Methodological Implications of Simultaneous Solid-Phase Peptide Synthesis. 1. Comparison of Different Coupling Procedures

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A new method of simultaneous solid-phase peptide synthesis has been developed and used, in combination with a competition method, to assess the relative efficiencies of a wide range of coupling reagents. Results from these studies have provided insight into the mechanism of action of carbodiimide and (benzotriazolyloxy)tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) reagents. Use of BOP in the presence of HOBt was found to provide an efficient coupling method of great use in Fmoc-mediated syntheses. This variation and a number of other methods are more effective than the use of preformed symmetrical anhydrides. Conclusions have been confirmed by syntheses of the acyl carrier protein 65-74 sequence.

Introduction

Many procedures have been recommended for the formation of amide bonds during the solid-phase assembly of peptides. This paper proposes a combination of methods that allow valid comparisons to be made between both the efficiency and the effectiveness of different coupling procedures. Subsequent papers in this series will compare different support media, support linkages, side-chain protection, and N-protection methods.

Simultaneous solid-phase peptide synthesis methods[1-3] are mostly used for the preparation of peptides for immunological applications and in the development of structure-activity relationships. Their use for comparing different synthetic procedures has received little attention. Simultaneous synthesis allows exact and significant comparisons to be made between a large number of procedures all performed under identical and carefully controlled conditions. The simultaneous syntheses and comparison experiments were performed by using polypropylene column reactors of approximately 1-mL internal volume equipped with sealable end fittings containing disks of polypropylene mesh to retain the support material. All reactions occur under conditions identical with those pertaining in the usual larger scale batch reactors; and reaction rates are not influenced by diffusion through porous membranes or other physical phenomena which are limiting factors in other methods.[2,3] Groups of columns may be filled individually for all steps or linked in series or in parallel to facilitate implementation of common treatments.

A competition method has been developed which provides a simple means of assessing the relative efficiencies of different coupling procedures under the excess and concentration conditions employed in actual syntheses. Originally competition experiments were used to determine the relative reactivities of different amino acids in solid-phase peptide synthesis,[4,5] and subsequently they were used to assess many factors influencing a polyethylene glycol based liquid-phase synthesis method.[6,7] Recently the same idea has found application in the determination of the relative rates of coupling of *tert*-butyloxycarbonyl (Boc), [(fluorenylmethyl)oxy]carbonyl (Fmoc), and dithiasuccinoyl (Dts) amino acids.[8] In the procedure adopted herein, Boc-Tyr(Bzl)-OH and Boc-Phe-OH, in equimolar amounts, were used as the components in the competition experiments and, after activation, were mixed and reacted with a Leu resin. The ratio of Tyr to Phe incorporated was then determined after resin hydrolysis. When the same reagent was used for activation of both Tyr and Phe components, the ratio of incorporation for these was 30:70. When one of the activation reagents was altered, then a variation in the incorporation ratio reflected the differing efficiency of activation of the new reagent. Mixing and addition to the support were performed rapidly (typically within 20 s) to diminish the danger of exchange of active species. In any event, exchange would result in a diminution of the observed differences in incorporations. Symmetrical anhydrides (preformed by literature methods[9-15] (PFSAs) were selected to act as reference substances in later parts of this study because they were considered to be unlikely to undergo exchange processes with the reagents and conditions used.

Results and Discussion

Initial experiments (Table I) were designed to illuminate the relative efficiency for the formation of symmetrical anhydrides provided by either diisopropylcarbodiimide (DIPCDI) or dicyclohexylcarbodiimide (DCCI) reagents under a variety of conditions. The incorporation figures showed (Table I) that when the same reagent was used for each component (1A and 1B), then the Tyr:Phe ratio was determined to be very close to 30:70. Similar ratios were obtained with use of DIPCDI and DCCI for individual components of the competition mixture (1C and 1D). Experiments 1E-1K were designed to determine the effect of dimethylformamide (DMF) on the completeness of anhydride formation in dichloromethane (DCM) solution (Table I, sol SA represents formation in solution of the symmetrical anhydride using one-half of an equivalent of

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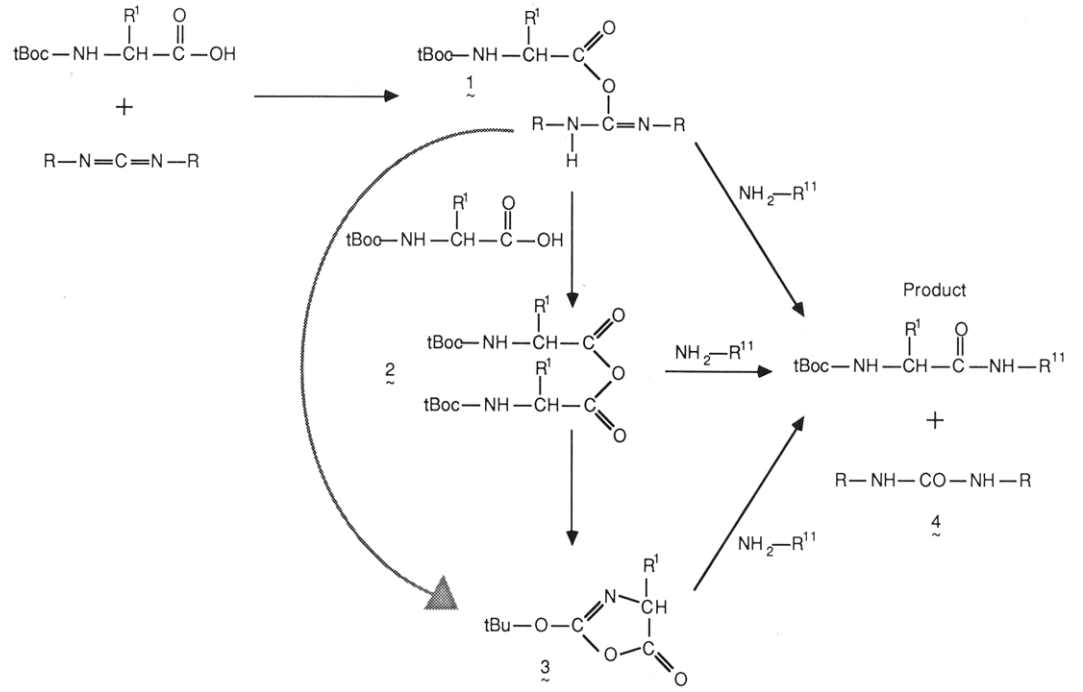

Figure 1. Mechanism of carbodiimide-mediated coupling reactions.

Table I. Comparison of Procedures for Symmetrical Anhydride Formation

	Tyr			Phe				
Ref. No.	Method	Reagent	Activation Conditions	Method	Reagent	Activation Conditions DCM/DMF	Reaction Solvent (DCM/DMF)	% Tyr Inc.
1A	PFSA	DIPCDI	DCM 15 min	PFSA	DIPCDI	DCM 15 min	1:1	29.1
1B	PFSA	DCCI	DCM 15 min	PFSA	DCCI	DCM 15 min	1:1	29.5
1C	PFSA	DCCI	DCM 15 min	PFSA	DIPCDI	DCM 15 min	1:1	29.7
1D	PFSA	DIPCDI	DCM 15 min	PFSA	DCCI	DCM 15 min	1:1	30.5
1E	PFSA	DIPCDI	DCM 15 min	sol SA	DIPCDI	3:1 DCM/DMF 10 min	2:1	30.8
1F	sol SA	DIPCDI	3:1 DCM/DMF 10 min	PFSA	DIPCDI	DCM 15 min	2:1	29.2
1G	PFSA	DCCI	DCM 15 min	sol SA	DIPCDI	3:1 DCM/DMF 10 min	2:1	33.3
1H	sol SA	DIPCDI	3:1 DCM/DMF 10 min	PFSA	DCCI	DCM 15 min	2:1	29.1
1J	PFSA	DIPCDI	DCM 15 min	sol SA	DIPCDI	1:1 DCM/DMF	1:1	37.7
1K	sol SA	DIPCDI	1:1 DCM/DMF 10 min	PFSA	DIPCDI	DCM 15min	1:1	22.7

carbodiimide). The incorporation ratios determined in experiments 1E–1H indicated completeness of symmetrical anhydride formation after 10 min in 3:1 DCM/DMF. Experiments 1J and 1K, performed in 1:1 DCM/DMF, showed that the presence of a greater proportion of DMF led to incomplete symmetrical anhydride formation (as judged by diminished incorporations relative to those obtained with the preformed standards). In summary, these initial experiments showed that DIPCDI and DCCI were equally effective in the formation of symmetrical anhydrides and that isolation of the anhydrides was unnecessary when the proportion of DMF present in the activation solvent mixture did not exceed 25%. Pathways of carbodiimide couplings[16,17] are represented in Figure 1; coupling may be mediated by *O*-acylisourea (OAIU) **1**, symmetrical anhydride **2**, or 2-alkoxy-4-alkyl-5(4*H*)-oxazolone **3** intermediates. DMF present in greater than 25% may

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Table II. The Relative Efficiency of Direct DIPCDI Activation Compared to the Use of Symmetrical Anhydrides

	Tyr			+ Phe				
Ref. No.	Method	Equivs. DIPCDI	Preactivation Conditions	Method	Equivs. DIPCDI	Preactivation Conditions	Reaction Solvent (DCM/DMF)	%Tyr Inc.
2A	PFSA	0.5	Standard	PFSA	0.5	Standard	1:1	29.5
2B	Direct	1	2 min 1:1 DCM/DMF	Direct	1	2 min 1:1 DCM/DMF	1:1	29.9
2C	PFSA	0.5	Standard	Direct	1	1 min 1:1 DCM/DMF	1:1	40.0
2D	Direct	1	1 min 1:1 DCM/DMF	PFSA	0.5	Standard	1:1	16.5
2E	PFSA	0.5	Standard	Direct	1	2 min 1:1 DCM/DMF	1:1	26.3
2F	Direct	1	2 min 1:1 DCM/DMF	PFSA	1	Standard	1:1	40.0

exert a solvent effect on the rate of formation of **1** and/or on conversion of **1** to **2**; it may also accelerate decomposition of **1** to an inactive *N*-acylurea. Nevertheless, the presence of a high proportion (≥50%) of a polar solvent, such as DMF, is desirable in coupling to decrease inter- and intrachain hydrogen bonding.

The next series of experiments (Table II), in conjunction with the previous studies, was designed to determine the most efficient procedure with which to perform the direct carbodiimide method and then to compare the efficiency of this procedure with that obtained by using preformed or solution-generated symmetrical anhydrides. Comparisons were made to the control values obtained with the mixed symmetrical anhydrides (Table II, 2A) and with both components activated by the direct DIPCDI procedure (2B). Experiments 2C and 2D showed diminished coupling efficiency when 1 equiv of DIPCDI was mixed with the appropriate amino acid in 1:1 DMF/DCM, **left to preactivate for just 1 min**, then mixed with the corresponding PFSA, and coupled. Conversely, when this preactivation process was performed **for 2 min** (2E and 2F), **then more efficient coupling was achieved than that that would have been obtained had the same amount of amino acid been converted into a symmetrical anhydride**. These observations are in direct contradiction with the widely held belief that the use of preformed symmetrical anhydrides provides an order of magnitude increased activity and efficiency over that obtained with the direct carbodiimide method.[18] Since the literature studies have mainly made comparison to the use of DCCI in neat DCM, the improvement reported by the use of preformed symmetrical anhydrides may simply have resulted from elimination or reduction of the pore-blocking precipitation of dicyclohexylurea **4**, whose presence diminishes coupling rates by a physical process, rather than because of any intrinsic lack of reactivity provided by direct use of the carbodiimide reagent. Under the conditions reported above, involving 2-min preactivation with 1 equiv of DIPCDI in 1:1 DCM/DMF, no precipitation is observed even for reaction times longer than 2 h.

It has also been believed, irrespective of the stoichiometry employed, that the direct carbodiimide method is mediated by symmetrical anhydrides.[16] This is demonstrably true in deuteriochloroform[19] and is probably true in neat DCM, but, when the coupling is performed as described, the data presented contradict this interpretation. Experiments 2E and 2F could not have resulted in more efficient coupling than would have been obtained with the preformed symmetrical anhydrides if a significant proportion of the reactive intermediate(s) present was not other than the symmetrical anhydride. Results 1J and 1K showed, from the inefficient coupling obtained, that symmetrical anhydride formation was incomplete after 10 min in 1:1 DCM/DMF. Clearly, after 2 min in that same solvent cocktail, only a small proportion of symmetrical anhydride can have been formed with 1 equiv of DIPCDI, and the majority of the coupling must have been mediated by the initially formed adduct, the *O*-acylisourea **1**, either alone or in conjunction with the 2-alkoxyoxazolone generated from it[17] (Figure 1). The OAIU intermediate has not been unambiguously identified[19] although apparently erroneous NMR evidence of its existence has been published.[20] Despite this uncertainty, the method is designated in subsequent discussions by the abbreviation OAIU.

To compare the effectiveness of OAIU and PFSA procedures in actual peptide synthesis, the simultaneous macrocolumn meethod was next employed to prepare the acyl carrier protein (ACP) sequence 65–74. This test peptide has been accepted as a standard since its synthesis requires a number of sterically hindered couplings and these are combined with segments that promote interchain aggregation with a corresponding dramatic reduction in amino group accessibility.[21] Racemization during coupling of the two isoleucine residues is simply detected. The syntheses, using the Fmoc-protection strategy, were performed with single brief couplings either in 1:1 DCM/DMF (OAIU method) or in neat DMF (PFSA method). The reverse phase HPLC chromatograms of the two crude products obtained after cleavage are depicted in Figures 2a and 2b. Both syntheses were closely similar in regard to their yields and in the purity of product obtained. Between the two procedures, the OAIU method was unquestionably far simpler to perform, since brief mixing of solutions was all that was required for activation. The PFSA procedure required lengthy preactivation, filtration, evaporation, redissolution, and addition processes. The

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Table III. The Effect of Additives on the Efficiency of the Direct DIPCDI Procedure

	Tyr		+ Phe		
Ref. No	Method	Conditions	Method	Conditions	%Tyr Inc.
3A	PFSA	Standard	PFSA	Standard	29.1
3B	DIPCDI + HO-Dhobt, 2 + 10 min		PFSA	Standard	38.1
3C	PFSA	Standard	DIPCDI + HO-Dhobt, 2 + 10 min		24.0
3D	DIPCDI + HO-Dhobt, 2 + 10 min		DIPCDI + HO-Dhobt, 2 + 10 min		33.0
3E	DIPCDI + HOBt, 2 + 10 min		PFSA	Standard	38.8
3F	DIPCDI + tetrazole, 2 + 10 min		PFSA	Standard	7.6
3G	DIPCDI + benzotriazole, 2 + 10 min		PFSA	Standard	8.2
3H	DIPCDI + HO-PFP, 2 + 10 min		PFSA	Standard	24.2

merits and application of the OAIU method in Boc-mediated syntheses of cecropin analogues have been discussed elsewhere.[22]

Table III shows results obtained when the OAIU method was performed in the presence of a variety of additives (see methods section). With both 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine[23,24] (HO-Dhobt, 3B and 3C) and 1-hydroxybenzotriazole (HOBt, 3E), efficiencies of coupling were 1.33 times that observed with the symmetrical anhydrides. It was found, however, that HOBt gave cleaner products when used for actual peptide synthesis, and HOBt was judged to be superior. The HPLC chromatogram of the product from DIPCDI/HOBt-mediated synthesis of the ACP 65–74 sequence is reproduced in Figure 2c. Activation was implemented by simply mixing the stable stored DMF solutions of the Fmoc-amino acids dissolved in the presence of HOBt with an equal volume of an identically concentrated solution of DIPCDI in DCM. Fmoc-amino acids are far more soluble in DMF than they are in DCM, and their solutions are stable (in the absence of amines) for at least 7 days. These facts further emphasize the advantage of applying the DIPCDI/HOBt method (or the OAIU method) with this protection strategy. Other additives (Table III) suppressed efficiency. The addition of 1*H*-tetrazole[25] or 1,2,3-benzotriazole (3F and 3G) caused a dramatic reduction. In situ formation of pentafluorophenyl active esters by the method resulted in a slightly lower coupling efficiency (3H, pentafluorophenol is abbreviated as HO-PFP).

Competition experiments were next used to assess, relative to symmetrical anhydrides, the coupling efficiencies of some of the numerous reagents available (Table IV). Also included, for comparison purposes, are summarized results from Tables I–III. Except with these, the coupling reagents were used in neat DMF in the presence of, as appropriate, of 1 or 2 equiv of *N*-methylmorpholine. Ten-minute preactivation at room temperature was allowed.

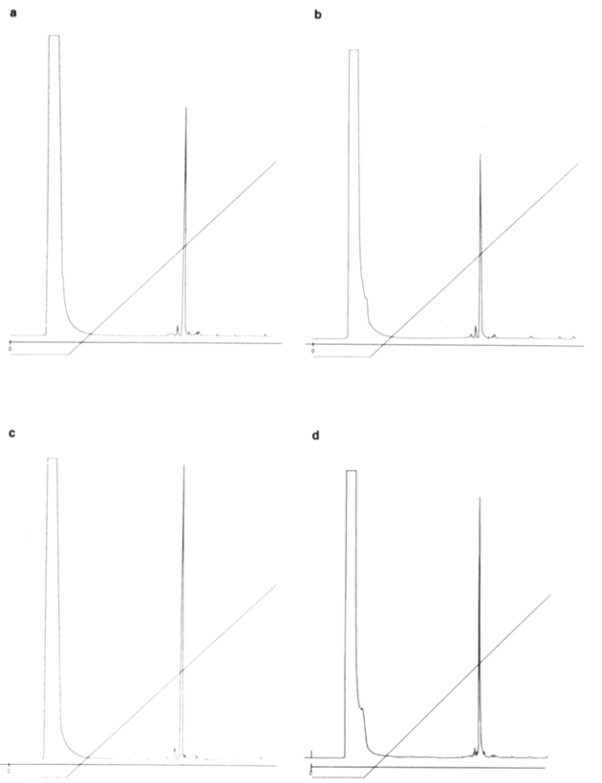

Figure 2. HPLC chromatograms from syntheses of the acyl carrier protein 65–74 sequence H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-OH. (Note: The offscale peak on all four chromatograms occurring 2–3 min after injection is caused by DMF and acetic acid added to solubilize the samples.) Runs 2a and 2b were performed by the simultaneous synthesis method either with coupling performed with preformed symmetrical anhydrides in DMF (2a) or by direct diisopropylcarbodiimide coupling in 1:1 DCM/DMF (2b). Runs 2c and 2d were performed by automated synthesis either using the DIPCDI/HOBt additive procedure (2c) or using BOP reagent (2d).

These conditions differ significantly in most cases from those advocated by originators of the methods. As previously noted, the use of polar solvents is helpful to reduce hydrogen bonding and peptide chain aggregation.[21] Although the DBTO reagent (Table IV, 1) gave the highest

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Table IV. Relative Coupling Efficiency of Activation Methods for Solid-Phase Peptide Synthesis

		Relative Efficiency	Reference
1.	Dibenzotriazolyl oxalate, DBTO	1.8	27
2.	BOP + HOBt	1.6	-
3.	OAIU method	1.4	22
4.	BOP + 10 minute activation	1.3	-
5.	DIPCDI + HOBt	1.3	23, 24
6.	DIPCDI + HO-Dhobt	1.3	23, 24
7.	BOP (0.5 equivs., act. 2 min)	1.0	-
8.	BOP (1.0 equivs., act. 2 min)	1.0	-
9.	Preformed Symmetrical Anhydride	1.0	9
10.	Symmetrical Anhydrides formed by DIPCDI in 3:1 DCM/DMF	1.0	-
11.	DIPCDI + HO-PFP	0.9	-
12.	Bop-Cl	0.8	28
13.	IIDQ	0.34	31
14.	DIPCDI + 1-H tetrazole	0.27	25
15.	DIPCDI + 1, 2, 3-benzotriazole	0.27	-
16.	Bates Reagent + HOBt	0.24	32
17.	EEDQ	0.23	33, 34
18.	Diphenyl phosphoryl azide, DPPA	0.16	35, 36
19.	Bates Reagent	<0.05	32
20.	Woodwards Reagent K	<0.05	37
21.	Woodwards Reagent L	<0.05	38, 39

efficiency, its use in synthesis of the ACP decapeptide sequence gave significantly less pure product than obtained with other methods; the analyses indicated approximately 5% chain termination at each coupling step. Under the conditions used, 1-(isobutoxycarbonyl)-2-isobutoxy-1,2-dihydroquinoline (IIDQ), the related 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ), (μ-oxo)bis[tris(dimethylamino)phosphonium] bis(tetrafluoroborate) (Bates reagent), diphenylphosphoryl azide (DPPA), 2-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K), and 2-*tert*-butyl-5-methylisoxazolium perchlorate proved to provide unsatisfactory coupling efficiency (Table IV, 13, 17, 16 and 19, 18, 20 and 21 respectively).

Recently the usefulness of bis(2-oxo-3-oxazolidinyl)-phosphinic chloride reagent (Bop-Cl)[28] has been reexamined,[29,30] relatively inefficient coupling being observed in DMF. Results from this study (Table IV, 12) confirm these findings. The similarity of the Tyr incorporation ratios obtained with Bop-Cl to those obtained with the PFSAs may be an indication that symmetrical anhydride intermediates were involved. This reasoning applies to the efficiency of coupling resulting from use of the (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophsphate (BOP) reagent of Castro[26] under certain conditions (Table IV, 7 and 8), but not others (Table IV, 2 and 4). Time-course studies (Figure 3) showed that when one-half of an equivalent of BOP reagent was used for activation, then the incorporation ratio rapidly attained a value closely similar to that found for the PFSA standards; this figure then remained essentially unchanged. With one full equivalent of BOP, a slightly more rapid initial activation was observed, followed by a linear further increase over the 10-min time span studied. When a mixture of BOP and HOBt was used, this resulted in a dramatic improvement in rate and magnitude of activation.

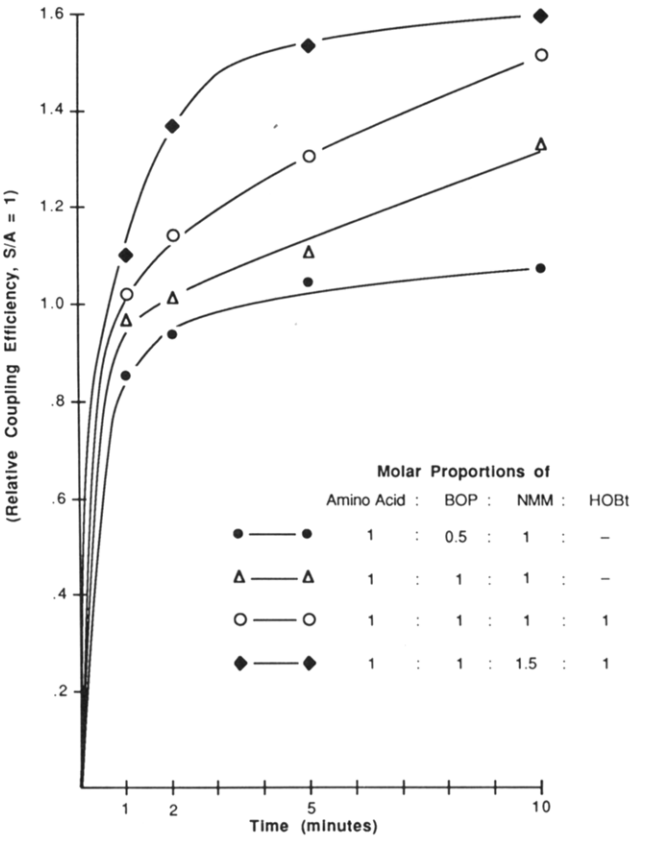

Figure 3. Time course of activation with BOP and BOP + HOBt reagents (NMM stands for *N*-methylmorpholine).

These results are in accord with the speculative mechanism displayed in Figure 4. With 0.5 or 1 equiv of BOP, the initially formed highly reactive (acyloxy)phosphonium salt intermediate **5** reacts rapidly with amino acid carboxylate to form the symmetrical anhydride **2′**. The second-phase activation, seen with 1 equiv, is presumed to result from a relatively slow reaction of the liberated ionized hydroxybenzotriazole with **2′**. Regenerated starting material is recycled, resulting in eventual conversion to the more effective benzotriazolyl active ester **6**. If HOBt is added initially, this competes with carboxylate in attack on the (acyloxy)phosphonium intermediate **5**, forming a mixture of **2′** and **6**. When sufficient NMM is added to ionize 50% of the added HOBt, then the direct pathway to the active ester predominates. This explanation is supported by the previous findings (Table III) that HOBt esters provide more efficient coupling than do symmetrical anhydrides.

Thus, in its simplest form, the use of BOP reagent offers a method for the rapid and convenient formation, without precipitation or solvent exchange, of symmetrical anhydrides in DMF. The method, in all variations, is ideally suited for Fmoc-mediated syntheses where the intransigent solubilities of several of the derivatives is a major problem for other coupling methods. All commonly used Fmoc

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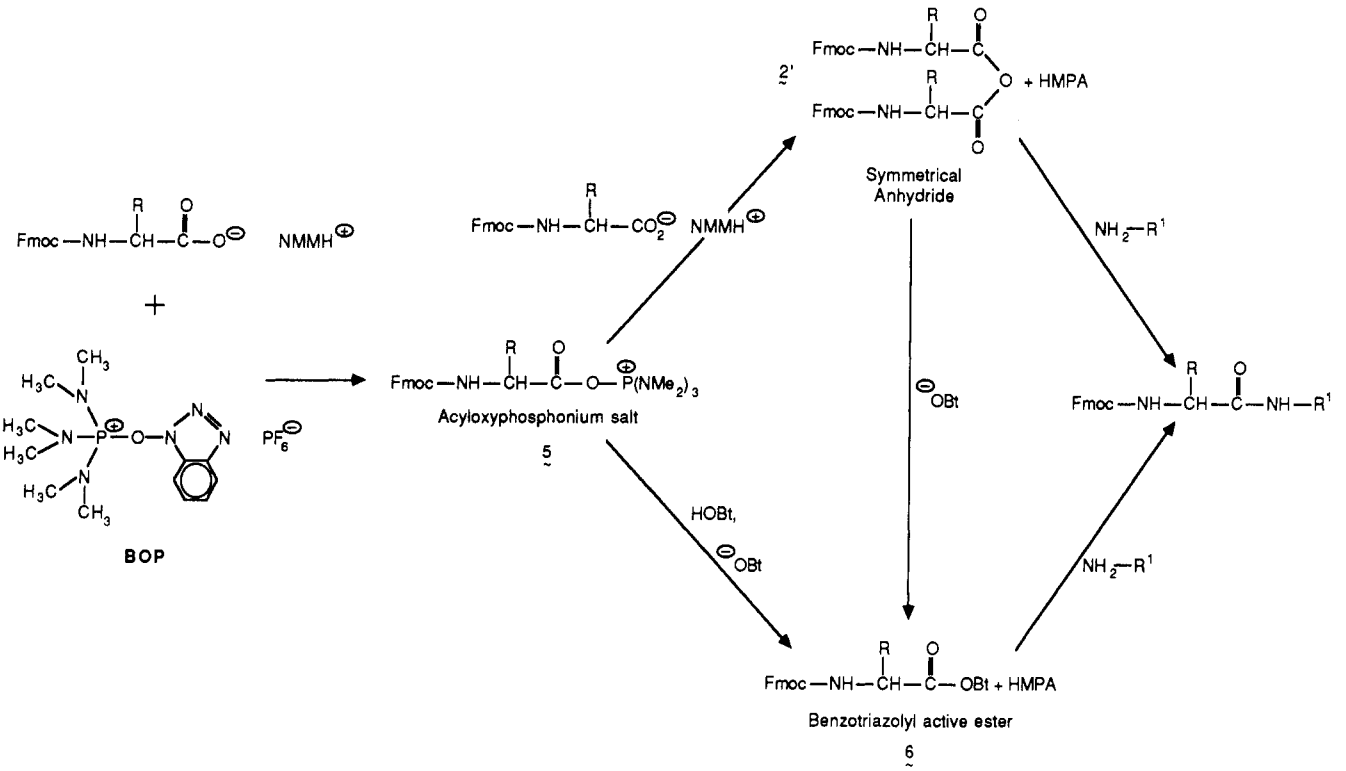

Figure 4. Mechanism of action of BOP and BOP + HOBt reagents.

derivatives are rapidly and completely solubilized in DMF as their *N*-methylmorpholinium salts. Solid stable mixtures of BOP combined with the Fmoc-amino acids (optionally with added HOBt) may simply be dissolved and activated by the addition of 0.2 or 0.3 M NMM in DMF. The HPLC chromatogram of the crude product from synthesis of ACP 65–74 (Figure 2d) illustrates the usual purity of product obtained with use of BOP reagent. This synthesis was performed with 1 equiv of BOP and with 10-min preactivation before coupling. Inclusion of 1 equiv of HOBt represents the optimal variation of this method (Table IV, 2 and Figure 4). It has been used to synthesize the ACP 65–74 sequence in high purity and has subsequently been used in correspondingly excellent syntheses of a variety of peptides, including substance P and human gastrin I.

The practical value of the recommended methods (OAIU, DIPCDI + HOBt, BOP + HOBt) is further enhanced by the lack of racemization that results from their use. This is evidenced by the absence of D-alloisoleucine in the amino acid analyses of the decapeptide products.[40] This represents, under actual solid-phase conditions, a simple yet rigorous test since both isoleucine residues are involved in slow couplings, which might be expected to be particularly prone to racemization. Additionally, numerous peptides have been synthesized by the OAIU method and have been shown to be digested completely by aminopeptidase M.[41] Reduction of racemization by addition of HOBt to carbodiimide couplings is well documented.[23-24,42-46] Absence of racemization with BOP has been reported[47] in the standard Anderson test[48] and in the fragment condensation of peptides with C-terminal Phe (expected to be particularly prone to racemization). These findings coupled with our results indicate that the use of BOP in the presence of HOBt not only provides highly efficient coupling in solid-phase synthesis but also preserves optical integrity.

Conclusions

The combination of simultaneous syntheses and competition experiments has allowed comparison of various methodologies for solid-phase synthesis, has given insight into the mechanism of two distinctly different classes of coupling reagent, and has defined several efficient procedures for solid-phase synthesis. It is expected that studies of this type should become a standard in methodological studies. It will no longer be sufficient for new approaches to be different from established procedures; they will need to be compared and demonstrated to be at the very least fully equivalent to established procedures in regard to the efficiency of activation determined by competition experiments, and in the yield and purity of products obtained

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by simultaneous synthesis methods.

Experimental Section

Chemicals and Equipment. Biosearch Macroscale DNA synthesis columns (Macrocolumns, 8600C-0227; an assembly drawing is available as supplementary material) were used for simultaneous syntheses packed with 50–100 mg of support; in the competition experiments this was 50 mg of a standard Merrifield Leu polystyrene resin (Biosearch, AA7576, 0.3 mmol/g) in a free amino form. Columns were filled and emptied by using disposable polypropylene syringes (Aldrich, Milwaukee, WI, Cat. No. Z11,686-6, 5 mL). Freshly opened bottles of dimethylformamide (Merck Omnisolve, EM Sciences, assay >99.9%, water <0.02%, dimethylamine <0.01%) were used for preparation of amino acid and reagent solutions. Boc- and Fmoc-amino acids were from Biosearch; other solvents were of HPLC grade. Biosearch supplied 1-hydroxybenzotriazole (HOBt, AA7600) and BOP reagent (AA7650); Fluka supplied IIDQ (58625), dibenzotriazolyl oxalate (DBTO, 75710), EEDQ (02541), Bates reagent (11855), Woodward's reagent K (95440), Woodward's reagent L (95450), and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HO-Dhobt, 37305); Aldrich supplied diphenylphosphoryl azide (17,875-6) bis(2-oxo-3-oxazolidinyl)phosphinic chloride (Bop-Cl, 27,096-2), *N*-methylmorpholine (M5,655-7), piperidine (10,409-4), and diisopropylcarbodiimide (D12,540-7).

Analytical Methods. In all competition experiments, the reactions were left for 2 h and then the contents of the macrocolumns washed with DMF (2×), 10% diisopropylethylamine in DMF, 1:1 DCM/DMF (4×), DCM (6×), and methanol (2×). The thoroughly dried resin was removed from the column, and weighed samples were used in a quantitative ninhydrin assay[49] and for amino acid analysis. For the latter, samples (1.5 mg) were hydrolyzed, along with a standard, at 130 °C for 2 h in 1:1 propionic acid/12 M hydrochloric acid containing 0.1% phenol. Tyrosine incorporations are given as a percentage of the nanomoles of Tyr determined divided by the total nanomoles of Tyr and Phe in the sample. Peptide samples were hydrolyzed at 110 °C for 18 h in 6 M HCl containing 0.1% phenol. All analyses were performed in duplicate with an LKB Model 4151 amino acid analyzer.

Reverse phase HPLC analyses were performed on Vydac 218TP54.6 columns at 1.7 mL/min, monitored at 230 nm, buffer A = 0.05% TFA/H_2O, buffer B = 0.05% TFA/CH_3CN, gradient 0–3 min, 5% B; linear gradient to 100% B 3–23 min.

Procedures Used for Experiments Listed in Table I. Solutions of preformed symmetrical anhydrides were prepared by using 0.4 M *t*-Boc-Phe-OH in DCM (1.06 g made up to 10 mL) and 0.4 M *t*-Boc-Tyr(Bzl)-OH in DCM (1.484 g made up to 10 mL). Samples (1.5 mL, 0.6 mmol) of each were rapidly mixed with 0.2 M carbodiimide reagent (1.5 mL, 0.3 mmol prepared from either 0.41 g of DCCI or 0.252 g of DIPCDI in DCM made up to 10 mL). After 15 min at room temperature, each mixture was filtered through a glass-wool plug, the precipitate and original vessel were washed with further DCM (2 × 2 mL), and the combined filtrates were evaporated in an ambient temperature bath under a stream of nitrogen. The residues were further dried under high vacuum for 1 h and then made up to 3 mL with 1:1 DCM/DMF or neat DMF, and 0.5-mL aliquots were taken, rapidly mixed as required, drawn into the macrocolumn, in which the Leu support had been preequilibrated with the appropriate solvent, and shaken. Agitation in a horizontal plane was provided by a New Brunswick Model R-2 shaker operated at 250 rpm. Solution-prepared symmetrical anhydrides were produced by dissolving the same masses of the amino acid derivatives in 1:1 DCM/DMF and treating 1.5-mL samples with 0.2 M DIPCDI (1.5 mL, either in neat DCM, experiments 1E–1H, or in 1:1 DCM/DMF, experiments 1J and 1K). After 10 min, samples (0.5 mL) were removed, mixed with samples of the competitive PFSA (0.5 mL), and rapidly taken up in the macrocolumn containing the preequilibrated Leu support.

Procedures Used for the Experiments Listed in Table II. Direct DIPCDI (OAIU) components were prepared from 0.4 M solutions of the amino acid in 1:1 DCM/DMF rapidly mixed with an equal volume of freshly prepared 0.4 M DIPCDI in the same solvent cocktail. Samples (0.5 mL) were removed at 1- and 2-min time intervals, mixed with the PFSA solutions, and treated as previously. Results are given as the average of two separate experiments.

Procedures Used for Simultaneous Peptide Syntheses. Both syntheses of the ACP sequence 65–74 (products shown in Figures 2a and 2b) used 0.2 mmol of each Fmoc derivative (note Asn and Gln were added as their pentafluorophenyl esters in the presence of 0.2 mmol of HOBt in DMF). In the PFSA run (2A), these intermediates were prepared during the coupling stage of the previous cycle by adding DCM (0.5 mL) to the Fmoc-amino acid (0.2 mmol), followed by the minimum of DMF required for solubilization (ca. 0.1 mL), and then adding 0.2 M DIPCDI in DCM (0.5 mL). After 15 min, the mixture was evaporated under nitrogen (without prior filtration) and dried further in a vacuum desiccator until required, at which junction it was dissolved in neat DMF (1 mL) and added to the column reactor. In the direct DIPCDI run (2b), the Fmoc-amino acid (0.2 mmol) was dissolved in DMF (0.5 mL), activated by the addition of 0.4 M DIPCDI in DCM (0.5 mL), and added directly to the reactor after 2 min. Asn and Gln, in both syntheses, were added via their pentafluorophenyl esters, in the presence of 1 equiv of HOBt, in neat DMF. The synthesis protocol started from 50 mg of Fmoc-Gly-hydroxymethylphenoxyacetyl substituted 4-methylbenzhydrylamine resin (Biosearch, AA77710-01). The support was washed with DMF (3×) and then treated with piperidine/DMF/toluene (6:7:7) for 1 min and then for 10 min with fresh reagent. After 6 DMF washes, coupling of Asn was achieved by using Fmoc-Asn-OPFP (104 mg, 0.2 mmol) and HOBt (31 mg, 0.2 mmol) in DMF (1 mL). In subsequent cycles, the OAIU activated and PFSA amino acids were prepared as described above (except for Gln, which in both syntheses was added as its PFP ester). Coupling times were as folllows: Asn, 1 h; Ile, 1 h; Tyr, 1 h; Asp, 30 min; Ile, 45 min; Ala, 30 min; Ala, 30 min; Gln, 1 h; Val, 1 h. After three DMF washes, Fmoc removal in the next cycle was initiated. On completion of chain assemblies, the last Fmoc group was removed, and then the resins were washed with DMF (6×), DCM (6×), and methanol (2×). After thorough drying, the resins were treated with TFA/dimethyl sulfide/DCM (14:1:5, 2 mL) for 2 h. The resin was removed by filtration, the filtrate evaporated, and the residue lyophilized from glacial acetic acid. Run 2A: yield 19 mg; amino acid analysis, Asx 1.92 (2), Glx 1.09 (1), Gly 1.24 (1), Ala 2.00 (2), Val 1.03 (1), Ile 2.18 (2), Tyr 1.17 (1). Run 2B: yield 20 mg; amino acid analysis, Asn 1.99 (2), Glx 0.98 (1), Gly 1.13 (1), Ala 2.00 (2), Val 1.05 (1), Ile 2.47 (2), Tyr 1.32 (1). D-Alloisoleucine was absent from both analyses.

Procedures Used in Additive Experiments Listed in Table III. These were performed analogously to the Table II experiments except that, after a 2-min preactivation, an aliquot of the solution (1 mL) was removed and added to the solid additive (0.2 mmol), the mixture was sonicated to effect dissolution, and after 10 min, 0.5 mL was removed and used in the competition experiment.

Automated Syntheses of ACP 65–74. These were performed, either by solution sampling techniques, as with the DIPCDI/HOBt synthesis (Figure 2C), or by solid storage and in reservoir dissolution and activation procedures, as with the BOP-mediated synthesis (Figure 2D), by using a Biosearch Model 9600 peptide synthesizer with standard operating programs. The amino acid analysis of the BOP product, obtained in 85% overall yield, as follows: Asx 2.00 (2), Glx 1.00 (1), Gly 1.07 (1), Ala 2.04 (2), Val 0.93 (1), Ile 1.92 (2), Tyr 1.02 (1). The amino acid analysis of the DIPCDI/HOBt product, obtained in 85% yield, was as follows: Asn 1.84 (2), Glx 1.03 (1), Gly 1.02 (1), Ala 2.21 (2), Val 1.02 (1), Ile 2.32 (2), Tyr 1.10 (1). D-Alloisoleucine was absent from both analyses.

Procedures Used for Comparison of Alternative Activators (Table IV). These were performed by competition of alternately activated Tyr with Phe PFSA (prepared as previously). All were performed analogously, except it should be noted that Woodward's reagent K proved insoluble under the activation conditions described. A representative example, activation with 0.5 equiv of BOP, follows. Boc-Tyr(Bzl)-OH (74.2 mg, 0.2 mmol) and BOP (44.2 mg, 0.1 mmol) were dissolved in 0.2 M *N*-methylmorpholine in DMF (1 mL). After 10 min, a sample (0.5

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mL) was removed, added to Phe PFSA (0.5 mL), and loaded into the syringe reactor in which the Leu resin had previously been thoroughly washed with DMF. After reaction, workup, and hydrolysis, the Tyr incorporation was determined to be 28.7%, compared with the standard PFSA mixture in DMF which gave 26.0% incorporation (average of four determinations).

Procedures Used in BOP Time-Course Studies (Figure 3). A typical series, using 0.5 equiv of BOP, was performed as follows. Boc-Tyr(Bzl)-OH (74.2 mg, 0.2 mmol) in 0.2 M *N*-methylmorpholine in DMF (1 mL) was treated with a solution of BOP (44.2 mg, 0.1 mmol) in DMF (1 mL). Samples (0.5 mL) were removed at 1-, 2-, 5-, and 10-min time intervals, mixed with Phe PFSA (0.5 mL, prepared from Boc-Phe-OH (53 mg, 0.2 mmol) in DCM (0.5 mL) treated with 0.2 M DIPCDI in DCM (0.5 mL) for 15 min, evaporated, dried, and dissolved in DMF (2 mL)), and rapidly added to the reactor. Tyr incorporations were determined to be as follows: 1 min, 21.3%; 2 min, 23.1%; 5 min, 26.0%; 10 min, 26.1%. Tyr PFSA + Phe PFSA standards gave 25% Tyr incorporation (average of four determinations). A duplicate series confirmed the results.

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Registry No. DBTO, 89028-37-5; BOP, 56602-33-6; DIPCDI, 693-13-0; Bop-Cl, 68641-49-6; IIDQ, 38428-14-7; EEDQ, 16357-59-8; DPPA, 26386-88-9; HO-Dhobt, 28230-32-2; HO-PFP, 771-61-9; HOBt, 2592-95-2; DCCI, 538-75-0; ACP 65-74, 66851-75-0; Bates reagent, 55881-03-3; Woodwards reagent K, 4156-16-5; Woodwards reagent L, 10513-45-8; 1*H*-tetrazole, 288-94-8; 1,2,3-benzotriazole, 95-14-7.

Supplementary Material Available: An assembly drawing of Biosearch Macroscale columns used for simultaneous syntheses and for competition experiments (1 page). Ordering information is given on any current masthead page.

Hydroxycarbonylation of Aryl Halides with Formate Salts Catalyzed by Palladium Complexes

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Substituted aryl halides were hydroxycarbonylated under 50 psi pressure of carbon monoxide in the presence of various formate salts and a homogeneous palladium catalyst to give aromatic acids in good yields. Selectivity of formate salts in hydroxycarbonylation reactions depends on the metallic counter-ion and the reaction conditions. Calcium formate was found to give the highest selectivity for hydroxycarbonylation. A mechanism consisting of the generation of mixed anhydrides as intermediates is suggested.

Introduction

The preparation of aromatic acids by a catalyzed hydroxycarbonylation of aryl halides under low carbon monoxide pressure, in the presence of a strong inorganic base such as aqueous sodium hydroxide[1] or calcium hydroxide[2] (eq 1), has been mentioned in the literature.

$$\mathrm{ArBr + CO + M(OH)_n \xrightarrow{catalyst} ArCO_2M + MBr + H_2O} \quad \begin{matrix} M = Na, Ca \\ n = 1, 2 \end{matrix} \qquad (1)$$

Such reactions were homogeneously catalyzed by transition-metal complexes with the help of either a phase-transfer catalyst,[2a] photostimulation,[2d] or the combination of both of them.[2c] No aromatic acids were formed under similar conditions when the strong inorganic base was replaced by a Brønsted base such as a tertiary amine or potassium carbonate.[2b]

Since the heating of alkali or alkali earth hydroxides under carbon monoxide reaches an equilibrium with the formic acid salt,[3] we have considered the possibility of replacing the aqueous base by a formate salt, serving both as a base and as an hydroxyl donor. Direct use of formate would also avoid an excess of alkali hydroxide and enable reaction at a milder pH and under anhydrous conditions (eq 2). We report here that under suitable conditions, an

$$\mathrm{ArBr + MO_2CH + C^*O \xrightarrow{catalyst} ArC^*O_2H + MBr + CO} \qquad (2)$$

hydroxycarbonylation reaction between aryl halides and formate salts occurred under 1–3 atm of CO pressure, giving the corresponding carboxylic acids in fair to good yields.

Results and Discussion

The catalyzed hydroxycarbonylation reaction is in competition with the reductive formylation by formates[4] (eq 3). Indeed, it was found that the formylation reaction

$$\mathrm{ArBr + MO_2CH + CO} \begin{cases} \xrightarrow{\text{path A}} \mathrm{ArCHO + MBr + CO_2} \\ \xrightarrow{\text{path B}} \mathrm{ArCO_2H + MBr + CO} \end{cases} \qquad (3)$$

(path A) is accompanied by the much slower hydroxycarbonylation reaction (path B). The reaction of sodium formate with 4-chlorobromobenzene, in an anhydrous aprotic dipolar solvent (e.g. DMF) and in the presence of 5 mol % homogeneous palladium catalyst, results in 4-chlorobenzaldehyde (70%), chlorobenzene (4%), and 4-

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