Application of the Continuous-flow Polyamide Method to the Solid-phase Synthesis of a Multiple Antigen Peptide (MAP) based on the Sequence of a Malaria Epitope

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Application of the 'flow-polyamide' method to the synthesis of the title compound, a high molecular weight (18 947) branched peptide, was successful without requiring any deviation from the standard protocol.

Multiple antigen peptides (MAPs) are branched compounds based on a core of lysine residues containing both α- and ε-amide linkages between themselves. The terminal amino groups represent the starting points for the assembly of multiple copies of a linear sequence (see Figure 1). MAPs have recently been described by Tam[1,2] and, owing to their many advantages over conventional peptide-carrier conjugates, are rapidly gaining application in immunological studies, both as prototype synthetic vaccines and as antigens in solid-phase immunoassays. In view of the characteristics of the MAP, namely high density and high molecular weight (M) we believed that the 'continuous-flow polyamide' method for solid-phase peptide synthesis, developed by Sheppard and co-workers,[3] is ideally suited for this synthesis.

Here we describe the assembly of a MAP based on the sequence of the immunodominant epitope of the Circumsporozoite protein of the *Plasmodium malariae* sporozoite, one of the causative agents of malaria in man, performed for the first time by 'continuous-flow polyamide' solid-phase synthesis. This assembly was successful without requiring any variation of the standard protocol.[4]

The antigenic sequence of the MAP is (Asn-Ala-Ala-Gly)$_6$ [(NAAG)$_6$], and the target peptide is [MAP$_8$(NAAG)$_6$], where the subscript 8 refers to the fact that the lysine core is octameric.

In order to distinguish between the problems arising from the macromolecularity of the system and those coming from the linear sequence alone, (Asn-Ala-Ala-Gly)$_6$Asn-Ala was previously assembled on the same support.† The synthesis was performed on an LKB 4170 Biolynx synthesizer, according to the standard protocol and essentially in agreement with the manufacturer's instructions, apart from the excess of acylating species, which was decreased to 0.25 mmol per gram of resin.

† The additional Asn-Ala residues at the C-terminus were included to prevent Ala-Gly dioxopiperazine formation.[5]

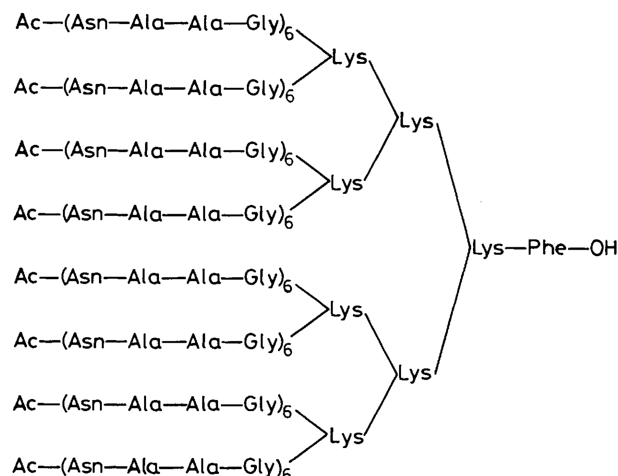

Figure 1. Structure of the octameric multiple antigen peptide [MAP$_8$(NAAG)$_6$] based on the sequence (Asn-Ala-Ala-Gly)$_6$, (NAAG)$_6$.

Thus, a 2.5-fold excess of Fmoc-amino acid pentafluorophenyl esters (Fmoc = fluoren-9-ylmethoxycarbonyl), together with 1 equiv. of 1-hydroxybenzotriazole (HOBt), were used throughout (coupling time 50 min), except for the initial esterification step, which was carried out by Fmoc-Ala symmetrical anhydride catalysed by 0.1 equiv. of 4-dimethylaminopyridine (DMAP). No resin samples were removed for

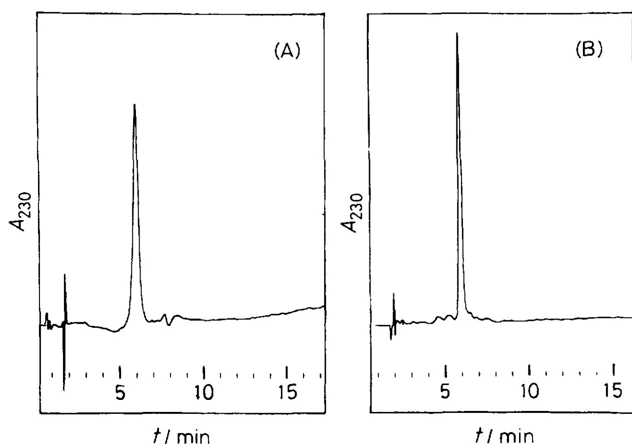

Figure 2. Analytical HPLC of (A) [$MAP_8(NAAG)_6$] and (B) $(Asn\text{-}Ala\text{-}Ala\text{-}Gly)_6Asn\text{-}Ala$; *conditions*: eluant a = 0.1% TFA, eluant b = MeCN (0.1% TFA), flow 1.5 ml min^{-1}; (A) column Vydac 218TP (300 Å), RP_{-18} (5 μm, 15 × 0.4 cm), linear gradient 10—70% b (15 min), (B) column Lichrosorb RP-18 (10 μm, 25 × 0.46 cm), linear gradient 10—20% b (15 min).

analytical control, and the whole synthesis was totally unattended. A post-assembly inspection of the spectrophotometric traces collected by the instrument during acylation and deprotection showed no evidence of incomplete reactions, as judged by the consistency of the area of the deprotection peak. The final crude peptide, cleaved by 90% aqueous trifluoroacetic acid (TFA), was 89% pure after desalting on Sephadex G-15 [see Figure 2(B)]; it showed the expected amino acid composition [Asn, 6.61 (7); Ala, 12.78 (13); Gly, 6.00 (6)] and its identity was confirmed by FAB MS analysis (calculated mass 2082, found M^+ + H 2083.0).

[$MAP_8(NAAG)_6$] was synthesized by the same procedure. The only precaution that we took, according to Tam's suggestion, was to decrease the starting load of the resin, by reducing the efficiency of the esterification step; thus, $(Fmoc\text{-}Phe)_2O$ (0.25 mmol/g of resin), in the presence of DMAP, was reacted with the resin (commercial Ultrosyn A resin; Pharmacia-LKB), already functionalized with Nle and the linkage agent 4-hydroxymethylphenoxyacetic acid for just 15 min, yielding a functionalization of 0.02 mequiv. g^{-1}. The octavalent lysine core was obtained by sequential coupling of 0.06, 0.12, and 0.25 mmol of Fmoc-Lys(Fmoc)-OH, which were incorporated through dicyclohexylcarbodiimide (DCC)/HOBt coupling in a batchwise operation. In a repeat assembly of [$MAP_8(NAAG)_6$], the incorporation of the lysine residues was achieved by BOP/HOBt coupling [BOP = benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate],[6] so that the whole synthesis of the MAP could be performed in a flow operation. The stepwise addition of each residue was monitored by the usual colour tests,[7,8] which showed that the acylations were complete after the 50 min recirculation period; no recoupling was necessary at any point in the synthesis. The *N*-terminal amino groups were acetylated by reaction with Ac_2O (0.25 mmol) in *N,N*-dimethylformamide (DMF) in the presence of 1 equiv. of DMAP. The peptide was cleaved from the resin by treatment with 90% (aq.) TFA (3 h, room temp.), the solvent was removed *in vacuo*, and the peptide obtained was directly applied to a Sephadex G-50 column (eluant 0.1 M acetic acid); the fractions corresponding to the first eluting peak were pooled and freeze-dried.

Peptide purity was checked by analytical HPLC both in the reverse-phase mode [see Figure 2(A)] and in the gel permeation mode on a TSK-GEL SW-3000 column, calibrated with a series of known molecular weight standards, according to Kato *et al.*;[9] the MAP eluted as a single peak with the expected *M* (found: 19000; calc. 18947). Given the large difference between the content of Asn, Ala, and Gly with respect to Lys and Phe, amino acid analysis gave satisfactory amino acid ratios [Asn, 0.92 (1), Gly, 1.00 (1), Ala, 1.95 (2), Lys 0.14 (0.14), Phe 0.03 (0.02)].

Our results suggest that, in the case of MAP synthesis, the 'flow-polyamide' method may be more suited than the standard 'Boc-polystyrene' protocol. (i) A major point raised by Tam,[1] namely the use of DMF instead of CH_2Cl_2 as the coupling solvent, is an inherent feature of the former method; moreover, the use of DMF throughout the assembly, together with the homogeneity of the peptide–resin system with regard to swelling properties, is probably beneficial in preventing aggregation of the densely packed peptide chains, thus explaining the observed smoothness of the acylations (no double coupling was necessary, and standard acylation kinetics were observed, using just a 2.5-fold excess of the acylating species). (ii) The use of the Fmoc group for temporary N^α protection, and t-butyl based side chain protection (when needed), together with a TFA-labile peptide–resin bond, avoids the addition of aromatic scavengers to the cleavage mixture (unless Trp and/or Arg residues are present); these compounds tend to adhere strongly to the MAP, and require dialysis against 8 M urea for efficient removal.[1] In our case the peptide could be recovered by simple freeze-drying of the aqueous solution (before or after the gel-permeation column chromatography).

A final comment concerns the use of the Kieselghur-poly(dimethylacrylamide) resin for the synthesis of very high molecular weight peptides (see ref. 10 for a recent discussion): though perhaps not conclusive, owing to the particular branched nature of [$MAP_8(NAAG)_6$], our results strongly suggest that this resin is as effective as the corresponding gel polymer within the practical size limits of the solid-phase method.

An immunoassay based on [MAP_8 $(NAAG)_6$], useful for the seroepidemiology of *P. malariae* malaria, is described in a patent application,[11] and will be the subject of a forthcoming publication.

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