SOLID PHASE SYNTHESIS OF THE PROTECTED 27–42 HEXADECAPEPTIDE OF THE HEAVY CHAIN FROM MYELOMA IMMUNOGLOBULIN M603

Elimination of Side Reactions Associated with Glycyl-2-oxypropionyl-resin

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A fully protected 27–42 hexadecapeptide of the variable region of myeloma immunoglobulin M603 was synthesized on a 2-bromopropionyl-resin by the solid phase method. Side reactions due to cyclization of glycyl-2-oxypropionyl-resin were studied under different reaction conditions. The loss of peptide chains at the dipeptide and tripeptide stages due to diketopiperazine formation was also examined. These side reactions were circumvented by using a combination of fragment and stepwise coupling methods. The synthesized protected peptide was removed from the resin in 85% yield by photolysis, and purified by crystallization and by chromatography on a Sephadex LH-60 column.

Key words: haloacylated polystyrene resin; protected 27–42 segment of immunoglobulin M603; α-methylphenacyl linkage; photolytic cleavage; side reactions of glycyl-2-oxypropionyl-resin; solid phase peptide synthesis.

In the course of the chemical synthesis of the VH domain of an antibody by a solid phase-fragment strategy (Wang & Merrifield 1972; Yajima et al., 1974), protected 16-residue peptide I has been prepared. This peptide, which corresponds to the 27–42 segment of the phosphorylcholine-binding IgA mouse myeloma protein M603 (Potter & Leon, 1968; Rudikoff & Potter, 1974), is of particular biological and synthetic interest.

Boc-Phe-Thr(Bzl)-Phe-Ser(Bzl)-Asp(OBzl)-Phe-Tyr(2,6-Cl2 Bzl)-Met-Glu(OBzl)-Trp(For)-Val-Arg(Tos)-Gln-Pro-Pro-Gly-OH

Studies by X-ray crystallography (Segal et al., 1974; Davies et al., 1975) have established that the combining site of the M603 antibody is a continuous surface formed by hypervariable regions of both the light and heavy chains. A large cavity, in which phosphorylcholine and antigens bearing this hapten are found to bind, was formed in the middle of this surface by the hypervariable residues located in the L1 and L3 regions of VH and VL, variable region of heavy chain and light chain, respectively; H1, H2, H3 and L1, L3, hypervariable regions of heavy chain and light chain, respectively.
regions of \( V_H \) (Kabat & Wu, 1971; Padlan et al., 1976). In this cavity, the phosphorylcholine residue was bound with the choline group buried deep in the interior and the phosphate group near the exterior. Of the six residues of \( V_H \) that are involved in antigen binding, two — Tyr\(^{33}\) and Glu\(^{35}\) — are found in the 27–42 segment of the first hypervariable region (H1). The hydroxyl group of Tyr\(^{33}\) forms a hydrogen bond with one of the phosphate oxygens, while the acid group of Glu\(^{35}\) interacts electrostatically with the positively-charged tetraalkylammonium group. In addition, there is extensive Van der Waals interaction between the phenyl ring of Tyr\(^{33}\) and the antigen. Finally, Phe\(^{32}\) and Met\(^{34}\) may have conformational roles that serve to ensure the proper positions of Tyr\(^{33}\) and Glu\(^{35}\) in the antigen binding cavity.

The segment 27–42 presented four separate synthetic problems which we have studied and report here. The initial synthetic strategy involved a stepwise solid-phase synthesis (Merrifield, 1963) with an \( \alpha \)-methylphenacyl ester anchoring bond (Fig. 1) (Mizoguchi et al., 1970). It was selected because this support would allow us to cleave the completed peptide from the resin in a fully protected form by photolysis (Wang, 1976). However, in this and several syntheses of other protected peptides with carboxyl-terminal glycine on the pro- pionyl-resin, we have observed a decreased incorporation of the second amino acid due, probably, to the cyclization of the glycyl-2-oxopropionyl-resin 2, with formation of Schiff base 3a or its tautomer, 2-oxo-2,3-dihydro-6-methyl-1,4-oxazinyl-5-resin 3b (Tam et al., 1977; Birr et al., 1977).

This cyclization reaction could also give rise to two further potential side reactions: (a) an acylation of the imino group of 3b by subsequent activated Boc-amino acids, and (b) a ring-opening reaction later in the synthesis with generation of a free amino group. The first would give peptides which would not be released from the resin by photolysis, while the latter would result in deletion peptides.

In our preliminary synthesis of the 27–42 segment by the stepwise procedure, further decreases of Gly and Pro led us to assume that there were some losses of the peptide segments, Pro-Gly and Pro-Pro, due to diketopiperazine formation at the dipeptide 4 and tripeptide 5 stages (Fig. 2).

It also has been reported that during Boc-removal from resin-bound N-terminal glutaminyl peptides with acid, pyroglutamyl peptides can be formed. Weak acids promote the cyclization

![FIGURE 1](image-url)

Formation of 2-oxo-2,3-dihydro-6-methyl-1,4-oxazinyl-5-resin 3b from glycyl-2-oxopropionyl-resin 2. \( \oplus \) designates the polystyrene-1%-divinylbenzene crosslinked resin. However, one of the aromatic rings is illustrated here to show the \( \alpha \)-methyl-phenacyl ester linkage.
more extensively than strong acids. Beyerman et al., (1973) have shown, using Boc-Gln-Gly-O-CH₂- as a model, that no pyroglutamyl peptide was formed, even after 70 h, when 4 N HCl/dioxane was used to deprotect the Boc group, while 11% of pyroglutamyl peptide was obtained when the same peptide-resin was treated with 50% TFA/CH₂Cl₂ (v/v) for the same length of time.

We have carried out a careful quantitation of the extent of each of these four side reactions during the stepwise synthesis of the C-terminal 38–42 pentapeptide-resin, Aoc-Arg(Tos)-Gln-Pro-Pro-Gly-2-oxypropionyl-resin. To circumvent the side reactions, a modified strategy was designed for the synthesis of the 27–42 segment of M603 heavy chain.

RESULTS AND DISCUSSION
To study the extent of terminated glycine due to cyclization of the Gly-2-oxypropionyl-resin and the effect of acid and base on this side reaction, an indirect test was carried out in which several samples of Boc-Gly-2-oxypropionyl-resin were treated alternately with 50% TFA/CH₂Cl₂ and 5% DIEA/CH₂Cl₂ for different lengths of time, followed by DCC-mediated coupling of Boc-Pro-OH. Based on the incorporation yield of Boc-Pro-OH onto Gly-2-oxypropionyl-resin, the relative extent of glycine termination was calculated. To test whether the coupling conditions used in this study were sufficient, Boc-Pro-OH was coupled to the conventional H-Gly-O-CH₂-
**TABLE 1**

**Effect of acid on the cyclization of Gly-2-oxypropionyl-resin: amino acid analysis of Boc-Pro-Gly-2-oxypropionyl-resin**

<table>
<thead>
<tr>
<th>Length of acid treatment of Gly-2-oxypropionyl-resin</th>
<th>Residue</th>
<th>30min</th>
<th>1.5h</th>
<th>3h</th>
<th>6h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>0.85</td>
<td>0.65</td>
<td>0.64</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>Termination of Gly [%]</td>
<td>15</td>
<td>35</td>
<td>36</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>

*Boc-Gly-2-oxypropionyl-resin samples (100 mg) were treated with 10 ml of 50% (TFA/CH₂Cl₂ (v/v) for different lengths of time followed by neutralization with 5% DIEA/CH₂Cl₂ (v/v, 5 min) and DCC-mediated coupling of Boc-Pro-OH (1 h).*

The study showed that at the beginning stage, with increasing length of time of the acid treatment an increase of glycine termination occurred as indicated by a steadily decreasing incorporation of Boc-Pro. However, after 90 min of acid treatment the cyclization reaction reached a maximum which did not change up to 6 h, as shown in the Table 1. At this stage 35% of glycine had been terminated.

In contrast, during treatment of the Gly-2-oxypropionyl-resin with base the extent of termination increased steadily and did not reach a maximum within 6 h (Table 2). These findings are well in accord with the observation of Birr et al. (1977) on oxyacetyl-resin. It is known that the reaction to form a Schiff base between an amine and carbonyl compound is most favorable at the pK of the nucleophilic reagent. Therefore, the extent of the cyclization reaction under neutral condition was also estimated by shaking Gly-2-oxypropionyl-resin in CH₂Cl₂ for 3 h after the neutralization step. Quantitative amino acid analysis of the resulting Boc-Pro-Gly-2-oxypropionyl resin indicated 89% termination. These results, together with those of Birr et al. (1977), support the hypothesis that intramolecular Schiff base formation and rearrangement to a dihydro-1,4-oxazine-2-one had occurred. The mechanism was further supported by the finding that the corresponding imino acid derivative, Pro-2-oxypropionyl-resin did not undergo termination.

**Stepwise synthesis of the protected pentapeptide-resin**
To study the levels of the four side reactions under the conditions of a normal stepwise solid phase synthesis, the pentapeptide-resin 38–42, Aoc-Arg(Tos)-Gln-Pro-Pro-Gly-2-oxypropionyl-resin was prepared by a stepwise procedure. Boc-Gly was attached to 2-bromopropionyl-resin, the Boc-group was removed by 50% TFA/CH₂Cl₂ (30 min), the resulting TFA salt was neutralized by two treatments with 5% DIEA/CH₂Cl₂ (2 min and 5 min), and a double coupling of the next amino acid was carried out. The couplings were mediated by DCC in CH₂Cl₂ except for Boc-Gln, which was coupled via its 4-nitrophenyl ester in DMF. The reactions were monitored for completeness by picric acid titration. At the second and third stages of the synthesis the filtrates and washes of the deprotection and neutralization steps were collected, hydrolyzed and examined by amino acid analysis. In addition, at each step of the synthesis a small sample of the peptide-resin was taken for amino acid analysis.

**TABLE 2**

**Effect of base on the cyclization of Gly-2-oxypropionyl-resin: amino acid analysis of Boc-Pro-Gly-2-oxypropionyl-resin**

<table>
<thead>
<tr>
<th>Length of base treatment of Gly-2-oxypropionyl-resin</th>
<th>Residue</th>
<th>5 min</th>
<th>30 min</th>
<th>3 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>0.85</td>
<td>0.79</td>
<td>0.63</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Termination of Gly [%]</td>
<td>15</td>
<td>21</td>
<td>37</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

*Boc-Gly-2-oxypropionyl-resin samples (100 mg) were treated with 50% TFA/CH₂Cl₂ (v/v, 30 min) and then 10 ml of 5% DIEA/CH₂Cl₂ (v/v) for different lengths of time followed by DCC-mediated coupling of Boc-Pro-OH (1 h).*
This synthesis showed that only 82% of Boc-Pro could be incorporated onto the H-Gly-2-oxypropionyl-resin under these conditions, which means that 18% of the chains had been terminated due to the cyclization reaction. The results also showed that the losses of Pro-Gly and Pro-Pro, due to diketopiperazine formation, were 0.17% and 1.06% respectively. While the small loss of Pro-Gly could be tolerated, the loss of Pro-Pro was more significant and could not be ignored. Finally, acid hydrolysis of the pentapeptide-resin showed that the Arg content was approximately 3% lower than Glu. Since picric acid titration had shown that no free amino groups remained after the coupling of Aoc-Arg(Tos), the amino acid data were interpreted to mean that 3% of the N-terminal glutaminyl peptide had also been terminated due to pyroglutamyl formation following removal of the Boc-group by 50% TFA/CH_2Cl_2.

**A modified strategy for the synthesis of peptides on 2-bromopropionyl-resin**

Based on this study, a modified strategy for the synthesis of the heavy chain segment 27-42 was designed as outlined in Fig. 3.

By utilizing this strategy the first side reaction, due to cyclization of Gly-2-oxypropionyl-resin, should be overcome via direct esterification of the first dipeptide, Boc-ProGly-PH, onto the resin. To avoid the diketopiperazine formation of Pro-Pro at the tripeptide stage, the plan was to couple Boc-Gln-Pro-OH to the H-Pro-Gly-2-oxypropionyl-resin. Although this strategy would not prevent the diketopiperazine formation of Pro-Gly at the dipeptide stage, we felt that this side reaction could be tolerated since our preliminary study showed that the loss of Pro-Gly was only 0.17%. Finally, by using 4 N HCl in peroxide-free dioxane to remove the Boc-group from the N-terminal glutaminyl peptide.
peptide, the fourth side reaction due to pyroglutamyl formation should be avoided.

**Fragment synthesis of the protected pentapeptide-resin**

In order to be able to test and to compare this synthetic strategy with the completely stepwise strategy, the pentapeptide-resin 38–42 was prepared by the new method. Boc-Pro-Gly-OH was obtained by saponification of Boc-Pro-Gly-OMe, which had been prepared from Boc-Pro and Gly-OMe with DCC coupling, and then esterified to the 2-bromopropionyl-resin by the cesium salt method of Gisin (1973). The Boc-group was removed with 50% TFA/CH₂Cl₂ to give H-Pro-Gly-2-oxypropionyl-resin. Attempts to synthesize Boc-Gln-Pro-OMe from Boc-Gln and Pro-OMe by the DCC/HOBt or the symmetrical anhydride methods were not satisfactory, but it was readily prepared from Boc-Gln-ONp and Pro-OMe (Bodanszky, 1955). It was purified on Sephadex G-10 with CH₃OH:H₂O (1:1) as eluent. The ester was saponified with 1 N NaOH at 0°C to give Boc-Gln-Pro, which was then coupled with Pro-Gly-2-oxypropionyl resin by the DCC/HOBt method to form the tetrapeptide. Finally, the Boc group was removed by treatment for 1 h with 4 N HCl in dioxane and Aoc-Arg(Tos) was added by DCC coupling to yield the desired protected pentapeptide-resin. The amino acid analyses of both pentapeptide-resins, after acid hydrolysis in 12 N HCl/propionic acid (1:1) at 130°C for 4 h in sealed tubes, are shown in Table 3.

The data indicated that the simple stepwise procedure led to 18% cyclization of Gly, 0.17% Pro-Gly diketopiperazine, 1.06% Pro-Pro diketopiperazine and 3% pyroglutamyl formation. In marked contrast, the modified fragment procedure did not give measurable amounts of any of these byproducts and, therefore, was able to circumvent each of these side reactions.

The photolytic cleavage yield of Aoc-Arg(Tos-Gln-Pro-Pro-Gly-2-oxypropionyl-resin was found to be the same (91%) whether it had been synthesized by the stepwise or fragment method, indicating that no detectable acylation of the imino group of the dihydrooxazinone had occurred. Th quantitative level of amino acids at each cycle of the stepwise synthesis also indicated that there was no significant accumulation of deletion peptides due to reversible ring-opening reaction during the synthesis.

**Synthesis of the 16-residue protected peptide 27–42**

After showing that the side reactions could be avoided by this strategy, the synthesis was continued via the stepwise coupling procedure until completion of the 16-residue peptide 27–42. The quantitative amino acid analysis

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**Table 3**

Amino acid analysis of Aoc-Arg(Tos)Gln-Pro-Pro-Gly-2-oxypropionyl-resin prepared by stepwise and fragment methods

<table>
<thead>
<tr>
<th>Synthetic step</th>
<th>Substitution of Gly [mmol/g]</th>
<th>Amino acid ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gly</td>
</tr>
<tr>
<td>Boc-Gly</td>
<td>0.409</td>
<td></td>
</tr>
<tr>
<td>Boc-Pro-Gly</td>
<td>0.417</td>
<td></td>
</tr>
<tr>
<td>Boc-Pro-Pro-Gly</td>
<td>0.416</td>
<td></td>
</tr>
<tr>
<td>BocGln-Pro-Pro-Gly</td>
<td>0.408</td>
<td></td>
</tr>
<tr>
<td>Aoc-Arg(Tos)-Gln-Pro-Pro-Gly</td>
<td>0.405</td>
<td></td>
</tr>
<tr>
<td>(Fragment method)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Corrected for the peptide weight and expressed as mmol per gram of polystyrene resin.
<table>
<thead>
<tr>
<th>Synthetic step</th>
<th>Subst. of Gly [mmol/g]a</th>
<th>Amino acid ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boc-Pro-Gly-HCl</td>
<td>0.324</td>
<td>Gly 1.00</td>
</tr>
<tr>
<td>Boc-Gln-Pro-Gly-HCl</td>
<td>0.330</td>
<td>Pro 2.01</td>
</tr>
<tr>
<td>Aoc-Arg(Tos)-Gln-Pro-Pro-Gly-HCl</td>
<td>0.337</td>
<td>Glu 2.06</td>
</tr>
<tr>
<td>Boc-Glu(OBzl)-Trp(For)-Val-Arg(Tos)-Gly-Pro-Pro-Gly-HCl</td>
<td>0.341</td>
<td>Arg 1.00</td>
</tr>
<tr>
<td>Boc-Asp(OBzl)-Phe-Tyr(2,6-Cl2_Bzl)-Met-Glu(OBzl)-Trp(For)-Val-Arg(Tos)-Gln-Pro-Pro-Gly-HCl</td>
<td>0.339</td>
<td>Val 1.90</td>
</tr>
<tr>
<td>Boc-Phe-Ser(Bzl)-Asp(OBzl)-Phe-Tyr(2,6-Cl2_Bzl)-Met-Glu(OBzl)-Trp(For)-Val-Arg(Tos)-Gln-Pro-Pro-Gly-HCl</td>
<td>0.332</td>
<td>Trp 1.05</td>
</tr>
<tr>
<td>Boc-Phe-Thr(Bzl)-Phe-Ser(Bzl)-Asp(OBzl)-Phe-Tyr(2,6-Cl2_Bzl)-Met-Glu(OBzl)-Trp(For)-Val-Arg(Tos)-Gln-Pro-Pro-Gly-HCl</td>
<td>0.328</td>
<td>Met 1.05</td>
</tr>
<tr>
<td>Boc-Phe-Thr(Bzl)-Phe-Ser(Bzl)-Asp(OBzl)-Phe-Tyr(2,6-Cl2_Bzl)-Met-Glu(OBzl)-Trp(For)-Val-Arg(Tos)-Gln-Pro-Pro-Gly-HCl</td>
<td>0.322</td>
<td>Tyr 1.03</td>
</tr>
<tr>
<td>Boc-Phe-Thr(Bzl)-Phe-Ser(Bzl)-Asp(OBzl)-Phe-Tyr(2,6-Cl2_Bzl)-Met-Glu(OBzl)-Trp(For)-Val-Arg(Tos)-Gln-Pro-Pro-Gly-HCl</td>
<td>0.321</td>
<td>Phe 1.06</td>
</tr>
<tr>
<td>Boc-Phe-Thr(Bzl)-Phe-Ser(Bzl)-Asp(OBzl)-Phe-Tyr(2,6-Cl2_Bzl)-Met-Glu(OBzl)-Trp(For)-Val-Arg(Tos)-Gln-Pro-Pro-Gly-HCl</td>
<td>0.320</td>
<td>Asp 0.97</td>
</tr>
<tr>
<td>Boc-Phe-Thr(Bzl)-Phe-Ser(Bzl)-Asp(OBzl)-Phe-Tyr(2,6-Cl2_Bzl)-Met-Glu(OBzl)-Trp(For)-Val-Arg(Tos)-Gln-Pro-Pro-Gly-HCl</td>
<td>0.319</td>
<td>Ser 0.97</td>
</tr>
<tr>
<td>Boc-Phe-Thr(Bzl)-Phe-Ser(Bzl)-Asp(OBzl)-Phe-Tyr(2,6-Cl2_Bzl)-Met-Glu(OBzl)-Trp(For)-Val-Arg(Tos)-Gln-Pro-Pro-Gly-HCl</td>
<td>0.318</td>
<td>Thr 0.97</td>
</tr>
</tbody>
</table>

aCorrected for the peptide weight and expressed as mmol per gram of polystyrene resin.
bObtained from alkaline hydrolysis of the protected peptide in 4.2 N NaOH in the presence of thiodiglycol in a sealed polypropylene tube. n.a., not analyzed.
after acid hydrolysis of the peptide-resin at different stages of synthesis showed that no significant loss of peptide chains had occurred during the synthesis (Table 4), which indicated that the peptide-resin ester bond was stable under the conditions used. The peptide was removed from the resin in fully protected form by irradiation at >350 nm in DMF. The cleavage yield was 84% based on the quantitative amino acid analysis of the peptide-resin remaining after photolysis. The protected peptide, which was insoluble in all solvents tested except DMF and HOAc, was crystallized from acetic acid by addition of water and washed with methanol. It was further purified on a Sephadex LH-60 column using DMF as eluent. The main component shown by monitoring at 275 nm was homogeneous when rerun under identical conditions (Fig. 4). The peptide was also homogeneous on thin-layer chromatography in two solvent systems and gave excellent amino acid analyses after both acid and alkaline hydrolysis (Table 4). The u.v.-absorption of the purified peptide showed two maxima, at 292 and 300 nm, that are characteristic for $N^\alpha$-formyltryptophan-containing peptides (Ohno et al., 1972) (see Fig. 5).

The combination of the fragment coupling and stepwise synthetic approach has enabled us to prepare the 16-residue protected peptide segment 27-42 of the heavy chain of the M603 myeloma protein and to circumvent several of the side reactions associated with a completely stepwise synthesis of this peptide on the 2-bromopropionyl-resin.

**EXPERIMENTAL SECTION**

All melting points are uncorrected. Spectroscopic grade DMF was purchased from Matheson, Coleman & Bell. Dichloromethane and DIEA were redistilled from Na$_2$CO$_3$ and NaH respectively. Trifluoroacetic acid was purchased from Halocarbon Products Inc. Boc-amino acid derivatives and Aoc-Arg(Tos) employed in these experiments were purchased from either Chemical Dynamic Corp. or Bachem, and their purity was tested by thin-layer chromatography prior to use. Dicyclohexylcarbodiimide was obtained from Pierce Chemical Co. and 1-hydroxybenzotriazol and 2-bromopropionyl chloride from Aldrich Chemical Company. Polystyrene-1%-divinylbenzene crosslinked resin (Bio-Beads S-XI,
200-400 mesh) was purchased from Bio-Rad Labs and washed (Mitchell et al., 1978). Dioxane was purified on an aluminum oxide column and tested for peroxide prior to use.

Photolysis was done at room temperature in a closed glass vial placed 3 cm from a water cooled jacketed photochemical immersion well containing a 450 W Hanover high pressure mercury vapor lamp 6515-34 (Ace Glass, Inc., Vineland, N.J.). The lamp was surrounded by a cylindrical uranyl glass filter to remove light with a wavelength shorter than 350 nm.

Amino acid analyses were obtained on a Beckman 121 amino acid analyzer after hydrolysis of the samples with 6 N HCl in sealed evacuated tubes at 110° for 24 h for cleaved peptides and with 12 N HCl-propionic acid (1:1, v/v) at 130° for 4 h for peptide-resins. Base hydrolysis was performed in 4.2 N NaOH in the presence of thiodiglycol mixture was cautiously added to a suspension at 110° for 24 h (Hugli & Moore, 1972).

T.l.c. was performed on silica gel plates and the compounds were visualized by spraying with ninhydrin in acetone followed by heating in an evacuated oven, or with Cl₂ and KI/starch solution. The following solvent systems were used: chloroform/methanol/acetic acid; 85:10:5 (system A); n-butanol/acetic acid/water; 8:1:1 (system B); n-butanol/acet acid/water; 3:1:1 (system C); n-butanol/acet acid/water/pyridine; 60:6:24:20 (system D). Elemental analyses were performed at the Microanalytical Laboratory, Rockefeller University.

2-Bromopropionyl-resin (Wang, 1976)
To a suspension of 4.9 g (0.035 mol) of AlCl₃ in 50 ml of CH₂Cl₂, which was placed in a dropping funnel, 2-bromopropionyl chloride (6.25 g, 0.035 mol) was slowly added. The solid dissolved after shaking for a brief time. This mixture was cautiously added to a suspension of 30 g Bio-Beads S-XI (200-400 mesh) in
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300 ml CH₂Cl₂ during a period of 10 min under protection from light and stirred overnight under nitrogen atmosphere. The resin was filtered and washed successively with CH₂Cl₂, benzene, tetrahydrofuran, methanol, CH₂Cl₂ and methanol to give 30.8 g of product; Br, 3.7% (0.45 mmol/g resin).

**Boc-Pro-Gly-OMe**

Boc-Pro-OH (10.7 g, 0.050 mol) and 6.3 g (0.050 mol) of Gly-OMe·HCl were dissolved in 150 ml of DMF. To this mixture 6.5 g (0.050 mol) DIEA and 10.3 g (0.050 mol) DCC dissolved in 50 ml CH₂Cl₂ were added. After stirring overnight at room temperature, the mixture was filtered and evaporated. The residue was dissolved in ethyl acetate and refiltered. The organic layer was washed three times with 10% citric acid and water, and three times with 10% sodium bicarbonate and water. The ethyl acetate solution was dried over anhydrous sodium sulfate, filtered, evaporated to a small volume, and crystallized with petroleum ether. Yield, 11.8 g (82%), m.p. 55-56°.

**Boc-Pro-Gly-OH**

Boc-Pro-Gly-OMe (13.8 g, 0.050 mmol) was dissolved in 200 ml of ethanol and 32 ml of water. N NaOH (60 ml, 1.2 equiv.) was added slowly with stirring at 0° and the mixture was stirred overnight at 4°. The saponification reaction was followed by thin-layer chromatography (solvent system A). The reaction mixture was neutralized with 1 N HCl and the ethanolic solution was evaporated. The aqueous layer was acidified to pH 2.0 with 1 N HCl and the product was extracted into ethyl acetate (3 x 50 ml). The ethyl acetate layer was extracted into 10% sodium bicarbonate solution (4 x 50 ml). The solution was again acidified with 1 N HCl and extracted twice into ethyl acetate (3 x 50 ml). The ethyl acetate layer was dried over anhydrous sodium sulfate, filtered and crystallized with petroleum ether. Yield, 3.5 g (49%), hygroscopic; Rₜ 0.60 (system B).

**Pro-OMe·HCl**

To 250 ml anhydrous methanol, 65 ml thionyl-chloride was added slowly at -10° under stirring (25 min). L-Pro-OH (28.7 g, 0.25 mol) was added to this solution and stirring was continued for another 24 h. Methanol was removed on the rotary evaporator and the residual oil was dried over P₂O₅ and KOH in vacuo overnight. The oily product solidified and was used without further purification. Thin-layer chromatography of the product gave only one ninhydrin-positive spot with Rₜ 0.35 (solvent system A). Yield: 41.2 g (100%); m.p. 71° (Lit. 71°, Guttmann, 1961).

**Boc-Gln-Pro-OMe**

Boc-Gln-ONp (7.3 g, 20 mmol) and 3.6 g (22 mmol) H-Pro-OMe·HCl were dissolved in 25 ml DMF. DIEA (2.8 g, 22 mmol) was added under stirring at 0°. The stirring was continued for another 2 days at room temperature. To the mixture 200 ml water was added and the aqueous layer was extracted with chloroform (3 x 50 ml). The chloroform layer was washed with 10% NaHCO₃ (3 x 30 ml), water (30 ml), 10% citric acid (3 x 30 ml) and water (30 ml). After removal of chloroform, the crude product which still contained Boc-Glu-ONp and p-nitrophenol, was purified on a Sephadex G-10 column with methanol/water (1:1, v/v) as eluent. The crude product was divided into four 2-ml samples and chromatographed on the Sephadex G-10 column (90 x 1.8 cm) equilibrated with 50% methanol/water. The flow rate was 21 ml/h, and fractions of 3.5 ml were collected. The desired fractions were pooled and evaporated. The product solidified after drying over phosphorus pentoxide in vacuo. Yield, 3.5 g (49%), hygroscopic; Rₜ 0.60 (system B).

**Boc-Gln-Pro-OH**

To 3.5 g (10 mmol) Boc-Gln-Pro-OMe dissolved in 75 ml ethanol and 12 ml water, 12 ml (1.2 eq.) 1 N NaOH was added slowly under stirring at 0°. The reaction mixture was stirred overnight at 4°. Thin-layer chromatography (in system A) of the reaction mixture indicated that some starting material remained, so an additional 6 ml of 1 N NaOH was added and stirring was continued for another day. The
reaction mixture was neutralized with 1 N HCl and worked up as previously described. Yield, 3.1 g (91%); m.p. 176–178°; Rf 0.69 (system A). Anal. calc. for C_{18}H_{25}N_{3}O_{6}: C, 52.46%; H, 7.34%; N, 12.23%. Found: C, 52.47%; H, 7.54%; N, 12.10%.

Study on termination of Gly-2-oxypropionyl-resin under acidic, neutral and alkaline conditions

Boc-Gly-2-oxypropionyl-resin was prepared from 2-bromopropionyl-resin using the cesium salt method (Gisin, 1973). The substitution of Gly on the resin was determined by the picric acid titration method and found to be 0.410 mmol per gram of resin.

A. Acid treatment. Four samples (A, B, C, D) of 100 mg of Boc-Gly-2-oxypropionyl-resin were suspended in 10 ml dichloromethane for 10 min in four separate reaction vessels. The following schedule was performed for the coupling of Boc-Pro-OH onto Gly-2-oxypropionyl-resin: 1) 50% trifluoroacetic acid/CH_{2}Cl_{2}, 10 ml (A, 0.5 h; B, 1.5 h; C, 3 h; D, 6 h); 2) CH_{2}Cl_{2}, 10 ml (2 x 1 min); 3) isopropanol, 10 ml (1 x 2 min); 4) CH_{2}Cl_{2}, 10 ml (3 x 1 min); 5) 5% DIEA/CH_{2}Cl_{2}, 10 ml (1 x 5 min); 6) CH_{2}Cl_{2}, 10 ml (4 x 1/2 min); 7) 0.205 mmol Boc-Pro-OH, CH_{2}Cl_{2}, 3 ml (1 min); 8) 0.205 mmol DCC, 2 ml CH_{2}Cl_{2} (1 h); 9) CH_{2}Cl_{2}, 10 ml (5 x 1 min); 10) isopropanol, 10 ml (2 x 1 min); 11) CH_{2}Cl_{2}, 10 ml (2 x 1 min); 12) isopropanol, 10 ml (2 x 1 min); 13) CH_{2}Cl_{2}, 10 ml (5 x 1 min). The finished peptide resins were dried over P_{2}O_{5} in vacuo and subjected to acid hydrolysis. The amino acid analyses of hydrolysates are shown in Table 1. To check whether the coupling time applied in these tests was sufficient Boc-Pro-OH (5 equiv.) was coupled to 100 mg Gly-2-oxypropionyl-resin by the stepwise method as described previously with changes in the following steps: 1) 50% TFA/CH_{2}Cl_{2}, 10 ml (30 min); 5) 5% DIEA/CH_{2}Cl_{2}, 10 ml (A, 5 min; B, 30 min; C, 3 h; D, 6 h). The amino acid analyses of the peptide-resins are shown on Table 2.

B. Base treatment. Another four samples (A, B, C, D) of 100 mg Boc-Gly-2-oxypropionyl-resin were placed in four separate reaction vessels and subjected to the same schedule as described previously with changes in the following steps: 1) 50% TFA/CH_{2}Cl_{2}, 10 ml (30 min); 5) 5% DIEA/CH_{2}Cl_{2}, 10 ml (A, 5 min; B, 30 min; C, 3 h; D, 6 h). The amino acid analyses of the peptide-resins are shown on Table 2.

C. Treatment under neutral conditions. Boc-Gly-2-oxypropionyl-resin (100 mg) was treated the same as described previously with changes in the following steps: 1) 50% TFA/CH_{2}Cl_{2}, 10 ml (30 min); 6) CH_{2}Cl_{2}, 10 ml (5 x 1 min, 1 x 3 h). Amino acid analysis: Pro, 0.107; Gly, 1.00.

Termination of H-Gly-2-oxypropionyl-resin was: 89.3%.

Aoc-Arg(Tos)-Gln-Pro-Pro-Gly-2-oxypropionyl-resin by the stepwise method

Boc-Gly-2-oxypropionyl-resin (0.50 g, 0.191 mmol), prepared as previously described, was placed in a reaction vessel and suspended in 15 ml CH_{2}Cl_{2}. Aoc-Arg(Tos)-Gln-Pro-Pro-Gly-2-oxypropionyl-resin was assembled by the stepwise method via DCC mediated couplings, except for Boc-Gln, which was coupled as the 4-nitrophenyl active ester. The synthesis was monitored by picric acid titrations. The program used for the first coupling consisted of: 1) 50% TFA/CH_{2}Cl_{2}, 15 ml (2, 30 min); 2) CH_{2}Cl_{2}, 15 ml (3 x 1 min); 3) isopropanol, 15 ml (2 x 1 min); 4) CH_{2}Cl_{2}, 15 ml (2 x 1 min); 5) 5% DIEA/CH_{2}Cl_{2}, 15 ml (2 x 1 min); 6) CH_{2}Cl_{2}, 15 ml (3 x 1 min); 7) 0.955 mmol Boc-Pro-OH, CH_{2}Cl_{2}, 5 ml (1 min); 8) 0.955 mmol DCC, 2 ml CH_{2}Cl_{2} (1 h); 9) CH_{2}Cl_{2}, 15 ml (5 x 1 min); 10) isopropanol, 15 ml (2 x 1 min); 11) CH_{2}Cl_{2}, 15 ml (5 x 1 min). The second coupling was identical to the above program beginning with the neutralization step. For the active ester coupling the following changes were undertaken: 7) DMF, 15 ml (3 x 1 min); 8) 0.955 mmol Boc-Gln-ONp, 5 ml DMF (24 h); 9) DMF, 15 ml (3 x 1 min). The peptide-resin was dried in vacuo and subjected to acid hydrolysis (Table 3). At the Pro-Gly and Pro-Pro-Gly stages, the filtrates and washes of the deprotection and neutralization steps were collected, evaporated, checked by t.l.c., and hydrolyzed. Amino acid analyses gave
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0.325 μmol (0.17%) Pro-Gly and 2.02 μmol (1.06%) Pro-Pro.

Aoc-Arg(Tos)-Gln-Pro-Pro-Gly-2-oxypropionyl-resin by the fragment method

Boc-Pro-Gly-2-oxypropionyl-resin. Boc-Pro-Gly-OH (2.2 g, 8.4 mmol) was dissolved in 9 ml ethanol and 3 ml water. Because of the insolubility of this peptide 5 ml tetrahydrofuran was added and the pH of the mixture was adjusted to 7.0 with 20% Cs₂CO₃ solution. The neutral solution was then flash evaporated. After repeated evaporation to dryness with benzene, the product was dried over phosphorus pentoxide in vacuo (24 h) and coupled onto 7.4 g (0.4 mmol Br per g of resin) 2-bromo-propionyl-resin. in 50 ml DMF at 50° for 18 h. The peptide resin was filtered and washed with 4 x 100 ml each of DMF, DMF/H₂O (9:1), DMF, ethanol and dried over P₂O₅. Substitution of Gly was 0.325 mmol per g resin, based on picric acid titration and quantitative amino acid analysis. No bromine could be detected.

Boc-Gln-Pro-Pro-Gly-2-oxypropionyl-resin. Boc-Gln-Pro-Pro-Gly-2-oxypropionyl-resin (3.4 g (1.1 mmol) was placed in a reaction vessel attached on a shaker and 40 ml CH₂Cl₂ added to swell the resin for 15 min. Boc-Gln-Pro-OH was coupled onto the peptidyl-resin via DCC/HOBt activation, with the following program: (1) 50% TFA/CH₂Cl₂, 40 ml (2.30 min); 2) CH₂Cl₂, 40 ml (5 x 1 min); 3) isopropanol, 40 ml (2 x 1 min); 4) CH₂Cl₂, 40 ml (5 x 1 min); 6) 7% DIEA/CH₂Cl₂, 40 ml (3.34 min); 7) CH₂Cl₂, 40 ml (5 x 1 min); 8) DMF, 40 ml (2 x 1 min); 9) 0.75 g (2.2 mmol) Boc-Gln-Pro-OH and 0.50 g (3.3 mmol) HOBt, DMF, 25 ml (5 min); 10) 0.45 g (2.2 mmol) DCC, CH₂Cl₂, 5 ml (40 h); 11) DMF, 40 ml (5 x 1 min); 12) isopropanol, 40 ml (2 x 1 min); 13) CH₂Cl₂, 40 ml (2 x 1 min); 14) isopropanol, 40 ml (2 x 1 min); 15) CH₂Cl₂, 40 ml (5 x 1 min). Picric acid titration indicated a quantitative coupling.

Aoc-Arg(Tos)-Gln-Pro-Pro-Gly-2-oxypropionyl-resin. Boc-Gln-Pro-Pro-Gly-2-oxypropionyl-resin (1.1 mmol) was suspended in dioxane (15 min). The program used in this synthesis was identical to the above program with the following changes: 1) 4 N HCl/dioxane, 40 ml (2 min, 1 h); 2) dioxane, 40 ml (5 x 1 min); 8) deleted; 9) 2.50 g (5.65 mmol) Aoc-Arg(Tos)-OH: CH₂Cl₂, 25 ml; DMF, 1 ml (5 min); 10) 1.16 g (5.65 mmol) DCC; CH₂Cl₂, 10 ml (2 h); 11) CH₂Cl₂, 40 ml (5 x 1 min). Since the second coupling did not go to completion, as shown by picric acid titration, a third coupling was carried out.

Boc-Phe-Thr(Bz1)-Phe-Ser(Bz1)-Phe-Tyr(2,6-C₁₂Bz1)-Met-Glu(Bz1)-Trp(For)-Val-Arg(Tos)-Gln-Pro-Pro-Gly-2-oxypropionyl-resin

After showing that the side reactions could be circumvented by the fragment coupling strategy, the synthesis was continued from the pentapeptide stage in a stepwise manner until completion of the fully protected hexadecapeptide (27–42). The same program as described above was used with the following changes: 8) deleted; 9) 5.65 mmol Boc-amino acid, CH₂Cl₂, 40 ml (5 min); 10) 5.65 mmol DCC, CH₂Cl₂, 10 ml (2 h); 11) CH₂Cl₂, 40 ml (5 x 1 min).

The second coupling was identical to the above program beginning with the neutralization step. The peptide resin was dried over phosphorus pentoxide in vacuo. Dry weight, 5.1 g.

Photolytic cleavage of the peptide from resin. The peptide-resin (2.02 g) was placed in a pyrex tube (2.5 x 15 cm) and suspended in 40 ml DMF. The suspension was bubbled with nitrogen for 2 h, sealed with a screw cap, and irradiated at a wave length of 350 nm at room temperature for 64 h. The resin was removed by filtration and washed with 40 ml of warm DMF (50°C, 4 x 2 min). The filtrate was evaporated on a rotary evaporator at 40° with an oil pump. The residue was redissolved in 10 ml DMF and 90 ml water was added. The colloidal suspension was centrifuged after 2 h standing in the cold room and the solvent was decanted. The precipitate was washed three times with 40 ml water and twice with methanol. The product was recrystallized from acetic acid by addition of water. Yield, 0.52 g.
The cleavage yield based on glycine analyses was 84%.

Chromatographic purification on Sephadex LH-60. The partially purified protected peptide (26 mg) was chromatographed on Sephadex LH-60 in a 2.5 x 40 cm Altex-column equilibrated with DMF. The flow rate was 12 ml/h and fractions of 2 ml were collected and read at a wavelength of 275 nm. The desired fractions (71–81) were pooled and evaporated to dryness. The product was rechromatographed on the same column under similar conditions and crystallized by addition of water. The precipitate was filtered and washed with methanol and dried over P2O5 in vacuo. Yield: 75%, m.p. 238° (decomposed); Rf 0.58 (system C); Rf 0.70 (system D).

Anal. calc. for C164H172N21O30Cl2S2: C, 61.54; H, 6.13; N, 10.47; Found: C, 60.77; H, 6.04; N, 10.52. The amino acid analyses of the purified product after alkaline and acid hydrolysis are shown in Table 4.

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