere.

Experimental

N^{α}-Fmoc amino acid activated species and Kieselguhr supported poly(dimethylacrylamide) resin with 4-hydroxymethylphenoxyacetic acid as the handle (Ultrosyn A) were purchased by LKB-Biochrom, Cambridge, England. Dimethylformamide (DMF) (Ultrosyn Chemicals, LKB) was freshly distilled from ninhydrin and phosphorus pentoxide at reduced pressure (b.p. 55–60 °C). Washing solvents such as t-pentyl alcohol, acetic acid and diethyl ether were obtained from Merck (p. A.). TFA was supplied by LKB Biochrom (Ultrosyn Chemicals) and scavengers such as anisole, thioanisole and ethanedithiol were from Merck. Stearic acid was purchased from Merck (Biochemistry grade), cholanic acid from Sigma and tripalmitoylcysteine from Boehringer Mannheim. Water (MilliQ, reagent grade) was filtered through 0.22 μm membranes. Iodine (p. A.) and DIEA (z. S.) were also from Merck. Coupling reagents BOP and PyBOP were obtained from Fluka and Novabiochem (Läufelfingen, Switzerland), respectively.

Amino acid analyses were carried out in a Pico-Tag system (Waters). HPLC was performed in a Perkin-Elmer series 250 LC pump connected to a LC-235 diode array detector and a LCI-100 integrator. Analytical columns (250 × 4.6 mm) with Spherisorb ODS-2 (10 μm) were eluted at a flow rate of 0.9–1.2 ml min^{-1} with acetonitrile–H_2O or THF–H_2O and 0.1% TFA. Semipreparative RP-HPLC was carried out in a 300 × 7.8 mm Spherisorb ODS-2 column eluting as above. A Lobar MPLC system with glass columns (250 × 10 mm) containing Lichroprep C18 packing material was also used.

Solid Phase Peptide Synthesis.—The synthesis of the peptides [Tyr^{148}]*S*(139–148) and pre*S*(120–145) was essentially straightforward using a standard Fmoc–Bu^t scheme of protection in a continuous-flow synthesizer Biolynx 4170 LKB. A functionalised (3 g; 0.093 mequiv. g^{-1}) acid labile poly(dimethylacrylamide) resin Ultrosyn A was used. Side chain protection was afforded by the following: Mtr for arginine, Acm or trityl (Trt) for cysteine, t-butyl for serine, threonine, aspartic acid and tyrosine and Boc for lysine and histidine. The C-terminal residue of the peptide was esterified manually onto the linkage agent using the pentafluorophenyl esters with 0.1 equiv. of DMAP as the catalyst.[18] Each of the steps involved in the synthetic cycle was controlled by an Olivetti M-240 personal computer. Four-fold molar excesses of activated species were employed throughout the synthesis. The reaction protocol was as follows: pre-wash with DMF (5 min), amino acid coupling (60 min), washing with DMF (10 min), Fmoc deprotection with

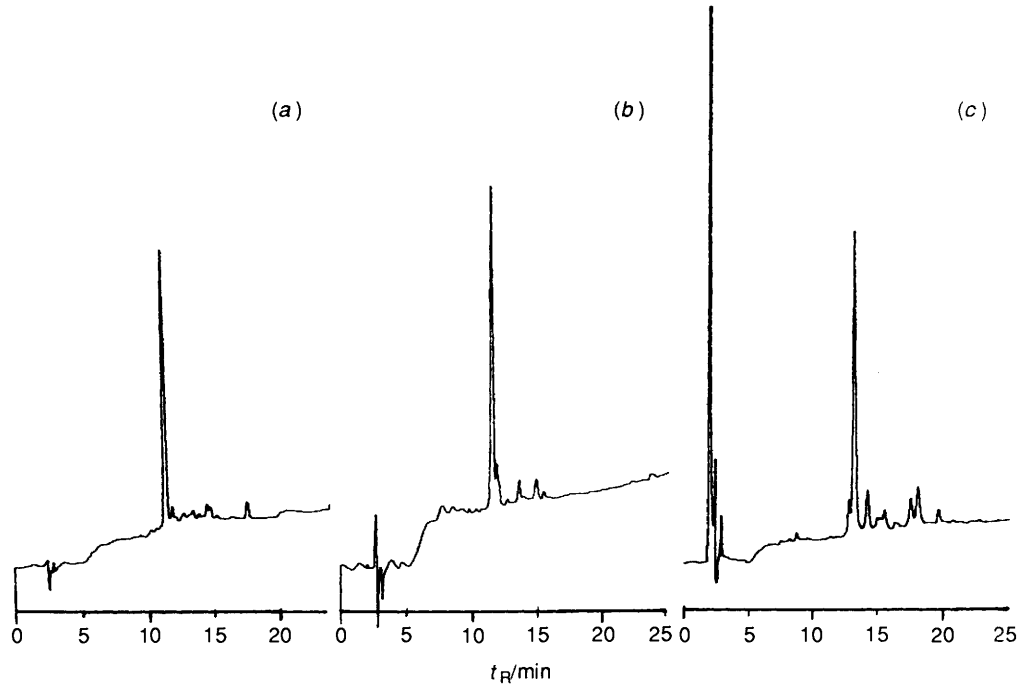

Fig. 6 HPLC elution profiles of some crude *S* decapeptides: (*a*) [Tyr^{148}]*S*(139–148); (*b*) [Cys(Acm)^{139}, Cys(Acm)^{147}, Tyr^{148}]*S*(139–148); (*c*) cyclic [Tyr^{148}]*S*(139–148). Gradient from 10% to 60% B in 30 min; A = H_2O–0.1% TFA; B = acetonitrile–0.1% TFA; flow 1.2 ml min^{-1}; detection at 215 nm.

20% piperidine in DMF (10 min) and post-wash with DMF (10 min). The flow rate of the synthesizer's pump was set at 4 ml min^{-1}. The reaction column effluent was subjected to continuous UV monitoring by passage through an Ultrospec 4050 spectrophotometer at 304 nm for the piperidine–dibenzofulvene adduct, at 320 nm for the DHBT derivatives of serine and threonine and at 330 nm for the DHBT derivative of arginine. The synthesis was programmed to pause after each acylation for removal of resin beads and subsequent testing for the presence of free amino groups by the Kaiser's test.[29] No repeat couplings were required during the assembly.

The assembly of the pre*S*(120–145) peptide was carried out in a single column whereas the [Tyr^{148}]*S*(139–148) peptide was synthesized in two-column mode. This allowed the synthesis of two differently protected *S* peptides: [Cys(Acm)^{139}, Cys(Acm)^{147}, Tyr^{148}]*S*(139–148) and [Cys(Trt)^{139}, Cys(Trt)^{147}, Tyr^{148}]*S*(139–148). At the completion of the last cycle, the peptide–resin was removed from the reaction column, washed well with DMF, t-pentyl alcohol (or isopropyl alcohol), acetic acid, t-pentyl alcohol and anhydrous diethyl ether, and dried under reduced pressure. Peptide resins were stored in nitrogen atmosphere at −30 °C until further use.

N$^{\alpha}$-*Acylation with Stearic or Cholanic Acid.*—Dry peptide–resin (400 mg; *ca.* 0.04 mequiv.) of pre*S*(120–145) or [Cys(Trt)^{139}, Cys(Trt)^{147}, Tyr^{148}]*S*(139–148) was well swelled in DMF for at least 1 h, and the solvent was decanted off. Stearic acid (22.8 mg, 0.08 mmol) or cholanic acid (28.9 mg, 0.08 mmol) was dissolved in a minimum amount of solvent [1 ml of DMF–dichloromethane (1:1), or DMF, respectively] followed by addition of Castro's coupling reagent PyBOP (41.6 mg, 0.08 mmol). The solution was then poured onto the soaked peptide–resin and DIEA (37.5 μl, 0.22 mmol) was finally added. The mixture was set aside at room temperature and swirled occasionally. The reaction was complete within 30 min as judged by the ninhydrin colour test. The resin was filtered and washed sequentially with DMF, t-pentyl alcohol, acetic acid, t-pentyl alcohol, anhydrous diethyl ether and dried *in vacuo*. Alternatively, stearic acid was also activated as its preformed symmetrical anhydride with DCC following the protocol described.

Synthesis of Pam$_3$Cys-Ser-Ser-peptide–resins.—Peptide–resins of pre*S*(120–145) and [Cys(Trt)^{139}, Cys(Trt)^{147}, Tyr^{148}]*S*(139–148) sequences (400 mg) were introduced in two different columns and two additional serines (0.5 mmol) were coupled in parallel according to the continuous-flow Fmoc-polyamide protocol. Once both Ser-Ser-peptide–resins were prepared, coupling of Pam$_3$Cys was carried out either with PyBOP or BOP–HOBt following the protocol described above. The BOP–HOBt method involved the following proportions: peptide–resin (70 mg, *ca.* 0.005 mequiv.), Pam$_3$Cys (10 mg, 0.011 mmol), BOP (4.9 mg, 0.011 mmol), HOBt (1.7 mg, 0.011 mmol) and DIEA (5.6 μl, 0.033 mmol) in DMF–dichloromethane (1:1). The reaction was set aside overnight (20 h). Work up was as usual.

Cleavage and Deprotection.—Dry protected peptide–resins (70–600 mg) were treated with TFA solution containing appropriate scavengers (10 ml; 100 mg peptide–resin). Peptide [Cys(Trt)^{139}, Cys(Trt)^{147}, Tyr^{148}]*S*(139–148) and its lipid derivatives were cleaved with TFA–ethanedithiol (95:5) whereas pre*S*(120–145) peptide and its related lipopeptides were treated with TFA–thioanisole–ethanedithiol–anisole (90:5:3:2). The mixture was maintained at room temperature and swirled from time to time. Reaction times were dependent on the peptide and established according to pilot cleavage and characterization experiments (10–50 mg of peptide–resin). Aliquots of 50–200 μl from the pilot cleavage mixtures were removed and TFA was blown off with a nitrogen stream in Eppendorf vials. The crude peptide was precipitated with dry diethyl ether. The sample was sonicated and centrifuged (5 min; 3000 rpm) and the supernatant liquid was decanted off. This last step was repeated 3–6 times with dry diethyl ether or ethyl acetate until the scavengers were removed. Finally, the crude peptide was dissolved in water, water–methanol or THF–water and subjected to HPLC analysis.

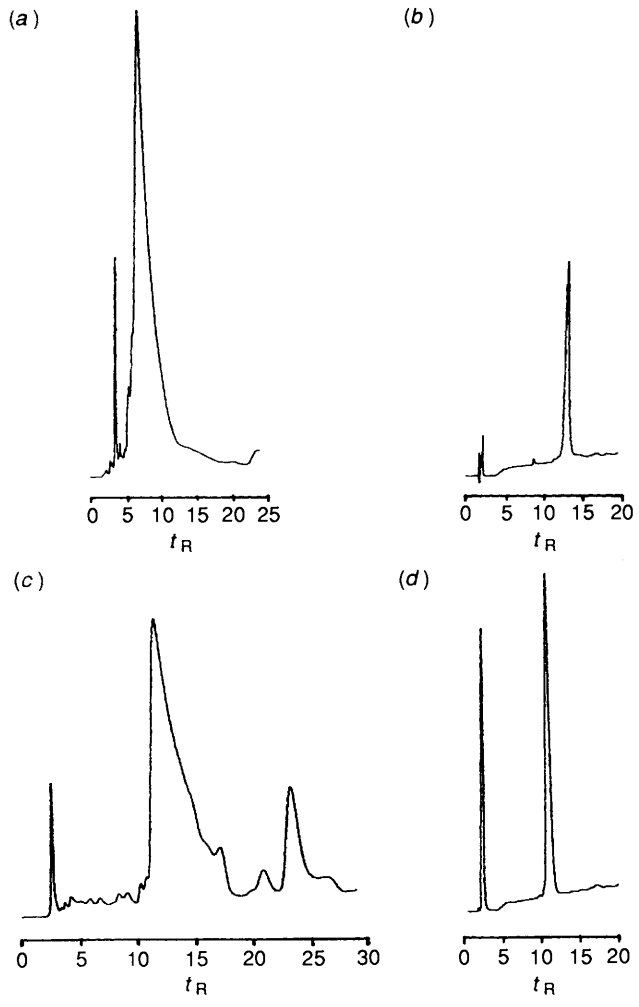

Fig. 7 Purification by semipreparative HPLC: (*a*) semipreparative HPLC of pre*S*(120–145). Elution conditions: isocratic 37% B; A = H_2O–0.1% TFA, B = acetonitrile–0.1% TFA; flow rate = 3 ml min^{-1}, detection at 275 nm; (*b*) analytical HPLC of purified pre*S*(120–145), elution conditions as in Fig. 2; (*c*) semipreparative HPLC of Pam_3Cys-Ser-Ser-pre*S*(120–145). Elution conditions: isocratic 29% B; buffers as in (*a*), detection at 275 nm; (*d*) analytical HPLC of purified Pam_3Cys-Ser-Ser-pre*S*(120–145), elution as in Fig. 2.

According to these small-scale experiments, a reaction time of 1.5 h was needed for the *S* peptides. On the other hand, reaction times for pre*S* peptides were strongly dependent on the lipid moiety. Thus, 8 h were needed for the pre*S*(120–145) peptide, 28 h for the stearoyl peptide, 35 h for the cholanoyl peptide and 30 h at room temperature and 16 h at 45 °C for the Pam_3Cys-Ser-Ser-peptide. The peptide was isolated from the cleavage mixture by filtration and washing the resin well with TFA. The filtrate was rotary evaporated and the oily residue transferred to a centrifugation tube of polypropylene. The crude peptide was precipiated with anhydrous diethyl ether (50 ml), sonicated and centrifuged (5–10 min; 3500 rpm) and the organic supernatant discarded (5 × 25 ml).

The extent of cleavage in each case was at least 95% according to amino acid analysis of the cleaved resins (norleucine as internal standard). Yields of crude peptides >85% were achieved according to the percentage of attachment.

Disulphide Bond Formation of [Tyr^{148}]*S*(139–148).—[$Cys(Acm)^{139}$, $Cys(Acm)^{147}$, Tyr^{148}]*S*(139–148) peptide–resin (200 mg) was cleaved with TFA–H_2O (95:5) for 1.5 h. The Acm-protected peptide was isolated from the cleavage mixture as above (extent of cleavage >95%, yield of crude peptide 92%). Disulphide bond formation of Cys(Acm)-Thr-Lys-Pro-Thr-Asp-Gly-Asn-Cys(Acm)-Tyr (12 mg, 0.01 mmol) was carried out in 40 ml of degassed AcOH–H_2O (8:2) with a 10-fold excess of iodine (25.4 mg, 0.1 mmol). The solution was magnetically stirred. Careful monitoring of the cyclization showed that the reaction had finished after 3.5 h. The mixture was then diluted with water (40 ml) and iodine was eliminated by extraction with carbon tetrachloride (5 × 100 ml). The aqueous solution was evaporated under reduced pressure at 45 °C to eliminate residual CCl_4 and AcOH. Finally, the solution was diluted with water (20 ml) and freeze dried (yield of crude peptide 83%).

Purification of the Peptides.—The peptides and lipopeptides were purified mainly by semi-preparative HPLC though when larger amounts of purified peptides were desired, a Lobar System (Merck) was used (Fig. 7).

Peptide pre*S*(120–145) and related lipopeptides as well as [Tyr^{148}]*S*(139–149)—cyclic or linear—were eluted with ACN/0.1% TFA–H_2O/0.1% TFA mixtures whereas the strongly lipophilic *S* lipopeptides required elution with THF–H_2O mixtures [B = THF/H_2O (8:2)–0.1% TFA; A = H_2O/THF (8:2)–0.1% TFA]. Purification was performed under isocratic conditions previously used on an analytical scale (target peptide elution k' = 4–8). Subsequent scale up was estimated according to the internal diameter and length of the column (300 × 0.78 mm semipreparative, 250 × 10 mm Lobar A). Thus, loads ranged from 2–3 mg for the semipreparative scale to 15–25 mg for the Lobar column. Flow rate was set at 3 ml min^{-1} and detection was at 275 nm (semipreparative) whereas the flow rate was set between 7 and 10 ml min^{-1} (6.5 bar) for the Lobar System and detection at 226 nm. Fractions were collected manually according to the chromatographic profile obtained simultaneously. Fractions of similar purity were pooled together and eventually the less pure purified again. The purity achieved was >90% with the Lobar System and >95% with the semipreparative HPLC (percentage of purity was estimated as the ratio of the integrated area of the peak over that of the total integrated areas. The solvent or loading artifact was excluded). Recoveries of peptides ranged from 26 to 55%. Homogeneity of the purified peptides was also established according to the diode array test. Three UV spectra (195–370 nm) were performed as the peak was being plotted. These spectra were compared and processed to give a purity index. This was always satisfactory for the peptides described which was indicative of their homogeneity.

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