Enhancement of peptide coupling reactions by 4-dimethylaminopyridine

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4-Dimethylaminopyridine (DMAP) was found to be useful in the enhancement of peptide coupling reactions mediated by dicyclohexylcarbodiimide or symmetrical anhydrides. In an automated synthesis of the model heptapeptide Boc-Ala-Cle-Ile-Val-Pro-Arg(Tos)-Gly-OCH2-Resin (Cle, cycloleucine), the efficiencies of various coupling methods such as dicyclohexylcarbodiimide, dicyclohexylcarbodiimide plus 1-hydroxybenzotriazole, and symmetrical anhydride were compared with that of dicyclohexylcarbodiimide plus 4-dimethylaminopyridine. Based on the amino acid composition of the peptide-resin samples and high pressure liquid chromatographic analyses of the protected heptapeptide amide obtained from the ammonolytic cleavage of the peptide-resin samples, it was concluded that only dicyclohexylcarbodiimide plus 4-dimethylaminopyridine gave the desired near quantitative couplings in those cycles involving the sterically hindered amino acid residues. Observations were also made that 4-dimethylaminopyridine was a useful additive in a modified symmetrical anhydride method of coupling. In the synthesis of the model tetrapeptide Leu-Ala-Gly-Val on a Pam resin, the anhydride couplings were accelerated by DMAP and the product was equivalent in homogeneity to that obtained by the best previous methods. In addition, no racemization was detectable by a sensitive chromatographic method. There also was no detectable racemization found in a DCC-DMAP coupling of Boc-Ile-OH with H-Val-OCH2-resin. However, significant racemization was observed during the coupling of Boc-Phe-OH with H-Glu(OBzl)-OCH2-resin. DMAP is recommended as an additive for coupling hindered amino acids, particularly Cα-substituted residues, where little or no racemization is expected.

Key words: hindered amino acids; 4-dimethylaminopyridine; peptide bond formation acceleration; racemization; solid phase peptide synthesis.

The organic base 4-dimethylaminopyridine (DMAP) is superior to pyridine as a catalyst for several acylation reactions. It can greatly accelerate the benzoylation of m-chloroaniline and facilitate the esterification of hindered alcohols with carboxylic acid anhydrides (1, 2). The compound has been utilized as an additive to the dicyclohexylcarbodiimide...
### TABLE 1

*Amino acid composition of Boc-Ala-Cle-Ile-Val-Pro-Arg(Tos)-Gly-OCMe-resins prepared by different methods*

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>DCC</th>
<th>DCC</th>
<th>DCC-HOBT</th>
<th>DCC-HOBT</th>
<th>Symm. anhyd.</th>
<th>DCC-DMAP</th>
<th>DCC-DMAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DCC</td>
<td>Double coupling</td>
<td>DCC-HOBT</td>
<td>Double coupling</td>
<td>Double coupling</td>
<td>DCC-DMAP</td>
<td>Double coupling</td>
</tr>
<tr>
<td>Gly</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Arg</td>
<td>0.81</td>
<td>0.86</td>
<td>0.94</td>
<td>0.91</td>
<td>0.62</td>
<td>0.96</td>
<td>0.93</td>
</tr>
<tr>
<td>Pro</td>
<td>1.02</td>
<td>1.02</td>
<td>0.92</td>
<td>1.10</td>
<td>1.02</td>
<td>1.34</td>
<td>1.23</td>
</tr>
<tr>
<td>Val</td>
<td>0.88</td>
<td>0.91</td>
<td>0.74</td>
<td>0.87</td>
<td>0.69</td>
<td>1.01</td>
<td>1.09</td>
</tr>
<tr>
<td>Ile</td>
<td>0.83</td>
<td>0.86</td>
<td>0.73</td>
<td>0.86</td>
<td>0.73</td>
<td>0.99</td>
<td>1.07</td>
</tr>
<tr>
<td>Cle</td>
<td>0.65</td>
<td>0.71</td>
<td>0.65</td>
<td>0.74</td>
<td>0.56</td>
<td>0.84</td>
<td>1.11</td>
</tr>
<tr>
<td>Ala</td>
<td>0.89</td>
<td>0.91</td>
<td>0.64</td>
<td>0.77</td>
<td>0.23</td>
<td>1.01</td>
<td>1.14</td>
</tr>
</tbody>
</table>

*aThe peptide-resin was hydrolyzed in 12 N HCl-propionic acid (1:1), 130°, 6 h. Hydrolysates in 12 N HCl-phenol-HOAc (2:1:1), 110°, 24 h gave almost identical amino acid analyses and they were unchanged after 96 h heating.

*bIncludes the D-alloisoleucine produced during acid hydrolysis.
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(DCC) mediated reaction in anchoring the first amino acid residue onto hydroxymethyl-polystyrene supports (3–5) for solid phase peptide synthesis (6, 7). Many other applications of DMAP and its analogs have been described (8–10). Here we wish to report the enhancement by DMAP of the efficiency of the DCC (11) and symmetrical anhydride methods (12) of peptide bond formation.

The automated solid phase synthesis (13) of Boc-Ala-Cle-Ile-Val-Pro-Arg(Tos)-Gly-OCH_2-copoly(styrene-1%-divinylbenzene) resin was chosen as the model system for comparing the coupling efficiencies of DCC, DCC plus HOBt, symmetrical anhydride and DCC plus DMAP methods, all programmed for single coupling procedures and also for double coupling procedures. The protected heptapeptide-resin samples obtained from each procedure were analyzed for their amino acid composition and the results are summarized in Table 1. It can be seen that only the DCC-DMAP procedure gave the desired near quantitative coupling in those cycles involving sterically hindered amino acids in the -Cle-Ile-Val- region. The results of the HPLC analyses of the crude protected heptapeptide amide Boc-Ala-Cle-Ile-Val-Pro-Arg(Tos)-Gly-NH_2 derived from the ammonolytic cleavage of the peptide resin samples (Fig. 1) also indicated that there were fewest impurities in the product prepared by the DCC-DMAP method. The homogeneity of the heptapeptide amide obtained by the symmetrical anhydride method was especially poor in this difficult synthesis.

Pyrrolidone carboxylic acid formation during the coupling of hindered amino acids to N-terminal glutaminyl peptide-resins was recently shown (14) to be catalyzed by weak acids and to be reduced by accelerated coupling methods. As shown in Table 2, the coupling of Boc-Ile to H-Gln-Gln-Ile-Phe-resin by the symmetrical anhydride method was markedly accelerated by the addition of 0.2 equiv. DMAP, and therefore is expected to lead to lower levels of chain termination due to pyrrolidone carboxylic acid formation.

The test peptide Leu-Ala-Gly-Val (6) was also prepared by solid phase synthesis using a modified DMAP procedure in which the symmetrical anhydride was first coupled for 20 min and then supplemented by the addition of 0.2 equiv. DMAP for an additional 20 min. This method was designed to accelerate the comp-

![Figure 1](image-url)

**FIGURE 1**

High pressure liquid chromatograms of crude Boc-Ala-Cle-Ile-Val-Pro-Arg(Tos)-Gly-NH_2 obtained from the Boc-Ala-Cle-Ile-Val-Pro-Arg(Tos)-Gly-OCH_2-resins prepared by different methods. The chromatography was carried out with 10-μg samples on an ultrasphere-ODS column (0.4 × 25 cm) with a linear gradient of 35–70% acetonitrile in 0.01 M KH_2PO_4.
TABLE 2
Coupling of Boc-Ile-OH to H-Gln-Gln-Ile-Phe-resin

<table>
<thead>
<tr>
<th>Run</th>
<th>Coupling step</th>
<th>Reagent</th>
<th>Time (h)</th>
<th>Uncoupled (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>S.A.</td>
<td>2</td>
<td>11.0</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>DCC-HOBt</td>
<td>2</td>
<td>7.2</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>DCC-HOBt</td>
<td>18</td>
<td>4.5</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>Ac₂O-pyridine</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>S.A.</td>
<td>2</td>
<td>11.0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>DMAP</td>
<td>0.3</td>
<td>3.8</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>S.A.-DMAP</td>
<td>2</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>S.A.-DMAP</td>
<td>2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

aSubstitution, 0.2 mmol/g. Solvent CH₂Cl₂.
bDetermined by amino acid analysis and ninhydrin monitoring.
cSymmetrical anhydride 2.5 equiv.
dBoc-Ile-OH, DCC and HOBt were all 2.5 equiv.
eBoc-Ile-OH, DCC, and HOBt were all 2.5 equiv. but in DMI.
fAfter 2 h with S.A., add 0.2 equiv. DMAP.
gSymmetrical anhydride 2.5 equiv. followed immediately by DMAP 0.2 equiv.

pletion of the synthesis and to minimize the exposure of the anhydride to basic conditions that can cause undesirable side reactions (15). The product and by-products of the synthesis of this peptide have been well characterized using a sensitive ion-exchange chromatographic system (16). It can be seen from Table 3 that 30-min coupling with symmetrical anhydride with the addition of an equivalent amount of DMAP gave a product comparable to the product obtained from a standard double coupling with DCC (2 h each) whereas single 30-min couplings with pre-formed symmetrical anhydrides led to noticeably more deletion peptides. Furthermore, less than 0.05% of racemized peptides containing D-Leu or D-Ala were detectable (17) (Table 3).

To study further whether the DCC-DMAP procedure can cause racemization, Boc-Ile-Val-

TABLE 3
Peptide products detected (mol %) after solid phase synthesis of Leu-Ala-Gly-Val (LAGV) under different conditions

<table>
<thead>
<tr>
<th>Coupling method</th>
<th>LAGV</th>
<th>LAV</th>
<th>LGV</th>
<th>AGV</th>
<th>GV + AV</th>
<th>LA</th>
<th>LAGGV</th>
<th>LLAGVe</th>
<th>LLAGVe</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Double coupling</td>
<td>99.0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.4</td>
<td>0.2</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Symm. anhyd.</td>
<td>98.0</td>
<td>0.4</td>
<td>0.3</td>
<td>0.7</td>
<td>0.3</td>
<td>0.1</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Symm. anhyd. + DMAP</td>
<td>98.9</td>
<td>0.05</td>
<td>0.05</td>
<td>0.4</td>
<td>0.2</td>
<td>&lt;0.05</td>
<td>0.2</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

aBoc-amino Acid and DCC, 3 equiv. each; 2 × 2 h coupling.
b3 equiv. symmetrical anhydride; 0.5 h coupling.
c3 equiv. each of symmetrical anhydride and DMAP; 0.5 h coupling.
dThe detection limit of the method was about 0.05% when the loading of the crude product to the ion-exchange column was 5 µmol.
eUnderlined residues are of D configuration.
Peptide coupling reactions

**TABLE 4**

*Racemization test for Boc-Ile-OH + H-Val-OCH₂-resin → Boc-Ile-Val-OCH₂-resin*

<table>
<thead>
<tr>
<th>Coupling method</th>
<th>Amino acid composition of Boc-Ile-Val-OCH₂-resina</th>
<th>Val</th>
<th>Ile</th>
<th>allo-Ile</th>
<th>allo-Ile/(Ile + allo-Ile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCCb</td>
<td>1.00 0.979 0.022 0.022</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCC-DMAPc</td>
<td>1.00 0.907 0.025 0.026d</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aSamples of Boc-Ile-Val-OCH₂-resin were hydrolyzed in propionic acid-12 N HCl (1:1) at 130° for 6 h.
b2 h coupling with 3 equiv. each of Boc-Ile-OH and DCC. Shown previously to proceed without detectable racemization.
c2 h coupling with 3 equiv. each of Boc-Ile-OH, DCC and DMAP.
dThe degree of racemization (0.026 - 0.022 = 0.004) is below the limit of precision of the method.

OCH₂-C₆H₄-resin was prepared from Boc-Ile-OH and H-Val-OCH₂-resin by the DCC-DMAP method and also by the standard DCC method, which has been shown to proceed without racemization under these conditions. The two dipeptide-resins thus obtained were hydrolyzed in propionic acid-12 N HCl (1:1) and their alloisoleucine content, which would reflect the extent of racemization during synthesis plus that due to acid hydrolysis, was determined (18). As summarized in Table 4, the dipeptide-resin sample prepared by the DCC method gave rise to 2.2% of alloisoleucine and the same dipeptide-resin prepared by DCC-DMAP method gave 2.6%. The difference was within the experimental error of this analytical procedure and thus we conclude that in each case the alloisoleucine was derived from the acid hydrolysis step and that little or no additional racemization took place when Ile was coupled to Val using the DCC-DMAP method. These results are in good agreement with the results of the experiment involving the synthesis of Leu-Ala-Gly-Val. However, significant racemization was observable when Boc-Phe-OH was coupled to H-Glu(OBz₁)-OCH₂-resin with the DCC-DMAP method (Table 5). In the control synthesis by the standard DCC method in CH₂Cl₂ very little D-Phe-L-Glu could be detected (0.4%) in the crude HF cleavage product by an ion-exchange chromatographic procedure similar to that of

**TABLE 5**

*Racemization test for Boc-Phe-OH + Glu(OBz₁)-OCH₂-resin to Boc-Phe-Glu(OBz₁)-OCH₂-resin*

<table>
<thead>
<tr>
<th>Run</th>
<th>Coupling methoda</th>
<th>DMAP (equiv.)</th>
<th>Solvent</th>
<th>D-Phe-L-Glu b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DCC</td>
<td>0</td>
<td>CH₂Cl₂</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>DCC</td>
<td>3</td>
<td>CH₂Cl₂</td>
<td>3.5</td>
</tr>
<tr>
<td>3</td>
<td>DCC</td>
<td>3</td>
<td>DMF</td>
<td>5.8</td>
</tr>
<tr>
<td>4</td>
<td>S.A.₅</td>
<td>3</td>
<td>CH₂Cl₂</td>
<td>5.2</td>
</tr>
<tr>
<td>5</td>
<td>S.A.₅</td>
<td>0.6</td>
<td>CH₂Cl₂</td>
<td>4.1</td>
</tr>
<tr>
<td>6</td>
<td>S.A.₅</td>
<td>0.03</td>
<td>CH₂Cl₂</td>
<td>0.8</td>
</tr>
<tr>
<td>7</td>
<td>S.A.₄</td>
<td>0.6</td>
<td>CH₂Cl₂</td>
<td>0.8</td>
</tr>
<tr>
<td>8</td>
<td>S.A.₆</td>
<td>0.6</td>
<td>CH₂Cl₂</td>
<td>17.9</td>
</tr>
</tbody>
</table>

a3 equiv. each of DCC and Boc-Phe, or 3 equiv. preformed symmetrical anhydride (S.A.), 2-h coupling times were used.
bSamples were cleaved from the resin by HF at 0°, and portions were analyzed by ion-exchange chromatography.

S.A. was added to the H-Glu(OBz₁)-OCH₂-resin, and followed immediately by the DMAP.

dS.A. was added to the H-Glu(OBz₁)-OCH₂-resin, and after 20 min mixing the DMAP was added.

eS.A. pretreated with DMAP for 2 min before adding to the resin.
Manning & Moore (19). In the corresponding sample prepared by the DCC-DMAP method using 3 equiv. each of Boc-Phe, DCC, and DMAP in CH₂Cl₂, 3.5% of D-Phe-L-Glu was present. Racemization during symmetrical anhydride coupling was reduced, but not eliminated, when the proportion of DMAP was decreased (Table 5, runs 4, 5, 6). Thus, 3 equiv. Boc-Phe anhydride and 0.03 equiv. DMAP gave only 0.8% of the D-Phe-L-Glu. If 0.6 equiv. DMAP was added 20 min after the anhydride, racemization was also reduced to 0.8% (run 7). However, if the anhydride and DMAP were premixed for only 2 min, racemization was sharply increased to 17.9% (run 8).

During the preparation of this manuscript a short communication appeared (20) showing that DMAP can cause racemization of Boc- or Fmoc-amino acids when they are esterified to hydroxymethyl-resins by the symmetrical anhydride method. Since the acylation of an alcohol is slower than an amine, the activated intermediate will be exposed to the base for a longer time and more racemization would be expected. Some of the limited data from our laboratory are in qualitative agreement with these findings. Thus, in a recent glucagon synthesis (21) Bpoc-Thr(Bu) was coupled to p-alkoxybenzylalcohol resin by 3 equiv. each of DCC and DMAP in CH₂Cl₂, and then released from the resin by TFA. Chromatographic analysis of the diastereomeric mixture produced by reaction with Boc-L-Leu-OSu showed that 0.2% of D-allothreonine was present. However, the Bpoc-Thr(Bu) control also showed 0.2% of D-allo-threonine and we conclude that no measurable racemization occurred during the esterification. In an esterification of Boc-Ile (3 equiv.) to hydroxymethyl-resin (1 equiv.) (Tam, J.P., unpublished results) racemization was 1.7% when 3 equiv. DMAP was present, and 1.2% when it was reduced to 0.3 equiv. However, with only 0.03 equiv. DMAP, racemization to D-alloisoleucine was only 0.6%. In the control experiment, Boc-Ile was found to contain 0.1% of D-allo-Ile. Under these conditions a 68% esterification was achieved in 2 h. Omission or further reduction of DMAP led to very slow and incomplete esterification.

It is clear that base-catalyzed racemization of activated Boc-amino acids by DMAP can occur under certain coupling conditions. Amino acids such as phenylalanine, which are more susceptible to abstraction of the α-proton, can give significant levels of racemization, whereas amino acids such as alanine and leucine were not racemized to a measurable extent under the conditions examined.

The addition of DMAP to the reaction mixture to enhance the coupling efficiency is most useful for those steps involving hindered amino acids, particularly Cα-substituted amino acids such as cycloleucine, where the reactions are usually slow and incomplete and where there is little or no danger of racemization. An indiscriminate application of the reagent in every coupling reaction cannot be recommended until it is shown that no significant side reaction occurs in that particular system under investigation.

EXPERIMENTAL PROCEDURES

Protected amino acid derivatives used were all of L-configuration unless otherwise stated. Cycloleucine (1-amino-1-cyclopentane carboxylic acid), 4-dimethylaminopyridine and 1-hydroxybenzotriazole hydrate were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. Di-tertiarybutyldicarbonate was from Fluka. Other chemicals and solvents used were reagent grade products from commercial sources. For the solid phase peptide syntheses, a Beckman 990B automated synthesizer was used. The microanalyses were carried out by Microlit Laboratories, Inc., Caldwell, New Jersey. For amino acid analyses, a Beckman 121 amino acid analyzer was used and for HPLC analyses, a Beckman Model 332 high pressure liquid chromatographic apparatus was used.

Boc-Cle-OH. Cyclo-leucine (4.6, 35.6 mmol) was mixed with 16.7 ml of Triton B (40% in MeOH, 35.6 mmol) and evaporated to dryness. The residue was then evaporated twice with DMF (each 50 ml) and the solid salt obtained was stirred with di-tertiarybutyldicarbonate (7.76 g, 35.6 mmol) in DMF for 24 h. A slightly turbid solution became clear when an additional portion of di-tertiarybutyldicarbonate (3.8 g) and 2 ml tetramethylguanidine were
added and the mixture was stirred for a few more hours. The solvent was removed by evaporation (oil pump, 45°) and the residue taken up in water (200 ml), washed with ether and acidified with dil. H₂SO₄ to pH < 3. The oily product separated, was then extracted into EtOAc, washed 3 times with H₂O, dried over Na₂SO₄ and evaporated to a smaller volume when crystallization began. Recrystallized from EtOAc and hexane: yield, 5.01 g (61%); m.p. 125–128°.


Automated synthesis of Boc-Ala-Cle-Ile-Val-Pro-Arg(Tos)-Gly-OCH₂-resin under different conditions

For the automated synthesis of Boc-Ala-Cle-Ile-Val-Pro-Arg(Tos)-Gly-OCH₂-resin, 1.28 g Boc-Gly-OCH₂-resin (0.39 mmol/g; 0.5 mmol) was placed in the reaction vessel of the automatic peptide synthesizer and the automatic synthesis carried out with a program which would perform the following steps in each cycle.

1. Wash 3 times with CH₂Cl₂,
2. Prewash 1.5 min with 40% TFA in CH₂Cl₂,
3. Deprotect with TFA (40% in CH₂Cl₂) for 28 min,
4. Wash 3 times with CH₂Cl₂,
5. Prewash 1.5 min and neutralize 8 min with 10% Et₃N in CH₂Cl₂,
6. Wash 3 times with CH₂Cl₂,
7. Add Boc-Arg(Tos)-OH (0.64 g, 1.5 mmol) and DCC (6 ml 0.25 M in CH₂Cl₂), then stirred for 120 min,
8. Wash 3 times each with CH₂Cl₂, EtOH-CH₂Cl₂ (1:1), and again with CH₂Cl₂. The synthetic cycle was repeated automatically with Boc-Pro-OH (0.32 g, 1.5 mmol) in step (7), followed by the remaining cycles each with Boc-Val-OH (0.33 g), Boc-Ile-OH (0.36 g), Boc-Cle-OH (0.34 g) and Boc-Ala-OH (0.28 g) in step (7). The protected heptapeptide resin was dried (1.71 g) and served as the sample for the DCC single coupling procedure. For the synthesis of the peptide-resin sample by the DCC-double coupling procedure, the synthesizer was programmed to perform steps 1 to 8 followed immediately by steps 5 to 8. In the second coupling, the same 3-fold excess of Boc-amino acid and DCC were used with identical coupling time of 120 min.

To prepare corresponding peptide-resin samples by DCC-HOBt or DCC-DMAP procedures, exactly identical programs were used except that HOBt (3 mmol) or DMAP (1.5 mmol) was added automatically into step 7 of every synthetic cycle. The symmetrical anhydride procedure sample was similarly synthesized with 1.5 mmol preformed Boc-amino acid symmetrical anhydride added into the reaction vessel at step 7. Starting from 1.28 g Boc-Gly-OCH₂-resin the weight of the Boc-Ala-Cle-Ile-Val-Pro-Arg(Tos)-Gly-OCH₂ obtained from the DCC single coupling procedure was 1.71 g; DCC-double coupling procedure, 1.72 g; DCC-HOBt single coupling, 1.77 g; DCC-HOBt double coupling, 1.73 g; DCC-DMAP single coupling, 1.79 g; DCC-DMAP double coupling, 1.85 g; symmetrical anhydride double coupling, 1.54 g.

All these protected heptapeptide resin samples (~ 50 mg) were hydrolyzed in propionic acid: 12 N HCl, 1:1 and the hydrolyzate analyzed on the amino acid analyzer. The results are tabulated in Table 1.

HPLC analyses of the crude Boc-Ala-Cle-Ile-Val-Pro-Arg(Tos)-Gly-NH₂ samples

Part (1 g) of Boc-Ala-Cle-Ile-Val-Pro-Arg(Tos)-Gly-OCH₂-resin from each resin sample was ammonolyzed in 50 ml MeOH saturated with NH₃ for 3 days in a tightly stoppered vessel. The resin was filtered off and the filtrate evaporated to dryness, giving 0.30–0.35 g crude heptapeptide amide, which was then analyzed on a high pressure liquid chromatographic apparatus. An Ultrasphere-ODS 5 μ column (Beckman 235329) was used with a linear gradient of 35–70% acetonitrile in 0.01 M KH₂PO₄ as eluent monitored at 210 nm. The flow rate was set at 1 ml/min. About 10–15-μg samples were applied. The results are summarized in Fig. 1.

Symmetrical anhydride method for the synthesis of Leu-Ala-Gly-Val with or without DMAP as compared with DCC method

Boc-Val-OCH₂-C₈H₄-CH₂-CO-NH-CH₂ resin (0.5 g, 0.1 mmol) (16, 22) was placed in a reaction vessel on a shaker and treated as follows for the incorporation of Boc-Gly:

1. CH₂Cl₂ (3 × 1 min), (2) 50% TFA-CH₂Cl₂
(2 × 2 min), (3) 50% TFA-CH₂Cl₂ (1 × 16 min), (4) CH₂Cl₂ (3 × 1 min), (5) 5% DIEA-CH₂Cl₂ (2 × 2 min), (6) CH₂Cl₂ (5 × 1), (7) 3 equiv. Boc-Gly in CH₂Cl₂ (1 min), 3 equiv. DCC in 5 ml CH₂Cl₂ (20 min) followed by 0.6 equiv. DMAP (20 min) (8) CH₂Cl₂ (3 × 1 min).

The cycle was repeated with Boc-Ala and Boc-Leu, but preformed symmetrical anhydrides were used in step (7). They were prepared by mixing Boc-amino acid (6 equiv.) with DCC (3 equiv.) in CH₂Cl₂ at 0°C for 10 min and then adding to the resin. After 20 min 0.6 equiv. DMAP was added for 20 min.

The Boc-Leu-Ala-Gly-Val-resin was washed 3 times with DMF, CH₂Cl₂, CH₃CN and vacuum dried. Amino acid analysis indicated that this material contained 0.18 mmol peptide/g of peptide-resin and had an amino acid composition of Leu₁, Ala₁, Gly₁, Val₀.₂. A portion of the peptide-resin (100 mg) was treated with 5 ml HF (containing 10% anisole, 0°C, 1 h). Crude H-Leu-Ala-Gly-Val-OH was obtained in 90% yield (0.016 mmol) which was dissolved in 1.00 ml water, and was applied on the long column (0.9 × 54 cm, AA-15 sulfonated polystyrene) of a Beckman 120B amino acid analyzer. For comparison, the same synthesis and analysis of products was carried out with (a) the DCC method without DMAP double coupling for 2 h each, (b) the DCC method without DMAP, single coupling, 30 min. The results of these syntheses are tabulated in Table 3.

Coupling of Boc-Ile-OH to H-Gln-peptide-resin
H-Gln-Gln-Ile-Phe-resin (0.45 g, 0.09 mmol) was coupled with (Boc-Ile)₂O in CH₂Cl₂ (5 ml, 0.23 mmol) for 2 h. A small sample was taken for quantitative ninhydrin analysis (23). The remainder of the peptide-resin was allowed to recouple with Boc-Ile-OH (0.23 mmol), DCC (0.23 mmol) and HOBt (0.23 mmol) in CH₂Cl₂ for 2 h. A sample was taken for ninhydrin analysis and the resin was allowed to react with fresh portions of Boc-Ile-OH, DCC, and HOBt for 18 h in DMF. Ninhydrin analysis showed 4.5% of unreacted amino group still present; the peptide-resin was finally treated with Ac₂O (2 ml) and pyridine (6 ml), which brought the free amino group content to below 0.5%. Another sample of this resin (0.1 g, 0.02 mmol) was coupled with (Boc-Ile)₂O (0.05 mmol) and DMAP (0.001 mmol) for 2 h. A sample was taken for analysis and the remaining resin recoupled with fresh portions of Boc-Ile and DMAP for another 2 h. Ninhydrin analysis indicated that there was 0.6% free amine left. The results are tabulated in Table 2.

Racemization tests for the DCC-DMAP coupling method
Boc-Ile-OH + H-Val-OCH₂-resin → Boc-Ile-Val-OCH₂-resin. Boc-Val-OCH₂-resin (2 g, 1 mmol) was placed in the automated synthesizer and the synthesis was carried out with 3 equiv. each of Boc-Ile-OH·1/2 H₂O (0.72 g), DMAP (0.36 g) and DCC (0.62 g). The coupling time was set at 120 min. The resultant dipeptide-resin was hydrolyzed in propionic acid-HCl (1:1) and analyzed for its allo-isoleucine content on an amino acid analyzer. As a control, the peptide synthesized without DMAP was hydrolyzed and analyzed.

Boc-Phe-OH + H-Glu(OBzI)-OCH₂-resin → Boc-Phe-Glu(OBzI)-OCH₂-resin. Boc-Glu(OBzI)-OCH₂-resin (2.85 g, 1 mmol) was placed in the automatic synthesizer and the synthesis was carried out with 3 mmol each of Boc-Phe-OH (0.79 g), DMAP (0.36 g) and DCC (0.62 g) in 30 ml of CH₂Cl₂, 2 h. The dipeptide-resin was then cleaved with HF (0°C, 30 min) (24) and the crude H-Phe-Glu-OH was analyzed on the amino acid analyzer according to the procedure of Manning & Moore (19). A 58-cm column of AA-15 resin was used with pH 4.25 citrate buffer as eluent, 56°C. The retention time for standard D-Phe-L-Glu was 127 min and for standard L-Phe-L-Glu was 145 min. The dipeptide sample prepared above had 3.5% of D-L and 96.5% of L-L isomers. When the same experiment was carried out without the addition of DMAP in coupling mixture, the crude Phe-Glu had 0.4% of D-L and 99.6% of L-L isomers.

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