Application of *N*-(*tert*-Butyloxycarbonyl)amino Acid *N*-Carboxyanhydrides in Solid-Phase Peptide Synthesis

Chu-Biao Xue and Fred Naider*

Department of Chemistry, College of Staten Island, City University of New York, Staten Island, New York 10301

Received March 24, 1992 (Revised Manuscript Received November 3, 1992)

A study of the utilization of *tert*-butyloxycarbonyl-protected amino acid *N*-carboxyanhydrides (Boc-NCAs) in solid-phase peptide synthesis revealed that coupling rates were favored in solvents with a high dielectric constant such as DMF. Tertiary amines such as DIEA are not required for efficient coupling in DMF. However, the use of 1 equiv of DIEA in DMF in the synthesis of a pentapeptide resulted in a cleaner crude product as compared with that obtained in the absence of DIEA. The rate of Boc–NCA coupling was comparable to that found for (benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate (BOP) coupling or 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) coupling as judged by kinetic analysis of coupling of BocIleNCA or BocValNCA to an Ala-PAM-resin or an Ile-Ala-PAM-resin. Coupling of Boc-L-ValNCA to a Phe-PAM resin or of Boc-L-PheNCA to an Ala-PAM resin resulted in 0.2%–0.25% formation of D-Val-Phe and D-Phe-Ala, respectively, indicating that racemization during UNCA coupling is comparable to that found using BOP or HBTU. Comparison of the Boc-NCA method with BOP or HBTU activation in the synthesis of Ala-Leu-Val-Ile-Ala, Gly-Val-Phe-Trp-Asp-Pro-Ala-Leu-Val-Ile-Ala, and Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala-Cys-Val-Ile-Ala using 4 equiv of protected amino acid and 1-h coupling in DMF showed that the Boc-NCA synthesis resulted in crude peptides which were of equal purity to those obtained from the BOP or HBTU methodologies.

Introduction

The synthesis of peptides of high purity by solid-phase procedures requires that the coupling, deprotection, and neutralization steps all go to completion without side reactions.[1] Most deprotection and neutralization strategies are generally assumed to fulfill this requirement. Therefore, much of the research on solid-phase synthesis during the last 20 years has been directed toward finding more efficient activation and coupling techniques.

Coupling of protected amino acids to solid-phase supports can be accomplished by either in situ activation or the use of preactivated derivatives. In situ activation is exemplified by carbodiimides such as dicyclohexylcarbodiimide[2] and diisopropylcarbodiimide.[3] Shortcomings of these reagents have been circumvented by the use of additives such as *N*-hydroxysuccinimide[4] and *N*-hydroxybenzotriazole[5] to minimize racemization and the formation of *N*-acylureas and to prevent dehydration of Asn and Gln residues.[6] Although hundreds of coupling reagents appear in the literature, several new reagents such as BOP,[7] HBTU,[8] and PyBroP[9] appear to result in especially efficient coupling and in minimal racemization and side reaction. BOP and HBTU are, at present, considered to be preferred reagents for rapid activation during solid-phase synthesis.[10]

Preactivated N-protected amino acids eliminate the need for a separate activation step and are, in principle, simpler to apply in solid-phase synthesis. Symmetrical anhydrides,[11] preformed HOBt-active esters,[12] and an extensive group of active esters have been evaluated as suitable reagents. The lability of most symmetrical anhydrides and of HOBt active esters necessitates their preparation immediately prior to coupling. In addition, symmetrical anhydrides require 2 equiv of protected amino acid to form 1 equiv of activated species. Structurally well-established active esters, such as nitrophenyl esters,[13] pentachlorophenyl esters,[14] and pentafluorophenyl esters,[15] provide little opportunity for side reactions. However, these esters usually react more slowly than DCC, DCC/HOBt, or symmetrical anhydride coupling.

Recently, a new class of preactivated amino acids, the urethane-protected amino acid *N*-carboxyanhydrides (UNCAs), was introduced by Fuller et al.[16] *N*-Carboxyanhy-

(1) (a) Barany, G.; Merrifield, R. B. In *The Peptides*; Gross, E., Meienhofer, J., Ed.; Academic Press: New York, 1980; Vol. 2, pp 1–284. (b) Stewart, J. M.; Young, J. D. *Solid Phase Peptide Synthesis*, 2nd ed.; Pierce Chemical Co.: Rockford, IL, 1984.

(2) Sheehan, J. C.; Hess, G. P. *J. Am. Chem. Soc.* **1955**, *77*, 1067.

(3) Sarantakis, D.; Teichman, J.; Lien, E. L.; Fenichel, R. L. *Biochem. Biophys. Res. Commun.* **1976**, *73*, 336.

(4) (a) Weygand, F.; Hoffmann, D.; Wunsch, E. *Z. Naturforsch., Teil B* **1966**, *21*, 426. (b) Zimmerman, J. E.; Anderson, G. W. *J. Am. Chem. Soc.* **1967**, *89*, 7151.

(5) (a) Konig, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788. (b) Windridge, G.; Jorgensen, E. *J. Am. Chem. Soc.* **1971**, *93*, 6318. (c) Bosshard, H.; Schechter, I.; Berger, A. *Helv. Chim. Acta* **1973**, *56*, 717.

(6) (a) Gish, D. T.; Katsoyannis, P. G.; Hess, G. P.; Stedman, R. J. *J. Am. Chem. Soc.* **1956**, *78*, 5954. (b) Ressler, C. *J. Am. Chem. Soc.* **1956**, *78*, 5956.

(7) Castro, B.; Dormoy, J. R.; Evin, G.; Selve, C. *Tetrahedron Lett.* **1975**, 1219.

(8) Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillessen, D. *Tetrahedron Lett.* **1989**, *30*, 1927.

(9) Frerot, E.; Coste, J.; Pantaloni, A.; Dufour, M.-N.; Jouin, P. *Tetrahedron* **1991**, *47*, 259.

(10) Fournier, A.; Wang, C.-T.; Felix, A. *Int. J. Peptide Protein Res.* **1988**, *31*, 86.

(11) (a) Wieland, T.; Birr, C.; Flor, F. *Angew. Chem.* **1971**, *83*, 333. (b) Hagenmaier, H.; Frank, H. *Hoppe-Seyler's Z. Physiol. Chem.* **1972**, *353*, 1973. (c) Blake, J.; Liu, C. H. *Int. J. Peptide Protein Res.* **1975**, *7*, 495.

(12) Mojsov, S.; Mitchell, A. R.; Merrifield, R. B. *J. Org. Chem.* **1980**, *45*, 555.

(13) Bodanszky, M. *Nature (London)* **1955**, *175*, 685.

(14) Kupryszewski, G.; Kaczmarek, M. *Rocz. Chem.* **1961**, *35*, 931; *Chem Abstr.* **1962**, *56*, 6081c.

(15) (a) Kisfaludy, L.; Roberts, J. E.; Johnson, R. H.; Mayers, G. L.; Kovacs, L. *J. Org. Chem.* **1970**, *35*, 3563. (b) Kovacs, L.; Kisfaludy, L.; Ciprini, M. Q. *J. Am. Chem. Soc.* **1967**, *89*, 183.

(16) (a) Fuller, W. D.; Cohen, M. P.; Shabankareh, M.; Blair, R. K.; Goodman, M.; Naider, F. R. *J. Am. Chem. Soc.* **1990**, *112*, 7414. (b) Fuller, W. D.; Cohen, M. P.; Naider, F. R.; Goodman, M. U.S. Patent No. 4,946,942, 1990.

0022-3263/93/1958-0350$04.00/0

drides (NCAs) have been used in the formation of peptide bonds since the late 1940's and were extensively applied to the synthesis of poly(α-amino acids).[17] NCAs are highly reactive toward the mildest nucleophiles, resist racemization, and generate only carbon dioxide as a byproduct. In the 1960's and 1970's, attempts were made to apply unprotected NCAs to the synthesis of small peptides of known sequence, and several notable successes were reported.[18] However, the inability to control the coupling reaction for unprotected NCAs frustrated the utilization of these reagents in the synthesis of large peptides and especially in solid-phase synthesis. Urethane-protected NCAs should result in readily controlled acylation reactions. These reagents were reported to be stable, crystalline solids and (9-fluorenylmethoxy)carbonyl-protected NCAs (Fmoc-NCAs) have been used in the successful synthesis of a decapeptide in a flow reactor.[16]

A detailed analysis of the relative coupling efficiencies of a wide range of coupling reagents has been reported by Hudson.[19] In this paper, we explore the optimal coupling conditions for *tert*-butyloxycarbonyl (Boc)-protected NCAs in the batch solid-phase synthesis of peptides by evaluating the coupling kinetics of sterically hindered amino acids in several solvents and in the absence and presence of diisopropylethylamine (DIEA). The rate of UNCA coupling is compared with that of BOP and HBTU activations using amino acid- and dipeptide-containing resins as the model chain ends. In addition, the relative efficacies of UNCA, BOP, and HBTU couplings are evaluated by the synthesis of three peptides containing from 5 to 15 amino acid residues.

Results

Kinetics. Effect of Coupling Solvent on Reaction of BocValNCA with Ala-PAM-Resin. The kinetics of coupling of BocValNCA in CH_2Cl_2, *N*-methylpyrrolidone (NMP), and DMF, the three commonly used solvents in solid-phase peptide synthesis, were evaluated by following the rate of addition of this activated amino acid to an Ala-PAM-resin. In preliminary studies, it was found that using either a 3- or a 4-fold excess of BocValNCA the coupling was more than 99% complete within 5 min in both CH_2Cl_2 and DMF (data not shown). In order to be able to make comparisons in different solvents, the reaction was carried out using only a 30% excess (1.3 equiv) of BocValNCA. Even under these conditions, nearly 97% of the free amine had reacted in NMP and more than 98% of the free amine had reacted in DMF in 5 min (Figure 1). Reaction culminated within 10–15 minutes in both of the solvents and leveled off at 98.2% in NMP and 99.2% in DMF. Reaction was appreciably slower in CH_2Cl_2 (Figure 1). Nevertheless, even in this solvent using only a 30% excess of the coupling reagent, nearly 95% of the chain ends reacted in 1 h and 97.4% completion of coupling was attained at 2 h.

Effect of Base on Coupling Rate. Since no information was available on the influence of base on UNCA coupling rates, we evaluated the coupling of BocIleNCA to Ala-PAM-resin in the absence and presence of DIEA in DMF and CH_2Cl_2. When a 20% excess (1.2 equiv) of BocIleNCA was used, 2 equiv of DIEA had no measurable effect on the coupling rate in DMF (Figure 2). Coupling yields reached 99.0–99.1% at 1 h and 99.1–99.3% at 2 h. In contrast, DIEA noticeably reduced both the rate and extent of UNCA coupling in CH_2Cl_2 (Figure 2).

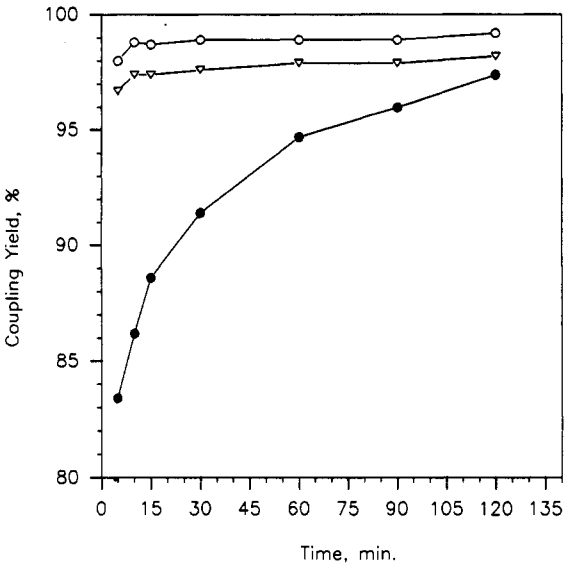

Figure 1. Coupling of BocVal to Ala-PAM-resin using 1.3 equiv of BocValNCA in DMF (-○-), NMP (-▽-), or CH_2Cl_2 (-●-).

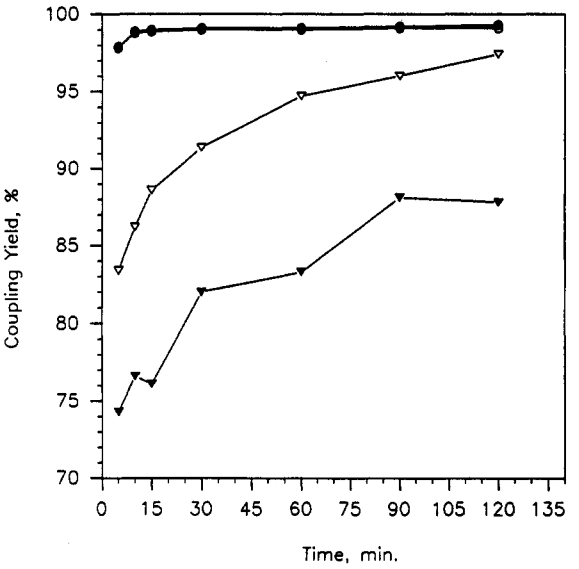

Figure 2. Coupling of BocIle to Ala-PAM-resin using 1.2 equiv of BocIleNCA in DMF in the presence of 2 equiv of DIEA (-●-) or without DIEA (-○-); coupling of BocVal to Ala-PAM-resin using 1.3 equiv of BocValNCA in CH_2Cl_2 in the presence of 2 equiv of DIEA (-▼-) or without DIEA (-▽-).

Comparison of UNCA Coupling with BOP and HBTU Couplings. Since BOP and HBTU are among the most efficient coupling reagents reported to date for peptide bond formation, we compared UNCA coupling with BOP- and HBTU-mediated couplings. Using a 20% excess (1.2 equiv) of BocValNCA, up to 15 min BocValNCA reacted somewhat more slowly with Ile-Ala-PAM-resin in DMF in the absence of DIEA as compared with the same amino acid activated with either BOP or HBTU reagent in DMF in the presence of 3 equiv of DIEA (Figure 3). By 1 h the reaction curves had crossed and the coupling yield with UNCA was slightly better than that of either the

(17) Katchalski, E. *Advances in Protein Chemistry*; Academic Press: New York, 1951; Vol. 6, p 123.
(18) Blacklock, T.; Hirschmann, R.; Veber, D. F. In *The Peptides*; Udenfriend, S., Meienhofer, J., Eds.; Academic Press: New York, 1987; Vol. 9, pp 39–102.
(19) Hudson, E. *J. Org. Chem.* 1988, *53*, 617.

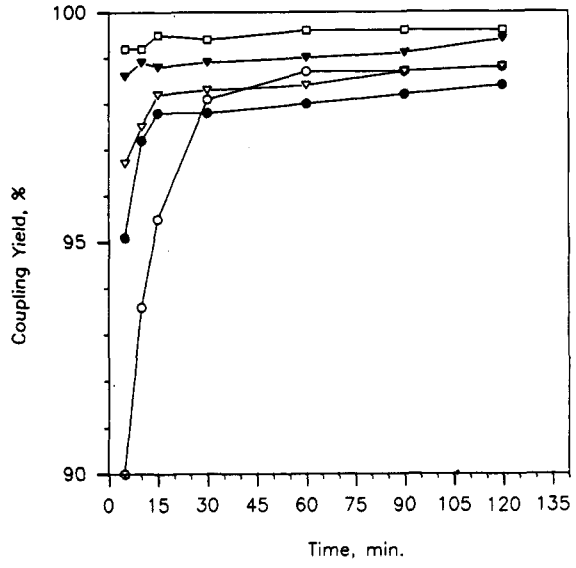

Figure 3. Coupling of BocVal to Ile-Ala-PAM-resin: -○-, 1.2 equiv of BocValNCA in DMF; -●-, 1.2 equiv of BocVal, 1.2 equiv of BOP, and 3 equiv of DIEA in DMF; -▽-, 1.2 equiv of BocVal, 1.2 equiv of HBTU, and 3 equiv of DIEA in DMF. Coupling of BocVal to Ala-PAM-resin. -□-: 4 equivalents of BocValNCA in DMF; -▼-: 4 equivalents of BocVal, 4 equivalents of BOP, and 8 equivalents of DIEA in DMF.

BOP or the HBTU-mediated reactions. It should be noted that when 1.5 or more equiv of reagent was utilized BocValNCA coupled at least as well as the BOP or the HBTU activation. Similarly, coupling of BocVal to Ala-PAM-resin using 4 equiv of BocValNCA in DMF in the absence of DIEA resulted in a slightly higher coupling yield than BOP activation using 4 equiv of BocVal, 4 equiv of BOP, and 8 equiv of DIEA (Figure 3).

Racemization. A major concern during the synthesis of peptides for biological assay or physicochemical analysis is the optical purity of the individual residues in the peptide chain. The amount of racemization during Boc-NCA coupling was determined by reacting Boc-L-ValNCA and Boc-L-PheNCA to a Phe-PAM-resin and an Ala-PAM-resin, respectively. After cleavage, the resulting dipeptides were analyzed using HPLC on a reversed-phase column. L-Val-L-Phe and D-Val-L-Phe elute from the column at 12.0 and 15.2 min, respectively. Elution times for L-Phe-L-Ala and D-Phe-L-Ala were 10.1 and 14.3 min, respectively. Integration of the peaks for the diastereomers indicated that the percentages of the D-isomers in Val-Phe and Phe-Ala were 0.2% and 0.25%, respectively. In parallel syntheses we evaluated racemization during formation of these dipeptides by coupling Boc-D-Val to a Phe-PAM-resin using HBTU and by coupling Boc-D-Phe to an Ala-PAM-resin using BOP. Racemization was 0.22% and 0.17% for BOP and HBTU, respectively. The racemization observed with the UNCAs represents the cumulative effect of both the synthesis of the Boc-NCA and the coupling reaction. The results show that racemization is at acceptably low levels with valine and phenylalanine and is comparable to that found with BOP and HBTU. The lack of significant impurities in the chromatograms of tetrapeptides and pentapeptides is additional evidence that significant racemization does not occur during the synthesis of peptides using UNCAs (see below).

Synthesis. Leu-Asn-Phe-Ala and Leu-Gln-Phe-Ala. As stated in the introduction, Asn and Gln have proved to be difficult residues during carbodiimide activation. To evaluate the utility of UNCA coupling for these residues, two tetrapeptides, Leu-Asn-Phe-Ala and Leu-Gln-Phe-Ala, were synthesized using Boc-NCAs. The Asn and Gln side-chain amide groups were protected as the trityl moiety in these preparations. The crude tetrapeptides obtained after cleavage from the PAM resin were grater than 97% homogeneous, indicating that serious side reactions do not occur during coupling of Asn and Gln UNCAs to a growing peptide chain.

Ala-Leu-Val-Ile-Ala. In order to evaluate the suitability of Boc-protected NCAs in solid-phase peptide synthesis, we synthesized a sterically hindered pentapeptide using either 4 equiv of UNCA or 4 equiv of UNCA in the presence of 1 equiv of DIEA. For comparison, this peptide was also synthesized using 4 equiv of Boc-amino acid and 4 equiv of BOP in the presence of 8 equiv of DIEA. In all cases, DMF was used as solvent and a single 1-h coupling was employed. We found that highly homogeneous crude Ala-Leu-Val-Ile-Ala was obtained regardless of the coupling procedure (Table I). However, as judged by the integrated area under the HPLC curve, the presence of 1 equiv of base led to a small increase in the purity and yield of the pentapeptide as compared with the UNCA alone (Table I). In addition, the base-catalyzed UNCA coupling resulted in a crude pentapeptide that was 98% homogeneous as compared to 97% homogeneity found in the BOP activated synthesis.

Gly-Val-Phe-Trp-Asp-Pro-Ala-Leu-Val-Ile-Ala. The 11-peptide Gly-Val-Phe-Trp-Asp-Pro-Ala-Leu-Val-Ile-Ala was synthesized using 4 equiv of Boc-amino acid NCA in the presence of 1 equiv of DIEA. For comparison, this peptide was also synthesized by BOP coupling using 4 equiv of Boc-amino acid and 4 equiv of BOP in the presence of 8 equiv of DIEA. In both cases, benzyl and formyl groups were used for protections of the Asp β-carboxyl and Trp indole, respectively, and a single 1-h coupling in DMF was employed. Pro was introduced using BOP coupling in the UNCA synthesis. The purity of the crude peptides obtained by either procedure was essentially identical (Table I), and the isolated products comigrated on reversed-phase HPLC.

Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala-Cys-Val-Ile-Ala. In order to make a comparison of UNCA coupling with HBTU coupling, we synthesized a 15-peptide using either 4 equiv of Boc-amino acid NCA in the presence of 1 equiv of DIEA in DMF or 4 equiv of Boc-amino acid and 4 equiv of HBTU in the presence of 6 equiv of DIEA in DMF. In both cases, the side-chain protecting groups were 4-methoxybenzyl for Cys, benzyl for Asp, formyl for Trp, 2-chlorobenzoyloxycarbonyl (ClZ) for Lys, and 2-bromobenzyloxycarbonyl (BrZ) for Tyr. In the UNCA synthesis, the Lys, Pro, and Tyr residues were introduced using HBTU coupling since BocLys(ClZ)NCA and Boc-Tyr(BrZ)NCA were not available. Up to the Gly^5 residue, each residue was introduced into the peptide chain using a single 1-h coupling. For Lys^1, Ile^2, Ile^3, and Tyr^4 residues, a single 1-h coupling was insufficient for complete reaction as judged by the Kaiser test,[20] and a second 1-h coupling was required in both the UNCA- and HBTU-mediated syntheses. The crude peptides from HF cleavage were treated with aqueous piperidine to remove the formyl group. Dithioerythritol was present to reduce disulfide formation under the basic deprotection conditions. The

(20) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Chem.* **1970**, *34*, 595.

Table I. Comparative Peptide Synthesis Using UNCA and BOP or HBTU Couplings[a]

	Ala-Leu-Val-Ile-Ala			Gly-Val-Phe-Trp-Asp-Pro-Ala-Leu-Val-Ile-Ala		Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala-Lys-Val-Ile-Ala	
coupling method	UNCA	UNCA + DIEA	BOP	UNCA + DIEA	BOP	UNCA + DIEA	HBTU
purity of crude product[b]	96	98	97	91	90	81	75
crude yield[c]	98	97	95	90	88	90	92
yield of purified product	70	73	71	54	47	32	29

[a] All values represent percentages. [b] Determined by HPLC. [c] After HF cleavage.

15-peptide from the UNCA synthesis comprised nearly 81% of the crude mixture. This was somewhat better than the crude product from HBTU synthesis (Table I).

Discussion

Val and Ile are two of the most sterically hindered amino acids and were chosen for kinetic studies on the reactivity of UNCAs. Kinetic assays in three different solvents (DMF, NMP, and CH_2Cl_2) indicated that UNCA coupling is favored in solvents with a high dielectric constant. The fastest coupling rate was found in the highly polar but aprotic solvent DMF, and the coupling rate was significantly slower in a less polar solvent such as CH_2Cl_2 (Figure 1). These results are consistent with the previous finding that polar solvents lead to optimal peptide-resin solvation and thus enhance the accessibility of the resin-bound amine to the acylating species.[21] It is also possible that polar solvents directly facilitate the acylation process. For example, in a study of the reaction of BocPheNCA with *N*-MeAib-PheOBzl (Aib = α-aminoisobutyric acid) better coupling was observed in DMF than in THF (Goodman, personal communication). For these reasons, DMF was employed as the coupling solvent throughout the syntheses in this investigation.

Kinetic analyses in the absence and presence of a tertiary amine such as DIEA revealed that DIEA has no appreciable influence on the coupling rate in DMF and significantly reduced the coupling rate in CH_2Cl_2 (Figure 2). These results imply that a tertiary amine is not required for the efficient formation of peptide bonds using Boc-NCAs. In contrast to the above results, the synthesis of a pentapeptide in the presence of 1 equiv of DIEA in DMF resulted in a crude peptide which was more homogeneous than that obtained in the absence of DIEA (Table I). One possible explanation for the difference between the kinetic studies and the actual peptide synthesis is that DIEA in the coupling step neutralizes some residual protonated α-amino groups on the resin which were not accessible during the neutralization step in CH_2Cl_2. For this reason, we routinely used 1 equiv of DIEA in synthetic strategies with UNCAs.

Kinetic studies also revealed that although in the difficult coupling of BocVal to Ile-Ala-PAM-resin the rate of UNCA coupling was slower than that of BOP and HBTU couplings up to 15 min using 1.2 equiv of BocValNCA (Figure 3), the rate of UNCA coupling was equal to that of the BOP and HBTU couplings when greater than 1.5 equiv of BocValNCA was used (Figure 3). It was observed also that in NMP and CH_2Cl_2, coupling goes to over 99.5% completion after 15 min when 4 equiv of UNCA was used in the coupling of BocVal or BocIle to Ala-PAM-resin (data not shown). However, as with BOP and HBTU couplings, the UNCA coupling did not go to completion (less than 99%) even after a 2-h reaction using 1.2 equiv of UNCA (Figure 3) or after 15 min using 3 equiv of UNCA (data not shown) in the coupling of two sterically hindered residues (Val to Ile). In order to assure that all coupling reactions went to completion, we utilized 4 equiv of UNCA and 1-h coupling as a standard procedure in the synthesis of the tetra-, penta-, undeca-, and pentadecapeptides. Using this procedure, complete coupling in each cycle was found by the Kaiser test[20] except for the Tyr[1], Ile[2], Ile[3], and Lys[4] residues in the synthesis of the 15-peptide. (It should be noted that the Tyr and Lys residues were introduced into the peptide chains using HBTU coupling in the UNCA synthesis of the 15-peptide.) The necessity for double couplings of these four residues in the synthesis of the 15-mer was also encountered using the HBTU procedure. It is probable that either peptide aggregation or other conformational effects are responsible for the sluggish coupling reactions at these chain lengths.[22]

In all of the above syntheses we observed that, as judged by the quality of the crude peptide and the yield of the purified product (Table I), UNCA coupling is equally efficient to BOP or HBTU activation. A similar conclusion is obtained concerning the stereochemical purity of dipeptides synthesized by these methods as judged by the % DL dipeptide found during synthesis of Val-Phe and Phe-Ala (approximately 0.2%). Phenylalanine is usually considered to be one of the amino acid residues which is highly prone to undergo racemization during activation and coupling. The low racemization found for Boc-Phe suggests that racemization will not be a problem associated with the UNCA strategy. The high quality of crude Leu-Asn-Phe-Ala and Leu-Gln-Phe-Ala indicates that the UNCA procedure is also compatible with Asn and Gln, residues which have required special attention during carbodiimide-mediated coupling. It should be noted, however, that the trityl group used for protection of the β- and γ-amides of BocAsnNCA and BocGlnNCA is slowly cleaved during removal of the Boc protecting group. This resulted in the production of a trityl carbocation but did not affect the quality of the resulting crude peptide.

In conclusion, our study shows that Boc-NCAs are efficient reagents for solid-phase synthesis and, as judged by direct tests of racemization and the quality of crude peptides, do not lead to significant losses in chirality at the α-carbon of amino acid residues. Since UNCAs are easy to handle and only generate carbon dioxide as a byproduct they can be readily applied to both laboratory-scale and large-scale synthesis of peptides. They offer an efficient and important alternative to existing methods for peptide bond formation.

Experimental Section

Materials and Methods. Boc-amino acid *N*-carboxyanhydrides were supplied by BioResearch, Inc. (San Diego, CA). The synthesis of Boc-NCAs has been described in a general manner

(21) Fields, G. B.; Fields, C. G. *J. Am. Chem. Soc.* **1991**, *113*, 4202.

(22) Pillai, V. N. R.; Mutter, M. *Acc. Chem. Res.* **1981**, *14*, 122.

in a publication by Fuller and co-workers and in more detail in a patent by this group.[16] An alternative procedure for the synthesis of Boc-NCAs from *N,N*-bis(alkoxycarbonyl)amino acids has also been reported recently.[23] *N*-BocAla-PAM-resin and *N*-Boc-amino acids were purchased from Bachem, Inc. (Torrance, CA). BOP and HBTU were from Richelieu Biotechnologies, Inc., and from AminoTech, Inc., respectively. All solvents used were of HPLC grade and were from Fisher Scientific, and all other reagents used were of reagent grade and from Aldrich Co.

Reversed-phase HPLC was performed on a Waters system. Preparative HPLC was run on a Waters μ-Bondapak-C_{18} column (19 × 150 mm). Detection was at 220 or 279 nm. Analytical HPLC was run on a Waters μ-Bondapak-C_{18} column (3.9 × 300 mm). Detection was at 220 nm. The relative percentages of L-Val-L-Phe and D-Val-L-Phe and of the corresponding Phe-Ala diastereomers were determined using HPLC processing software IEEE-488 version 2.04 from Autochrome Incorporated. Under the conditions used any peak with an area greater than 0.05% of the major peak was integrated. Amino acid analysis was carried out at the Analytical Services Laboratory at the University of Tennessee or at the Wistar Institute at Philadelphia, PA. FAB-MS was carried out at the University of Tennessee on a VG ZAB EQ mass spectrometer equipped with a flow FAB ion source of an Ion Tech fast atom gun. UV absorbance was measured on a Varian DMS 300 UV–vis spectrometer.

Kinetic Studies. The Boc group of BocAla-PAM-resin or BocIle-Ala-PAM-resin was removed with 50% TFA in CH_2Cl_2 for 30 min, and the resin was washed with CH_2Cl_2, neutralized with 10% DIEA in CH_2Cl_2, washed with CH_2Cl_2, methanol, and CH_2Cl_2, and dried overnight. Samples of 30 or 50 mg of the appropriate resin were placed in disposable microcentrifuge tubes. Solutions of BocValNCA or BocIleNCA in DMF, NMP, or CH_2Cl_2, BocVal or BocIle and BOP in DMF, and BocVal and HBTU in DMF were each freshly prepared at a concentration to provide a final volume of 0.12 mL/mg of resin in each microcentrifuge tube. The solutions were immediately pipetted into the tubes, and DIEA was added where applicable. The tubes were shaken rapidly and at 5, 10, 15, 30, 60, 90, and 120 min, the solution from one tube was poured into a fritted funnel, and the resin was washed with methanol, CH_2Cl_2, and methanol and dried. Two samples of 5 mg of resin were weighed into two test tubes, and ninhydrin assays were performed using the procedures described by Sarin et al.[24] The residual amine content was calculated using an extinction coefficient ϵ' of 1.5×10^4 M^{-1} cm^{-1}. Coupling yield at each time point was taken from the average of two separate assays.

Racemization Studies. Val-Phe and D-Val-Phe. BocPhe-PAM-resin (0.5 g, 0.18 mmol) was treated with 50% TFA/CH_2Cl_2 for 30 min, and the resin was washed with CH_2Cl_2, neutralized with 10% DIEA in CH_2Cl_2 for 10 min, and washed with CH_2Cl_2 and DMF. BocValNCA (175 mg, 0.72 mmol) and DIEA (0.18 mmol, 31 μL) or Boc-D-Val (156 mg, 0.72 mmol), HBTU (273 mg, 0.72 mmol), and DIEA (188 μL, 1.08 mmol) in 6 mL of DMF were added to the resin. After shaking for 2 h, the resin was washed with DMF and CH_2Cl_2, treated with 50% TFA/CH_2Cl_2, washed with CH_2Cl_2, MeOH, and CH_2Cl_2, and dried overnight. The dipeptide was cleaved from the resin using 5 mL of HF at 0 °C for 1 h. After evaporation of HF, the resin was washed with precooled ether and the peptide was extracted using water–acetonitrile. The relative percentages of D,L and L,L isomers were directly analyzed on a C_{18} column using a linear gradient of water (0.025% TFA) and acetonitrile (0.025% TFA) with water from 90% to 40% over 25 min.

Phe-Ala and D-Phe-Ala. The Boc group of BocAla-PAM-resin (0.5 g, 0.3 mmol) was deprotected with 50% TFA/CH_2Cl_2, and the resin was washed with CH_2Cl_2, neutralized with 10% DIEA in CH_2Cl_2 for 10 min, and washed with CH_2Cl_2 and DMF. BocPheNCA (349 mg, 1.2 mmol) and DIEA (52 μL, 0.3 mmol) or Boc-D-Phe (318 mg, 1.2 mmol), BOP (530 mg, 1.2 mmol), and DIEA (313 μL, 1.8 mmol) in 6 mL of DMF were added to the resin. After shaking for 2 h, the resin was washed with DMF and CH_2Cl_2, treated with 50% TFA/CH_2Cl_2, washed with CH_2Cl_2, MeOH, and CH_2Cl_2, and dried overnight. The dipeptide was cleaved from the resin using 5 mL of HF at 0 °C for 1 h. After evaporation of HF, the resin was washed with precooled ether, and the peptide was extracted with water–acetonitrile. The crude product was directly analyzed on a C_{18} column using a linear gradient of water (0.025% TFA) and acetonitrile (0.025% TFA) with water from 95% to 45% over 25 min.

Solid-Phase Synthesis. The solid-phase syntheses of Ala-Leu-Val-Ile-Ala, Gly-Val-Phe-Trp-Asp-Pro-Ala-Leu-Val-Ile-Ala, and Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala-Cys-Val-Ile-Ala were carried out manually starting with BocAla-PAM-resin (0.5 mmol/g of resin). The Boc group was used for all N-α-protections. The side-chain protecting groups were Asp(Bzl), Trp(For), Cys(Mob), Lys(ClZ), and Tyr(BrZ). The following protocol was used for the introduction of each residue into a peptide chain (10 mL of solvent/g of resin for coupling and 15–20 mL of solvent/g of resin for other procedures): (1) CH_2Cl_2, 3 × 1 min; (2) 45% TFA, 2% DMS in CH_2Cl_2, 1 × 1 min; (3) 45% TFA, 2% DMS in CH_2Cl_2, 1 × 30 min; (4) CH_2Cl_2, 5 × 1 min; (5) 10% DIEA in CH_2Cl_2, 1 × 2 min; (6) 10% DIEA in CH_2Cl_2, 1 × 5 min; (7) CH_2Cl_2, 2 × 1 min; (8) methanol, 1 × 1 min; (9) DMF, 2 × 1 min; (10) for UNCA coupling, Boc-amino acid *N*-carboxyanhydride (4 equiv) in DMF was added to the vessel followed by DIEA (1 equiv) where applicable. For BOP coupling, Boc-amino acid (4 equiv) and BOP (4 equiv) in DMF were added to the vessel followed by DIEA (8 equiv). For HBTU coupling, Boc-amino acid (4 equiv) and HBTU (4 equiv) in DMF were added to the vessel followed by DIEA (6 equiv). Coupling time was 1 h; (11) DMF, 3 × 1 min. All couplings were found complete by the Kaiser test after step 1 except for residues Tyr^1, Ile^2, Ile^3, and Lys^4 in the synthesis of the pentadecapeptide using both UNCA and HBTU couplings. For these residues, a second coupling was applied by repeating steps 9 and 10. In step 10, one-fourth of the amount of all reagents was used. The synthesis of each peptide reported in this paper was carried out one time. The data reported in Table I reflect one independent synthesis for each method evaluated.

After peptide chain assembly was completed, the amine terminal Boc group was deprotected with 45% TFA in CH_2Cl_2 containing 2% DMS, and the resin was washed with CH_2Cl_2, methanol, and CH_2Cl_2 and dried overnight. One g of resin was mixed with 1 mL of anisole in a Kel-F HF reaction vessel, and HF was condensed at −78 °C under reduced pressure to a total volume of 12 mL (HF + anisole + resin). Cleavage reaction proceeded at 0–2 °C for 1.2 h. After evaporation of HF, the residue was washed with ether, the peptide was extracted with 25% aqueous acetic acid, and the extract was lyophilized.

Purification and Characterization. Leu-Asn-Phe-Ala. The crude tetrapeptide was dissolved in water–acetonitrile and purified by semipreparative reversed-phase HPLC using a linear gradient of water (0.025% TFA) and acetonitrile (0.025% TFA) with water from 100% to 40% over 60 min to give over 99% homogeneous Leu-Asn-Phe-Ala in 74% yield: FAB-MS calcd 464, found 464; amino acid analysis Ala 1.03 (1), Glx 1.00 (1), Leu 0.95 (1), Phe 1.01 (1).

Leu-Gln-Phe-Ala. The crude product from lyophilization was purified using the same procedure as for Leu-Asn-Phe-Ala. Pure peptide was isolated in 77% yield: FAB-MS calcd 478, found 478; amino acid analysis Ala 1.07(1), Asx 0.99 (1), Leu 0.97 (1), Phe 0.97 (1).

Ala-Leu-Val-Ile-Ala. The crude pentapeptide from the UNCA synthesis in the absence or presence of DIEA or the Bop synthesis was dissolved in acetic acid–water and purified by semipreparative reversed-phase HPLC using the same gradient as for Leu-Asn-Phe-Ala. The purified pentapeptide from either purification was over 99% homogeneous and coinjection of the products from three syntheses gave one peak: FAB-MS calcd 486, found 486; amino acid analysis Ala 2.15 (2), Val 0.68 (1), Ile 0.79 (1), Leu 1.00 (1).

Gly-Val-Phe-Trp-Asp-Pro-Ala-Leu-Val-Ile-Ala. The crude undecapeptide from lyophilization was treated with 30% piperidine in water–acetonitrile (1:1) for 2 h to remove the formyl group on tryptophan. The solution was concentrated under reduced pressure, and the residue was taken up in acetic acid–water and purified by semipreparative HPLC using a linear

(23) Savrda, J.; Wakselman, M. *J. Chem. Soc., Chem. Commun.* **1992**, 812.

(24) Sarin, V. K.; Kent, S. H. B.; Tam, J. B.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147.

gradient of water (0.025% TFA) and acetonitrile (0.025% TFA) with acetonitrile from 0 to 60% over 60 min. Purified products from the UNCA synthesis and the BOP synthesis comigrated on an analytical C_{18} column: FAB-MS calcd 1187, found 1188; amino acid analysis Asp 1.00 (1), Gly 1.00 (1), Ala 2.11 (2), Pro 1.10 (1), Val 1.35(2), Ile 0.71 (1), Leu 0.97(1), Phe 0.75(1).

Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala-Cys-Val-Ile-Ala. The crude pentadecapeptide from the UNCA synthesis or the HBTU synthesis was treated with 30% piperidine in water–acetonitrile (1:1) for 2 h to remove the formyl group on tryptophan. Under this condition, the sulfhydryl group on cysteine was found by HPLC to be easily oxidized, and 10% by weight of dithioerythritol relative to the peptide was added to reduce the disulfide formed. The solution was concentrated under reduced pressure, and the residue was taken up in acetic acid–water and purified by semipreparative HPLC using a linear gradient of water (0.025% TFA) and acetonitrile (0.025% TFA) with acetonitrile from 0–70% over 60 min. Coinjection of the purified products from the UNCA synthesis and the HBTU synthesis resulted in one peak: FABMS calcd 1695, found 1695; amino acid analysis Asp 1.09 (1), Gly 1.12 (1), Ala 2.03 (2), Pro 1.10 (1), Tyr 1.07 (1), Val 1.94 (2), Ile 2.65 (3), Phe 0.97 (1), Lys 0.99 (1).

Acknowledgment. This work was supported by grants GM-22086 and GM-46520 from the National Institutes of Health. The authors are indebted to Dr. William D. Fuller and his colleagues at BioResearch, Inc., San Diego, CA, who provided all Boc-amino acid NCAs as a gift. We thank Kelsey Cook and Al Tuinman of the University of Tennessee for running FAB mass spectrometry. The UTK Chemistry Mass Spectrometer Center is funded by the Science Alliance, a State of Tennessee Center of Excellence. The NSF Chemical Instrumentation Program also contributed to the acquisition of the ZAB-EQ (Grant no. CHE-86-09251). We are grateful to Dr. Shabbir Khan at Wistar Institute, Philadelphia, PA, for his help in the amino acid analysis.

Abbreviations: Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; HBTU, *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPLC, high-pressure liquid chromatography; NCA, *N*-carboxyanhydride; PyBroP, bromotrispyrrolidinophosphonium hexafluorophosphate; TFA, trifluoroacetic acid; UNCA, urethane-protected amino acid *N*-carboxyanhydride.

Supplementary Material Available: HPLC profiles for the peptides synthesized (4 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.