Solid phase synthesis of gastrin I

Comparison of methods utilizing strong acid for deprotection and cleavage

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A successful synthesis of human gastrin I in 60% overall yield based on the first residue attached to a benzhydrylamine-resin was achieved by the stepwise solid-phase method. The synthesis was carried out on a 1% crosslinked polystyrene support, using conventional benzyl-based side chain protecting groups and final deprotection with different acidic protocols. Several improvements in this general approach were applied, including new scavengers, new resin attachment and especially a new technique that allows the strong acid reactions to occur by an \( S_N 2 \) mechanism.

Key words: benzhydrylamine resin; gastrin 1–17; glutamic acid side reaction; low-high HI deprotection; multidetachable resin; solid phase peptide synthesis

The chemical synthesis of the heptadecapeptide amide, gastrin I, has a long history of being difficult to achieve by the strategy of utilizing strong acid deprotecting agents. In gastrin I, 10 out of 17 residues, including Glu, Asp, Trp, Tyr and Met, can be sensitive to the usual conditions of deprotection by strong acids. The first total synthesis of hog gastrin I in 1964 (1) and human gastrin I (Fig. 1) 2 years later (2), adopted a strategy incorporating the use of tert.-butyl side chain protecting groups and trifluoroacetic acid (TFA) as the deprotecting agent, and thus purposely avoided many attendant disadvantages associated with the use of strong acids (3).

Subsequent syntheses of gastrin I and its analogs have also led to the recommendation of this principle (4–13). For example, in a recent comparison of solid-phase synthesis (14) of human gastrin I by the tert.-butyl/TFA and benzyl/TFA and benzyl/HF strategies, the synthesis using the benzyl/HF method was considered a failure, yielding a negligible quantity of gastrin, while the synthesis by tert.-butyl/TFA strategy yielded 11% of gastrin I (10). Similarly, in a series of publications dealing with the synthesis of gastrin I and its analogs by the strategy utilizing the base labile \( N^\alpha \)-fluorenylmethoxycarbonyl (Fmoc) and tert.-butyl side chain protecting group on a polyamide resin support, good yields were obtained and the authors strongly emphasized that the gastrin sequence remained inaccessible to synthesis with the benzyl/HF method (10–13).

There is no doubt that these tert.-butyl- and Fmoc-based syntheses were successful and useful approaches to the synthesis of this hormone. There is considerable doubt, however, that the dogmatic statements concerning the synthesis based on strong acid steps are correct. There is good reason to believe that the experiments were inadequately performed and that the observed poor results should have been attributed to other causes.
In this paper we wish to report a satisfactory stepwise synthesis of human gastrin I on a 1% crosslinked polystyrene support, using conventional benzyl-based side chain protecting groups and cleavage with the standard 90% HF/10% anisole reagent. We have gone on to develop several improvements in this general approach, including new scavengers, new resin attachments, and especially a new technique that allows the strong-acid reactions to occur by an S_N_2 mechanism (15–17) which avoids many of the side reactions known to be associated with strong acid under the usual S_N_1 conditions. A comparison between the conventional and the improved HF method is reported.

MATERIALS AND METHODS

Materials

Commercial protected amino acids were obtained from Peninsula Laboratories, San Carlos, CA. The purity was assessed by melting points and thin-layer chromatography. Diisopropylethylamine was distilled twice from CaH_2 and then once through a fractionating column (Vigreaux). Dichloromethane (Eastman) was distilled from Na_2 CO_3 and dimethylformamide (Aldrich, Spectrophotometric grade) was stored over molecular sieve for a week and filtered through an aluminium oxide column before use. Trifluoroacetic acid (Halocarbon Products), HF (Matheson), dicyclohexylcarbodiimide (Pierce), and acetonitrile (HPLC grade, Burdick and Jackson) were used without purification.

General methods

Analytical high-pressure liquid chromatography of the peptide was on a reverse-phase μBondapak C-18 column (4 × 300 mm) in a Waters Associate instrument fitted with a Schoeffel variable-wavelength UV-photometer and a Wisp injector. The chromatograms were recorded on a Hewlett-Packard 3380A integrator (1 mV full scale). Solution A contained 950 mL H_2 O, 50 mL CH_3 CN, and 1 mL 85% H_3 PO_4, titrated to pH 6.5 by triethylamine; solution B contained 500 mL H_2 O, 500 mL CH_3 CN, and 1 mL 85% H_3 PO_4, titrated to pH 6.5 by triethylamine. The linear gradient was from 20 to 50% solution B into solution A in 30 min, 2 mL/min.

Preparative HPLC of the peptide was on a C-18 reverse phase column (25 × 300 mm). Solution A contained 900 mL H_2 O, 100 mL CH_3 CN, and 0.5 mL CF_3 CO_2 H; solution B contained 500 mL H_2 O, 500 mL CH_3 CN, and 0.5 mL CF_3 CO_2 H. The linear gradient was from 10 to 70% solution B into solution A in 6 h, 1 mL/min.

Protected gastrin-I multidetachable benzhydrylamine resin

N-tert. Butyl-4-hydroxybenzhydrylamine (0.45 g, 1.5 mmol) prepared as described (18, 19), dicyclohexylcarbodiimide (DCC, 0.33 g, 2.5 mmol) and N,N-dimethyl-4-aminopyridine (0.12 g, 0.98 mmol) were added successively to the carboxymethyl resin (3 g, 0.75 mmol) in 30 mL CH_2 Cl_2 and dimethylformamide (1:1, v/v). After 4 h, the reaction was terminated by the addition 0.33 g DCC and 0.5 mL EtOH for 1 h. Both elemental nitrogen analysis and the quantitative ninhydrin analysis (20) (after the removal of the N-Boc group and neutralization of the TFA salt by DIEA) showed that the substitution was 0.21 mmol/g. The multidetachable benzhydrylamine resin, placed in a silanized reaction vessel, was carried manually through 17 synthetic cycles in a mechanical shaker. All amino acids were protected with N"-tert.-butyloxy carbonyl (Boc). Side chain protecting groups were: Asp(OBzl), Trp(For) (22, 23), Glu(OBzl), Met(O) (24), and Tyr(BrZ) (25).

Each synthetic cycle consisted of (i) a 20-min deprotection with 50% trifluoroacetic acid/CH_2 Cl_2, (ii) neutralization with 5% diisopropylethylamine/CH_2 Cl_2, and (iii) double coupling with preformed symmetrical anhydrides (1.89 mmol, 3 equiv. of the Boc-amino acid) for 1 h each. First coupling was in CH_2 Cl_2 and second in dimethylformamide (DMF). Boc-Gly-OH was coupled with dicyclohexylcarbodiimide alone. All couplings were monitored by the quantitative ninhydrin test and, if necessary, additional couplings of symmetrical anhydride in DMF for 2 h were used to give >99.8% completion. Portions of protected peptide resin were removed after cycles 4, 9, 13 and 15 for Edman degradation and HF cleavage to examine the progress and the results of the synthesis. Edman degradation
and preview analysis was performed as described (16).

Cleavage of peptide-resin
Protected gastrin I-resin (0.2–0.5 g) was first treated with 50% trifluoroacetic acid in CH₂Cl₂ (10 mL) for 5 min to remove the extraneous materials adsorbed or precipitated on the resin. The dried peptide-resin was treated with the low-high HF method of deprotection (17). For the low HF treatment the peptide-resin was mixed with dimethylsulfide, p-thiocresol and p-cresol, and then liquid HF at −78° was added to give a final volume of 5 mL (65:2.5:7.5:25, by vol.). The mixture was equilibrated to 0° with stirring in an ice bath. After 2 h, the HF and dimethylsulfide were removed in vacuo. The high HF treatment was initiated by recharging the reaction vessel at −78° with 9.5 mL of liquid HF to give a total volume of 10 mL of HF-p-cresol-p-thiocresol. The reaction was carried out at 0° for 1 h. After evaporation of HF at 0° and washing with cold ether in the presence of mercaptoethanol (ether:thiol 99:1, v/v, 10 mL) to remove p-thiocresol and p-cresol and to prevent oxidation of Met to Met(O), the crude gastrin I mixture was dissolved in buffer A and purified by preparative HPLC. The cleavage yield was 90% based on the back hydrolysis of the HF-treated resin by 12 N HCl-phenol-acetic acid (2:1:1, by vol., 2 mL).

For comparison, two other HF deprotections were also performed. The high-low HF treatment of the gastrin peptide resin (0.2 g) was carried out essentially in the reverse order of the low-high HF treatment except the high HF treatment was performed in HF-p-cresol (9:1, v/v) for 1 h. After the removal of HF, DMS and p-thiocresol and then fresh HF were added to the reaction vessel to continue the low HF treatment for 2 h. Another portion of the peptide-resin (0.1 g) was also treated by the high HF reagent alone. Note: p-thiocresol should be avoided in the high HF treatment when Trp(For) is present in the peptide resin. Similarly, anisole should be avoided in the low HF treatment. See text for a detailed explanation.

RESULTS
Synthesis of gastrin-I on multidetachable benzhydrylamine resin
The resin (3 g) was placed in a silanized reaction vessel and carried manually through 17 synthetic cycles on a mechanical shaker. The conventional combination of tert.-butyloxycarbonyl for the Nα-amine and benzyl derivatives for the side chains was adopted as the differential acid-labile protecting group strategy; methionine was incorporated as the sulfoxide (22) and tryptophan as N'-formyl derivative (23, 24). Both modifications avoid the tert.-butylation side reactions during the repetitive TFA deprotection of the Nα-Boc group. The last, N-terminal, residue was pyrrolidone carboxylic acid (pGlu). Double coupling for 1 h with 3 equiv. of preformed symmetrical anhydrides was used first in CH₂Cl₂ and then in dimethylformamide (DMF). Boc-Gly was coupled with the standard dicyclohexylcarbodiimide method to avoid urethane acylation and dipeptide formation (28). All couplings to the Glu(OBzl) residues were mediated by symmetrical anhydrides in DMF or N-methyl-pyrrolidinone to minimize pyroglutamyl formation (29, 30). Both CH₂Cl₂ and 1-hydroxy-pGlu—Gly—Pro—Trp—Leu—Glu—Glu—Glu—Ala—Tyr—Gly—Trp—Met—Asp—Phe—NH₂

FIGURE 1
Human gastrin I.
benzotriazole were found to be inducive to pyroglutamyl formation and were omitted. All couplings were monitored by the quantitative ninhydrin method (21) and no apparent difficulties were found throughout the synthesis. The monitoring level at each cycle indicated 99.6–99.9% completion. Portions of protected peptide resin were removed after synthetic cycles 4, 8, 12 and 15 (peptide 2–17) for Edman degradation and preview analysis and for HF cleavage and deprotection to examine the progress of the synthesis. When protected peptide fragment 2–17 was subjected to the preview analysis (26), an average level of 0.28% of preview was obtained (4.2%/15 = 0.28%). A random background of 0.1–0.3% of preview was observed by exhaustive acylation of the protected gastrin peptide-resin, indicating a level of true deletion peptides not higher than 0.1%. The coupling efficiency was therefore greater than 99.9% by this technique. This result was consistent with the data obtained from the quantitative ninhydrin monitoring, indicating the coupling efficiency at each step was near quantitative. Both analyses are summarized in Table 1.

### Deprotection by HF

The synthetic protected gastrin-resin was deprotected and cleaved under a variety of conditions in order to provide comparative data on the yield and purity of the product. For all experiments the peptide was treated with 50% trifluoroacetic acid/CH₂Cl₂ for 5 min to remove the extraneous materials absorbed or precipitated on the resin. After washing with CH₂Cl₂, the peptide-resin was dried, and weighed samples (0.5 g) were deprotected and cleaved under the following conditions:

**Run A, high HF/anisole**

This first cleavage was in 5 mL of what we have recently termed “high HF”, i.e. the usual 90% HF containing 10% of anisole (v/v) as scavenger for carbocations (31, 32). This provided the control for the series and offered a chance to compare our data directly with those reported earlier for this benzyl/HF strategy. Following the HF treatment at 0° for 1 h the liberated peptides were extracted into 10% aqueous acetic acid. The crude mixture was immediately analyzed by analytical HPLC to assess the heterogeneity of the product (Fig. 2, part A).

A main peak was obtained at 13 min, corresponding to [Met(O)₁⁵ Trp(For)₈₋₁⁴] - gastrin I. It represented approximately 50% of the u.v. absorbing material. Many of the slower moving components were due to the known HF-induced reactions including alkylation by products of Tyr and especially the acylation products of the five Glu residues.

### TABLE 1

Quantitative ninhydrin monitoring and preview analysis of gastrin-I

<table>
<thead>
<tr>
<th>Analytical test</th>
<th>Average unreacted amine (%/step)</th>
<th>Average background (%)</th>
<th>Average coupling efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ninhydrin monitoring residues</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12–17</td>
<td>99.70</td>
<td>0.10</td>
<td>99.8</td>
</tr>
<tr>
<td>8–17</td>
<td>99.70</td>
<td>0.20</td>
<td>99.9</td>
</tr>
<tr>
<td>4–17</td>
<td>99.65</td>
<td>0.20</td>
<td>99.8</td>
</tr>
<tr>
<td>2–17</td>
<td>99.60</td>
<td>0.25</td>
<td>99.8</td>
</tr>
<tr>
<td><strong>Preview analysis residues</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12–17</td>
<td>0.17</td>
<td>0.14</td>
<td>99.9</td>
</tr>
<tr>
<td>8–17</td>
<td>0.26</td>
<td>0.17</td>
<td>99.9</td>
</tr>
<tr>
<td>4–17</td>
<td>0.26</td>
<td>0.20</td>
<td>99.9</td>
</tr>
<tr>
<td>12–17</td>
<td>0.28</td>
<td>0.26</td>
<td>99.9</td>
</tr>
</tbody>
</table>

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deprotection in two more steps using 5% mercaptoethanol and dithiothreitol at pH 8 (55°, 30 h) and then 1 M NH₂OH (Fig. 2. Part B). Gastrin I (18 min) was the main component, representing about 38% of total product. The two additional steps introduced several other components.

**Run C, high HF/p-cresol**
This cleavage was also in high HF, but the 10% anisole was replaced with 10% p-cresol. The partially protected [Met(O), Trp(For)] gastrin I was again obtained (Fig. 2, part C). The pattern was similar to Run A, but lower levels of by-products were present and the main component was increased to 60%.

**Run D, high/low HF**
After cleavage as in run C, the remaining protecting groups were removed by Sn₂ treatment under “low HF” conditions. The reagent was 25% HF in 65% dimethylsulfide (DMS) containing 7.5% p-cresol and 2.5% p-thiocresol. This was achieved by evaporating the HF after run C on a water aspirator and then adding 6.5 mL DMS, 0.25 mL p-cresol (to the 0.5 mL remaining after evaporation of the HF) and 0.25 mL p-thiocresol. The reaction vessel was cooled to -78° and 2.5 mL HF was collected, after 2 h at 0° the mixture was evaporated to dryness on a vacuum pump. Following the ether-mercaptoethanol and acetic acid extractions the peptide mixture was examined as before by HPLC (Fig. 2, part D). The low HF step gave complete deprotection of Met(O) and Trp(For) but did not introduce further contaminants. It should be noted that the low HF step in this experiment served only to reduce Met(O) to Met and to deprotect Trp(For) to Trp in the cleaved peptide which already had been freed of benzyl-type protecting groups. Such a manipulation eliminated the conventional aqueous thiolytic reduction of Met (O) and the deprotection of Trp(For) under strongly basic conditions. This was important because it avoided loss of material due to handling and the attendant side reactions of thiolytic reduction as well as N-formyl transfer during the basic deprotection (23, 24).

![FIGURE 2](image_url)

Analysis of crude and unpurified gastrin I peptides by HPLC. Elution profile of the first 30 min of each chromatogram (not shown) run at isocratic condition of 10% CH₃CN to remove one or more of the following peaks: p-cresol, dimethylsulfide and its sulfonium salts, mercaptoethanol (reduced and oxidized), tetrahydrothiophene and its sulfonium salts, N-formyl oxime, and other p-thiocresol adducts. Amino acid-containing products were not found in this region. To simplify the comparison, only the elution profile of the last 30 min of HPLC is shown and gastrin I and gastrin peptides are eluted in this portion of the chromatogram. (A) high HF treatment alone (anisole as scavenger), (B) A followed by thiolytic and basic treatment, (C) high HF treatment, p-cresol as scavenger, (D) C + low HF treatment, (E) low-high HF treatment and (F) low HF treatment + hydrazinosis + 2.5% TFMSA-TFA. Synthetic samples of gastrin 1 obtained from other sources co-elute with sample B, D, to F.
Gastrin I synthesis

Run E, low-high HF
A more appropriate method to deprotect the gastrin peptide-resin was by the new low-high HF procedure which was developed in this laboratory (15–17). The reagents for both the low and high HF treatment were essentially the same as just described but were used in the reversed order and the effects were different. Following the washing of the synthetic protected peptide-resin, it was dried and 0.50 g was placed in the HF reaction vessel. Dimethylsulphide (3.25 mL), p-cresol (0.375 mL) and p-thiocresol (0.125 mL) were added and then 1.25 mL HF was condensed in the evacuated vessel at $-78^\circ$. After stirring for 2 h at $0^\circ$, the HF and DMS were evaporated at $0^\circ$ on a water aspirator and then on the high vacuum pump. Direct removal of the HF-DMS mixture on the high vacuum pump will cause uncontrollable bumping and splattering of peptide resin. Under such circumstances, most resin will not be in direct contact with the subsequent HF step and hence incomplete deprotection and heterogeneity of products will result. At the same time it is also important to remove all DMS at this step in the shortest possible time. Usually the evaporation time should not exceed 2 h to avoid unwarranted side reactions. Fresh HF (9 mL) was then condensed into the vessel at $-78^\circ$ and the high HF reaction was carried out at $0^\circ$ for 1 h. The HF was evaporated in vacuo at $0^\circ$ and reagents and scavengers were removed by ether-mercaptoethanol extraction. Finally, the peptide and by-products were extracted into 10% aqueous acetic acid for analysis by HPLC (Fig. 2, part E). Under these conditions the main gastrin I peak was increased to 70% of total U.V. absorbing material, but low levels of by-products were still present. Most of these were formed during the high HF step where some acylium ion is formed from the glutamyl residues and acylates the aromatic scavengers. Under these low HF conditions, the DMS served to reduce the acidity function of the mixture from $-11$ to $-6.8$ and also served as an effective nucleophile. All benzyl-based side chain protecting groups were removed by an $S_N^2$ mechanism and thus the generation of carbocations and acylium ions and the resultant alkylation and acylation side reactions were minimized. Concomitantly, Met(O) and Trp(For) were also deprotected. However, the peptide, though deprotected, still remained attached to the polymeric support through its more acid-resistant benzhydrylamine linkage and required the high HF-treatment for its release.

In the low HF procedures anisole was intentionally replaced by p-cresol and p-thiocresol for three reasons: 1) both p-cresol and p-thiocresol are not as volatile as anisole and remain in the reaction during the low and high treatments; 2) p-thiocresol was required to deprotect $N^1$-formyl tryptophan (16); 3) p-cresol is less readily acylated via the acid generated acylium ion of glutamyl residues than is anisole; and 4) anisole was found to be an alkylating agent during the low HF treatment (17). Note: p-thiocresol should be avoided in the high HF treatment when Trp(For) is present in the peptide-resin since p-thiocresol will react with Trp(For) to give about 30% of side products (25). However, once the $N^1$-formyl group is removed (such as after being treated in the low HF step) the presence of p-thiocresol is usually harmless. Similarly, anisole should be avoided in the low HF treatment since alkylation of methionine by anisole at the low HF step does occur.

Run F, low HF/nucleophile/dilute acid
To avoid completely a high HF step, the special properties of the multidetachable benzhydrylamine support were utilized. The sample was first fully deprotected under the low-HF conditions as before. The resin-bound peptide chain, still containing the benzhydrylamine handle, was washed thoroughly with ether-mercaptoethanol to remove reagents and scavengers and then cleaved from the resin at the phenyl ester bond by treatment with 5% hydrazine hydrate in DMF at $25^\circ$ for 1 h. The resulting peptide benzhydryl amide, containing the $p$-hydroxyl group, was precipitated from the acidified DMF solution. It was much more susceptible to acid than the original ester-containing derivative and could be cleaved by treatment with 5 mL trifluoroacetic acid containing 0.15 mL trifluoromethanesulfonic acid for 1 h. The crude product (Fig. 2 part F) contained 75% of a single main peak of gastrin I and only small amounts of several minor
### TABLE 2

Comparative yields from deprotection and cleavage of synthetic protected gastrin-resin

<table>
<thead>
<tr>
<th>Run</th>
<th>First reagent (% by vol.)</th>
<th>Additional reagents</th>
<th>Product</th>
<th>Yield main product in crude extract (%)</th>
<th>Overall yield isolated pure product (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>HF-anisole 90 10</td>
<td>-------</td>
<td>[Met(O),Trp(For)] gastrin1</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>B</td>
<td>HF-anisole 90 10</td>
<td>HSCH₂CH₂OH; NH₂OH</td>
<td>Gastrin I</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>C</td>
<td>HF-cresol 90 10</td>
<td>-------</td>
<td>[Met(O), Trp(For)] gastrin I</td>
<td>60</td>
<td>45</td>
</tr>
<tr>
<td>D</td>
<td>HF-cresol 90 10</td>
<td>HF-DMS-cresol-thiocresol 25 65 7.5 2.5</td>
<td>Gastrin I</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>E</td>
<td>HF-DMS-cresol-thiocresol 25 65 7.5 2.5</td>
<td>HF-cresol-thiocresol 90 7.5 2.5</td>
<td>Gastrin I</td>
<td>70</td>
<td>55</td>
</tr>
<tr>
<td>F</td>
<td>HF-DMS-cresol-thiocresol 25 65 7.5 2.5</td>
<td>Hydrazine; TFMSA-TFA-cresol 2.5 87.5 10</td>
<td>Gastrin I</td>
<td>75</td>
<td>60</td>
</tr>
</tbody>
</table>
components. This was the cleanest of the crude deprotection mixtures obtained in this study.

**Purification and characterization**

The crude peptide mixtures obtained from the HF deprotection and cleavage experiments were purified either by preparative HPLC or by DEAE-Sephadex ion exchange chromatography. Fig. 3 shows the HPLC purification of gastrin I obtained from run F under conditions described in the methods section. The gastrin obtained from the main peak represented a 60% overall yield based on the first residue attached to the benzhydrylamine resin. Alternatively, purification of another sample of the same run F preparation by the DEAE-Sephadex produced a main peak which gave 43 mg isolated peptide; 57% overall yield. The other preparations (Run A-E) were purified by similar procedures. The yields are recorded in Table 2.

All of the preparations gave products containing only one peak on the analytical HPLC column. Amino acid analysis (Table 3) revealed that the purified products all had the composition required for the gastrin I sequence. The integrity of the two-tryptophan residue was shown in the u.v.-chromatograms in which the deprotection of both \(^{N\text{a}}\) formyl tryptophans was evidently completed (Fig. 4). This was consistent with the HPLC analysis (Fig. 3) in which little product was detected by monitoring at 310 nm (the \(\lambda_{\text{max}}\) for Trp(For)).

Finally synthetic gastrin I was found to give a principal mass ion of 2119.85 (M+Na\(^+\), calculated 2119.9) obtained by the Californium fission fragment mass spectrometry (33, 34).

The identity of the product with natural human gastrin I was further confirmed by demonstrating that our synthetic gastrin I coeluted with each of two separately obtained synthetic samples (8, 9) that were prepared in other laboratories by strategies and protocols different from ours and which had been shown to contain full biological activities of gastrin I.

**DISCUSSION**

The present synthesis of human gastrin I is significant at two levels. Firstly, it represents a

### TABLE 3

**Amino acid analysis of synthetic hgastrin I (1–17)**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Theory</th>
<th>Crude</th>
<th>Purified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>1</td>
<td>1.10</td>
<td>1.09</td>
</tr>
<tr>
<td>Glu</td>
<td>6</td>
<td>5.58</td>
<td>6.00</td>
</tr>
<tr>
<td>Gly</td>
<td>2</td>
<td>1.94</td>
<td>1.91</td>
</tr>
<tr>
<td>Ala</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Met</td>
<td>1</td>
<td>0.87</td>
<td>0.94</td>
</tr>
<tr>
<td>Leu</td>
<td>1</td>
<td>0.90</td>
<td>0.99</td>
</tr>
<tr>
<td>Tyr</td>
<td>1</td>
<td>0.96</td>
<td>0.97</td>
</tr>
<tr>
<td>Phe</td>
<td>1</td>
<td>0.95</td>
<td>1.00</td>
</tr>
<tr>
<td>Trp</td>
<td>2</td>
<td>1.40</td>
<td>2.10</td>
</tr>
<tr>
<td>Pro</td>
<td>1</td>
<td>0.96</td>
<td>0.87</td>
</tr>
</tbody>
</table>

| Total 17   | Avg. 0.92 | 0.99 |

\(^{a}\)4.0 N Methanesulfonic acid + 0.2% 3-(2-aminoethyl) indole, 110°, 22 h.
successful synthesis by a stepwise solid phase method using the Boc/benzyl protecting group strategy and HF as the final deprotecting agent. For unexplained reasons, previous attempts using this approach by others were unsuccessful (11). Thus, our results dispel the misconception that the gastrin sequence is inaccessible to this solid-phase method. Secondly, our results have shown that the difficulties associated with the synthesis of this peptide are due to several causes and can be minimized in several ways. With a basic understanding of the mechanism of the potential side reactions and the introduction of the necessary precautions, successful syntheses of gastrin could be achieved. For example, a chemically unambiguous benzhydrylamine resin is used to avoid low cleavage yield and to reduce other side reactions due to uncertainties in the preparation of benzhydrylamine resin directly on a polymeric support (18, 19). In a typical benzhydrylamine resin preparation, only approximately a third of the benzophenone sites are converted to the benzhydrylamine moiety. In our solution model studies, these remaining sites were shown to be benzhydryl alcohol, benzhydryl chloride, and other unidentified materials. Both benzhydryl alcohol and benzhydryl chloride are known to be susceptible to trifluoroacetic acid, possibly transferring trifluoroacetyl groups that will lead to termination and other modifications.

The resin support for the synthesis of gastrin I was modified benzhydrylamine resin, which was introduced by us recently as a multidetachable resin (18, 19). The \( p \)-acyloxybenzhydrylamine resin 2 contains a benzhydrylamine with a \( p \)-acyloxy substituent which is also a phenyl ester linkage (Fig. 5). The electronic and chemical properties of such a substituent provide the acid stability required for the repetitive acid treatment in the synthesis but retain the sensitivity to cleavage by HF. The support also allows flexibility of synthetic design by its susceptibility to cleavage from the resin by several other types of reagents and conditions needed for different purposes (18, 19). In the gastrin synthesis, both advantages were utilized. Another advantage of this resin is that the benzhydrylamine moiety, being prepared and purified prior to its attachment to the resin support, is a chemically defined and unambiguous benzhydrylamine moiety covalently connecting the peptide and the resin support. We specifically emphasize this point to justify the direct comparisons between the different deprotection processes.

![FIGURE 5](image-url)

**FIGURE 5**
Synthesis and structure of the multidetachable benzhydrylamine resin. The polymeric support, copoly-(styrene-1% divinylbenzene) beads (Bio-Rad Laboratories, 200–400 mesh), is represented by “R”.

![FIGURE 4](image-url)

**FIGURE 4**
U.v.-spectrum of purified gastrin I.
Gastrin synthesis

In the normal solid-phase protocol, there was little loss of peptide chains from the resin support. For example, in the synthesis of I, greater than 98% of the starting peptide chains were found to remain on the resin support after 16 cycles of acid and base treatment. However, prolonged treatments with nucleophilic additives such as pyridine and 1-hydroxybenzo triazole in the coupling step should be avoided since the phenyl ester linkage is susceptible to such reagents.

Another precaution that we have taken and vigorously enforced concerns the completion of the coupling steps of the synthesis. The coupling steps in this synthesis, as monitored by the quantitative ninhydrin test (20, 35) and assessed by preview sequence analysis, were shown to proceed at a very high completion rate (99.9% per step). Furthermore, several peptide fragments at the intermediate stages have also been examined by HPLC before any purification and in each case were found to contain a single major peak accounting for greater than 80% of the crude peptide content. These results confirm that the gastrin peptide has been assembled correctly with minimal deletion or termination of peptide chains. In human gastrin I, there are five consecutive glutamic acid residues and difficulties in coupling at these positions could lead to termination products. Recently, we have examined the mechanism of coupling activated amino acids to N-terminal glutamyl or glutaminyl residues (29, 30). Our findings point to the ability of weak acids such as Boc-amino acid or 1-hydroxybenzotriazole in nonpolar solvents such as CH₂Cl₂ to associate as ion pairs with the N-terminal amino group and to catalyze pyroglutamic acid formation. Coupling at these residues using activated amino acids such as preformed symmetrical anhydride (36) in DMF or other polar solvents minimizes this side reaction. In the Fmoc/tert-butyI/polyamide strategy for the synthesis of gastrin (10–13) all the couplings were performed in DMF because it was a more compatible solvent for the polyamide support. For the reasons given above it is probable that this solvent was largely responsible for the good results obtained by this strategy. Finally, in regard to the controversy concerning the use of HF as the final deprotecting agent for an acid sensitive peptide such as gastrin, we have compared several HF procedures: the high, high-low and low-high HF procedures, and found significant differences in homogeneity of the crude products by HPLC analysis. The differences, however, were not as great as we had anticipated from the literature data. The poorest of the procedures, i.e. the standard 90% HF/10% anisole, gave an overall yield of 40% pure product, and approximately 50% of the crude cleavage mixture was the desired product. Cleavage in 90% HF in the presence of 10% p-cresol increased the yield by 8% over the one using anisole as scavenger. The low-high HF procedure, which was specifically designed to overcome side reactions due to carbonium ion intermediates, improved the yield of the main product by another 14%.

The five consecutive glutamic acid residues in the middle portion of little gastrin are probably responsible for most of the observed by-products in these syntheses. Strong acid such as 90% HF causes the formation of acylium ions from the γ-benzyl ester or from the free γ-carboxylic acid (38, 39). This very active electrophile can readily acylate the phenyl ring of the usual anisole scavenger and lead to stable ketone adducts. The effect of the high HF was obvious when the products of the gastrin syntheses were examined by HPLC and were shown to contain a number of slower moving peaks following the gastrin peak. Although the low-high HF procedure (Run E) gave a rather high, 70%, overall yield there still were present small amounts of several by-products. Ideally then, the deprotection of gastrin should not involve a high HF step at all. Since the benzhydrylamine linkages previously in use are all resistant to low HF conditions and require a high HF step for their cleavage, the avoidance of the high HF step in the synthesis of gastrin could best be achieved by an alternative resin support. The multidetachable benzhydrylamine support reported here meets most of the requirements. It is resistant to cleavage under the low-HF conditions, so that all the undesirable scavengers and deprotecting reagents could be washed from the peptide-resin prior to nucleophilic release of the gastrin.
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from the resin support. The gastrin peptide so obtained was shown to be a pseudo diastereomeric pair in HPLC since it contained the racemic benzhydrylamine handle. To remove the handle, the peptide was then treated under moderate acid concentration (2.5% trifluoromethane-sulfonic acid in TFA for 45 min). With this combination of low-HF treatment and multidetachable resin support, gastrin I could be obtained in 75% purity with few of the other side products seen in the previous cleavage procedures involving a high HF step. The final isolated yield of isolated homogenous gastrin was 60% based on the first residue attached to the resin.

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