CHAPTER III

RESULTS AND DISCUSSION

3.1 Synthesis of Reagents

In order to search for an effective coupling reagent for peptide synthesis, several activating parts (A) and auxiliary nucleophiles (B) were screened first. In this study, three classes of reagents were selected to examine their effectiveness in peptide coupling reactions including carbonates, oxalates, and sulfonates. These reagents were easily prepared in good yield (> 80%) by the reaction of acid chloride or sulfonyl chloride with strongly acidic OH compounds such as HOBt and electron-deficient phenols such as 4-nitrophenol, 2,4,5-trichlorophenol, pentafluorophenol, and pentachlorophenol under basic condition using pyridine or triethylamine/ dichloromethane mixture according to the well known procedure.[38] Structures of the reagents prepared and tested are as shown in Figure 3.1.

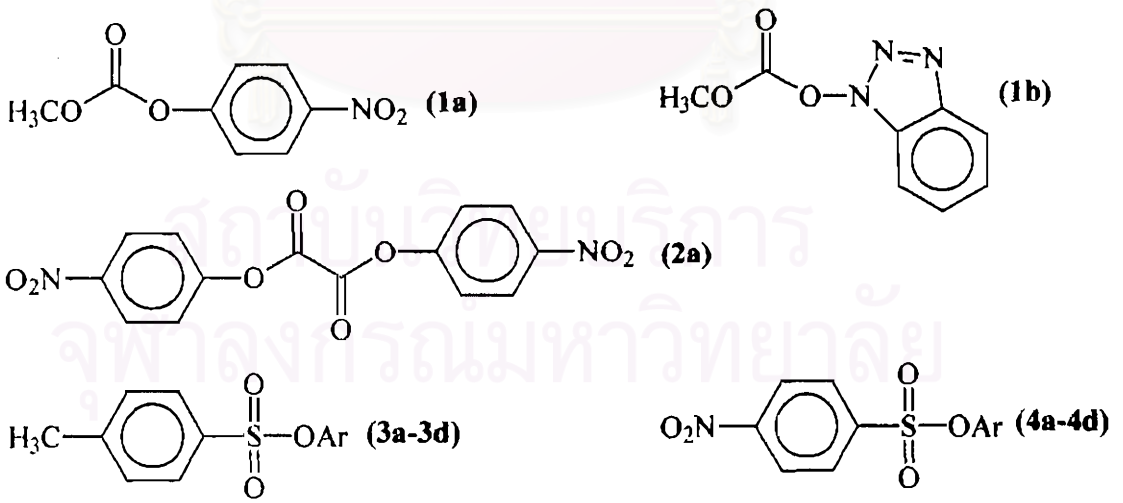


Figure 3.1: The structure of A-B reagents tested in this study

Generally pyridine is an effective base (Method I) except for the synthesis of benzotriazolyl methyl carbonate (**1b**), which gave low yield (<50% yield) because the product seemed to decompose during aqueous work up due to the susceptibility to hydrolysis of the product. So aqueous work up was considered unsuitable for preparing this reagent and a condition employing an equivalent amount of triethylamine in dichloromethane (Method II) provided better results. The arylsulfonates are stable crystalline solids which can be stored at room temperature for prolonged periods of time without deterioration, but aryl methyl carbonates and aryl oxalate (type I and II) decompose easily within 2-3 weeks. The physical data of these reagents are shown in Table 3.1.

Table 3.1 Physical data of reagents

Type	Reagent	Method[a]	m.p. (°C)	% yield
I	**methyl carbonates**			
	4-nitrophenyl methyl carbonate (**1a**)	I	108-110	80
	benzotriazolyl methyl carbonate (**1b**)	II	77-79	82
II	**oxalate**			
	bis(4-nitrophenyl) oxalate (**2a**)	II	268-270	89
III	**p-toluenesulfonates**			
	4-nitrophenyl *p*-toluenesulfonate (**3a**)	I	93-94	87
	2,4,5-trichlorophenyl *p*-toluenesulfonate (**3b**)	I	92-93	90
	pentafluorophenyl *p*-toluenesulfonate (**3c**)	I	63-64	85
	pentachlorophenyl *p*-toluenesulfonate (**3d**)	I	154-156	88
IV	**4-nitrobenzenesulfonates**			
	4-nitrophenyl 4-nitrobenzenesulfonate (**4a**)	I	156-157	86
	2,4,5-trichlorophenyl 4-nitrobenzenesulfonate (**4b**)	I	143-144	81
	pentafluorophenyl 4-nitrobenzenesulfonate (**4c**)	I	108-109	83
	pentachlorophenyl 4-nitrobenzenesulfonate (**4d**)	I	195-196	86

[a] Method I : phenol 1.1 eq + acid chloride or sulfonyl chloride 1.0 eq in pyridine at 4°C.

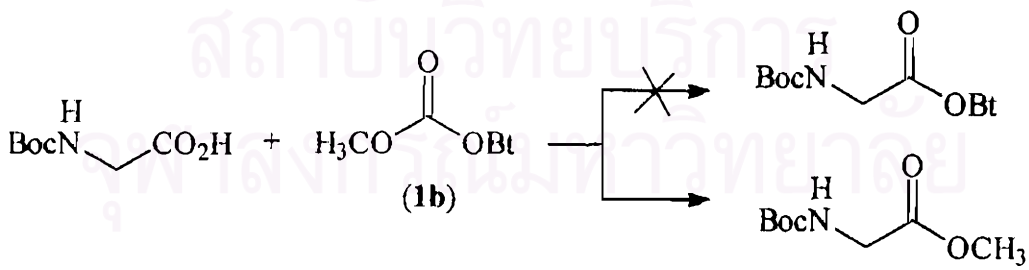
Method II : phenol 1.0 eq + acid chloride of sulfonyl chloride 1.0 eq + Et_3N 1.0 eq in CH_2Cl_2.

3.2 Screening the Reagents for Peptide Synthesis

The purpose of this study was to evaluate potential application of synthesized reagents as coupling reagents. The study focused on the reactivity of activating part (A) and auxiliary nucleophile (B). Therefore the first criteria for the reagents to pass the test is their ability to convert the hydroxy group of amino acids into a good leaving group such as aryloxy or benzotriazol-1-yloxy group.

3.2.1 Reagent Type I: Methyl carbonates

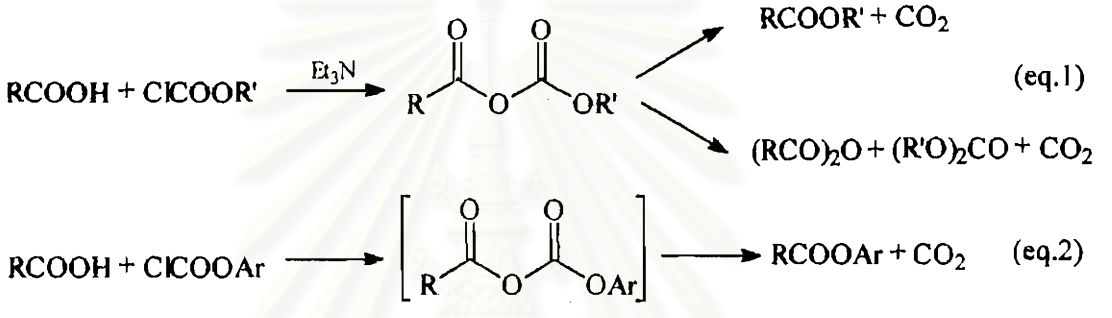
For the reagent of this type, two compounds, 4-nitrophenyl methyl carbonate (**1a**) and benzotriazolyl methyl carbonate (**1b**), were synthesized. When Boc-Gly-OH was treated with reagent (**1a**) in the presence of triethylamine in acetonitrile at room temperature, no reaction had occurred (indicated by TLC). But when reagent (**1b**) was used in place of reagent (**1a**) under similar condition, the reaction proceeded smoothly and was completed within 1 hour to give only one product which was isolated as a white solid. ^{1}H NMR of the product revealed resonance signal of a methoxy group (OCH_3) appeared a singlet peak at 3.74 ppm and no signal of benzotriazolyl group (around 7.0-8.0 ppm) was observed, which suggested that the isolated product was Boc-Glycine methyl ester and not the anticipated product (Boc-Glycine benzotriazol-1-yl ester).



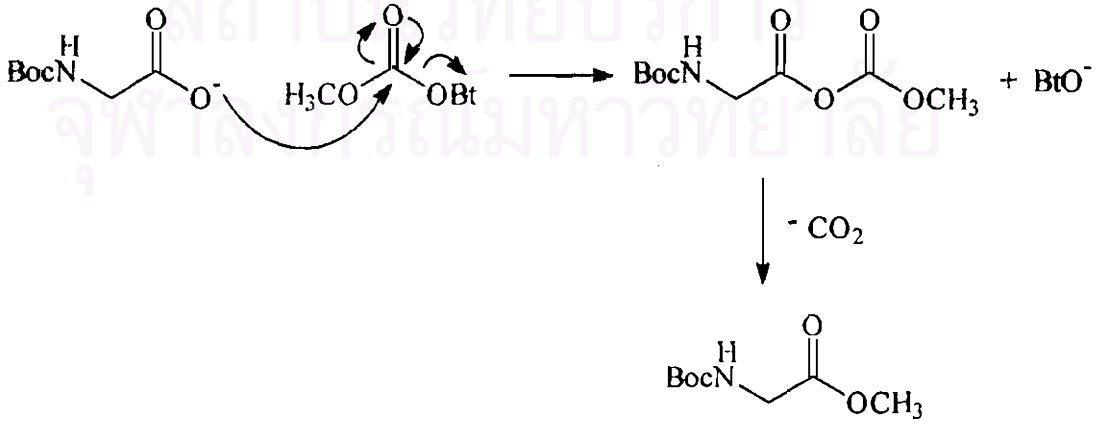
Bt = benzotriazolyl

Scheme 3.1: Reaction of Boc-Gly-OH with reagent (**1b**)

The formation of methyl ester instead of the expected hydroxybenzotriazole ester suggested that this reaction took place *via* the formation of mixed carboxylic-carbonic anhydride. In general, the decomposition of mixed carboxylic-carbonic anhydride proceeded by two different pathways yielding two types of products, the ester and the symmetrical acid anhydride and the carbonate (eq 1). It has been reported that several aryl chloroformates can be used to esterify carboxylic *via* the intermediacy of the mixed anhydrides (eq 2).[39]



The formation of the methyl ester can therefore be explained as followed. In the reaction of Boc-Gly-OH with reagent (**1b**), the mixed carboxylic-carbonic anhydride was formed in the first step, followed by releasing carbon dioxide (CO_2) before the benzotriazolyl anion came back to attack on carbonyl. The mechanism was proposed in Scheme 3.2. Because no expected product was obtained, it was decided to abandon this type of reagent and more efficient reagents were sought.



Scheme 3.2: A proposed mechanism of reaction of Boc-Gly-OH with reagent (**1b**)

3.2.2 Reagent Type II: Aryl oxalate

Bis(4-Nitrophenyl) oxalate (**2a**) was prepared by the reaction of oxalyl chloride and 4-nitrophenol in triethylamine/dichloromethane mixture. Reaction of reagent (**2a**) with benzoic acid under various conditions at room temperature was investigated. The results are presented in Table 3.2.

Table 3.2 Reaction of reagent (**2a**) with benzoic acid under various conditions

Condition	Base	Solvent	Time	Product (%)
1	Et_3N	CH_2Cl_2	overnight	no reaction
2	pyridine	pyridine	overnight	no reaction
3	Et_3N	acetonitrile	overnight	28
4	Et_3N	DMF	overnight	46

According to Table 3.2, it was found that condition 4, using DMF as a solvent and triethylamine as a base, was the most effective condition. However, reagent (**2a**) was not good enough to use as a coupling reagent because high yield was not obtained and the reaction time was too long. Therefore synthesis of bis(pentachlorophenyl) oxalate which should be more reactive reagent due to the presence of more electron withdrawing groups was attempted by using pentachlorophenol as auxiliary nucleophile instead of 4-nitrophenol. Disappointingly bis(pentachlorophenyl) oxalate could not be synthesized under similar condition therefore the reagents of this type were not further investigated.

3.2.3 Reagent Type III: Aryl *p*-toluenesulfonates

It has been reported that some sulfonates of strongly acidic *N*-hydroxy compounds such as 6-chloro-1-*p*-chlorobenzenesulfonyloxybenzotriazole possessed potential activity as coupling reagents while the methanesulfonates of strongly acidic phenols showed far less activity.[25,26] Being encouraged by these results, a wide variety of *p*-toluenesulfonates of possible strongly acidic phenols were prepared from

the reaction of tosyl chloride with phenols and their potentials examined. Initially their reactions with benzoic acid was selected as a model experiment. When benzoic acid (1 eq) was treated with 4-nitrophenyl *p*-toluenesulfonate (**3a**) (1 eq) in the presence of triethylamine (1 eq) in a variety of solvents (dichloromethane, pyridine, acetonitrile, and DMF) at room temperature, no reaction took place as indicated by TLC. Under the same condition, similar results were obtained when reagent (**3b**) and (**3d**) were utilized. In contrast, when pentafluorophenyl *p*-toluenesulfonate (**3c**) was used, it was observed that the reaction took place when DMF was used as solvent ,but the product, pentafluorophenyl benzoate, was obtained only 50% yield and the starting material was recovered according to ^{1}H NMR of the crude product. The results are shown in Table 3.3.

Table 3.3 Reaction of aryl *p*-toluenesulfonates with benzoic acid

Reagent	Base	Solvent	Time	Product (%)
3a	Et_3N	CH_2Cl_2	overnight	no reaction
	pyridine	pyridine	overnight	no reaction
	Et_3N	acetonitrile	overnight	no reaction
	Et_3N	DMF	overnight	no reaction
3b	Et_3N	CH_2Cl_2	overnight	no reaction
	Et_3N	DMF	overnight	< 10[a]
3c	Et_3N	CH_2Cl_2	overnight	no reaction
	Et_3N	DMF	overnight	50
3d	Et_3N	CH_2Cl_2	overnight	no reaction
	Et_3N	DMF	overnight	< 10[a]

[a] The yield was shown as judged by TLC.

The results indicated that aryl *p*-toluenesulfonates were unreactive toward a benzoic acid without any additive. In addition, it was observed that DMF was the best solvent after extensive screening and it is usually employed in peptide synthesis. Next, the effect of additives was explored.

The effect of catalysts

In order to promote the reaction, the same reaction in the presence of a variety of catalysts was tried. In this experiment, two commercially available nucleophilic catalysts namely 1-hydroxybenzotriazole (HOBt) and 4-dimethylaminopyridine (DMAP) were chosen to compare their ability as a catalyst in this carboxylic acid activation reaction. Both compounds were reported to be good acylating catalyst. HOBt are usually employed as an additive in coupling reaction of peptide synthesis, especially in conjunction with DCC.[6,7] As for DMAP, it is a catalyst for acylation of alcohols or amines, especially for acylation of tertiary or hindered alcohols and phenols and for macrolactonization.[40-41] In addition, it is used to catalyse direct esterification of carboxylic acids and alcohols in the presence of DCC (Steglich-Hassner esterification).[42] The effect of these catalysts was examined by the reaction of reagent (**3a**) with benzoic acid under the same reaction condition using DMF as solvent. The result are presented in Table 3.4.

Table 3.4 Effect of catalysts in sulfonation reaction

Catalyst	Equivalent	Reaction Time (min)	Product (%)
HOBt.H_2O	1.0	30	90
DMAP	1.0	overnight	no reaction

As shown in Table 3.4, when benzoic acid was allowed to react with reagent (**3a**) in the presence of an equimolar amount of HOBt.H_2O in DMF, the reaction proceeded smoothly and was completed within a few hours at room temperature to give the desired product, 4-nitrophenyl benzoate. After simple aqueous work up by acid-base extraction, the practically pure product was obtained in 90% yield. Thus HOBt could be employed as a good catalyst in this carboxylic acid activation reaction. Furthermore, HOBt used here is not necessary to be anhydrous, the inexpensive monohydrate worked just fine. Under similar condition, DMAP was totally

ineffective in catalysing this reaction. This was confirmed by allowing reagent (**3a**) to directly react with DMAP under the same condition and no reaction was observed.

Next, an appropriate amount of $HOBt.H_2O$ in catalysing sulfonation reaction was investigated by using the same reaction. It was found that decreasing the amounts of $HOBt.H_2O$ added to 0.5, 0.1 eq did not cause negative effects both in yield and the reaction time but no reaction took place in the absence of $HOBt.H_2O$. The results are tabulated in Table 3.5.

Table 3.5 Effect of the amounts of $HOBt.H_2O$ in catalysing carboxylic acid activation reaction

Equivalent of $HOBt.H_2O$	Reaction Time (min)	Product (%)
1.0	30	90
0.5	30	91
0.1	30	90

3.2.4 Reagent Type IV: Aryl 4-nitrobenzenesulfonates

It was though that replacing the 4-methyl group in aryl *p*-toluenesulfonate by an electron withdrawing group would give an even more reactive reagent. Therefore, another type of sulfonyl derivatives, aryl 4-nitrobenzenesulfonates (type IV) were also synthesized and their ability as coupling reagents in the synthesis of active ester was examined. They were prepared from the reaction of 4-nitrobenzenesulfonyl chloride with phenols under basic condition similar to the preparation of aryl *p*-toluene-sulfonates. When benzoic acid was treated with reagent (**4a**) in the presence of $HOBt.H_2O$ (0.1 eq) as a catalyst in DMF, the reaction was completed within 20 min at room temperature to give 4-nitrophenyl benzoate as when reagent (**3a**) was used. Other aryl *p*-toluenesulfonates (**4b-4d**) reacted similarly with benzoic acid under the same condition by using dichloromethane instead of DMF giving the corresponding aryl benzoates in good yield (Table 3.6). The results in Table 3.6 indicated that aryl 4-nitrobenzenesulfonates are more reactive than aryl *p*-toluenesulfonates as expected.

Furthermore, highly polar solvent such as DMF is not necessary because they still reacted with benzoic acid to give the corresponding aryl benzoates in high yields despite utilizing dichloromethane as solvent. However, of all four aryl 4-nitro-benzenesulfonates, reagent (**4a**) possesses the least reactivity, because its reaction in dichloromethane provided only 50% of the desired product as indicated by TLC which probaly due to the poorest ability of 4-nitrophenoxide ion as a leaving group.

Table 3.6 Reaction of benzoic acid with aryl 4-nitrobenzenesulfonates

Reagent	HOBt.H_2O (eq.)	Solvent	Reaction Time (min)	Product (%)	m.p. (°C)
4a	0.1	DMF	20	92	88-89
	0.1	CH_2Cl_2	overnight	50	88-89
4b	0.1	CH_2Cl_2	20	90	145-146
4c	0.1	CH_2Cl_2	20	97	74-75
4d	0.1	CH_2Cl_2	20	87	163-164

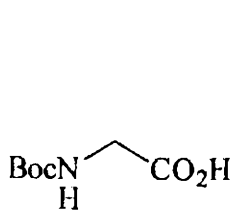
From the aforementioned experimental results, the preferred conditions for carboxylic acid activation were as follows : carboxylic acid (1 eq), aryl 4-nitro-benzenesulfonates (1 eq), triethylamine (1 eq) and HOBt.H_2O (0.1 eq) as a catalyst in DMF or dichloromethane using the reaction time about 20-30 min. This standard conditions were used in all further experiments.

3.3 Synthesis of Aryl Esters of *N*-protected amino acids

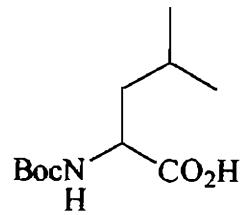
Before applying the newly developed reagents for peptide synthesis, the synthesis of active esters of *N*-protected amino acids was examined. Active esters of amino acid derivatives represent one of the most important classes of activation for peptide coupling because they are at low level of activation, side-reactions during coupling including racemization which are generally less problematic than with most peptide bond forming procedures.[43-45] In this study, the choice of *N*-protecting group was limited to only two popular urethane-type protecting group, namely *t*-butoxy-carbonyl (Boc) and 9-fluorenylmethoxycarbonyl (Fmoc). The Boc group was chosen first because it is less expensive and more stable towards nucleophiles and bases although nowadays its uses are much less frequent than Fmoc group for modern peptide synthesis.

3.3.1 Synthesis of aryl esters of *N*-Boc protected amino acids

Firstly, protection of amino acids were achieved by treatment of the amino acids with di-t-butyl dicarbonate (Boc_2O) in the presence of 10% excess NaOH in *t*-butanol/water (1:1) mixture. The reaction of Boc-Gly-OH and Boc-L-Leu-OH with aryl 4-nitrobenzenesulfonates (**4a-4d**) under standard condition was subsequently examined. The results are shown in Table 3.7. Since Boc-amino acids contained no chromophore, it was not possible to follow the reaction by UV. However, they could be monitored by ninhydrin test after the Boc group was cleaved by trifluoroacetic acid (TFA) vapour to reveal the free $-NH_2$ group as red to purple spots.



Boc-Gly-OH



Boc-L-Leu-OH

Table 3.7 Aryl esters of *N*-Boc protected amino acids from aryl 4-nitrobenzene-sulfonates

Acid	Reagent	$HOBt.H_2O$ (eq.)	Product (%)	$[\alpha]_D^{26}$ ($CHCl_3$)	m.p. (°C)
Boc-Gly-OH	**4a**	0.5	81	-	55-57
	4b	0.1	91	-	104-105
	4c	0.1	93	-	74-75
	4d	0.1	80	-	132-133
Boc-Leu-OH	**4a**	0.5	65	-[a]	64-66
	4b	0.1	90	-23.9 (*c*=1.42)	62-63
	4c	0.1	80	-16.4 (*c*=2.34)	oil
	4d	0.1	27	-[a]	oil

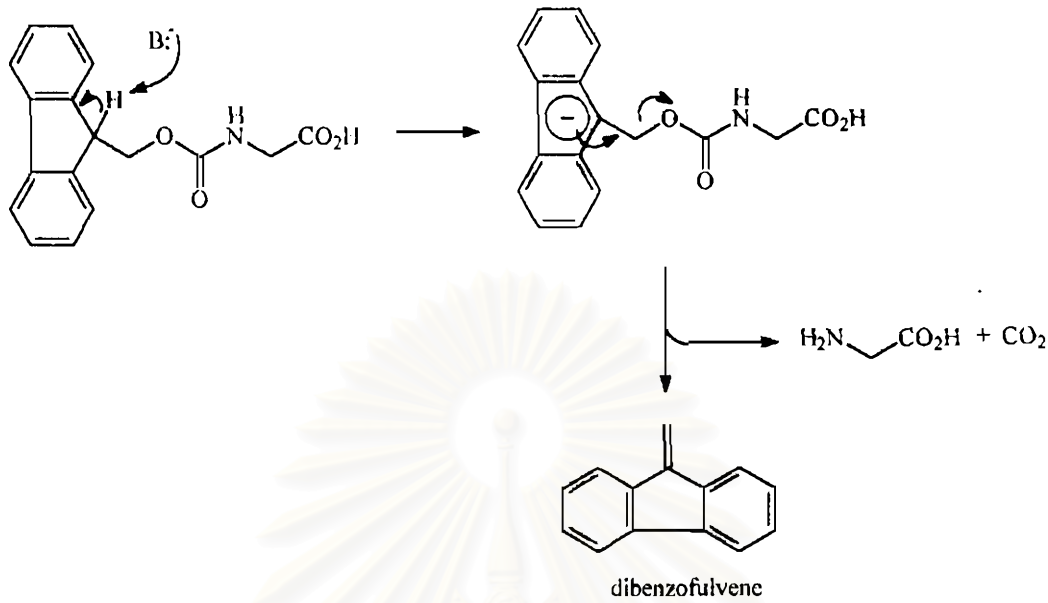
[a] $[\alpha]_D$ value was not measured.

According to Table 3.7, it was observed that reagent (**4b**) and (**4c**) , 2,4,5-trichlorophenyl and pentafluorophenyl 4-nitrobenzenesulfonate, are the best reagent. The expected aryl esters of both Boc-Gly-OH and Boc-Leu-OH were obtained in good to high yields (over 80%). Considering the reaction with Boc-Gly-OH, reagent (**4a**) was less reactive than other reagents, since up to 0.5 eq of HOBt had to be used for catalysing the reaction. In case of Boc-L-Leu-OH, both reagent (**4a**) and (**4d**) afforded the poor yield. Especially the reaction with reagent (**4d**) provided only 27% of the desired product. The reason to explain this result was possibly due to steric effect. Reagent (**4d**) contains large pentachlorophenyl group, so the reaction was difficult to occur when amino acids carrying large side-chain such as leucine were employed.

From the aforementioned reasons, only reagent (**4b**) and (**4c**) were powerful reagents for efficient synthesis of aryl esters of amino acids. Incidentally, only pentafluorophenyl and trichlorophenyl esters of amino acids are widely used in modern practice of peptide synthesis. Reagent (**4b**) and (**4c**) were therefore selected to utilize in the next application. Because of the poor reactivity and yield, reagent (**4a**) and (**4d**) were not tested in the next experiments.

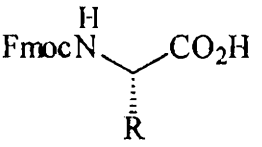
3.3.2 Synthesis of aryl esters of *N*-Fmoc protected amino acids

N-Fmoc protected amino acid was the next target to be investigated. The Fmoc group can be removed by basic reagents under mild conditions and yet is quite resistant to acidolysis.[45] It is therefore completely orthogonal to the Boc group. Based on the observed reaction of aryl 4-nitrobenzenesulfonates with *N*-Boc protected amino acids, the reaction of *N*-Fmoc protected amino acid with reagent (**4b**) and (**4c**) was attempted. The reaction of Fmoc-Gly-OH with reagent (**4b**) was first carried out under standard condition as follows : Fmoc-Gly-OH (0.3 mmol), reagent (**4b**) (0.3 mmol), triethylamine (0.3 mmol) and HOBt.H_2O (0.03 mmol) as a catalyst in DMF (3 mL). Unfortunately, no desired product was obtained at all, but only extensive cleavage of the Fmoc group was observed. The only product isolated by column chromatography was dibenzofulvene. This implies that triethylamine was strong enough base to induce cleavage of the Fmoc group. The mechanism of the cleavage is E_1CB *via* the stabilized dibenzocyclopentadienide anion as shown in Scheme 3.3.



Scheme 3.3: The mechanism of the Fmoc group removal

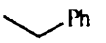
Next, the sterically hindered diisopropylethylamine (DIEA) was chosen in place of the more common trietylamine because it has been previously shown that the Fmoc group is only very slowly deprotected by this base even at a relatively high concentration.[48] The reaction of Fmoc-Gly-OH with reagent (**4b**) was thus tried again by using DIEA as a base under the same condition. It was found that the reaction took place smoothly and was completed within 30 min to give the desired product, Fmoc glycine 2,4,5-trichlorophenyl ester, in 98% yield. Most importantly, no elimination of the Fmoc group was observed. A variety of *N*-Fmoc protected amino acids reacted similarly with reagent (**4b**) and (**4c**) under the same condition giving the 2,4,5-trichlorophenyl esters and pentafluorophenyl esters in good yield as shown in Table 3.8. All products have been characterized by ^{1}H NMR and microanalysis (C, H, N). Other physical data including melting point and optical rotation are also consistent with literature values, suggesting that no signification racemization took place during the reaction.

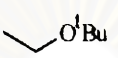


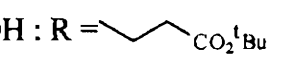
Fmoc-Gly-OH : R = H

Fmoc-Val-OH : R = 

Fmoc-Met-OH : R = 

Fmoc-Phe-OH : R = 

Fmoc-Ser(O^{t}Bu)-OH : R = 

Fmoc-Glu(O^{t}Bu)-OH : R = 

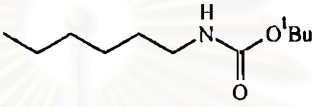
Fmoc-Lys(Boc)-OH : R = 

Table 3.8 Aryl esters of *N*-Fmoc protected amino acids from reagent (**4b**), (**4c**)

Amino acid	Reagent	Product (%)[a]	$[\alpha]_D^{26}$ ($CHCl_3$)	m.p. (°C)
Fmoc-Gly-OH	**4b**	98	-	143-144
	4c	87	-	154-156
Fmoc-Val-OH	**4b**	96	-30.2 (*c*=1.32)	145-146
	4c	80(95)	-22.4 (*c*=1.32)	119-121
Fmoc-Met-OH	**4b**	88(90)	-15.7 (*c*=1.36)	152-153
	4c	93(95)	-11.9 (*c*= 1.32)	110-111
Fmoc-Phe-OH	**4b**	93(97)	-29.4 (*c*= 1.15)	184-185
	4c	91(94)	-20.7 (*c*= 1.42)	148-149
Fmoc-D-Ser(O^{t}Bu)-OH	**4b**	83(97)	+16.0 (*c*= 1.76)	oil
	4c	65(87)	+11.4 (*c*=1.84)	oil
Fmoc-Ser(O^{t}Bu)-OH	**4b**	87(99)	-16.2 (*c*= 3.28)	oil
	4c	80(94)	-11.1 (*c*= 2.66)	oil
Fmoc-Glu(O^{t}Bu)-OH	**4b**	79(95)	-18.5 (*c*= 1.05)	140-141
	4c	61(93)	-17.7 (*c*=1.06)	116-117
Fmoc-Lys(Boc)-OH	**4b**	93	-17.7 (*c*= 1.37)	109-110
	4c	88(90)	-15.0 (*c*=1.41)	103-104

[a] yield after purification by passing through a short siliga gel column (crude yield in parenthesis)

In summary, aryl esters of Boc- and Fmoc-protected amino acids could be easily prepared by using reagent (**4b**) and (**4c**) and HOBt as a catalyst in the presence of triethylamine or DIEA for Fmoc amino acids in DMF at room temperature.

3.4 Synthesis of Dipeptides

The next goal was to extend the application of our reagents in peptide synthesis without prior isolation of the active ester intermediate. Kinetic studies have shown the superiority of pentafluorophenyl ester compared to other coupling reagents with respect to speed of the coupling[48] and pentafluorophenyl ester has been used as preformed active ester for coupling reaction in both the solution and the solid phase peptide synthesis.[49] Therefore, only pentafluorophenyl 4-nitrobenzenesulfonate (**4c**) was selected as a reagent to synthesize dipeptides in this section.

By using reagent (**4c**) in the presence of catalytic amounts of HOBt, dipeptides could be prepared from *N*-protected amino acids and amino acid ester hydrochlorides under the same condition as that for preparing aryl ester of *N*-protected amino acid except that two equimolar amounts of base was used. One equivalent of base was employed to generate the carboxylate anion in the first step and the other was added in order to generate the free amine from the salt. Since this coupling reaction would occur *via* the formation of active ester, the carboxy component would be activated by coupling reagent before addition of the amino component. So the procedure is as follows : a solution of amino acid (0.3 mmol), reagent (**4c**) (0.3 mmol) and HOBt.H_2O (0.03 mmol) in the presence of base (triethylamine or DIEA) (0.3 mmol) in DMF was stirred about 15 min, followed by adding the solution of amino acid ester hydrochloride (0.3 mmol) and base (0.3 mmol) in DMF and the stirring was continued until starting materials was used up. Monitoring the reaction by TLC revealed that all reactions were completed within 1 hour in most cases. Many Boc and Fmoc dipeptide esters were synthesized with excellent yield including those with sterically hindered carbonyl and/or amino part such as Fmoc-Val-Sar-OEt as shown in Table 3.9. An alternative method involving mixing amino acid (0.3 mmol), amino acid ester hydrochloride (0.3 mmol), reagent (**4c**) (0.3 mmol) and HOBt.H_2O (0.03 mmol) together gave the same results. Interestingly, although -NH_2 group is better

nucleophile than carboxyl group, but the carboxyl group reacted with the reagent first as evidenced by the absence of *N*-nitrobenzenesulfonyl derivative of the amino component.

Table 3.9 Dipeptide esters from reagent (**4c**) with HOBt

Dipeptide ester	Product (%)	$[\alpha]_D^{20}$ ($CHCl_3$)	m.p. (°C)
Boc-Gly-Gly-OEt	90	-	oil
Boc-Gly-Ala-OMe	82	+10.7 (*c*= 1.00)	oil
Boc-Gly-Sar-OEt	92	-	oil
Boc-Leu-Gly-OEt	95	-20.0 (*c*= 1.02)	76-78
Boc-Leu-Ala-OMe	84	-31.7 (*c*= 1.00)	97-99
Boc-Phe-Leu-OMe	94	+15.1 (*c*=1.01)	98-100
Fmoc-Gly-Gly-OEt	96	-	115-116
Fmoc-Val-Gly-OEt	97	-18.0 (*c*= 1.00)	197-198
Fmoc-Val-Ala-OMe	91	-18.3 (*c*= 1.02)	208-209
Fmoc-Val-Sar-OEt	95	-14.5 (*c*= 1.03)	oil
Fmoc-Phe-Gly-OEt	94	-14.2 (*c*= 1.02)	185-187
Fmoc-Lys(Boc)-Sar-OEt	92	+0.50 (*c*= 1.02)	oil
Fmoc-Ser(O^{t}Bu)-Leu-OMe	96	+22.5 (*c*=1.01)	105-106
Fmoc-Trp(Boc)-Gly-OEt	94	-10.3 (*c*=1.11)	80-81

All dipeptides gave clean ^{1}H NMR spectra and provided satisfactory elemental analysis (C, H, N). It should be noted that ^{1}H NMR spectra of dipeptides containing sarcosine, which is secondary amino acid, showed two sets of signals for each proton, notably the two singlets of the CH_3 group. This was attributed to the presence of two conformations due to the restricted rotation around the C-N bond (Figure 3.2). The two rotamers coexist in equilibrium and give rise to different sets of signals fo protons in each of them.

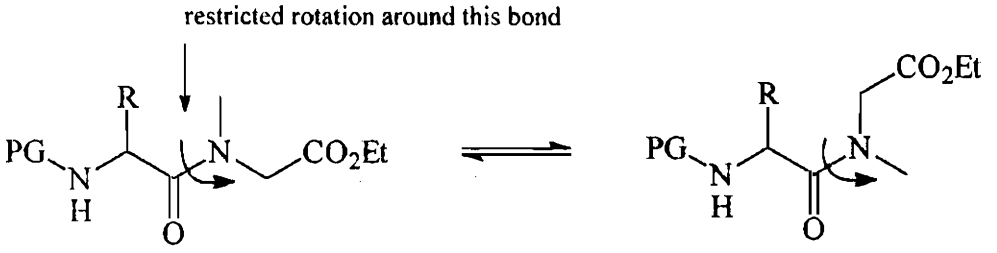
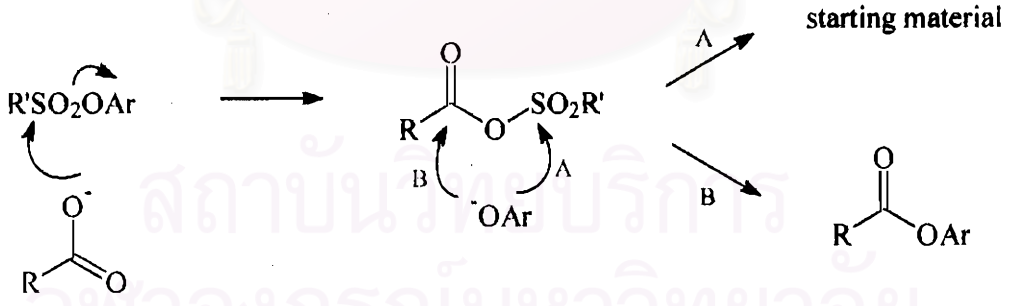


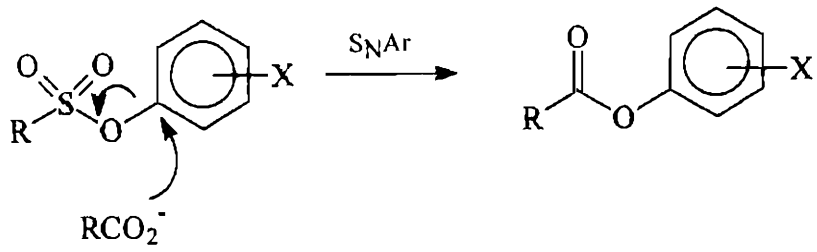
Figure 3.2: Restricted rotation around C-N bond in dipeptides containing sarcosine

3.5 The Study of Mechanism

The mechanism of sulfonate esters as peptide coupling reagents was believed to take place *via* the formation of aryl esters.[25-28] Two reaction pathways may be proposed. One involves the formation of a mixed carboxylic-sulfonic acid anhydride which is obtained from nucleophilic substitution by carboxylate ion at the sulfur atom and is further attacked by phenoxide ion liberated from the previous step as shown in Scheme 3.4. Another mechanism may proceed *via* S_NAr mechanism by direct nucleophilic attack of the carboxylate ion at the *ipso*-C of the reagent as shown in Scheme 3.5.

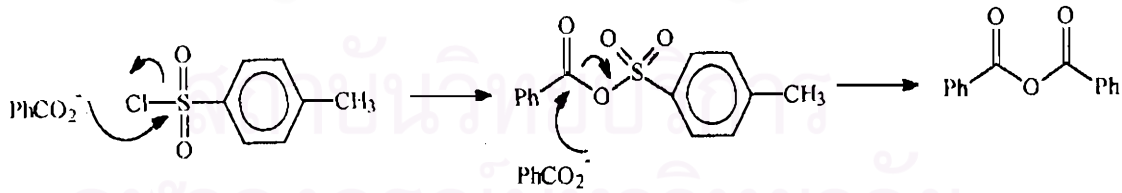


Scheme 3.4: The mechanism of aryl sulfonate as coupling reagent *via* the formation of a mixed carboxylic-sulfonic acid anydride



Scheme 3.5: The mechanism of aryl sulfonate as coupling reagent *via* S_NAr mechanism

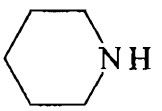
In this study, attempts have been made to confirm the former proposed mechanism. Synthesis of a mixed carboxylic-sulfonic acid anhydride, which is believed to be the intermediate of this reaction, by the reaction of tosyl chloride with benzoic acid under basic conditions[31] failed. Only benzoic anhydride, an unexpected product, was obtained as indicated by IR spectrum of the product compared to that of DCC-mediated coupling of benzoic acid. It was also observed that only half-equivalent amount of tosyl chloride was consumed in the reaction. This indicated that benzoic acid probably first reacted with tosyl chloride to form a mixed anhydride, a highly reactive acylating agent, which further reacted with another benzoate ion to give benzoic anhydride as shown in Scheme 3.6.



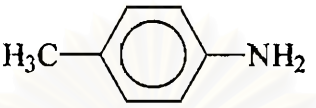
Scheme 3.6: Reaction of benzoic acid with tosyl chloride

Since the existence of mixed carboxylic-sulfonic acid anhydride could not be proven by direct isolation, evidences against the alternative mechanism, *ie*, direct S_NAr were saught. Uncatalyzed S_NAr-type nucleophilic displacement also previously observed, for example, reactions between 4-nitrophenyl 4-nitrobenzenesulfonate with morpholine under harsh conditions gave 4-nitrophenylmorpholine together with a

considerable amounts of 4-nitrobenzenesulfonyl morpholine.[50] Thus, the reactions of 2,4,5-trichlorophenyl 4-nitrobenzenesulfonate (**4b**) with various nucleophiles such as piperidine, *p*-toluidine and morpholine under the same conditions were explored (Table 3.10).



piperidine



p-toluidine

morpholine

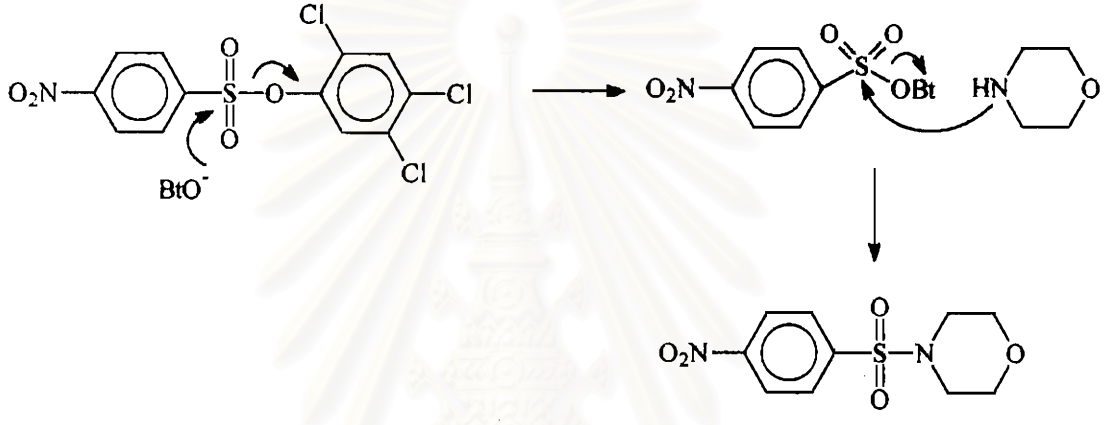
Table 3.10 Reaction of reagent (**4b**)with various nucleophiles in DMF

Nucleophile	pK_a[31]	Condition	Reaction time (min)	Product (%)
piperidine	11.12	no $HOBt.H_2O$	30	87
		$HOBt.H_2O$ 0.1 eq	10	83
p-toluidine	5.08	no $HOBt.H_2O$	overnight	no reaction
		$HOBt.H_2O$ 0.1 eq	overnight	no reaction
morpholine	8.33	no $HOBt.H_2O$	120	< 10[a]
		$HOBt.H_2O$ 0.1 eq	30	90

[a] The yield was shown as judged by TLC.

As shown in Table 3.10, piperidine and *p*-toluidine were unsuitable nucleophile for this study. Piperidine was too strong nucleophile, since the reaction was rapid and completed within minutes even in the absence of HOBt. But *p*-toluidine was too weak nucleophile, since no reaction occurred in both cases. As expected on the basis of pK_a value, morpholine gave good results. When morpholine reacted with reagent (**4b**) in the presence of HOBt, 4-nitrobenzenesulfonyl morpholine was the only product obtained and no 2,4,5-trichlorophenylmorpholine, the S_NAr product, was observed at all. In addition, when morpholine reacted with reagent (**4b**) in the absence of HOBt, the reaction proceeded slowly and not completed which

emphasize the role of HOBt in catalysing the sulfonation reaction. We proposed that HOBt, a strong nucleophile, probably catalyzed this reaction by first reacting with 2,4,5-trichlorophenyl 4-nitrobenzenesulfonate to give the benzotriazolyl 4-nitro-benzenesulfonate. Since OBt^- is a very good leaving group, this reactive intermediate will further react with morpholine to form 4-nitrobenzenesulfonyl morpholine as the product (Scheme 3.7).

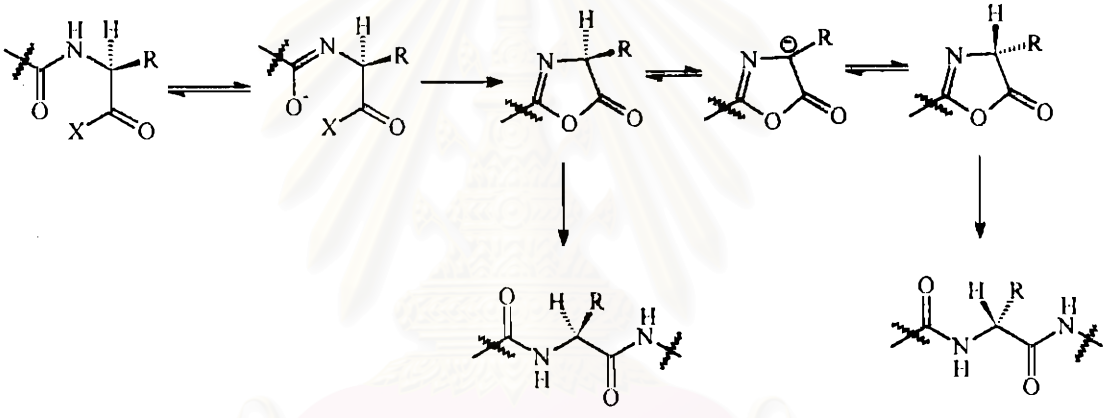


Scheme 3.7: A proposed mechanism of reaction of reagent (**4b**) with morpholine in the presence of HOBt

Therefore, based on the evidences provided above we proposed the mechanism of aryl sulfonates mediated peptide coupling in the presence of HOBt to involve HOBt-catalysis in the first step by reacting with aryl sulfonates to give the corresponding benzotriazolyl sulfonates. Such compounds were known to react with carboxylate ions to give mixed carboxylic-sulfonic acid anydrides,[26,27] which could react further with phenoxide ions generated in the first step to form aryl esters as previously described. Dipeptides would be obtained after adding an amine component. In order to prove this hypothesis, the reaction of reagent (**4a**) with HOBt was attempted. In spite of the complete disappearance of the starting materials, no benzotriazolyl 4-nitrobenzenesulfonate could be isolated. The explanation is that it may decompose during the reaction or during work up due to its high reactivity.

3.6 The Study of Racemization

Racemization is one of the most serious problem in peptide synthesis. In particular, when an *N*-acyl-protected amino acid was activated for coupling with the amino component, racemization of such *N*-protected amino acid occurs to some extent through an oxazolone intermediate[4] (Scheme 3.8). The oxazolone so formed are themselves activated towards aminolysis, and reaction with amino components leads ultimately to peptides, but since their racemization *via* stabilized anions is usually fast compared to the rate of peptide bond formation, any peptide thus produced is largely racemized.



Scheme 3.8: Racemization of peptide by oxazolone intermediate

3.6.1 The study of racemization of *N*-acyl-protected amino acid

The model peptide coupling between Bz-L-Phe-OH and L-Leu-OMe was chosen because this model system suffers extremely large degree of racemization and ester signals of diastereomeric pair of resulting peptide on ^{1}H NMR spectrum separate clearly as shown in Figure 3.3.

In order to evaluate the effectiveness of aryl 4-nitrobenzenesulfonates as compared to other reagents, Bz-L-Phe-L-Leu-OMe was synthesized by various coupling reagents such as DCC, DCC+HOBt, HBTU and reagent (**4c**)+HOBt under the same condition by using DMF as solvent and triethylamine as base. The degree of

racemization was calculated from the ratios of -OCH_3 peaks of the two diastereomer at 3.56 and 3.68 ppm on ^1H NMR spectrum of the crude product after aqueous work up (Figure 109) and are tabulated in Table 3.11.

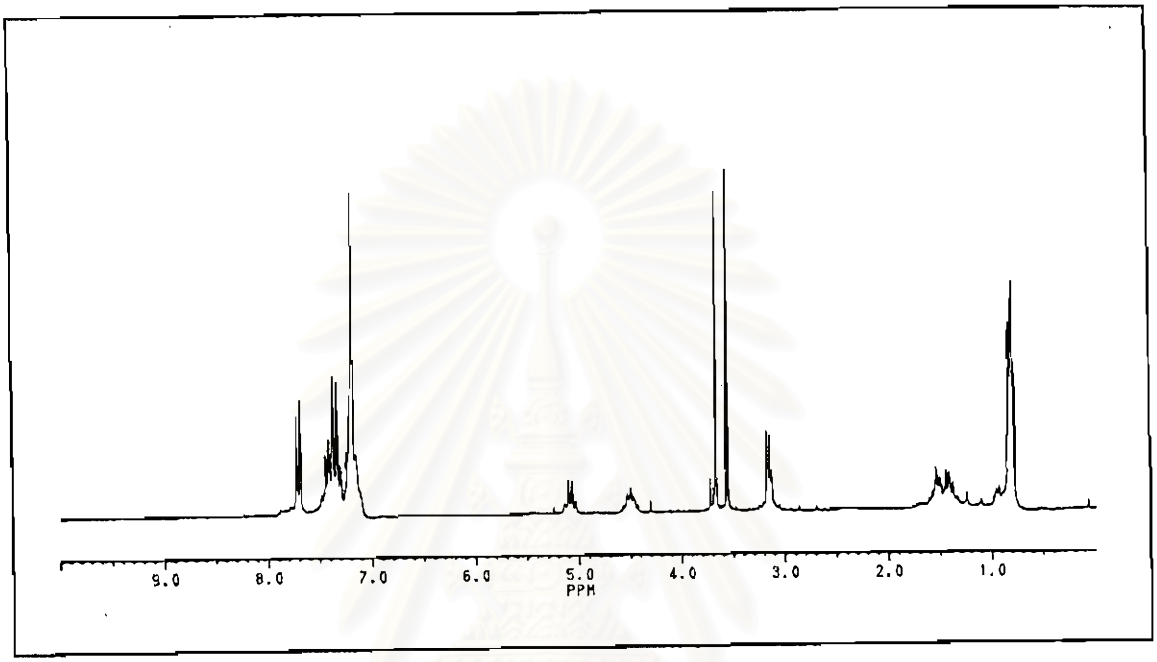


Figure 3.3: ^1H NMR (200 MHz) spectrum of Bz-DL-Phe-L-Leu-OMe

Table 3.11 Racemization during the coupling of Bz-L-Phe-OH with L-Leu-OMe

Reagent	degree of racemization (%)
DCC	-[a]
DCC + HOBt.H_2O 1 eq	70
HBTU	68
(**4c**) + HOBt.H_2O 0.1 eq	100

[a] The degree of racemization could not be determined.

As can be seen from Table 3.11, HBTU and DCC+HOBt showed by far the lowest racemization, whereas reagent (**4c**)+HOBt gave completely racemized product. When DCC alone was used as coupling reagent, the degree of racemization could not

be determined because it gave complicated ^{1}H NMR spectrum which resulted from by-products.

Since reagent (**4c**)+HOBt gave unsatisfactory result, variation of the reaction conditions were therefore attempted by changing the solvent, the base and the amounts of HOBt. But it was found that completed racemization occurred in all cases as shown in Table 3.12. This indicated that the intermediate mixed carboxylic-sulfonic acid anhydride are too reactive and underwent racemization before the auxiliary nucleophile have a chance to react with it, a common phenomena observe for this type of activating reagent based on sulfonate esters.

Table 3.12 Racemization during the coupling Bz-L-Phe-L-Leu-OMe with reagent (**4c**)+HOBt under various conditions.

Reagent	HOBt.H_2O (eq)	Base	Solvent	degree of racemization (%)
4c	0.5	Et_3N	DMF	100
	1.0	Et_3N	DMF	100
	1.0	DIEA	DMF	100
	1.0	Et_3N	acetonitrile	100
	2.0	Et_3N	DMF	100

3.6.2 The study of racemization of urethane-protected amino acid

Boc-L-Phe-OH are chosen a representative of urethane-type acylamino acid in this experiment. The model peptide, Boc-L-Phe-L-Leu-OMe, was selected as a model because ^{1}H NMR spectrum (500 MHz, $CDCl_3$) showed two sets of isopropyl signals of diastereomeric pair as shown in Figure 3.4. In the same manner as *N*-acyl protected amino acid, Boc-L-Phe-L-Leu-OMe was prepared from DCC, DCC+HOBt, HBTU and reagent (**4c**)+HOBt. The degree of racemization was determined by ^{1}H NMR and are reported in Table 3.13.

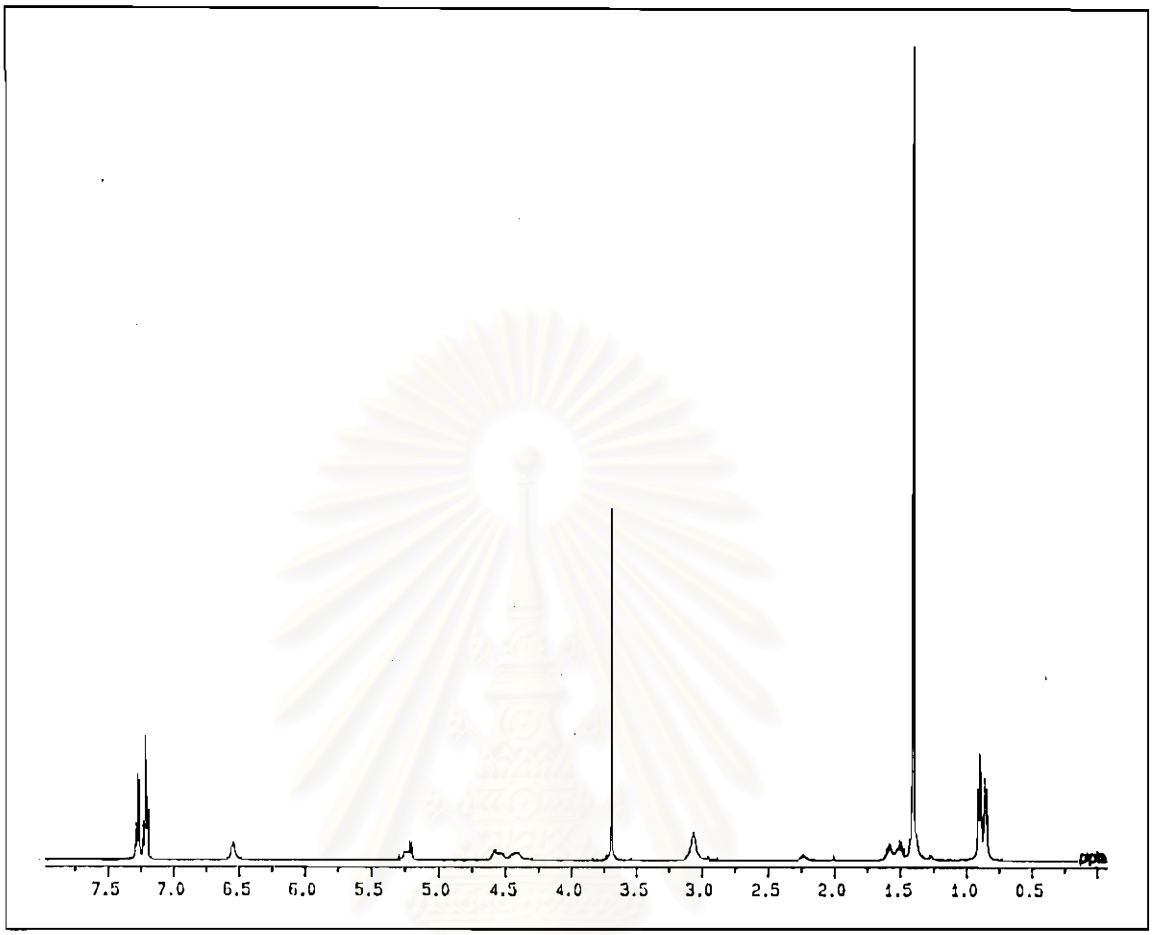


Figure 3.4: ^{1}H NMR (500 MHz) spectrum of Boc-DL-Phe-L-Leu-OMe

Table 3.13 Racemization during the coupling of Boc-PheOH with LeuOMe

Reagent	Reaction time (min)	degree of racemization (%)
DCC	overnight	-[a]
DCC + HOBt.H_2O 0.1 eq	overnight	<5
HBTU	60	<5
(4c) + HOBt.H_2O 0.1 eq	60	<5

[a] The degree of racemization could not be determined.

As shown in Table 3.13, HBTU, DCC+HOBt, and reagent (4c)+HOBt showed no significant racemization during this coupling reaction , whereas DCC gave the complicate ^{1}H NMR spectrum , therefore degree of racemization could thus not be determined. All reactions were completed within 1 hour except DCC+HOBt required a long time before the reaction was completed.

From ^{1}H NMR spectra as shown in Figure 111, reagent (**4c**)+HOBt gave the cleanest spectrum, while DCC+HOBt showed complicated signals around 1.0-2.0 ppm and douplet peak at 3.0 ppm due to the presence of dicyclohexylurea (DCU). Both HBTU and reagent (**4c**)+HOBt afforded clean spectrum. Therefore, reagent (**4c**)+HOBt performs at least as good as HBTU, which is one of the most effective coupling reagent, during coupling reaction of urethane-protected amino acid.