BIOORGANIC CHEMISTRY **24,** 59–68 (1996)
ARTICLE NO. 0007

Application of Inverse Substrates to Trypsin-Catalyzed Peptide Synthesis

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Received May 25, 1995

Trypsin-catalyzed peptide synthesis has been studied by using ''inverse substrate,'' i.e., *p*-amidinophenyl ester derived from α-amino acid derivative as an acyl donor component. Inverse substrate can afford acyl trypsin in a very specific manner, liberating the site-specific *p*-amidinophenyl moiety as the leaving group. Thus a variety of α-amino acid residues which are a part of *p*-amidinophenyl ester can be involved in the trypsin-catalyzed coupling reaction. The method has been shown to be successful as expected. In conclusion, the method was proposed as a new procedure which overcomes the disadvantage of enzymatic peptide synthesis.

INTRODUCTION

It is recognized that enzyme-catalyzed peptide synthesis is more advantageous than chemical synthesis in many respects (*1*, *2*). The enzymatic method is highly stereoselective, racemization-free, and requires minimal side-chain protection. The enzymatic method is at an advantage when discriminating enantiomers and amino acid side-chain residue and at a disadvantage as a general procedure of peptide synthesis. The reactants used as acyl coupling component are limited. Only the amino acid or peptide derivatives which meet the substrate specificity of the enzyme are applicable to the coupling reaction. This is because the enzymatic peptide coupling is considered to proceed via enzyme–substrate complex and subsequent acyl enzyme formation. In the case of trypsin-catalyzed reaction, for example, the acyl donor is limited to positively charged arginine or lysine residue at the carboxyl end position.

In our previous work (*3*), it has been shown that esters of *p*-amidinophenol are specifically hydrolyzed by trypsin and trypsin-like enzymes. In these esters the site-specific group for the enzyme, a charged amidinium group, is liberated during acylation to produce an acyl enzyme intermediate. Kinetic analyses showed that the binding affinity and catalytic efficiency at the acylation step of these esters are comparable to those for normal-type specific substrates, and so a new term, ''inverse substrates,'' was proposed for these esters. Thus the compounds provide a general method for efficient synthesis of acyl-enzymes without recourse to the structure of

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0045-2068/96 $18.00

the acyl component. The compounds are applicable to trypsin-catalyzed peptide synthesis for the same reason. The method is extremely useful since the acyl component is not restricted to cationic residues such as lysine and arginine.

Noting the characteristic feature of inverse substrate Schellenberger *et al.* (*4*) have tested the applicability of *p*-guanidinophenyl ester as tool for trypsin-catalyzed peptide synthesis. The paper prompted us to report the results on the general use of our inverse substrate, *p*-amidinophenyl esters, to tryptic peptide synthesis in detail.

EXPERIMENTAL PROCEDURES

Synthetic Chemistry

General. Dimethylformamide (DMF), ethyl acetate, and diethyl ether were obtained as the anhydrous solvents by usual manner. *N-tert*-butyloxycarbonylamino acid (Boc-amino acid) and *N,N′*-dicyclohexylcarbodiimide(DCC) were purchased from Peptide Institute, Inc. All other reagents were used without further purification from Aldrich Chemical Co., Inc., Wako Pure Chemical Industries, LTD., and Kanto Chemical Co., Inc. Melting points were determined on a Yanaco MP-500D apparatus and are uncorrected. Infrared spectra were recorded on a JASCO VALOR-III FT-IR spectrometer. NMR spectra were recorded on a JEOL JNM-FX400 FT NMR spectrometer.

General procedure for synthesis of inverse substrates. Inverse substrates, i.e., Boc-amino acid *p*-amidinophenyl esters, were prepared as follows according to our previous paper (*5*). DCC (454 mg, 2.2 mmol) was added to a solution of Boc-amino acid (2.0 mmol), *N*-benzyloxycarbonyl-*p*-amidinophenol (*5*) (540 mg, 2.0 mmol), and 4-dimethylaminopyridine (24 mg, 0.2 mmol) in 3 ml of DMF and 6 ml of ethyl acetate. The reaction mixture was kept at 0°C for 30 min and subsequently at room temperature overnight. The precipitated dicyclohexylurea was removed by filtration and the filtrate was evaporated *in vacuo.* The resulting *N*-benzyloxycarbonyl-*p*-amidinophenyl ester was recrystallized from benzene–hexane. Isolation yields of 64–80% resulted. Structure of the ester was confirmed by NMR spectra, ir spectra, and elemental analysis. Optical purity of each enantiomer was analyzed by optical rotation measurement. The ester (1.0 mmol) was dissolved in methanol (20 ml) containing *p*-toluenesulfonic acid monohydrate (190 mg, 1.0 mmol) and hydrogenated in the presence of 10% Pd-charcoal (10 mg). After the catalyst was removed, the filtrate was evaporated to dryness *in vacuo* and the residue was washed with dry ether. Recrystallization from ethanol–ether gave pure *p*-amidinophenyl ester tosylate.

p-Amidinophenyl esters prepared according to the general procedure are summarized in Table 1.

Enzyme Assay

Substrates and enzymes. Alanine *p*-nitroanilide (Ala-*p*NA) and leucine *p*-nitroanilide (Leu-*p*NA) were purchased from Peptide Institute, Inc. Glycine *p*-nitroanilide (Gly-*p*NA), D-alanine *p*-nitroanilide (D-Ala-*p*NA), and D-leucine *p*-nitroanilide (D-Leu-*p*NA) were prepared following the reported procedure (*6–8*).

TABLE 1
Physical Data of the Inverse Substrates (*N-tert*-Butyloxycarbonylamino Acid *p*-Amidinophenyl Ester Tosylate) Used for Trypsin-Catalyzed Coupling Reaction

Inverse substrate tosylate	mp(°C)	$[\alpha]_D^{20}$ (c = 1, methanol)	Formula	Analysis: calculated(found) C	H	N	S
Boc-GlyOAm	170–172		$C_{21}H_{27}N_3O_6S$ 4/3H_2O	52.65 (52.37	6.00 5.70	8.77 8.53	6.69 7.04)
Boc-AlaOAm	162–164	−41.8	$C_{22}H_{29}N_3O_7S$	55.10 (55.04	6.10 6.07	8.76 8.72	6.69 6.71)
Boc-D-AlaOAm	163–164	+41.2	$C_{22}H_{29}N_3O_7S$ 1/2H_2O	54.09 (54.00	6.19 5.94	8.60 8.48	6.56 6.69)
Boc-PheOAm	161–162	−7.2	$C_{28}H_{33}N_3O_7S$ 1/2H_2O	59.56 (59.56	6.07 5.80	7.44 7.46	5.68 5.96)
Boc-D-PheOAm	161–163	+7.0	$C_{28}H_{33}N_3O_7S$ 1/2H_2O	59.56 (59.68	6.07 5.93	7.44 7.49	5.68 5.99)
Boc-LeuOAm	153–154	−40.0	$C_{25}H_{35}N_3O_7S$ 2/3H_2O	56.27 (56.24	6.86 6.66	7.65 7.65	6.01 6.26)
Boc-D-LeuOAm	153–155	+39.2	$C_{25}H_{35}N_3O_7S$ 2/3H_2O	56.27 (56.37	6.86 6.63	7.65 7.71	6.01 6.43)

Bovine pancreatic trypsin (EC 3.4.21.4) purchased from Worthington Biochemical Corp. (twice recrystallized, lot TRL) was further purified by affinity chromatography (*9*), using Benzamidine Sepharose 6B obtained from Pharmacia. Enzyme concentration was determined from active site titration using *p*-nitrophenyl *p*-guanidinobenzoate (*10*).

Trypsin-catalyzed peptide coupling reaction. Peptide coupling reaction was carried out at 25°C in 50 mM 4-morpholinepropanesulfonic acid buffer (MOPS), pH 8.0, containing dimethylsulfoxide (DMSO). Concentrations of acyl donor (inverse substrate), acyl acceptor (amino acid *p*-nitroanilide), and trypsin were 1 mM, 20 mM, and 5μM, respectively. The progress of the coupling reaction was monitored by HPLC on a Hitachi Model L-6200 equipped with a Hitachi UV-VIS detector. The chromatograph was equipped with a Wakosil 5C18-200 column (4.0 × 250 mm). The mobile phase was acetonitrile-0.1% aqueous trifluoroacetic acid and the flow rate was 1.0 ml/min. Analysis was performed using isocratic system. An aliquot of the reaction mixture was injected and peaks were detected at 310 nm for *p*-nitroanilide moiety. Peak identification was made by correlation with authentic samples which were chemically synthesized (*11, 12*). Peak intensities were used to calculate relative concentration.

RESULTS

Trypsin-catalyzed coupling reaction of *N-tert*-butyloxycarbonylalanine *p*-amidinophenyl ester (Boc-Ala-OAm) and Ala-*p*NA to give Boc-Ala-Ala-*p*NA was determined in aqueous DMSO and aqueous DMF. The coupling reaction was instantaneous in the media less than 60% organic solvent and was completed within several minutes. During the reaction period the peak of Boc-Ala-OAm completely disappeared from the HPLC elution diagram. Only two peaks which were assigned as the coupling product and the hydrolysate newly emerged. Control experiments in the absence of trypsin showed that hydrolysis of Boc-Ala-OAm is so slow that no appreciable amount of the ester is consumed during the period that enzymatic reaction is complete. The rate constant for the spontaneous hydrolysis at pH 8.0 in 50% DMSO was determined to be 1.3×10^{-5} s^{-1} which is equal to 14.8 h of half-life time. In the presence of the acyl acceptor aminolysis takes place and affords coupling product even in the absence of the enzyme though the extent of the reaction is not large. It was found that the nonenzymatic aminolysis is sufficiently less than the enzymatic reaction; i.e., coupling yield of Boc-Ala-Ala-*p*NA in the absence of trypsin is 3% after 0.1 h. Thus the enzymatic hydrolysis of acyl donor (inverse substrate) is the only reaction competitive to the peptide synthesis, and the rest of the coupling is nearly equal to the part of hydrolysis.

Effects of DMSO and DMF concentration on coupling yields were shown in Fig. 1. Coupling yields higher than 65% were observed at the DMSO concentration range of 20–60%, and the best yield (85%) was obtained at 50% DMSO. Effect of DMF was nearly the same as that of DMSO but the coupling yield in aqueous DMF was less than that in aqueous DMSO. The diminished coupling yield at the low concentration of organic solvent could be due to the enzyme-catalyzed hydrolysis of acyl donor as mentioned previously. A higher concentration of organic solvent is not completely advantageous for the coupling, although it is expected to retard the hydrolysis. The high concentration of organic solvent results in the separation of the catalyst as well as the decrease of enzymatic activity.

The effect of pH of the buffer component in the medium on the coupling yields was analyzed. Reaction yields were determined for 50% DMSO solutions, changing pH of the buffer solution. The pH dependency of the yield at the reaction period of 10 min was determined as shown in Fig. 2. The pH dependency was also determined for 50% DMF in a similar manner. The pH dependency was not different for both cases though the reaction yield in 50% DMF was relatively low. The observed dependency was similar to that of trypsin-catalyzed hydrolysis of the specific ester substrates as reported previously in which the catalytic rate was increased at the higher pH and reached the limit at around pH 9 (*13*). Effect of cosolvent on the coupling yield was analyzed. Among six solvents so far tested, DMF and especially DMSO were found to be advantageous, as shown in Table 2. The analysis was made in MOPS buffer (pH 8.0) containing 50% organic solvent. For each case the coupling reaction was completed within 2 h. As shown in Table 2 the best result was obtained with DMSO after all. The observation was not different from that of enzyme-catalyzed coupling procedure by means of conventional substrates (*1*, *2*).

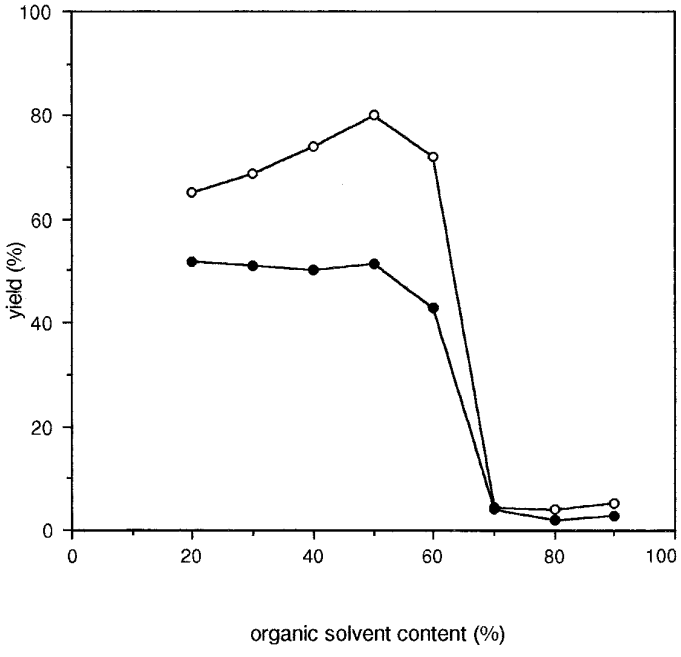

FIG. 1. Effect of organic solvent on trypsin-catalyzed condensation of *t*-butyloxycarbonylalanine *p*-amidinophenyl ester and alanine *p*-nitroanilide. Reaction was carried out in 50 mM MOPS buffer (pH 8.0) containing DMSO (○) or DMF (●) at 25°C. Product yield was analyzed after reaction period of 10 min in which the coupling was completed. Boc-Ala-OAm, 1 mM; Ala-*p*NA, 20 mM; trypsin, 5 μM.

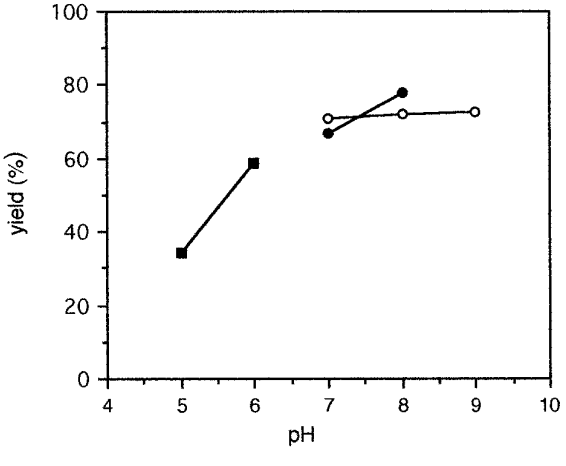

FIG. 2. pH dependency of trypsin-catalyzed condensation. Boc-Ala-OAm and Ala-*p*NA were reacted in 50 mM 4-morpholineethanesulfonic acid (MES) (■), 50 mM MOPS (●), and 50 mM Tris (○) buffers containing 50% DMSO at 25°C. Boc-Ala-OAm, 1 mM; Ala-*p*NA, 20 mM; trypsin, 5 μM. Coupling yield was determined after a reaction period of 10 min in which the coupling was completed.

TABLE 2
Effect of Water-Miscible Organic Cosolvent on Trypsin-Catalyzed Peptide Synthesis[a]

Cosolvent	Yield (%)
DMF	29
DMSO	58
Dioxane	9
Acetonitrile	18
THF[b]	5
2-Propanol	10

[a] Boc-Ala-OAm, 1 mM; Ala-*p*-NA, 10 mM; trypsin, 10 μM; cosolvent: MOPS(50 mM, pH 8.0), 1:1, at 25°C for 2 h.
[b] Precipitation of Ala-*p*NA resulted.

Acyl acceptor concentration influenced the coupling yield. The reaction was determined in aqueous DMSO (Fig. 3). The reaction yield was increasing to 80% when 40 mM acyl acceptor was used.

Coupling reactions were carried out for various combinations of acyl donor and acyl acceptor. As summarized in Table 3, the reaction involving D-acyl acceptor is not favorable. Reaction yields are very low for cases in which either L- or D-enantiomer was used as acyl donor. In contrast, the structure of acyl donor does not markedly affect the reaction yield. Acyl donors derived from Gly, Ala, Leu, and Phe react with Ala-*p*NA with comparable rates. Reaction rate for acyl donor

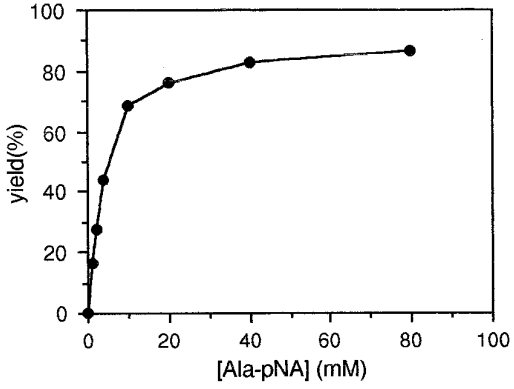

FIG. 3. Effect of acyl acceptor concentration on coupling reaction. Reactions were carried out in 50 mM MOPS (pH 8.0) containing 50% DMSO at 25°C, using different concentration of Ala-*p*NA (1–80 mM). Boc-Ala-OAm, 1 mM; trypsin, 5 μM.

TABLE 3
Trypsin-Catalyzed Peptide Coupling by Use of Inverse Substrate as Acyl Donor Component[a]

Acyl donor	Acyl acceptor	Product	Reaction time (h)	Yield (%)
Boc-Gly-OAm	Ala-*p*NA	Boc-Gly-Ala-*p*NA	0.1	77
Boc-Ala-OAm	Ala-*p*NA	Boc-Ala-Ala-*p*NA	0.1	77
Boc-D-Ala-OAm	Ala-*p*NA	Boc-D-Ala-Ala-*p*NA	1	65
Boc-Leu-OAm	Ala-*p*NA	Boc-Leu-Ala-*p*NA	0.1	85
Boc-D-Leu-OAm	Ala-*p*NA	Boc-D-Leu-Ala-*p*NA	2	68
Boc-Phe-OAm	Ala-*p*NA	Boc-Phe-Ala-*p*NA	0.1	82
Boc-D-Phe-OAm	Ala-*p*NA	Boc-D-Phe-Ala-*p*NA	2	64
Boc-Pro-OAm	Ala-*p*NA	Boc-Pro-Ala-*p*NA	2	80
Boc-D-Pro-OAm	Ala-*p*NA	Boc-D-Pro-Ala-*p*NA	12	76
Boc-Ala-OAm	Leu-*p*NA	Boc-Ala-Leu-*p*NA	0.1	74
Boc-D-Ala-OAm	Leu-*p*NA	Bod-D-Ala-Leu-*p*NA	0.5	54
Boc-Ala-OAm	Gly-*p*NA	Boc-Ala-Gly-*p*NA	0.1	47
Boc-D-Ala-OAm	Gly-*p*NA	Bod-D-Ala-Gly-*p*NA	6	53
Boc-Ala-OAm	D-Ala-*p*NA	Boc-Ala-D-Ala-*p*NA	0.1	8
Boc-D-AlaOAm	D-Ala-*p*NA	Boc-D-Ala-D-Ala-*p*NA	3	7
Boc-Ala-OAm	D-Leu-*p*NA	Boc-Ala-D-Leu-*p*NA	1	Trace
Boc-D-Ala-OAm	D-Leu-*p*NA	Boc-D-Ala-D-Leu-*p*NA	4	Trace
Boc-*β*-Ala-OAm	Ala-*p*NA	Boc-*β*-Ala-Ala-*p*NA	2	8

[a] Acyl donor, 1 mM; acyl acceptor, 20 mM; trypsin, 5 μM; DMSO: MOPS(50 mM, pH 8.0), 1:1.

derived from D-amino acid is more or less slower than that of corresponding L-enantiomer and the difference is reflected in the final reaction yields for both cases. As previously stated, the enzymatic hydrolysis of inverse substrate is the only process competitive to the peptide coupling, and the rate ratios of coupling reaction over hydrolysis directly determine the coupling yield. The observed small difference in the coupling yields for the reactions of the enantiomeric pair of acyl donor could be due to the respective rate ratio.

In Fig. 4 time courses of the reaction of Ala-*p*NA with Boc-Leu-OAm or Boc-D-Leu-OAm were shown. Both reactions were compared with nonenzymatic coupling reactions. It was shown that D-acyl donors are efficient substrates for the enzymatic coupling reaction though they are relatively less efficient than L-acceptors. The behavior of the D-acyl acceptors is distinct from that of D-acyl donors.

DISCUSSION

Our results in Table 3 show that the enantiomeric preference of acyl acceptor is rather strict. This is in contrast to the result obtained from the conventional enzymatic method. It is reported that the enantiomeric preference of acyl acceptor for the trypsin-catalyzed and chymotrypsin-catalyzed coupling reactions was less strict and substantial reaction yield resulted in the reaction involving D-acyl acceptor (*14*, *15*).

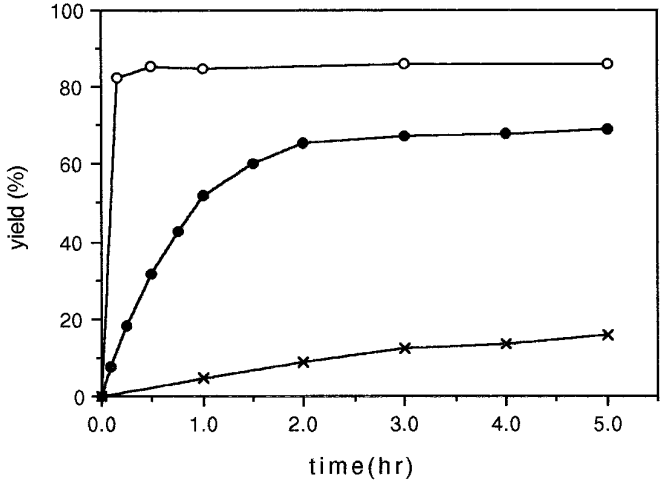

FIG. 4. Time course of the peptide coupling reactions. Reactions were carried out in 50 mM MOPS (pH 8.0) containing 50% DMSO at 25°C. Ala-pNA, 20 mM was reacted with Boc-Leu-OAm, 1 mM (○), or Boc-D-Leu, 1 mM (●) in the presence of trypsin, 5 μM. In the absence of trypsin the same reactions were carried out using Boc-Leu-OAm or Boc-D-Leu-OAm as acyl donor (×).

A variety of attempts have been carried out to overcome the shortcomings of enzymatic peptide synthesis. A narrow substrate specificity including L-specificity restricts its wide application to peptide synthesis. Margolin *et al.* (*16*) reported that L-specificity of subtilisin for acyl donor relaxed in anhydrous organic solvent. It was reported that coupling product was obtained in good yield from D-acyl donor in anhydrous *tert*-amyl alcohol. The method, however, is inadequate for a general procedure of peptide synthesis. The method requires use of high concentrations of acyl donor, acceptor, and subtilisin as well.

Another drawback of the enzymatic method is an undesirable proteolysis of growing polypeptide chain. Synthesis by use of nonprotease, lipase, was proposed for this solution (*17–19*). The method also requires use of a high concentration of the enzyme. The use of chemically modified enzymes was another approach to prevent undesirable proteolysis of the growing peptide chain. Thiolsubtilisin (*20*), methylchymotrypsin (*21*), and methylsubtilisin (*22*) are modified proteases in which the active site serine or histidine residue is modified. All the modified enzymes were analyzed to retain the catalytic activity for peptide coupling to some extent. In these instances, substantial improvement of the ratio of peptide coupling over hydrolysis resulted, though the enzymatic activity decreased greatly on the basis of the modification (*21*).

It should be noticed that in our case protease itself was used as a catalyst and no hydrolysate originating from the coupling product was included in the reaction mixture. This is another characteristic of the present method in addition to its remarkable feature of being free from the narrow substrate specificity. Inverse substrates exhibit strong binding affinity toward trypsin, so that the substrates

facilitate the formation of the coupling product. Furthermore, the resulting peptides lacking the site-specific group are no longer involved in its reverse process, thus hydrolysis of the resulting peptides are retarded.

It is necessary to compare our result with the finding of Schellenberger *et al.* (*4*) who noticed the versatility of inverse substrate as a tool for peptide synthesis and attempted the use of *p*-guanidinophenyl ester. Schellenberger's work, however, did not include any information on the reaction rate. Therefore the direct comparison of the practical applicabilities of both methods is inadequate. It was noticed that the experiments were carried out using much higher concentrations of enzyme and acyl acceptor than those of ours, 10-fold and 3-fold, respectively. The paper mainly concerned determination of ''partition constant,'' the rate ratio between hydrolysis and coupling. Calculation of the partition constants was carried out for our case. The values were determined to be 8.6–5 mM for the typical examples in Table 3 (for cases which afford 70–80% yield), and these values were 10-times smaller than those of Schellenberger *et al.* (*4*); i.e., ours are preferable for peptide synthesis. This is the reason why in our case a low concentration of acyl acceptor (one-third of that used by Schellenberger *et al.*) is applied to the coupling. Thus it may be concluded that *p*-amidinophenyl esters are much more suitable as a tool for peptide synthesis. One possible reason for this would be the difference in the acylation efficiencies of their respective substrates. An adequate explanation for this has to await detailed kinetic analysis and the study is now in progress.

ACKNOWLEDGMENTS

This work was supported in part by grants from The Akiyama Foundation and The Fugaku Trust for Medicinal Research.

REFERENCES

1. Schellenberger, V., and Jakubke, H.-D. (1991) *Angew. Chem. Int. Ed. Engl.* **30,** 1437–1449.
2. Wong, C.-H. (1989) *Science* **244,** 1145–1152.
3. Tanizawa, K., Kasaba, Y., and Kanaoka, Y. (1977) *J. Am. Chem. Soc.* **99,** 4485–4488.
4. Schellenberger, V., Jakubke, H.-D., Zapevalova, N. P., and Mitin, Y. V. (1991) *Biotechnol. Bioeng.* **38,** 104–108.
5. Fujioka, Y., Tanizawa, K., Nakayama, H. and Kanaoka, Y. (1980) *Chem. Pharm. Bull.* **28,** 1899–1905.
6. Oyamada, H., Saito, T., Inaba, S., and Ueki, M. (1991) *Bull. Chem. Soc. Jpn.* **64,** 1422–1424.
7. Kasafirek, E., Fric, P., Slaby, J., and Malis, F. (1976) *Eur. J. Biochem.* **69,** 1–13.
8. Tuppy, H., Wiesbauer, U., and Wintersberger, E. (1962) *Hoppe-Seyler's Z. Physiol. Chem.* **329,** 278–288.
9. Hixson, H. F., Jr., and Nishikawa, A. H. (1973) *Arch. Biochem. Biophys.* **154,** 501–509.
10. Chase, T., Jr., and Shaw, E. (1967) *Biochem. Biophys. Res. Commun.* **29,** 508–514.
11. Bieth, J., and Weremath, C. G. (1970) *Biochem. Biophys. Res. Commun.* **53,** 383–390.
12. Okada, Y., Tsuda, Y., Hirata, A., Nagamatsu, Y., and Okamoto, U. (1982) *Chem. Pharm. Bull.* **31,** 4060–4068.
13. Gutfreund, H. (1955) *Trans. Faraday Soc.* **51,** 441–446.
14. Oka, T., and Morihara, K. (1977) *J. Biochem.* **82,** 1055–1062.

15. Weast, J. B., and Wong, C.-H. (1986) *J. Org. Chem.* **51,** 2728–2735.
16. Margolin, A. L., Tai, D. F., and Klibanov, A. M. (1987) *J. Am. Chem. Soc.* **109,** 7885–7887.
17. West, J. B., Hennen, W., Lalonde, J. L., Bibbs, J. A., Zhong, Z., Meyer, E. F., Jr., and Wong, C.-H. (1990) *J. Am. Chem. Soc.* **112,** 5313–5310.
18. Gardossi, L., Bianchi, D., and Klibanov, A. M. (1991) *J. Am. Chem. Soc.* **113,** 6328–6329.
19. Kawashiro, K., Kaiso, K., Minato, D., Sugiyama, S., and Hayashi, H. (1993) *Tetrahedron* **49,** 4541–4548.
20. Nakatuka, T., Sasaki, T., and Kaiser, E. T. (1987) *J. Am. Chem. Soc.* **109,** 3808–3810.
21. West, J. B., Sholten, J., Stolowich, N. J., Hogg, J. L., West, A. I., and Wong, C.-H. (1988) *J. Am. Chem. Soc.* **110,** 3709–3710.
22. Zhong, Z., Bibbs, J. A., Yuan, W., and Wong, C.-H. (1991) *J. Am. Chem. Soc.* **113,** 2259–2263.