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INVESTIGATIONS OF A ONE-STEP AQUEOUS-PHASE CHLOROFORMATE DERIVATIZATION REACTION FOR THE ANALYSIS OF AMINO ACIDS BY GC/GC-MS AND SMALL PEPTIDES BY FAB-MS presented by

Jian Wang

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Chemistry

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INVESTIGATIONS OF A ONE-STEP AQUEOUS-PHASE CHLOROFORMATE DERIVATIZATION REACTION FOR THE ANALYSIS OF AMINO ACIDS BY GC/GC-MS AND SMALL PEPTIDES BY FAB-MS

By

Jian Wang

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Submitted to

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DOCTOR OF PHILOSOPHY

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1994

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ABSTRACT

INVESTIGATIONS OF A ONE-STEP AQUEOUS-PHASE CHLOROFORMATE DERIVATIZATION REACTION FOR THE ANALYSIS OF AMINO ACIDS BY GC/GC-MS AND SMALL PEPTIDES BY FAB-MS

By

Jian Wang

In gas chromatography (GC)-electron impact (EI) mass spectrometry (MS) of organic mixtures, one of the most severe restrictions is the requirement that sample has to be in the gas phase for separation by GC, ionization, and subsequent analysis in the mass spectrometer. Derivatization is generally used to reduce the polarity of analyte molecules by chemically replacing active hydrogens, thereby increasing volatility and promoting thermal stability. Although techniques of desorption ionization mass spectrometry, such as fast atom bombardment (FAB), have the capacity to analyze polar, nonvolatile, and thermally labile compounds (such as polypeptides, oligosaccharides, oligonucleotides, and other small biopolymers) by sampling analytes directly from a condensed phase, derivatization still can be utilized to augment the amount of information available from the analysis.

This dissertation focuses on: (1) derivatization-assisted amino acid analysis by GC-MS in EI, positive CI and negative CI modes and (2)

derivatization-assisted peptide analysis by FAB-MS. For the analysis of amino acids, the one-step aqueous medium chloroformate derivatization method introduced by Husek for analysis by GC has been extended to analysis by GC-MS, and the structurally diagnostic fragmentation of the amino acid alkyl chloroformate derivatives was also studied. Modification of the derivatization procedure has been conducted. An extended examination of the derivatization method with the combination of a variety of alkyl chloroformates and alcohols has been carried out. Fluorinated derivatives based on a modified reaction procedure, in combination with electron capture negative ionization (ECNI) MS analysis has been evaluated for increasing the sensitivity of analysis. A collaborative research project to quantitatively assess the incorporation of stable isotope-labeled amino acids into photosynthetic proteins with the chloroformate derivatization has been carried out. For peptide analysis, investigation of the chloroformate derivatization for small peptides prior to analysis by FAB-MS, and a comprehensive evaluation of the derivatization conditions have been completed. The advantages and the limitations of the one-step aqueous medium chloroformate derivatization method for these analytes have been further investigated.

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Thank the

school.

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I would like to thank my Dad, Mom, brother for all the support they have provided in my life. I would also like to thank my wife for her help and love contributed during the years I was in college and in graduate school.

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Finally, I would say, thank all my teachers and friends in my kindergarten, elementary school, middle schools, high school, and college. Thank them for all the help during different times of my twenty years in school.

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Chapter I

Introduction and objectives

L Introduction

The reasons for utilizing chemical derivatization in analyses by mass spectrometry have been summarized by Knapp [1] as follows: (1) enhancement of volatility; (2) degradation of the sample molecule to smaller subunits; (3) enhancement of detectability; (4) enhancement of separability; (5) modification of fragmentation: (a) enhancement of molecular weightrelated ions and (b) enhancement of structurally informative ions; (6) determination of functional groups.

In gas chromatography (GC)-electron impact (EI) mass spectrometry (MS) of organic mixtures, one of the most severe restrictions is the requirement that sample be in the gas phase for separation by GC, ionization, and subsequent analysis in the mass spectrometer. Derivatization is generally used to reduce the polarity of a molecule by chemically replacing active hydrogens, thereby increasing volatility and promoting thermal stability. Although desorption ionization mass spectrometry, such as fast atom bombardment (FAB), has the capacity to analyze polar, nonvolatile, and thermally labile compounds (such as polypeptides, oligosaccharides, oligonucleotides, and other small biopolymers) by sampling analytes directly from a condensed phase, derivatization still can be utilized to augment the amount of information available from the analysis.

The objectives of this first chapter are to 1) review and discuss the procedures and characteristics of chemical derivatization methods for amino acid analysis by GC and GC-MS, 2) introduce FAB ionization and

instrume derivatiza FAB, and II. Deriva A. Th prerequi hence, to an enzyr membra represer blocks o the cell vehicles particip in meta for bios compos transpo S a carbo fourth Particu in prote aromat ^{acids} an instrumentation, 3) review and discuss the different strategies and derivatization methods employed to enhance the analysis of peptides by FAB, and 4) state the research objectives of this dissertation.

II. Derivatization of amino acids for gas phase analysis by GC and GC-MS

A. Introduction

The determination of amino acid composition is a fundamental prerequisite to the definition of the structure of a protein or peptide and, hence, to the understanding of functional properties, whether the protein be an enzyme, a transport protein, or a protein crucial to the integrity of a cell membrane. Amino acids as inherent constituents of living matter represent more than 50% of the dry weight of a cell, mainly as building blocks of proteins. These protein not only constitute structural elements of the cell architecture, but also function as biocatalysts, as messengers, or as vehicles for the selective transport of biological fuels. Amino acids also participate in cell chemistry in their free forms, occurring as intermediates in metabolism, or serving as nutrients, neurotransmitters, or precursors for biosynthesis of cell constituents. A knowledge of the free amino acid composition of biological fluids is central to studies of metabolism and transport [2,3].

Structural features common to all amino acids are an amino group, a carboxyl group, and a hydrogen attached to a central (α) carbon atom. A fourth substituent of variable structure confers to each amino acid its particular chemical property. Out of twenty amino acids commonly found in proteins (Table 1.1), fifteen amino acids are neutral with an aliphatic or aromatic side chain determining their chemical properties. These amino acids are soluble as zwitter ions with isoelectric points between pH 5 and 7.

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Table 1.1. Structures of protein amino acids,

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STRUCTURE OF R	NAME	ABBRI VIATIO	NZ	STRUCTURE OF R	NAME	ABBI	-NOI
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Ť	Glycine	Gly	G	-CH_CONH_	Glutamine	5	: 0
-сн ²	Alanine	Ala	<			5	,
	Valine	Val	>				
-CH ₁ CH(CH ₃)	Leucine	Leu	-		Tryptophan	Trp	₹
-снсн,сн,	Isoleucine	lle	-) z—x			
ĊH,				0			
-cH ₁ -CH	Phenylalanine	Phe	u.	нос-сн-сн,	Proline	Pro	٩
				HN CH			
R contains a carboxyl group				CM2 (complete structure)			
-CHICOOH	Aspanic acid	Asp	٥				
-сн,сн,соон	Olutamic acid	Glu	w	R contains anOH group			
					Senne	Ser	ŝ
K contains a basic amino group				Нон	Threonine	Ĕ	►
-CHICHICHICHINH	Lysine	Lys	×	ĊН ,			
HN			(-CHOH	Turneine	Ě	>
-CH,CH,CH,NH-C-NH,	Arginine	Arg	Ľ		1 7103105	1	-
ZCH.	Histidine	His	I	0"			
)z-				нос-сн-сн	Hydroxyproline	Нур	P-OH
-1				HN CH.			
R contains sulfur				CH, OH			
-CH,SH	Cysteine	δ	v	(complete structure)			
-CH ₃ -S	Cystine	CyrC	2-0 X				
			1				
-CH3CH3SCH3	Methionine	Met	£				

Table 1.1. Structures of protein amino acids.

The thre meth amin aspar chain Any s separ difficu choser availa chrom ago [4] Moder purcha instrur • have l Determ mol usi HPLC (analyze amino ; relative not all t These side chains may also contain a hydroxyl group (as in serine, threonine, tyrosine), a sulfur-containing group (as in cysteine and methionine), or an amide group (as in asparagine and glutamine). Other amino acids carry an additional carboxyl group, as in acidic amino acids aspartic and glutamic acid, or a nitrogen-containing function on the side chain of basic amino acids arginine, histidine and lysine.

There is no analytical panacea applicable to assaying amino acids. Any single technique may confer advantages in specific situations, and a separation that is readily achieved by using one method may be very difficult if not impossible by using other means. Frequently, the technique chosen by the individual researcher or analyst is dictated by the equipment available.

The separation of the protein amino acids by ion exchange chromatography led to the first automated instruments some three decades ago [4]. A separation of the twenty protein amino acids took about 2 hours. Modern amino acid analyzers cut the analysis time in half. However, their purchase price and the cost of operation are high, and they are dedicated instruments with little flexibility for other tasks.

The advances in high-performance liquid chromatography (HPLC) have led to rapid and sensitive procedures for assaying amino acids. Determinations of amino acids can now be achieved at levels reaching 10⁻¹⁸ mol using fluorimetric [5-8] or voltameric detection [9-11] combined with HPLC or capillary electrophoresis [7,8]. Although one-purpose amino acid analyzers are no longer essential, routine automated measurements of amino acids with modern, flexible HPLC instruments are not possible with relatively inexpensive systems [11]. Furthermore, although in LC analysis not all the active hydrogen-containing groups need to be treated so that the

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average time for sample pretreatment is shorter than in GC, one hour analysis time is considered as an average for LC performed amino acid analysis.

Soon after the advent of gas liquid chromatography, its application to amino acid analysis was proposed. High sensitivity, great speed of analysis, high resolving power, low cost, and great versatility of the instrument are its expedient features. Perhaps the most important advantage offered by GC over other methods is unsurpassed resolution. The resolving power of HPLC columns is of the order of tens of thousands of theoretical plates per meter, yet it is rare indeed that a column even 1 m long is used. Unlike GC analysis, use of capillary columns in LC lengthens the total time of the chromatographic run [6,10]. Thus, the resolving power brought to bear on the analytical problem is only of the order of a few thousand theoretical plates. In this regard, no other technique offers the capability of long capillary GC column. Complete baseline resolution of the amino acids in a protein hydrolysate can be achieved in about 10 min using short capillary columns, but resolution of this order has not been achieved using any other method in an acceptable analysis time. Furthermore, even in standard amino acid assays, the analyst is faced with not only resolving a few components but also simultaneously resolving and precisely assaying about 20 components. Since the precision and accuracy of the analysis are a function of the separation of the sample components, the importance of achieving optimal resolution is axiomatic. The analysis is much more complex when the sample is a physiological fluid in which any one or more of hundreds of nonproteic amino acids may be present in addition to the standard protein amino acids. In this context, resolving power is a crucial factor in determining the success of the assay. In biological samples, the

nature o specific] from nor variety chromat flexibilit analysis. so many analysis and resol acids in p line coup rapid ide Al nonspeci specific s detector a (MS) pro unquestic chromato technique ^{establish}e ^{applied} to 0n

^{consequer ^{be} quanti ^{The} goal} nature of the sample matrix can often require the development of a tissuespecific procedure for sample cleanup prior to GC to eliminate interference from non-amino acid components. The analyst face with assaying a wide variety of sample types must be prepared to select derivatives and chromatographic columns to suit the purpose of the analysis. This flexibility is not always available when using other methods of amino acid analysis. Furthermore, no other technique offers the potential for resolving so many components in one analysis in less than 1 hour. An ion-exchange analysis of a physiological fluid typically requires 5 hours. The flexibility and resolving power make GC the method of choice for assaying free amino acids in physiological fluids [2]. Another advantage is the possibility of online coupling of a gas chromatograph to a mass spectrometer, enabling rapid identification of unknowns.

Although most gas chromatographic analyses are conducted using a nonspecific flame ionization detector, other detectors can be used to gain specific structural information. Thus a nitrogen- or phosphorus-specific detector could confirm the presence of specific element. Mass spectrometry (MS) provides structural information and a mass spectrometer is unquestionably the most powerful detector that can be coupled to a gas chromatograph. Advances in HPLC/MS in recent years have made this technique valuable for other classes of compounds, but it is not as well established or as generally available as GC/MS, nor has it been as widely applied to the identification of amino acids.

One perceived disadvantage of GC for amino acid analysis is a consequence of the inherent nonvolatility of amino acids. Derivatives must be quantitatively formed to block or remove the polar functional groups. The goal of these derivatization reactions is to reduce the polarity and

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increase the vapor pressure by chemically replacing active hydrogens, thereby increasing volatility and promoting thermal stability of amino acids. Vapor pressure of a compound is influenced by intermolecular attractions due to dispersion (van der Waals) forces, ionic interactions, and hydrogen bonds. The total dispersion forces increase with molecular size and little can be done with respect to these forces to increase volatility other than reduce the size of the molecule. Chemical derivatization for volatility enhancement is aimed at reducing ionic and hydrogen bond interactions by conversion of ionizable groups to nonionizable derivatives (e.g., carboxyl groups to esters); replacing hydrogens bound to heteroatoms (N-H, O-H, S-H) with alkyl, acyl, silyl or other groups; and reducing the polarity of hydrogen bond accepting groups

B. Review of derivatization methods of amino acids for gas phase analysis by gas chromatography

(1) Strategies of amino acids derivatization

Amino acids, as the name indicates, contain an amino and a carboxyl group. These and other polar groups on the side chains must be blocked to reduce intermolecular attractions and to convert amino acids into sufficiently volatile derivatives. This area of research has received considerable attention, as derivatization is decisive for success or failure of gas chromatographic amino acid analysis. It has been intensively studied since the early 1960s. Since the first report 36 years ago of the formation of fully blocked N-trifluoroacetyl amino acid methyl esters which are well suited for gas chromatography [12], a wide range of reagents has been used to mask the functional groups of amino acids. However, most derivatization schemes have either failed to be applicable to all the standard

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amino acids to be expected in a protein hydrolysate or, because of their chromatographic properties or the properties of the column used, failed to be sufficiently resolved during chromatography. It should be borne in mind that the protein amino acids do not represent a single homologous series. Thus, determining the combination of derivatives and chromatographic column to effect the resolution of these derivatives was not an easy task.

When summarizing criteria aimed at the establishment of a hypothetical, ideal procedure for amino acid determination, an "ideal" derivatization should fulfill the following requirements:

- 1. Formation of only one derivative per compound
- 2. Formation of derivatives of high chemical and thermal stability
- 3. Complete and reproducible derivatization over a wide range of concentrations
- 4. No interference with other derivatization
- 5. Derivatization of polyfunctional compounds in minimum number of steps
- 6. A rapid reaction proceeding at room temperature
- 7. Ability to derivatize in an aqueous medium
- 8. Reagents more volatile than derivatives
- 9. Short GC analysis with a good resolution for all the amino acid derivatives
- 10. Low reagent and instrumental cost

The main problem in GC analysis for amino acids is the need for derivatization, commonly involving laborious and multi-step procedures taking 1 hour or more [13], thereby losing the advantage of the speed of GC

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itself. Looking for more convenient and less time-consuming derivatization method has been the goal for many researchers in this area.

(2) Derivatization methods by other investigators

a) N-acyl amino acid alkyl ester

The foundation for the most commonly used derivatization procedure was laid by Gehrke, who developed the procedures for quantitative formation of N(O,S)-trifluoroacetyl (TFA) n-butyl amino acid esters [13-15]. These are but one set of N-acyl amino acid alkyl esters which represent the most commonly used class of derivatives for amino acid analysis by GC based the two-step reactions. Other variants by different research groups include the N-heptafluorobutyryl (HFB) isopropyl esters [16], the N-HFB isobutyl esters [17,18], the N-TFA n-propyl esters [19], and the N-TFA isopropyl esters [20]. The alkyl group has ranged from the methyl to the pentyl ester while the acyl group, usually derived from the corresponding anhydride, has been almost exclusively confined to the acetyl derivatives, its fluorinated analog, or a perfluorinated homolog. N-acyl amino acid alkyl esters are usually formed in two separate reactions, acid-catalyzed esterification followed by acylation. The typical procedure to form n-HFB amino acid isobutyl esters involves transferring the standard amino acid solution and internal standard solution to a sample tube and evaporating to dryness under nitrogen stream at 50°C. To the dried sample, 200 μ l isobutanol-3 N HCl is added, then sample tube is capped and heated for 45 min at 120°C. After cooling, the sample is evaporated to dryness under a nitrogen stream at 40°C, 80 μ l ethyl acetate and 20 μ l heptafluorobutyric anhydride (HFBA) are added, and the mixture is heated 20 min at 110°C. After cooling, evaporate excess HFBA at room temperature under nitrogen

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H₂NCH(I R stream [2,17,21]. Gehrke demonstrated that capillary GC analysis of amino acids using fused silica bonded-phase columns provides data with good precision and in general excellent agreement with ion-exchange analysis [21]. One problem of these procedures based on N-acyl amino acid alkyl esters is that they cannot be used to assay asparagine and glutamine because of conversion to aspartic and glutamic acids, respectively, during acid-catalyzed esterification [2,3]. A third reaction is usually necessary to block the imidazole ring for quantitation of histidine [3,22]. The solubility of the amino acids decrease in higher alcohols.

Naturally, complete derivatization of all functional groups in one step is most attractive. If volatile derivatives could be formed in a single reaction, significant benefits would accrue. These include simplification of the reaction mixtures. Consequent reduction of the number of highly purified reagents and solvents to be obtained or prepared would minimize the potential for introduction of impurities into the reaction. Presumably, the overall derivatization time would be as short as possible.

b) Silvlation

One of the one-step approaches is the silvlation method, a widely used method to make volatile derivatives. Under proper conditions, amino, carboxyl, hydroxyl, carbonyl, and thiol groups are converted to the corresponding trimethylsilyl (TMS) ether or ester by trimethylsilylation [23,24].(Eq.1.1):

 $\begin{array}{c} & \operatorname{N-Si}(\operatorname{CH}_3)_3 \\ & \operatorname{H}_2\operatorname{NCHCOOH} & + & \operatorname{CF}_3\operatorname{C-O-Si}(\operatorname{CH}_3)_3 \longrightarrow (\operatorname{CH}_3)_3\operatorname{Si-NHCHCOO-Si}(\operatorname{CH}_3)_3 + & \operatorname{CF}_3\operatorname{CONH}_2 \\ & \operatorname{I}_R & & \operatorname{I}_R \\ & & \operatorname{R} \end{array}$

(Equation 1.1)

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Silvlation of seventeen of the amino acids was achieved in a closed tube reaction in 15 min at 150°C using bis(TMS)-trifluoroacetamide (BSTFA) as the silvl donor. However, 2.5 hours at 150°C was necessary for the reproducible derivatization of glycine, arginine, and glutamic acid, and similar reaction conditions were recommended for all twenty protein amino acids [25]. Stable derivatives are also formed with glutamine and asparagine, enabling their distinction from glutamic acid and asparatic acid, respectively. The drawbacks are the inherent instability of the resulting TMS derivatives toward hydrolysis. Furthermore, the excessive silulation of nitrogen atoms lead to the formation of multiple derivatives for amino acids [25-27]: especially glycine, glutamic acid, lysine, arginine, histidine and tryptophan present difficulties [3]. Coinjection of excess TMS reagent is required in order to block active sites of the columns. This is often necessary for protection of N-TMS derivatives because trimethylsilylamines and imidazoles are potent reagents themselves and transfer the TMS group easily to hydroxyl groups [3].

The tert.-butyldimethylsilyl (TBDMS) function group was first employed as a more moisture stable alternative to the TMS group. The TBDMS is now widely used for the silylation of hydroxyl and carboxyl groups using N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) as silylating reagent. The TBDMS derivatives were found to have superior GC and mass spectral properties [28,29]. Multifunctional organic acids were quantitatively converted to their TBDMS derivatives, yielding a single peak for each organic acid [30]. In recent years, the TBDMS derivatization has been successfully extended to amino acid analysis, which have been shown to be suitable for assaying asparagine, glutamine, and most of the other protein amino acids [23,31-37].

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Quantitative silvlation has been achieved using TBDMS in dimethylformamide (DMF) by heating at 75°C for 30 min [22]. Mass spectral (EI, CI) analysis of TBDMS amino acids have been conducted. Mass spectra display a characteristic and unique [M-57] fragment ion for each amino acid which often dominates the EI mass spectrum [33], and predominant ions in the spectra reflect losses of part or all of the TBDMS moiety with or without the associated functional group [31]. However, two peaks are obtained for arginine and either derivatization is incomplete or the derivatives degrade in the chromatographic system [22]. A painstaking step for moisture removal from the hydrochloride salts of amino acids still is prerequisite for TMS derivatives. Meanwhile, relatively few nonproteic amino acids have been chromatographed as the TBDMS derivatives. Furthermore, it was pointed out that the *de novo* interpretation of the mass spectra of TBDMS amino acid derivatives is fraught with somewhat more potential for ambiguity than is the interpretation of the spectra of amino acid N-acyl alkyl esters. Therefore, these derivatives are perhaps less suitable for the identification of unknown compounds [2].

c) Others

The cyclic oxazolidinone derivatives of amino acids are formed by condensation with 1,3-dichlorotetrafluoroacetone at room temperature for 15 min in acetonitrile-pyridine solvent. Further treatment with either pentafluoropropionic anhydride (PFPA) or heptafluorobutyric anhydride (HFBA) in a benzene-methanol solvent for at least 30 seconds at room temperature results in derivatives of protein amino acids that can be separated in less than 10 min. Arginine, glutamine, and asparagine cannot be analyzed without additional acylation in heptane at 80°C for 2-3

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min [38,39]. Although the total reaction is not long, it actually involve three reactions.

A modified procedure using a mixture of PFPA and hexafluoroisopropanol (HFIP) was introduced to provide N-PFPA amino acid HFIP ester in one reaction [40,41]. The derivatization reaction needs one hour at 100°C. These derivatives do not form stable anions for all amino acids. They have been used for certain amino acids for special purpose but are not recommended for general analysis.

d) Derivatization in an aqueous medium

Prior to conversion to suitable volatile derivatives, amino acids are isolated from complex aqueous samples mainly by the multi-step ionexchange technique although its inherent drawbacks are well known [42]. There appears to be a need for improvement of sample purification.

Makita et al. [43-46] demonstrated that amino acids were quantitatively extracted with diethyl ether from aqueous media after selective blocking of the active hydrogens on the amino, thiol, imidazole, and phenolic hydroxyl groups by reaction with isobutyl chloroformate (iBuCF). The remaining carboxyl groups were then methylated with diazomethane to form N(O,S)-isobutoxycarbonyl amino acid methyl esters. For the chloroformate reaction, the amino acid solution was buffered by 2.5% sodium carbonate, iBuCF was added, and the reaction mixture was shaken for 10 min at room temperature. Arginine was an exception, in that it was converted into N-isoBOC ornithine methyl ester by treatment with arginase, followed by the above derivatization procedure.

Kim et al. [47,48] combined Makita's N(O,S)-isoBOC procedure for the selective purification of protein (and non protein) amino acids from aqueous

samples, amide or silylation 60°C for reaction i protein a derivatizat amino acid and amide derivatives was exclud

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samples, and the TBDMS derivatization of the carboxyl and remaining amide or aliphatic hydroxyl groups in the N(O,S)-isoBOC amino acids. The silylation reaction producing optimal overall derivatization by heating at 60°C for 15 min in acetonitrile and MTBSTFA. The aqueous isoBCF reaction is an alternative to the conventional ion-exchange clean-up for protein amino acids from aqueous samples prior to the TBDMS derivatization. The TBDMS derivatization of the resulting N(O,S)-isoBOC amino acids offers advantages over the methylation, since polar hydroxyl and amide functions as well as carboxyl groups are converted to TBDMS derivatives which give better results for the GC analysis. Again, arginine was excluded from the study.

e) One-step chloroformate derivatization of amino acids

in aqueous medium.

Husek [49-51] developed a one-step aqueous medium procedure for amino acids derivatization which is uniquely rapid. The derivatization can be performed in a few seconds, and the subsequent capillary GC analysis can be carried out in a few minutes. The total time of sample preparation and the analysis can be as short as 5 min. Amino acids were treated by ethyl (methyl) chloroformate in a water-ethanol(methanol)-pyridine medium (60:32:8) for 3-5 sec. In a single step, the amino, carboxyl, sulfhydryl, phenolic, and imino group on the side chain of histidine are derivatized. Carboxyls were converted to esters, while the other functional groups were converted to N,O- and S-eth(meth)oxycarbonyl derivatives (see Figure 1.1 for the reaction schemes). Arginine analyses requires an

Amino $R-NH_2 +$ Carbox R-COOH Phenoli Ph-OH + (Thiol gr R-SH + C Figure



Figure 1.1. Reactions of chloroformate with functional groups of amino acid.

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III. Derivat (FAB)-mass A. Int 0ver mass spect ionization (SIMS), f desorption ionization (to create g without the thermolysis successful. need for ch less critical researchers important r [57].

additional step such as that in Makita's method [43-46]. A similar procedure was also used to derivatize fatty acids [52-56]. This procedure has the benefits of simple sample handling, the ability to conduct derivatization in aqueous solutions, and the use of inexpensive reagents. A comment in an annual review, <Amino Acids and Peptides> V.24 (1991), was qouted as: "N-acyl derivatives are continuing in use after many years, and are being shadowed by N-alkoxycarbonyl derivatives that have the benefit of being formed from an alkyl chloroformate very rapidly".

III. Derivatization of peptides prior to analysis by fast atom bombardment (FAB)-mass spectrometry

A. Introduction

Over the past fifteen years, many new "soft ionization" techniques for mass spectrometry have emerged, among them desorption chemical ionization (DCI), field desorption (FD), secondary ion mass spectrometry (SIMS), fast atom bombardment (FAB), matrix assisted laser desorption/ionization (MALDI), ²⁵²Cf plasma desorption (PD), thermospray ionization (TSP), and electrospray (ES). The goal of all of these methods is to create gas-phase ions of polar or thermally fragile molecules, often without the introduction of excessive internal energy that would cause thermolysis or extensive fragmentation. The methods have been extremely successful. One might suppose that such methods would eliminate the need for chemical derivatization; certainly the volatility enhancement is less critical when using a desorption ionization method. However, many researchers are finding that chemical derivatization can still play an important role in increasing the sensitivity of ion production or analysis [57].

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B. Fast atom bombardment mass spectrometry [58-61]

Before the introduction of FAB-MS [62,63], in order to be analyzed by electron impact mass spectrometry (EI-MS), peptides had to be derivatized to increase volatility and thermal stability. In the late 1950s, Biemann developed a derivatization procedure using acetylation and reduction with LiAlH₄ to form polyamino alcohols which could be analyzed by GC-MS [64]. These derivatized peptides displayed improved volatility, and also fragmentation of specific bonds in the peptide backbone. Later, this derivatization procedure was modified to yield N-trifluoroethyl-Otrimethylsilyl polyamino alcohols. Another procedure employed the formation of N-acetyl-N,O-permethylated derivatives which were introduced by direct probe insertion for analysis by EI-MS [65]. Although it was possible to analyze peptides by EI-MS, mass spectrometry contributed little to peptide analysis until the 1980s.

(1) Principles

Fast atom bombardment (FAB) provided for the first time a technique for ionizing nonvolatile samples that is both simple to use and gives reproducible results. The FAB employs a neutral Ar or Xe atom beam, having several keV of transitional energy, to sputter ions (secondary ions) from a sample dissolved in a liquid matrix. When the fast atoms strike the probe, energy is transferred to the matrix causing molecules of both sample and matrix to undergo desorption and ionization. Figure 1.2 shows the process. FAB-MS allows the desorption, ionization, and analysis of nonvolatile and thermally labile compounds with derivatization. As result, FAB-MS can be used to analyze peptides without derivatization.



Figure 1.2. Schematic diagram for fast atom bombardment process.

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Liquid matrices, such as glycerol, or other suitable viscous organic compounds are mixed with the sample in order to maintain the fluidity of the sample during the introduction process into the high vacuum source chamber and throughout the analysis period. The matrix promotes a stable, reproducible ion current lasting for long periods of time. The sputtering process leading to the production of ions is dependent on the maintenance of a liquid surface. Figure 1.3 represents a closer view of the sample surface. This figure illustrates the surface destruction of a sample by the high-flux particle beam and the desorbed species produced. The viscous liquid matrix replenishes the destroyed area and provides additional analyte to the sample surface. Without this renovating effect, the analyte signal would not last nearly as long nor be as stable.

Although the FAB matrix provides benefits for desorption ionization, the presence of a viscous matrix material in a high concentration gives rise to several significant problems. These include poor sensitivity and limit of detection, high background ion counts at every mass, intense cluster ions from the matrix, and ion suppression effects whereby the formation of certain ions from the sample are inhibited by the presence of other compounds in the sample.

Although the mechanism of desorption and ion formation in FAB is not completely understood, it is based on a combination of factors. Among these mechanisms are desorption of preformed ions, including both protonated and metal ion (e.g. Na⁺, K⁺) adducts, and desorption of neutral molecules followed by gas-phase ionization in the high-pressure region ["selvedge"] directly above the vacuum-solution interface.
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Figure 1.3. View at surface of glycerol/sample solution during fast atom bombardment.

(MH⁺: positive ions, (M-H)⁻: negative ions, N:neutrals, G: glycerol)

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Fast atom bombardment of peptides provides abundant molecular weight information, usually in the form of a protonated molecule, $[M+H]^+$, in the positive mode. Limited fragmentation of peptides occurs along the backbone and at side chains, giving information about the amino acid chains and the amino acid sequence of the peptide. Peptide fragments can retain the charge at the N-terminus (producing $\mathbf{a_n}$, $\mathbf{b_n}$, $\mathbf{c_n}$, and $\mathbf{d_n}$ ions) or at the C-terminus (producing $\mathbf{x_n}$, $\mathbf{y_n}$, $\mathbf{z_n}$, $\mathbf{v_n}$, and $\mathbf{w_n}$ ions). This nomenclature is based on a system proposed by Roepstorff [66] (labeling fragment ions by upper-case letters) (Figure 1.4) and modified by Biemann [67,68] (labeling fragment ions by lower-case letters). The structures of these ions are shown in Figure 1.5, the subscript **n** represents the number of amino acid residues present in the fragment.

(2) Tandem mass spectrometry - collisionally induced dissociation (CID)

FAB is considered a soft ionization technique because very little fragmentation occurs in the desorption process. Methods of supplying an ion with enough energy to dissociate include collision with a surface, adsorption of energy from a laser beam, use of an electron beam, and the most commonly used method of collisions with an inert gas, collisionall induced dissociation (CID).

The use of tandem mass spectrometry (MS/MS) can increase the amount of structural information available from FAB ionization. The concept is illustrated in Figure 1.6. In MS/MS, a precursor ion is selected by the first mass analyzer and directed into a collision cell to collide with an inert gas, such as He or Ar, then the fragments (product ions) emerge from the collision cell will be analyzed by the second mass-selective device to

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$$H_{2}N - \begin{array}{c} X_{3} & Y_{3} & Z_{3} & X_{2} & Y_{2} & Z_{2} & X_{1} & Y_{1} & Z_{1} \\ R_{1} & 0 & 1 & 1 & 1 \\ 0 & 1 & 1 & 1 & 1 \\ C & 1 & 1 & 1 & 1 \\ H & 1 & 1 & 1 & 1 \\ H & 1 & 1 & 1 & 1 \\ H & 1 & 1 & 1 & 1 \\ H & 1 & 1 & 1 & 1 \\ H & 1 & 1 & 1 & 1 \\ H & 1 & 1 & 1 & 1 \\ H & 1 & 1 & 1 & 1 \\ H & 1 & 1 & 1 & 1 \\ H & 1$$

Figure 1.4. Peptide fragment ion designations proposed by Roepstorff and Fohlman [66].

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Figure 1.5. Fragment ion structures commonly observed in FAB mass spectra of peptides [61].

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provide additional characteristic data for the precursor ion. It is this requirement for two mass-selective devices that led to the terminology MS/MS. With a triple quadrupole instrument, a precursor ion selected with the first quadrupole undergoes collisions and fragmentation in the second quadrupole (collision cell), and the product ions are analyzed with the third quadrupole. When a two-sector instrument is used for tandem mass spectrometry analysis, CID occurs in the first field free region (between the ion source and the first sector) and analysis is performed by scanning the electric and magnetic sectors with the ratio between the magnetic and electric fields (B/E) held at a constant value determined by the mass of the precursor ion [58,60].

(3) Instrumentation

All mass spectrometers consist of three basic components: the ion source, the mass analyzer, and the detector. Ions are produced from the sample in the ion source, the mass analyzer separates ions according to their mass to charge ratio, m/z. Each ion strikes the detector and produces a signal proportional to its relative abundance.

Singly charged ions with mass of m, subjected to an accelerating voltage V, acquire a translation energy:

$eV = (1/2) mv^2$

where e is the electronic charge and v is the velocity of the ion m^+ .

Double focusing mass spectrometers have an electric sector (E) for selecting monoenergetic ions as well as a magnetic sector (B) to analyze the momentum (and thereby the mass) of the ions.



Product Ion Spectrum

Figure 1.6. Tandem Mass Spectrometry (MS/MS).

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Electric sector

Ions leave the ion source with a small spread of kinetic energy due to the ionization process and other factors. The electric sector with a fixed field E deflects the ions in a circular path. The deflection radius, R_e , is proportional to the energy. The electric sector acts as a device for dispersing ions according to their kinetic energies.

Magnetic sector

A magnetic analyzer separate ions according to their relative massto-charge ratios. When accelerated, m ions enter a magnetic field of strength B, the ions follow a circular path of radius R_m .

$R_m = mv/eB$

The radius taken by an ion in a magnetic field is proportional to its momentum. Combing these equations gives:

$$m/e = R_m^2 B^2/2V$$

If B is scanned at fixed value of R_m , ions of different m/z will pass through a detector slit to give a mass spectrum.

Linked scans

Double focusing instruments can be scanned in special ways so that metastable ion decomposition of a precursor ion can be observed. To obtain these product ions, a B/E linked scan in which both B and E are scanned together, such that the ratio of the magnetic field strength and electric field strength is held constant.

In the 1st field free region (between the ion source and the electric sector), fragmentation is induced by collisional activation to produce

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product ions $(m_2^+, m_3^+, m_4^+,...)$ from a precursor ion m_1^+ (with M_n , M_n' , M_n^- as the neutral loss):

$$m_2^+ + M_n$$

 $m_1^+ - \dots > m_3^+ + M_n'$
 $m_4^+ + M_n^*$

During fragmentation, m_1^+ discharges internal energy and adds kinetic energy. However, this energy is known to be approximately 1 eV or less, and is very small compared with the initial kinetic energy of m_1^+ (10 KeV when accelerated by 10 kV). The velocity of m_2^+ is approximately the same as that of m_1^+ (M_n: neutral loss).

When the ion m_1^+ fragments to m_2^+ , the conditions of the electric sector to pass m_1^+ and m_2^+ are:

for
$$m_1^+$$
 $eE_1 = m_1 v_1^{2/R_e}$
for m_2^+ $eE_2 = m_2 v_1^{2/R_e}$

thus, it can be easily shown that $m_2/m_1 = E_2/E_1$

Similarly, the conditions to pass m_1^+ and m_2^+ through the magnetic sector are:

for
$$m_1^+$$
 $eB_1 = m_1v_1/R_m$
for m_2^+ $eB_2 = m_2v_1/R_m$

giving rise to the conclusion: $m_2/m_1 = B_2/B_1$ Combining the two equations $m_2/m_1 = E_2/E_1 = B_2/B_1$ thus

$$B_1/E_1 = B_2/E_2 = constant$$

With this method, by simultaneously scanning (linked-scanning) the magnetic field strength B and electrostatic field strength E so that the ratio of B/E is maintained constant while the m_1 ⁺ precursor ions are detected, all of the product ions that are generated from the precursor ion can be detected.

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C. Derivatization of peptides prior to analysis by FAB-MS

Fast atom bombardment mass spectrometry has made a significant impact on the approach to the structure analysis of proteins. But, in fast atom bombardment mass spectrometry of peptides, some factors prevent successful detection and sequence analysis of peptides. One of them is the poor ion production and detection (ion desorption/ionization efficiency) of some hydrophilic peptides, and the second factor is the ambiguity in interpretation of the spectra when key sequence ions are weak or absent.

Investigators [69-72] soon realized that chemical modification of an analyte to enhance its hydrophobic and/or ionic character improved its signal-to-background ratio during FAB analysis.

In the analysis of peptides by FAB-MS, there are two independent advantages that result from derivatization: (i) enhancement of detectability, and (ii) modification of fragmentation (enhancement of structurally informative ions). These advantages result from forming more hydrophobic and/or precharged derivatives. Certainly, researchers are looking for derivatization methods to fulfill both of these aspects. Useful derivatization should be specific, modifying only the intended portion of the target molecule, and yield a single product. The modification procedure should be fast, simple, and applicable to small sample sizes. Ideally, the derivatization reagent should be safe and stable.

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(1) Enhancement of surface activity

The penetration depth of the incident particle into the liquid is believed to lie within 50 to 100 angstroms of the gas/liquid interface (73). When the sample volume is approximated as a portion of a sphere, the maximum depth of the sampled volume is equivalent to five molecular layers (74). All the processes associated with the energy deposition and collision cascade are centralized near the surface of the matrix. Since desorption depends on sputtering from the matrix surface, the surface concentration of the peptide is an important factor. The capability of an analyte to occupy the surface area is dependent upon interactions (e.g., hydrophobic, hydrophilic, hydrogen bonding,) between the matrix molecules and the analyte. Most of the matrices tend to be hydrophilic in nature. The more hydrophilic compounds, which are surface inactive, can interact favorably with the matrix molecules and distribute themselves throughout the entire matrix volume. Meanwhile, the more hydrophobic compounds, which are surface active, move themselves towards the surface and away from the interaction between the matrix molecules. Thus, the surface-active compounds will have a higher concentration of molecules near the surface than surface-inactive compounds.

When working on a series of related surfactants differing only in alkyl chain length by LSIMS, Ligon et. al. [75] observed differences in signal strength attributable to differences in surface activity; the spectra correspond to real surface concentrations. Ligon concluded that the observed ratio between two analyte molecules will depend on their bulk concentration and on their relative surface activity.

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hyd supj has , Physical and chemical parameters such as pH, surface tension, and the hydrophilicity/hydrophobicity of the matrix, charge, size, stability, and solubility of the peptide affect its surface activity [76].

Clench et. al. [77] found that if two dipeptides with grossly differing surface activity were present in a mixture, the one with lesser surface activity would not be observed. In the positive FAB spectrum of a 0.01 M Ala-Gly and 0.001 M Phe-Leu, the MH⁺ of Phe-Leu dominated the mixture spectrum despite the fact that Ala-Gly was present at 10 times the concentration of Phe-Leu.

Naylor et. al. [78] pointed out the limitation in the use of FAB-MS for analysis is that the hydrophilic peptides in a mixture are suppressed. (i) The hydrophilic peptides alone give a relative poor signal response. (ii) Hydrophilic peptides are further suppressed in the presence of hydrophobic peptides that initially occupy the surface of the matrix. These attributes result from the intrinsic tendency of even a pure hydrophilic peptide to avoid the matrix/"vacuum" interface; in a mixture of hydrophilic and hydrophobic peptides, the hydrophobic components occupying the interface tend to suppress any signal that might otherwise arise from the low concentrations of the hydrophilic component at or near the surface. (iii) Hydrophilicity/hydrophobicity index (ΔF values) can be used to indicate which peptide may be suppressed. These indices for peptides are calculated by adding ΔF values of Bull and Breese indices for amino acids [79], and each sum is divided by the number of amino acids in the peptide. This correlation of the FAB mass spectrometric sensitivity to a hydrophobicity/hydrophilicity scale seems useful to predict the peptide suppression. The peptide that is suppressed in the analysis of the mixture has a more positive ΔF value.

Ī V a 8 of ca m ma of p hyd laye hyd ana wate sens facto layer pepti activ homa differ mixtu The scale of Bull and Breese is derived from the preference or reluctance of an amino acid to transfer from aqueous solution to an airwater interface. Bull and Breese [79] measured the surface tensions of amino acids in 0.10 M NaCl as a function of the concentration of the amino acids at 30°C. From the experimental results, the free energies of transfer of the amino acid residues from the solution to the surface have been calculated to yield a hydrophobicity index for each residue. (Table 1.2) The more positive the index, the more hydrophilic is the amino acid.

Furthermore, the hydrophobicity/hydrophilicity index of a peptide may not be sufficient in itself to predict ion suppression effects. A number of physical/chemical properties of peptides, including their hydrophobic or hydrophilic nature, play important roles in their tendency to occupy surface layers of sample and therefore their capacity to form ions by FAB-MS. For hydrophilic peptides, other factors such as charge state of the peptide in the analysis matrix and its tendency to form secondary structure in the water/glycerol solution in addition to the hydrophilic index affect the sensitivity of the FAB measurements. Whatever the balance of these factors, it is clear that competition of the various species for the surface layers of the liquid sample remains the dominate factor [59]. One goal of peptide derivatization for analysis by FAB-MS is to enhance the surface activity of hydrophilic peptides. Derivatization inherently increases the homogeneity of molecular mixture. This technique can equalize differences in surface activity for individual components of a peptide mixture.

1 A A A A A A A A A A S C Y G I I G I Y H iss I I e Leu Lys Met Phe Pro Ser Thr Trp Tyr Val

Amino Acid	$\Delta \mathbf{F}$ (cal / mole)
Ala	+610
Arg	+690
Asn	+890
Asp	+610
Cys	+360
Gln	+970
Glu	+510
Gly	+810
His	+690
Ile	-1450
Leu	-1650
Lys	+460
Met	-660
Phe	-1520
Pro	-170
Ser	+420
Thr	+290
Trp	-1200
Tyr	-1430
Val	-750

Table 1.2. Hydrophilicity / hydrophobicity index (ΔF) of amino acid [79].

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an am con Various methods have been employed to increase the FAB signal response of hydrophilic peptides. One successful approach used to increase the FAB signal response has involved formation of hydrophobic derivatives of hydrophilic peptides. These derivatization methods include a variety of peptide modifications, ranging from esterification to the attachment of a single hydrophobic moiety to a specific site on the peptide

Although not widely used, esterification can greatly reduce the detection limits of hydrophilic peptides. Falick, et. al. [80] derivatized the hydrophilic peptides for LSIMS by preparing alkyl and benzyl esters of peptides. He reported a factor of 25 increase in sensitivity of hydrophilic peptides at 20 pmol level (Thr-Lys-Pro-Arg). The derivatives were prepared as follows: Peptides collected from the HPLC were lyophilized. A volume of 5 µl of dry alcohol 0.2 M in acetyl chloride was added. The reaction was allowed to proceed for 1 hour at 45°C. Relative yields of MH⁺ ions from peptides esterified with various alcohols (methanol, 2-propanol, 1-butanol, 1-hexanol, 1-octanol, and benzyl alcohol) were compared. Although the signal-to-background ratio increased for peptide esterified with alcohols of increasing alkyl chain length, the best combination of ion yield and ease of reagent removal was obtained with 1-hexanol. The alkyl chain increased the surface activity of peptides and eliminated the discrimination against hydrophilic peptides. A mixture of peptides of different hydrophilicity was analyzed after derivatization. The procedure did not affect side-chain amides. Partial derivatization was sometimes observed with peptides containing more than one carboxyl group.

Naylor et. al. [78] converted the peptides to their isopropyl esters by esterification with 1 M HCl in 2-propanol (at 37° C for 24 h). After the esterification, the more hydrophilic derivatives were detected in the complex enzymatic digests of proteins. This was possible because the derivatization converts $-CO_2^-$ to $-COOCH(CH_3)_2$, which will produce a maximum index change of about -1000 (the difference in the Bull and Breese value for aspartic acid and leucine).

Ligon et. al. [81] derivatized several dipeptides by treating them with dodecanal to attach a long hydrocarbon tail on the peptides to increase their hydrophobicity. It was also pointed out that the term "surface activity" included such phenomena as hydrophobicity, solubility, and solute-induced variations in surface tension. The more polar peptides were improved by derivatization. The samples were prepared by treating an aqueous solution (30 μ l, 0.1 M) of each dipeptide with an equal volume of a 0.1 M solution of dodecanal dissolved in methanol. The resulting solution was warmed to the boiling point of methanol. Under the conditions, dodecanal forms a Schiff base or imine with peptides having a primary amine function.

The analysis is greatly complicated if the aldehyde contains homologues. The analysis may fail entirely if the aldehyde has been partially oxidized to the corresponding acid, which can by itself entirely dominate the SIMS spectrum. Furthermore, it was found that relative hydrophobic peptides (and probably most large peptides) may not benefit from this derivatization.

Chai and Zhao [82,83] reported the analysis of positive FAB of seven amino acids, dipeptides, and tripeptides as di-isopropylphosphorylated derivatives. Results showed an improvement in sensitivity by factors of 4-29, mostly above 10, for the derivatized amino acids compared to that for the underivatized ones. Also improved sensitivity and decreased background noise from the glycerol matrix were observed after derivatization of peptides. It is probably because the combination of the enhanced surface activity and increased proton affinity by the derivatization. N-terminal fragment ions dominated the fragmentation of N-diisopropyloxy phosphoryl derivatized peptides, giving evidence of directed fragmentation. Derivatized peptides also displayed suppressed fragmentation of amino acid side chains. The dialkylphosphite reagent used for derivatization also can be used for peptide synthesis [84] and more studies are underway for this derivatization [85].

Baillie and Nelson [86-88], in their work on glutathione conjugates by FAB/MS, derivatized the amino terminus with ethyl or benzyl chloroformates and methylated the carboxyl group with HCl/MeOH to form the alkyloxycarbonyl (ethyl or benzyl) methyl ester. The derivatization procedure using chloroformate reagents was similar to that for amino acids [43,46]. (pH 9 by $0.5 \text{ M} \text{ Na}_2\text{CO}_3$ and $0.1 \text{ M} \text{ Na}\text{HCO}_3$, vortex for 1 min. The reaction was allowed to proceed at room temperature for 30 min.) The methylation was completed after 30 min at room temperature by methanolic HCl. The chloroformate derivative facilitated the purification of the conjugate which had better chromatographic properties in HPLC, and showed informative mass spectral fragmentation under CID conditions. N-benzyloxycarbonyl derivatized glutathione had higher sensitivity in FAB-MS detection compared to that of an underivatized species.

The two examples above [82,88] also demonstrate the effect of derivatization for modification of fragmentation of peptides.

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b) Enhancement of structure informative fragmentation (modification of fragmentation)

Renner et. al. [89,90] made derivatives of peptides with dansyl chloride (DnsCl) or 2-bromo-5-(dimethylamino)benzensulfonyl chloride (Bdbs-CI) at the amino terminus. The peptide (1 μ mol) is dissolved in 0.2 M NaHCO₃ (60 μ l). After addition of a 50 mM solution of Dns-Cl or Bdbs-Cl in acetone (mole ratio peptide: reagent=1:3), the mixture is incubated for 1.5 h at 40°C. The procedure is followed by distilling off the acetone and acidifying with 10% H₃PO₄. The derivatized peptide is isolated by HPLC. In contrast to those of underivatized peptides, the spectrum of the dansyl derivatives exhibit intense and structure-specific cleavage patterns, all of which relate to the derivatized end of the molecule and result from cleavage of the peptide bond.

Roepstorff [91] did an on-probe acetylation of the peptides by the addition of acetic anhydride with careful mixing into the matrix with a glass tip at ambient temperature for 10 min. The estimated degree of acetylation for some tri- to hexa-peptides were 40-100%, most of them were 60-70%. The acetylation conditions were insufficient for effective acetylation of the side-chain amino group of lysine. The spectrum of the acetylated peptides gave more sequence information than the spectra of the free peptides; especially the assignment of the N-terminal amino acid residue was facilitated by the acetylation.

The C-terminus derivatization of a peptide is also useful in the interpretation of MS/MS spectra because of the selective mass shift of all of the C-terminal fragments relative to the underivatized peptides, while leaving the N-terminal fragments unchanged [78,80].

spe FAI proc grou pept befor increa surfac molecu gas pl cation "prefor of large C been si enhance charge amino a appeara elucidat; When a ^{more} abu ^{ions} are fragment To increase the amount of sequence information from FAB-MS spectra, peptides converted to polyamino alcohols were analyzed by tandem FAB-MS [92]. The peptide YAGFL was reduced using diborane. In this procedure, the amide groups were converted to amines and the carboxyl group was reduced to an alcohol. The MS/MS spectra of the reduced peptide had complete $\mathbf{b_n}$, $\mathbf{y_n}$, and $\mathbf{z_n}$ ion series, which were incomplete before reduction. Therefore, the simplicity of spectral interpretation was increased markedly with reduction of peptides to polyamino alcohols.

The "preformed" ions produced with peptide modification are more surface active in the FAB matrix than the unmodified analytes. If molecules carry a charge initially, they are thought to be sputtered into the gas phase directly from the matrix by the primary beam. Protonation, cationation with metal ions, and chemical modification to produce a "preformed" charge are derivatization techniques to enhance the analysis of large, highly polar, and nonvolatile compounds.

Chemical derivatization to introduce a charge into a molecule has been suggested as means to improve the sensitivity in FAB-MS and to enhance the detectability of molecular-weight related ions. The localized charge has a stronger effect in directing fragmentation than any basic amino acids that may be present in the peptide, drastically altering the appearance of the MS/MS spectra of peptides [93] and facilitating structure elucidation by enhancing the formation of structurally informative ions. When a preformed charge is introduced into peptides, a_n and d_n ions are more abundant for N-terminally derivatized peptides, while v_n , y_n , and w_n ions are formed when peptides are derivatized at the C-terminus. The fragmentation pattern produced by derivatization with a fixed charge simplifies mass spectral interpretation. There are a number of derivatization schemes which attach a fixed charge to peptides [58,93-100].

c) Functional group determination and amino acid

detection

The presence of functional groups in a peptide can be identified by measuring the mass shift after reacting the peptide with a site-specific reagent.

In order to distinguish between the isobaric Gln and Lys residues, as well as to confirm a proposed structure, Kausler [101] methylated free carboxylic groups by treatment of methanol/HCl, resulting in a mass shift of 14 u. Peptides were mixed with 100 μ l methanol/2 N HCl and after heating at 100°C for 1 hour, the sample was lyophilized. The amide groups of Asn as well as Gln were also quantitatively converted to esters, indicated by a mass shift of 15 u per amide function.

Fragmentations of N-benzyloxycarbonyl-protected tripeptide ethyl esters have been investigated by negative-ion FAB-MS [102]. A significant difference was found among the intensities of the fragment ions formed by cleavage of the benzyloxycarbonyl group, depending on the numbers and positions of proyl residues in the derivatives. So the fragmentation pattern of N-benzyloxycarbonyl-protected tripeptide ethyl esters can be used to predict the numbers and positions of proline residues in the peptides.

FAB was employed to identify and quantitate dansyl amino acids obtained in the N-terminal analysis of proteins [103]. After a timeconsuming dansylation process, the protein was hydrolyzed for 12 hours at 105°C with 6 N HCl. The spectra of N-terminal dansyl amino acids are characterized by protonated molecules and fragment ions produced by cleavage of the bonds on either side of the sulfanyl group. The dansyl amino acids can be determined quantitatively at a level of 0.1 nmol with the response being a linear function of concentration up to approximately 10-20 nmol/ μ l.

d) Others

The t-butyloxycarbonyl (t-BOC) protected peptides [104,105] and amino acids [106] were investigated. Prominent peaks due to subsequent loss of C_4H_8 [M+H-56]⁺ and CO₂ [M+H-100]⁺ are always accompanied with [M+H]⁺ ions. The [M+H]⁺ ion abundances of t-BOC peptides are smaller than those of the corresponding underivatized species. Due to the facile fragmentation of the t-BOC group, especially during CID-MS/MS, the spectra are more complex and yield less sequence information than those of the underivatized peptides. Fragmentations of benzyloxycarbonyl protected amino acids and peptides with FAB ionization have also been investigated [107].

IV. Research objectives

This dissertation focuses on: (1) derivatization-assisted amino acid analysis by GC-MS in EI, positive CI and ECNI modes and (2) derivatization-assisted peptide analysis by FAB-MS.

For the analysis of amino acids, the one-step aqueous medium chloroformate derivatization method introduced by Husek [49-51] for analysis by GC has been extended to analysis by GC-MS, and the structurally diagnostic fragmentation of the amino acid alkyl chloroformate derivatives in EI-MS was also studied. Modification of the derivatization procedure has been conducted. An extended examination of the derivatization method with the combination of a variety of alkyl chloroformates and alcohols has been carried out. Fluorinated derivatives prepared with a modified reaction procedure, in combination with ECNI analysis has been evaluated for increasing the sensitivity of analysis. A collaborative research project to quantitatively assess the incorporation of stable isotope-labeled amino acids into photosynthetic proteins with the chloroformate derivatization has been carried out.

For peptide analysis, investigation of the chloroformate derivatization for small peptides prior to analysis by FAB-MS to enhance the FAB signal of peptides, and a comprehensive evaluation of the derivatization conditions have been completed.

The advantages and the limitations of the one-step aqueous medium chloroformate derivatization method have been further investigated.

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Chapter II

Simultaneous derivatization of functional groups of amino acids by an aqueous medium chloroformate reaction prior to analysis by GC and

GC-MS

L Introduction

The one-step aqueous medium ethyl chloroformate derivatization procedure for analysis of amino acids by GC [1-3] is a very attractive method based on the criteria for a hypothetical, ideal procedure for amino acid determination by GC. In this procedure, using an aqueous medium containing water / ethanol / pyridine, in a single step, the amino, carboxyl, sulfhydryl, phenolic and imino groups on the side chain of histidine are derivatized by ethyl chloroformate. Carboxyls are converted to esters, while the other functional groups are converted to N,O- and S-ethoxycarbonyl derivatives (Figure 2.1).

This chapter includes: 1) characterization of N-ethoxycarbonyl ethyl esters of amino acids by EI mass spectrometry; 2) a modification of the reaction procedure; 3) an extended examination of the derivatization method with a combination of a variety of alkyl chloroformates and alcohols; and 4) comparisons of EI, PCI, ECNI ionization for the fluorinated derivatives of amino acids formed through a modified reaction procedure.



Figure 2.1. Reactions of chloroformate with functional groups of amino acid.

A. Experimental

A solution of 20 amino acids in 0.1M HCl at a concentration of 0.5 μ g/ μ l each was prepared. The N(O,S) ethoxycarbonyl ethyl ester (ECEE) derivatives of amino acids were prepared by adding 10 μ l of the amino acid mixture to a H₂O / ethanol (EtOH) / pyridine (Py) (50 μ l / 30 μ l / 10 μ l) solution. Ethyl chloroformate (EtCF) (5-10 μ l) was then added and the reaction mixture was vortexed for 5-10 s and extracted with 100-200 μ l CHCl3. A 1- μ l aliquot of the CHCl3 layer was injected for analysis.

Analyses by GC-MS were carried out on a JEOL AX-505H double focusing mass spectrometer coupled to a Hewlett-Packard 5890J gas chromatograph. GC separation was achieved on a DB-1701 (15-m length x 0.25-mm i.d.) fused silica capillary column with a 0.25 μ m film coating from J. & W. Scientific (Rancho Cordova, CA). Direct (splitless) injection was used. Helium gas flow was approximately 1 ml/min. MS conditions were as follows: interface temperature 275°C, ion source temperature ca. 150-200°C, electron energy was 70 eV, scan rate of the mass spectrometer was 1 s/scan over the range of m/z 50-500.

B. Results and discussion

Interpretation of the spectra of this family of new derivatives can facilitate recognition of individual amino acids.

Table 2.1 lists the mass value of the characteristic peaks in the spectra of individual amino acid derivatives. The main fragmentation pathway for EtCF-EtOH amino acid derivatives are shown in Figure 2.2, two fragmentation routes (a and b) are possible through the rupture of a



Figure 2.2. Main fragmentation pathways for amino acid EtCF-EtOH derivatives in EI mass spectrometry

other ions, m/z			116	102	102	86	88	175, 129, 101, 86	175,101	258, 70	272, 226, 84	174, 114, 102,	74	220, 174, 102	188, 142, 129,	101, 61	142, 74, 70	156, 128, 84		174, 102	128	131, 102, 91,	74	264, 192	215	154, 81
M-145			22	88	88	8	88	8	74			148					116			89		120		208		182
M-73	102	116	144	158	158	142	158	132	146			8 2		367	176		188	202	22	141	173	192		280	ន្ត	254
Base peak m/z	102	116	144	158	158	142	158	132	129	142	156	କ୍ଷ		188	175		188	202	5 8	141	28	176		107	130	238
Derivative M+.	175	189	217	22	5	215	83	205	219	304	318	233		440	249		790	275	157	214	246	265		353	304	327
MM	75	88	117	131	131	115	131	105	119	132	146	121		240	149		133	147	129	132	146	165		181	204	155
Amino acid	Gly	Ala	Val	Leu	Ile	\mathbf{Pro}	Hpy	Ser	Thr	Orn	Lys	Cys		Cys-Cys	Met		Asp	Glu	p-Glu	Āsn	Gln	\mathbf{Phe}		Tyr	Trp	His

Table 2.1. Characteristic ion peaks in EI mass spectra of EtCF-EtOH derivatives of amino acids

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carbon-carbon bond α to the amine group. Generally, path **b** is preferred over **a** because 'CO₂Et, the fragment having higher ionization energy compared to that of the alkyl chain, is favored energetically to retain the unpaired electron and to become the neutral product. Subsequent fragmentation of the even-electron ions (EE⁺) thus formed is dominated by the loss of 72 u ('CO₂Et - H'), giving rise to ions m/z 102 and [M-145], respectively. For detailed fragmentation pathways for each type of amino acid (aliphatic, cyclic, hydroxyl, sulfur-containing, acidic, basic, and aromatic amino acids) see Ref. 4.

III. Modification of the reaction procedure

A. Background of chloroformate chemistry

(1) Reaction with amino and phenolic groups

Chloroformates have been used as the protecting group for Nterminus during peptides synthesis. The alkoxycarbonyl derivatives formed can be cleaved under mild conditions (by careful alkaline hydrolysis) [5,6]. Benzyl- and *tert*.-butyl chloroformate are generally used as reagents [5].

In analytical chemistry, chloroformates have been used extensively to treat amino, phenolic hydroxyl groups. Derivatization of amino groups in basic aqueous solution by chloroformates has been a commonly used approach (as reviewed for derivatization of amino acids in chapter I [7-11]). Other than those discussed in chapter I, applications for the derivatization of catecholamines in aqueous solution by methyl chloroformate have also been reported [12,13].

Chloroformate derivatization has also been applied in LC analysis. One example is that amino acids were derivatized at amino groups by 9fluorenylmethyl chloroformate (FMOC-Cl) separated by reverse-phase HPLC and quantitated based on the fluorescent properties of the derivatives [14-17]. The protein hydrolysates were dissolved in 0.1 M sodium bicarbonate, pH 8.0. The derivatization was achieved by the addition of 4.0 mM solution of FMOC-Cl in dry acetone. The mixture was quickly shaken and the reaction was allowed to proceed for 10 min at room temperature (22°C). The excess reagent was removed by extraction with pentane/ethyl acetate (90:10) [14].

A slightly different derivatization procedure was used by Betner [15]. Borate buffer (0.5 M boric acid solution adjusted to pH 7.7 with 30% sodium hydroxide solution) was used. The reaction mixture was incubated for 45 s after vortexing. 1-aminoadamantane (ADAM) of 40 mM in water-acetone (1:3, v/v) was added to react with the excess reagent by incubation for at least 45 s. An advantage of using 1-aminoadamantane (ADAM) was that the risk of partial extraction of the hydrophobic amino acids into the organic phase was eliminated [15].

Chiral chloroformates as reagents for the resolution of metoprolol enantiomers [18], separation of amino acid enantiomers and chiral amines using pre-column derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate [19] by reversed-phase liquid chromatography are also in the literature.

(2) Reaction with carboxyl groups

Previously, the only application of chloroformate to react with a carboxylic group was the formation of the mixed anhydride as an activated intermediate to separate carboxylic acid enantiomers by GC [20], and to determine the enantiomers of indoprofen [21] and ketoprofen [22] by HPLC. Both cases used ethyl chloroformate.

In 1990, Husek demonstrated a method to derivatize carboxvlic groups in fatty acid analysis [23-27], and to derivatize both amino and carboxyl groups in one reaction in an aqueous medium for analysis of amino acids by GC [1-3]. In this method, Husek derivatized short-chain fatty acids by methyl and ethyl chloroformate (MCF and EtCF) to form esters or alkoxycarbonyl esters which were analyzed by GC. The esters can be formed instantaneously and almost quantitatively, at least from the standpoint that no other volatile derivatives were produced. The reaction can proceed in both non-aqueous solvent and in solvent containing water In the case of non-aqueous solvent: 5 μ l of the appropriate [23]. chloroformate was added and the reaction medium was shaken for 2-3 s. The medium consists of acetonitrile/pyridine/alcohol (methanol, ethanol) in volume ratio of 22:2:1. In the case of solvent containing water: to 50 μ l of a water-pyridine (5:1) solution was added 50 μ l of an acetonitrile-methanol (5:1) solution for MCF treatment, or 50 μ l of an acetonitrile-ethanol (2:3) solution for EtCF treatment. The derivatizations were believed to proceed through the mechanism of decarboxylation of the intermediates: the mixed anhydrides.

Chloroformates have been known in organic chemistry as a possible source of mixed carboxylic-carbonic anhydride formation since the beginning of this century [28]. These reagents and the thermal decomposition of mixed carboxylic-carbonic anhydride were intensively investigated in the 1950s and 1960s. Esterification of carboxylic acids using chloroformates through mixed carboxylic-carbonic anhydrides with new catalysts was investigated in the 1980s. Thermal decomposition of mixed carboxylic-carbonic anhydride

The reaction of a chloroformate with a carboxylic acid leads the formation of an ester as the main product when heating and / or acidic or basic catalysts are employed [6]. The mechanism and kinetics of the thermal decomposition of the intermediate mixed carboxylic-carbonic anhydride have been investigated [6,29-35]. The mixed anhydrides are stable at room temperature, but decompose around 150-170 $^{\circ}$ C [32] in the absence of catalysts, and at lower temperature and faster in the presence of catalysts [29]. The decomposition proceeds by paths A and B (Figure 2.3). For certain mixed anhydrides, when the temperature is 250°C, however, the ester is the only decomposition product (path C) [34].



Figure 2.3. Thermal decomposition pathways for mixed anhydride.

Tarbell [6,29,30] suggested that the decomposition of mixed benzoic nbutyl carbonic anhydride is through a series of ionic chain reactions which are initiated by catalysts acting as nucleophiles. (Figure 2.4) OR⁻ generated according to (1) and (2) is the chain carrier; it can attack at either carbonyl, as in (3) and (4) to generate products. $C_6H_5COO^-$ generated in (2) and (4) attacks unchanged anhydride at the carboxylic carbonyl to form the acid anhydride in (5). The decomposition according to both path A and B proceeds by a single rate-determining step [29]. This rate-determining stage of thermal decomposition is the attack of nucleophile B:, according to (1) and (2). Reactions (3) and (4) are very fast. For this compound, the proportions of products (path A and B) are not altered by changes in solvent, temperature, or presence of catalysts.

It was also pointed out that for $C_6H_5COOCOOR$, path A is favored when the point of attachment of the alkyl group (from chloroformates) is a secondary or a primary carbon with extensive substitution on the β -carbon [32].

Another researcher, Windholz [34], reported that for a mixed anhydride $R_1COOCOOR_2$, structures of both carboxylic (R_1) and carbonic (R_2) components have directing influences on the possible path of decomposition. A number of mixed anhydrides derived from aliphatic acids decompose exclusively by path A while aromatic derivatives tend to decompose equally along path A and path B. The differences were explained by steric factors. The directing influences of alkyl radicals R_2 have also been examined. Experiments indicated that the more electronreleasing isopropyl group favors easier decomposition by path A, while phenyl as R₂ favors path B. For benzoic ethyl carbonic anhydride, it was reported that heating the mixed anhydride in the presence of triethylamine hydrochloride lowered the decomposition temperature and favored ester formation. Boron trifluoride etherate lowered the decomposition temperature considerably and caused exclusively ethyl benzoate formation [34]. Various catalysts have been investigated; the results have some controversy. Overall, the outcome of the mixed anhydride decomposition reaction was disappointing as a means for the preparation of esters.

$$C_{6}H_{5}-C-O-C-OR + B: = C_{6}H_{5}-C-O-C-OR \qquad (1)$$

$$B$$

$$C_{6}H_{5}-C-B + O-C-OR$$

$$C_{6}H_{5}-C-B + O-C-OR$$

$$C_{6}H_{5}-C-B + O-C-OR$$



$$C_6H_5$$
-C-O-C-OR + OR \rightarrow C_6H_5 -C-OR + CO_2 + OR (3)

$$C_6H_5$$
-C-O-C-OR + OR \rightarrow C_6H_5 -C-O + RO-C-OR (4)

$$C_{6}H_{5}-C-O-C-OR + C_{6}H_{5}-C-O^{-} \longrightarrow C_{6}H_{5}-C-O-C-C_{6}H_{5} + CO_{2} + OR^{-}$$
 (5)

Figure 2.4. Mechanism for mixed carboxylic-carbonic anhydride thermal decomposition proposed by Tarbell [29] - ionic chain reaction.

Esterification of carboxylic acids through mixed anhydride by catalysts without heating

In 1980s, several papers were concerned with forming esters of carboxylic acids through carboxylic-carbonic mixed anhydride intermediate by accelerating the reaction via path A [36-39].

Kim [36,37] demonstrated a simple and mild esterification method for carboxylic acids using chloroformate through the mixed carboxyliccarbonic anhydrides. Typically, a solution of equal molar amount of acid, alkyl chloroformate, and triethylamine in methylene chloride at 0°C was added 4-(dimethylamino)pyridine (DMAP) in a catalytic amount. The resulting solution was stirred at 0°C for 30 min. Simple aliphatic carboxylic esters were prepared in high yields by the reaction of acids with equal molar amounts of various chloroformates and triethylamine in the presence of a catalytic amount of 4-(dimethylamino)pyridine without contamination of the symmetrical acid anhydrides and the carbonates. Although aromatic acids gave a mixture of the ester, the acid anhydride, and the carbonate under normal conditions used, it was found that increasing the amount of 4-(dimethylamino)pyridine drastically decreased the formation of the acid anhydride and the carbonate. The method reached a limit with sterically hindered acids such as pivalic acid and mesitoic acid. Pavalic acid yielded approximately a 1:1:1 mixture of ester, anhydride and carbonate upon treatment with benzyl chloroformate. In the case of mesitoic acid, exclusive formation of mesitoic anhydride was obtained without the formation of a trace of the ester. The same method was applied to esterification of N-protected α -amino acids which gained high yields (88-98%) for ten amino acids.

The reaction mechanism was suggested as in Figure 2.5 which is similar, but different, from that suggested by Tarbell [29,30] for thermal decomposition of mixed anhydrides. The esterification proceeds via a mixed carboxylic-carbonic anhydride as the intermediate. The mixed anhydride is converted into an acylpyridinium species (2a) by nucleophilic attack of DMAP on the carboxyl carbonyl center of the mixed anhydride as a major pathway along with an alkoxycarbonylpyridinium species (3b) by nucleophilic attack of DMAP on the carbonate center as a minor pathway in most carboxylic acids. The carbon dioxide evolution would provide a driving force for the major pathway. Nucleophilic attack by the alcohol on the acyl group of 2a gives the ester and DMAP, which is reused in the formation of 2a. Also, the acid anhydride, which can be formed via nucleophilic attack by the carboxylate anion of **3b** on the carboxyl carbonyl center of the mixed anhydride, can be converted into 2b by DMAP to raise the yield of the ester, while the reaction of 3a with the alcohol affords the carbonate.

In the case of aromatic acids, it is assumed that increasing the amount of DMAP increases the reaction rate of conversion of the acid anhydride into 2b and would, therefore, prevent the formation of the carbonate by consuming the alcohol due to fast reaction of 2b with the alcohol. However, in the case of sterically hindered acids: the nucleophilic attack by DMAP on the two reactive carbonyl centers of the mixed anhydride occurs with roughly a 1:1 ratio and the acid anhydride is inert toward DMAP for pivalic acid. The reaction proceeds exclusively via the intermediate **3b** for mesitoic acid due to the steric hinderance on the carboxyl carbonyl center of the mixed anhydride.





3a, X = Cl; **3b**, X = RC00

2a, X = Cl; **2b**, X = RC00

Domagala [38] also described a convenient esterification of α -keto acids by chloroformate. Triethylamine was added to a dichloromethane solution of α -keto acid, then methyl chloroformate was added at 20°C. The loss of CO₂ was reported to be completed in 10 min with a 95% yield.

Boltanski [39] esterified the half ester of malonic acid using ethyl chloroformate. Triethylamine was added to the monomethyl malonate in dry THF. Ethyl chloroformate was added at 4°C and the reaction mixture was stirred for 30 min, during which time the loss of CO_2 was complete with a yield of 99%.

B. Modification of the reaction procedure

In Husek's approach, it was concluded that esters are formed though the decarboxylation of the mixed anhydride with pyridine as a catalyst. The role of the alcohol in the reaction medium was not clearly explained. Both ethyl and methyl esters were present in the derivatization products when fatty acids were derivatized with methyl chloroformate in a reaction medium containing 2% pyridine in chloroform which was "stablized" with 1% ethanol, and the GC signals of ethyl esters were more than double of those from methyl esters [23]. It was explained that ethyl esters were formed by activation of the trace amount of ethanol present by the HCl released from methyl chloroformate, as shown in reaction 2-1.

$$\begin{array}{c} 0 \\ \text{R-C-OH} + \text{HO-C}_2\text{H}_5 & \xrightarrow{\text{HCl}} & 0 \\ \text{(Reaction 2-1)} \end{array} \\ \end{array}$$

In Kim's esterification mechanism [36,37], the ester is believed to form through the nucleophilic attack by the alcohol on the acyl group of the intermediate **2a** (Figure 2.5). Experiments were designed to clarify the role of the alcohol in the reaction medium for Husek's derivatization method.

(1) Experimental

Derivatization:

a) Phe iBuCF-ROH

To five H₂O-alcohol-pyridine (60-10-10 μ l) solutions, 5 μ l of Phe (10 μ g / μ l) were added. The alcohols were isobutanol (iBuOH), trifluoroethanol (TFEtOH), n-pentafluoropropanol (PFPrOH), n-heptafluorobutanol (HFBuOH), and trimethylsilylmethanol (TMSCH₂OH). To each solution, 5 μ l of isobutyl chloroformate (iBuCF) were added and reaction mixtures were vortexed for 5-10 s. Each derivative was extracted by 200 μ l CHCl₃; the solvent was removed (evaporated) under N₂ stream. Each derivative was redissolved in 100 μ l CHCl₃.

b) Phe MCF-ROH

Similar to a), except that the iBuCF was replaced by methyl chloroformate (MCF). Three alcohols: MeOH, TFEtOH, and TMSCH₂OH, were used.

c) Phe EtCF-EtOH

Similar to a), but only ethyl chloroformate was applied, and EtOH was in the reaction medium.

d) Phe iBuCF-mixed alcohol with equal volume

Similar to a), but instead of using one alcohol in the reaction medium in each derivatization, a mixture of seven alcohols (MeOH, EtOH, iBuOH, TFEtOH, PFPrOH, HFBuOH, and TMSCH₂OH), each 10 μ l, was added to the reaction medium.

e) Phe iBuCF-mixed alcohol with equimolar ratio

i) 10 μ g of Phe were added to a solution of H₂Omixed alcohol-pyridine (80-30-10 μ l). The mixed alcohol contained seven alcohols (MeOH, EtOH, PrOH, TFEtOH, PFPrOH, HFBuOH, and TMSCH₂OH) with equal molar amount, iBuOH was not included. The reaction proceeded by vortexing for 10 s after adding 10 μ l of iBuCF. 200 μ l CHCl₃ were used to extract the derivatives and 1 μ l of the chloroform layer was injected to GC-MS for analyses.

ii) Similar to i), except that MCF was used instead of iBuCF. In the mixed alcohol, MeOH was replaced by iBuOH.

GC-MS:

Column: DB1701 (15 m x 0.25 mm I.D.) fused-silica capillary column with a 0.25 μ m film. Source temperature was ~200 °C, interface temperature was 275°C, electron energy 70 eV, scan rate of the mass spectrometer was 1 s/scan over the range of m/z 50-750.

(2) Results and discussion

a) In these series of experiments, Phe were derivatized with iBuCF in a solution containing different alcohols (TFEtOH, PFPrOH, HFBuOH, TMSCH₂OH, iBuOH) in each case.

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The results indicated that the type of ester formed during the derivatization process with chloroformate reagents is directly dependent upon the type of alcohol present in the reaction medium. When using an alcohol (TFEtOH, PFPrOH, HFBuOH, and TMSCH₂OH) with an alkyl group different from that in the alkyl chloroformate (iBuCF in these experiments), the alkoxy group found in the ester part of the major derivative corresponds to the alcohol in the reaction medium and not to the alkyl group of the chloroformate reagent. For example, when phenylalanine is reacted with isobutyl chloroformate (iBuCF) in an aqueous medium also containing HFBuOH, the major derivative produced is that in which the carboxylic group is esterified with the heptafluorobutyl group, not the isobutyl group. The TIC and the mass spectrum of this derivative and its structure are shown in Figure 2.6. Only a small amount of derivative is formed in which the alkyl group in the ester moiety is the same as that in the isobutyl chloroformate reagent. It is 4% by both the peak area and the peak height of the total intensity. Similarly, when Phe is derivatized with isobutyl chloroformate in the presence of trimethylsilylmethanol $((CH_3)_3SiCH_2OH=TMSCH_2OH)$ in an aqueous reaction medium, the ester derivative formed includes a trimethylsilylmethyl group. The TIC and the mass spectrum of this derivative and its structure are shown in Figure 2.7 The same results were found for Phe treated with iBuCF in the presence of TFEtOH (Figure 2.8) and PFPrOH (Figure 2.9). The TIC and mass spectrum of Phe derivatized with iBuCF in the presence of iBuOH is in Figure 2.10 for comparison.

The results presented in Figures 2.6 to 2.9 cannot be explained very well by the simple decarboxylation mechanism proposed by Husek, *et. al.* [1-3]. According to the Husek mechanism, the mixed anhydride formed by the



Figure 2.6. TIC (a) and EI mass spectrum (b) of Phe derivatized by reaction with iBuCF-HFBuOH.



Scan# (529) 7B Phe iBuCF-TMSCH₂OH R MW 351 el ative 60-Abundance M-NH₂COOiBu • 10.0 M/Z (b)

Figure 2.7. TIC (a) and EI mass spectrum (b) of Phe derivatized by reaction with iBuCF-TMSCH₂OH.



Figure 2.8. TIC (a) and EI mass spectrum (b) of Phe derivatized by reaction with iBuCF-TFEtOH.





Figure 2.9. TIC (a) and EI mass spectrum (b) of Phe derivatized by reaction with iBuCF-PFPrOH.



Figure 2.10. TIC (a) and EI mass spectrum (b) of Phe derivatized by reaction with iBuCF-iBuOH.

reaction between the alkyl chloroformate and carboxyl group should decarboxylate ($-CO_2$) to yield the ester containing the alkyl group derived from the alkyl chloroformate. Also, it does not seem to be possible to form the major ester product so exclusively in such a short time by the acid (released from the chloroformate) catalyzed esterification suggested by Husek [23].

It turned out that with a slight modification, the mechanism suggested by Kim [36] for the esterification of fatty acids with chloroformate can be used to explain our experimental results.

In Kim's mechanism (Figure 2.5), DMAP catalyst attacks the mixed anhydride on the carboxyl carbonyl center to form a acylpyridinium species (2a); then the attack by the alcohol released from the mixed anhydride on the acyl group of 2a gives the ester product. In that reaction medium, no additional alcohol is added. The reaction proceeds in CH_2Cl_2 with 1:1 ratio of fatty acid and ethyl chloroformate.

Following the idea of this mechanism, it is not hard to explain what we observed in our experiments. Since we added an alcohol in which the alkyl group was different from that in the chloroformate used; and also, the amount of this alcohol was in great excess relative to the amount of the analyte present, which would be converted to the mixed carboxylic-carbonic anhydride. The amount of alcohol released from the process of forming 2a was no more than the amount of the mixed anhydride, which was the same amount as the analyte if the yield of forming the mixed anhydride was one hundred percent. In this case, there were two alcohols in the reaction medium to compete the attack on the acyl group of 2a. The huge difference in the quantity of these two alcohols explain why the ester with the alkyl group from the added alcohol formed exclusively. The relative nucleophilic reactivity of the alcohols should also be a factor to affect the formation of the ester product.

We believe that this mechanism explains our experimental results better than the acid catalyzed esterification does, and the simple decarboxylation of the mixed anhydride [1-3] is not a complete and detailed explanation for ester formation during reaction of carboxyl groups with chloroformates.

Other information from the literature also supports our explanation. Mixed carboxylic-carbonic anhydrides are considered as active acylating agents. Their reaction with nucleophiles proceeds readily under mild conditions [6]. The use of chloroformates as esterification catalysts in the presence of alcohol has been reported [6,40-42]. Although no detailed mechanism was proposed, it was believed that the reaction was through the formation of the mixed anhydride which then reacted rapidly with the alcohol with tertiary amine base as the catalyst [40-42]. An attempt was made to avoid the competition of the alcohol released during ester formation by employing an excess of the alcohol to be esterified [40]. Kim's mechanism should also fit those experiments.

Combining all the information, we can conclude that for the esterification of the carboxyl groups with chloroformates in Husek's method, the overall result is an exchange reaction between the mixed anhydride and the alcohols in the reaction medium (Reaction 2-2). The mixed anhydride reacts with the alcohol in the reaction medium to undergo an exchange reaction as illustrated via path A leading to the principal product. A small amount of derivative in which the alkyl group in the ester moiety is the same as that in the alkyl chloroformate reagent is formed through path B as a minor product (since R"OH is in great excess relative to R'OH). R'OH is released from path A'and is also produced from the hydrolysis of the chloroformate reagent in the reaction medium.



(Reaction 2-2)

It also can be seen from the series of experiments of Phe derivatized with iBuCF and different alcohols (ROH) (Experimental **a**)), that fluorinated moieties increased the volatility of the derivatives. The Phe derivative from iBuCF-TFEtOH eluted at scan 423 (Figure 2.8); the Phe derivatives from iBuCF-PFPrOH (Figure 2.9) and iBuCF-HFBuOH (Figure 2.6) eluted at scan 411 and scan 420, respectively, while the iBuCF-iBuOH derivative of Phe eluted at scan 520 (Figure 2.10).

In the EI mass spectra of Phe derivatives from fluorinated alcohols, (M⁺.- NH₂COOiBu) ions (m/z 230 in Figure 2.8, m/z 280 in Figure 2.9, and m/z 330 in Figure 2.6) are more predominant than that in the spectrum of Phe derivative with iBuOH (m/z 204 in Figure 2.10). These spectra are also relatively simpler than that of Phe iBuCF-iBuOH derivative while the Phe iBuCF-TMSCH₂OH derivative produces more fragmentation in its spectrum (Figure 2.7). For these series experiments (Experimental a)), the peak area and peak height of the derivatives, intensity percentages of the minor products are in section I of Table 2.2.

b) In the series of experiments of Phe derivatized with iBuCF and different alcohols (ROH) (Experimental a)), the minor product is less than 4% of the total intensity by area for each derivative with iBuCF. In the second series of experiments (Experimental b)), MCF derivatives of Phe were made in the presence of TFEtOH (Figure 2.11), and in the presence of TMSCH₂OH (Figure 2.12) to compare the intensity percentages of the minor products in these two reactions with what was observed for the corresponding reactions from the iBuCF derivatization (Experimental a)). The MCF derivative of Phe in the presence of MeOH was also made as a control (Figure 2.13).

The results of MCF derivatization (Experimental b)) have the same trend as what was observed from iBuCF derivatization (Experimental a)) for Phe with different alcohols: the ester moiety of the major derivatization product is from the alcohol added in the reaction medium. But from the results summarized in section II of Table 2.2, we can determine that the relative intensity of the minor product by area is 9.7% for the Phe derivative with MCF-TFEtOH and 8.7% for the Phe derivative with MCF-TMSCH₂OH. These percentages are higher than those obtained for the corresponding derivatives from iBuCF. (3.6% for Phe iBuCF-TFEtOH derivative, and <1% for Phe iBuCF-TMSCH₂OH derivative.)

These differences may indicate the different nucleophilic reactivities of MeOH and iBuOH for the attack on the acyl group of the acylpyridinium intermediate and / or that MCF is easier to hydrolyze to produce more MeOH in the reaction medium.

c) EtCF-EtOH derivative of Phe was made for the comparison with other derivatives made from iBuCF (Experimental a)) and MCF (Experimental b)) in the presence of different alcohols. The result is in section III of Table 2.2.

From Table 2.2, except for the different retention times for the different derivatives, different percentages of the minor products for the different derivatives, we also can see that the derivatives from iBuCF-HFBuOH, -PFPrOH, and -iBuOH have higher responses by areas which are twice as much as that from the EtCF-EtOH derivative. This may indicate that iBuCF derivatives of amino acids provide higher sensitivity for detection than EtCF derivatives do. This aspect will be evaluated later in this chapter.

d) and e) Additional evidences for the direct involvement of alcohol constituents in the aqueous reaction medium containing the chloroformate reagent are from experiments in (Experimental d)) (Figure 2.14), and (Experimental e)) (Figure 2.15 and 2.16).

These data were obtained during the analysis of Phe after treatment with isobutyl chloroformate in an aqueous solution: (1) containing seven alcohols with equal volume (Experimental d)): pentafluoropropanol (PFPrOH), heptafluorobutanol (HFBuOH), trifluoroethanol (TFEtOH), methanol (MeOH), ethanol (EtOH), isobutanol (iBuOH), and trimethylsilylmethanol (TMSCH₂OH) (Figure 2.14); and (2) containing equimolar amounts of seven alcohols (the first experiment in Experimental e)): pentafluoropropanol (PFPrOH), heptafluorobutanol (HFBuOH), trifluoroethanol (TFEtOH), methanol, ethanol, propanol, and trimethylsilylmethanol (no iBuOH) (Figure 2.15).

In Figure 2.14, derivatives from all seven alcohols were produced. Figure 2.15 is more meaningful since each alcohol was present with an equimolar amount. Figure 2.15 is a reconstructed total ion current chromatogram resulting from analysis of the reaction mixture by GC-MS. Seven major peaks are obtained corresponding to the esters formed by reactions with the alcohols in the reaction medium. The different intensities of the peaks result from the differential nucleophilic reactivity of these alcohols with the acylpyridinium species and/or responses of the corresponding derivatives under EI-MS conditions. A minor peak is also present which derives from the esterification with the alcohol released from iBuCF.

The second experiment in (Experimental e)) resulted in Figure 2.16. In this experiment, Phe was derivatized by MCF with equimolar amounts of seven alcohols (no MeOH). The same explanation for Figure 2.15 is applicable to Figure 2.16


Figure 2.11. TIC (a) and EI mass spectrum (b) of Phe derivatized by reaction with MCF-TFEtOH.



Figure 2.12. TIC (a) and EI mass spectrum (b) of Phe derivatized by reaction with MCF-TMSCH₂OH.



Figure 2.13. TIC (a) and EI mass spectrum (b) of Phe derivatized by reaction with MCF-MeOH.

chloroformate	Area	Area	Percentage	Height	Height	Percentage	Scan No.	Scan No.
-alcohol	major	minor	of minor	major	minor	of minor	major	minor
	product	product	product	product	product	product	product	product
Section I								
iBuCF								
-iBuOH	3187	•	•	1205		•	520	•
-TFEtOH	2269	85	3.6%	561	56	10%	423	517
но-дяд-	3297	58	1.8%	798	29	3.6%	411	516
HPBuOH-	3191	124	3.9%	795	64	3.7%	420	517
-TMSCH20H	2610		<1%	1237	•	<1%	530	•
	2266	•	<1%	1148		<1%	530	
Section II								
MCF								
-MeOH	1659	٠	•	415			358	•
-TFEtOH	1846	179	9.7%	457	78	17%	318	353
-TMSCH20H	2145	186	8.7%	766	81	11%	474	354
Section III								
EtCF								
-EtOH	1549	•		412			418	

Table 2.2. Summary of results of Phe derivatives from different chloroformate and alcohol reagents.

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Figure 2.14. TIC of Phe derivatized by reaction with iBuCF in an aqueous solution containing an equal volume mixture of seven alcohols (MeOH, EtOH, iBuOH, TFEtOH, PFPrOH, HFBuOH, and TMSCH₂OH).



Figure 2.15. TIC of Phe derivatized by reaction with iBuCF in an aqueous solution containing an equimolar mixture of seven alcohols (MeOH, EtOH, PrOH, TFEtOH, PFPrOH, HFBuOH, and TMSCH₂OH). The peak labeled R=iBu represents a trace of the ester product formed with the alkoxyl group corresponding to the alkyl group of the chloroformate reagent.



Figure 2.16. TIC of Phe derivatized by reaction with MCF in an aqueous solution containing an equimolar mixture of seven alcohols (EtOH, PrOH, iBuOH, TFEtOH, PFPrOH, HFBuOH, and TMSCH₂OH). The peak labeled R=Me represents a trace of the ester product formed with the alkoxyl group corresponding to the alkyl group of the chloroformate reagent.

C. Effect of concentration of the alcohol in the reaction medium on the percentage of the minor derivatization product

(1) Experimental

Derivatization

a) Phe iBuCF-HFBuOH (0, 10, 30, 50, 70 µl)

To each of the five solutions containing 70 μ l H₂O, 10 μ l pyridine, and various amounts of HFBuOH: 0, 10, 30, 50, 70 μ l, were added 10 μ l of Phe (10 nmol/ μ l). To each solution, 10 μ l of iBuCF were added and the reaction mixtures were vortexed for 30 s. 200 μ l chloroform were used to extract the derivatives, and 2 μ l of chloroform layer was injected to GC-MS for analyses.

b) Leu iBuCF-HFBuOH (0, 10, 30, 50, 70 µl)

To each of the five solutions containing 60 μ l H₂O, 10 μ l pyridine, and various amounts of HFBuOH: 0, 10, 30, 50, 70 μ l, were added 20 μ l of solution containing Leu, Phe, Tyr, and Lys (each 2.5 nmol/ μ l). 10 μ l of methyl stearate (0.25 nmol/ μ l) were added as an internal standard. To each solution, 10 μ l of iBuCF were added and the reaction mixtures were vortexed for 30 s. 200 μ l chloroform were used to extract the derivatives, and 50 μ l of 1 M HCl were added as the counter phase. The chloroform solvent was evaporated under N₂ stream. The derivatives were redissolved in 100 μ l chloroform, and 2 μ l of solution were injected to GC-MS for analysis.

<u>GC-MS:</u>

Instrument: HP5892 MSDColumn: DB-5MS 30 m x 0.25mm I.D.Injection Temperature: 250-280°CInterface Temperature: 280°C

(2)Results and discussion

When Phe and Leu were treated with iBuCF with a different concentration of HFBuOH in the reaction medium, the percentages of the minor products (in which the ester moieties are from the iBuCF) were different. Increasing the amount of HFBuOH in the reaction medium, the amount of the minor products decreased. Under the experimental conditions specified, when HFBuOH was more than 30 μ l in the reaction medium, the percentages of the minor products were below 1% for both derivatives. This indicated that the major products were formed exclusively with higher concentration of alcohols. See Table 2.3.

Table 2.3. Effect of HFBuOH concentration in the reaction solution (H₂O 80 μ l, pyridine 10 μ l, iBuCF 10 μ l) on the percentage of minor derivatization product.

	Percentage of minor derivatization product		
HFBuOH (μl)	Phe	Leu	
0	-	-	
10	10%	8.9%	
30	3.2%	3.0%	
50	<1%	<1%	
70	<1%	<1%	

IV. Investigation of different chloroformate derivatives of amino acids

A. Amino acid derivatives formed by selected combinations of various chloroformates and alcohols

(1) Experimental

A solution of 20 amino acids in 0.1M HCl at a concentration of 0.5 μ g/ μ l each was prepared. The ethyl chloroformate (in the presence of different alcohols) derivatives of amino acids were prepared by adding 10 μ l to the amino acid mixture to a H₂O-alcohol-pyridine (Py) (50 μ l-30 μ l-10 μ l) solution. 10 μ l of ethyl chloroformate (EtCF) were then added to each solution and the reaction mixtures were vortexed for 5-10 s and extracted with 200 μ l CHCl₃. A 2- μ l aliquot of the CHCl₃ layer was injected for analysis. Other chloroformate (in the presence of different alcohols) derivatives were prepared by adding 10 μ l of the amino acids and 10 μ l of the chloroformate reagent to a solution of H₂O-alcohol-Py (70 μ l-30 μ l-10 μ l) following the same procedure. See group 1 to group 5 in Table 2.4 for the different combinations of chloroformates and alcohols from which the derivatives of the amino acids were made.

Analyses by GC-MS were carried out on a JEOL AX-505H double focusing mass spectrometer coupled to a Hewlett-Packed 5890J gas chromatograph. GC separation was achieved on a DB-1701 (15-m length x 0.25-mm i.d.) fused silica capillary column with a 0.25 μ m film coating from J. & W. Scientific (Rancho Cordova, CA). Direct (splitless) injection was used. Helium gas flow was approximately 1 ml/min. MS conditions were as follows: interface temperature 275°C, ion source temperature ca. 150-200°C, electron energy was 70 eV, scan rate of the mass spectrometer was 1 s/scan over the range of m/z 50-750. GC-FID (flame ionization detection) was carried out on the same gas chromatographic column with

Reagents Derivatives Ι EtCF-EtOH N(O,S)-ethoxycarbonyl ethyl ester PrCF-PrOH N(O,S)-propoxycarbonyl propyl ester iBuCF-iBuOH N(O,S)-isobutoxycarbonyl isobutyl ester II EtCF -TFE N(O,S)-ethoxycarbonyl trifluoroethyl ester -PFP N(O,S)-ethoxycarbonyl pentafluoropropyl ester -HFB N(O,S)-ethoxycarbonyl heptafluorobutyl ester III PrCF -TFE N(O,S)-propoxycarbonyl trifluoroethyl ester -PFP N(O,S)-propoxycarbonyl pentafluoropropyl ester -HFB N(O,S)-propoxycarbonyl heptafluorobutyl ester IV iBuCF-TFE N(O,S)-isobutoxycarbonyl trifluoroethyl ester -PFP N(O,S)-isobutoxycarbonyl pentafluoropropyl ester N(O,S)-isobutoxycarbonyl heptafluorobutyl ester -HFB V iBuCF-TMSCH₂OH N(O,S)-isobutoxycarbonyl trimethylsilymethyl ester N(O,S)-isobutoxycarbonyl trimethylsilyethyl ester -TMS(CH₂)₂OH $-TMS(CH_2)_3OH$ N(O,S)-isobutoxycarbonyl trimethylsilypropyl ester

Table 2.4. Chloroformate-alcohol reagents studied for amino acid derivatization.

injector and detector temperatures 260°C and 280°C; N₂ was the carrier gas.

(2) Results and discussion

The one-step chloroformate derivatization of amino acids in an aqueous medium has been extended with the use of a variety of alkyl chloroformate and alcohol reagents. In Husek's method, only methyl and ethyl chloroformate were applied and the application of other chloroformate derivatives were not investigated. In the previous section, we discovered that the ester moiety of the amino acid derivatives is directly dependent upon the type of alcohol used in the aqueous reaction medium. These results provide new insight into the one-step derivatization reaction and provide the basis for preparing a variety of chloroformate derivatives that can be assessed for optimizing the analysis of amino acids by GC with FID or by GC-MS. We also noticed that isobutyl chloroformate derivatives of Phe showed higher responses than that of ethyl chloroformate derivative of Phe (Table 2.2). Based on these results, various combinations of chloroformate reagents and alcohols were used to generate a wide variety of N(O,S)alkoxycarbonyl amino acid alkyl esters for analyses by GC or GC-MS with the objectives of obtaining higher sensitivity for detection, optimizing the chromatographic separation of the amino acid derivatives, and evaluating the influence of the alkyl group of the chloroformate (alkyl carbamate in the derivative) and also the structure of the alcohol (alkoxyl group in the ester of the derivative) in the reaction medium on the response of the derivatives detected by FID or by EI-MS. Chloroformate with larger alkyl groups (propyl and isobutyl), fluorinated alcohol (TFEtOH, PFPrOH, and

HFBuOH), and trimethylsilyl alcohols (TMSCH₂OH, TMS(CH₂)₂OH, and TMS(CH₂)₃OH) have been used for the investigation.

Figure 2.17 to 2.21 show the reconstructed TIC of each group of chloroformate-alcohol derivatives of 20 amino acids. In group 1, the derivatives formed by the reagents generate a response in MS analysis and by FID that increases slightly with the size of the alkyl groups for the groups studied: (isobutyl > propyl > ethyl) in the chloroformate reagent as well as in the derivatizing alcohol. Although it is not true for each amino acid, the responses of the iBuCF-iBuOH amino acid derivatives are higher than those of EtCF-EtOH derivatives. Meanwhile, in other series, the trend is not very clear, and not the same for every amino acid, or the difference is not significant. Generally, for each alcohol (HFBuOH, PFPrOH, and TFEtOH), the responses of the PrCF and iBuCF derivatives are higher than those of EtCF derivatives. For each chloroformate (EtCF, PrCF, and iBuCF), the responses from HFBuOH derivatives are higher, although the factors of difference are generally within 2-3. The overall highest detectability is produced by iBuCF-iBuOH, iBuCF-HFBuOH, and iBuCF-TMSCH₂OH derivatization reagents. Figure 2.22 shows that the GC-FID responses of the 20 amino acids derivatized with iBuCF-iBuOH, iBuCF-HFBuOH, and iBuCF-TMSCH₂OH are higher than those prepared with EtCF-EtOH. Tyr and Hyp also can be derivatized and separated from the other 20 amino-acids (even though these two were not included in the mixture represented in Figure 2.17-2.22. The guanidino group on the side chain of Arg is not derivatized by the reaction mixture described here as verified by detection with FAB; Arg in this form cannot be eluted from the GC column. A quantitative comparison of the detector response to various derivatives is given in Table 2.5, which lists the ratio of GC-FID peak area

produced by the derivatives made from PrCF-PrOH, iBuCF-iBuOH or iBuCF-HFBuOH to those made from EtCF-EtOH. Table 2.6 compares the reconstructed TIC responses of different groups of derivatives relative to those prepared from EtCF-EtOH. In nearly all cases, derivatives prepared from EtCF-EtOH gave a lower response. The mass spectra of the amino acid derivatives reported herein are produced through fragmentation pathways similar to those described earlier for mass spectra of EtCF-EtOH derivatives of amino acids in section II of this chapter and Ref. 4. The amino acid derivatives described here are expected to have recoveries similar to those of amino acids derivatized with EtCF-EtOH [1-3]. The inclusion of a suitable internal standard, such as norleucine (Figure2.17-2.22) or stable isotope labeled amino acids, would make the approach described here suitable for quantitative analyses.

In group 5, with TMS alcohols, it is found that an increase of the size of the alcohol (TMS(CH₂)₃OH>TMS(CH₂)₂OH>TMSCH₂OH) causes greater production of the derivatives esterified with the alkyl group of the chloroformate reagent. The increased competition from this side reaction may result from the weaker nucleophilic attack on the intermediate acylpyridinium species for the bulkier TMS alcohols. In general, the formation of minor products through path B of Reaction 2-2 are typically much less than 10% as indicated by the total ion current chromatogram of the derivatives; however, the yields of side products were not systematically investigated for each amino acid with each derivatization reagent combination.

Fast GC temperature programs were applied to EtCF-EtOH and iBuCF-iBuOH derivatives of amino acids. Complete separation of the



Figure 2.17. TICs of 20 amino acid derivatives prepared from different chloroformate-alcohol reagents (a) EtCF-EtOH, (b) PrCF-PrOH, (c) iBuCF-iBuOH. GC-MS analysis of an aliquot of the reaction mixture containing 50 ng of each amino acid on to a 15-m, 0.25-mm i.d. column containing a 0.25- μ m film of DB-1701. GC temperature programs: (a) from 100°C to 200°C at 10°C / min, then 20°C / min to 280°C; (b) and (c) from 120°C to 200°C at 10°C / min, then 20°C / min to 280°C.

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Figure 2.18. TICs of 20 amino acid derivatives prepared from EtCF with (a) TFEtOH and (b) HFBuOH. Temperature programs: from 100°C to 200°C at 10°C / min, then 20°C / min to 280°C.



Figure 2.19. TICs of 20 amino acid derivatives prepared from PrCF with (a) TFEtOH, (b) PFPrOH, and (c) HFBuOH. Temperature programs: (a) from 100° C to 200° C at 10° C / min, then 20° C / min to 280° C; (b) and (c) from 120° C to 200° C at 10° C / min, then 20° C / min to 280° C.



Figure 2.20. TICs of 20 amino acid derivatives prepared from iBuCF with (a) TFEtOH, (b) PFPrOH, and (c) HFBuOH. Temperature program: from 120°C to 200°C at 10°C / min, then 20°C / min to 280°C.



Figure 2.21. TIC of 21 amino acid derivatives prepared from iBuCF-TMSCH₂OH. Temperature program: from 120°C to 180°C at 10°C / min, then 20°C / min to 280°C. GC column OV1701 10-m, 0.25-mm i.d., 0.2- μ m film. (280 ng for each amino acid. Cys was not identified.)



 140° C to 200° C @ 10° C/min, then 30° C/min to 280° C

Figure 2.22. GC-FID chromatograms of 20 amino acid derivatives prepared from different chloroformate-alcohol reagents (1) EtCF-EtOH, (2) iBuCF-iBuOH, (3) iBuCF-HFBuOH, and (4) iBuCF-TMSCH₂OH. The chromatograms result from injection of an aliquot of the reaction mixture containing 50 ng of each amino acid on to a 15-m, 0.25-mm i.d. column containing a 0.25- μ m film of DB-1701.



 150° C to 200° C @ 10° C/min, then 40° C/min to 280° C.

Figure 2.22. GC-FID chromatograms of 20 amino acid derivatives prepared from different chloroformate-alcohol reagents (1) EtCF-EtOH, (2) iBuCF-iBuOH, (3) iBuCF-HFBuOH, and (4) iBuCF-TMSCH₂OH. The chromatograms result from injection of an aliquot of the reaction mixture containing 50 ng of each amino acid on to a 15-m, 0.25-mm i.d. column containing a 0.25- μ m film of DB-1701.



Figure 2.23. TIC of 20 amino acid derivatives prepared from iBuCF-iBuOH. GC separation is done within 6 min. GC temperature program: 120°C to 280°C at 40°C/min.

	PrCF-PrOH	iBuCF-iBuOH	iBuCF-HFBuOH
	/EtCF-EtOH	/EtCF-EtOH	/EtCF-EtOH
Ala	1.5	2.0	1.4
Gly	1.8	2.6	2.4
Val	1.2	1.6	1.5
Leu	1.3	1.7	1.4
Ile	1.1	1.6	1.4
n-Leu	1.5	1.7	1.9
Pro	1.2	1.7	1.2
Thr	1.3	1.7	1.5
Ser	1.8	1.3	2.0
Asn	1.5	1.9	1.6
Asp	0.5	3.1	2.9
Met	1.7	1.5	1.4
Glu	1.3	2.2	4.3
Phe	1.2	1.5	1.4
Cys	1.3	1.4	2.0
Gln	4.1	3.2	4.5
Orn	1.1	1.2	1.4
Lys	1.0	1.1	1.2
His	0.7	0.4	5.7
Trp	0.9	1.2	2.7

Table 2.5. Ratio of peak area on GC-FID of indicated derivatives relative to those prepared with EtCF-EtOH: (response for EtCF-EtOH derivatives were the average of results from triplicate analyses; responses for the indicated derivatives were the average of results from duplicate analyses).

	PrCF-PrOH		iBuC	iBuCF-iBuOH		iBuCF-HFBuOH	
	/EtCl	F-EtOH	/EtCI	/EtCF-EtOH		F-EtOH	
	A	H	A	Н	A	H	
 Ala	0.99	0.95	 1.79	2.09	2.05	 1.93	
Gly	1.13	1.13	1. 9 8	2.24	3.33	3.02	
Val	0.90	0.81	1.09	1.21	1.70	1.57	
Leu	0.97	1.01	1.49	1.58	2.24	2.96	
Ile	0.78	0.81	0.87	0.91	1.37	1.75	
Leu	0.98	0.92	1.42	1.45	2.01	1.83	
Pro	1.06	1.16	1.60	1.92	1.88	1.88	
Thr	1.14	1.39	1.58	1.70	1.87	2.02	
Ser	0.85	0.67	1.10	1.08	1.05	1.43	
Asn	1.23	1.18	1.69	1.72	2.49	2.42	
Asp	0.37	0.37	1.88	2.66	3.51	2.81	
Met	1.81	1.55	1.52	1.53	1.84	1.52	
Glu	1.20	1.30	0. 94	0.83	2.33	2.16	
Phe	1.22	1. 79	1.41	1.90	1.83	1.82	
Cys	1.48	1.85	0.62	0.83	2.02	2.25	
Gln	2.03	1.96	1.70	1.68	1.40	1.22	
Orn	1.22	1.87	1.01	1.58	1.02	1.27	
Lys	1.07	1.42	0.95	1.14	0.71	0.67	
His	1.68	1.65	1.17	0.83	2.93	3.17	
Trp	1.10	0.84	1.20	0.86	1.52	1.05	

Table 2.6. Ratio of both peak area and peak height of reconstructed TIC corresponding to the indicated derivatives relative to those made with EtCF-EtOH from analyses by GC-MS (EI).

A=Area; H=Height

derivatives of 20 amino acids can be accomplished within 6 minutes in both cases (Figure 2.23).

B. Evaluation of reaction conditions

The reaction conditions of iBuCF-iBuOH derivatives of amino acids have been evaluated.

(1) Effect of iBuOH concentration in the reaction medium a) Experimental

A solution of 50 μ l of 24 amino acids (2 μ g/each) was added to each of the six solutions containing 30 μ l H₂O, 10 μ l pyridine, and various amount of iBuOH (0, 10, 20, 30, 40, 50 μ l). To each solution, 10 μ l of iBuCF were added and reaction mixtures were vortexed for 30 s. The derivatives were extracted by chloroform twice, (200 μ l and 100 μ l for each time); the solvent was removed (evaporated) under N₂ stream. Derivatives were redissolved in 100 μ l chloroform. A 2- μ l aliquot of chloroform solution were injected for analysis. The GC-MS conditions were the same as those in the previous experiments.

b) Results and discussion

The TIC responses of the derivatives of Phe, 4-Cl-Phe, Tyr, nLeu, Asn, Trp were approximately constant over the entire range of iBuOH (0 to 50 μ l) added in the reaction solution (Figure 2.24). The TIC responses of the derivatives of Ala, Gly, Val, Leu, Ile, Pro, Thr, and Asp increased when the amount of iBuOH added increased from 0 to 20 μ l, and their responses did not change when the amount of iBuOH added increased from 20 to 50 μ l (Figure 2.25). The TIC responses of Orn, Lys, and Met decreased as the amount of iBuOH added increased (Figure 2.26). These results indicated that the addition of 20-40 μ l of iBuOH in the reaction solution containing 80 μ l of water was appropriate to achieve good responses from all the amino acids. In these experiments, His, Gln and Cys-Cys derivatives were not observed. Cys was strange in these experiments. The response of Cys derivative was good when no iBuOH was added in the reaction solution, but Cys peak disappeared in the case of 10 and 20 μ l of iBuOH were added. Cys peak was detected again when 30, 40, 50 μ l of iBuOH were added. In the other parallel experiments, when the amino acid mixture was newly made from each amino acid solution, the responses of Cys derivative was approximately constant over the entire range of 0 to 50 μ l of iBuOH added. Also from the newly prepared amino acid mixture, response of Hyp derivative was approximately constant when the amount of iBuOH added was from 10 to 40 μ l while its response was relatively lower in the case that no iBuOH was added.

(2) Effect of concentration of H₂O in the reaction mediuma) Experimental

A solution of 10 μ l of 20 amino acids (0.5 μ g/ μ l) were added to each of six solutions containing 30 μ l iBuOH, 10 μ l pyridine, and various amounts of H₂O (0, 10, 20, 30, 40, 50 μ l). To each solution, 10 μ l of iBuCF were added and reaction mixtures were vortexed for 30 s. The derivatives were extracted by chloroform twice, 200 μ l and 100 μ l for each time; the solvent was removed (evaporated) under N₂ stream. Derivatives were redissolved in 200 μ l chloroform. A 2- μ l aliquot of chloroform solution was injected for analysis. The GC-MS conditions were the same as those in the previous experiments.



Figure 2.24. Effect of iBuOH concentration in the reaction medium for formation of iBuCF-iBuOH derivatives of amino acids (1).



Figure 2.25. Effect of iBuOH concentration in the reaction medium for formation of iBuCF-iBuOH derivatives of amino acids (2).



Figure 2.26. Effect of iBuOH concentration in the reaction medium for formation of iBuCF-iBuOH derivatives of amino acids (3).

b) Results and discussion

The results of TIC responses from these experiments did not follow very smooth curves, but the trend of the TIC responses for each amino acid derivative as a function of the volume of H2O added in the reaction solution still can be described. The overall trend of TIC responses for most of the derivatives was that the TIC responses increased slightly when the amount of H₂O added increased, and then the responses were about constant over a range of volume of water added. The ranges were different for different derivatives, but each amino acid derivative was among one of the several groups. Approximately, the TIC responses of Asn, Cys, Met, Thr, Ser, Glu, Orn, and Trp were about constant when 20 to 100 μ l of water were present in the reaction medium. The corresponding range for constant responses was from 40 to 100 µl for Ala, Gly, Val, and Leu, and was from 60 to 100 µl for Phe, nLeu, and Pro, while the range was 20 to 60 μ l for Lys. The TIC responses of Ile and Asp decreased slightly over the range of 10 to 100 μ l of water. The results are summarized in Table 2.7. His and Gln derivatives were not detected in these experiments. These experiments indicated that 40 to 80 μ l of H₂O were appropriate for the reaction solution containing 30 μ l of iBuOH, and the amount of H_2O in the reaction solution did not seem to be critical for the chloroformate derivatization of amino acids.

(3) Effect of iBuCF concentration in the derivatizationa) Experimental

A solution of 50 μ l of 24 amino acids (2 μ g/each) were added to each of five solutions containing 30 μ l H₂O, 30 μ l iBuOH, and 10 μ l pyridine. To each solution, various amounts of iBuCF (5, 10, 15, 20, 30 μ l) were added and reaction mixtures were vortexed for 30s. The derivatives were

Table 2.7. Effect of H_2O volume in the reaction medium (X μ l H_2O , 30 μ l iBuOH, and 10 μ l pyridine) on the TIC responses of iBuCF-iBuOH derivatives of amino acids.

Amino acids	Range of volume of H_2O added to
	produce constant TIC responses of
	amino acid derivatives
Asn, Thr, Ser, Met, Glu, Cys, Orn,	20 - 100 µl
Trp	
Ala, Gly, Val, Leu	40 - 100 µl
nLeu, Pro, Phe	60 - 100 µl
Lys	20 - 60 µl
Ile, Asp	slightly decrease from 10 - 100 µl

extracted by chloroform twice, 200 μ l and 100 μ l for each time; the solvent was removed (evaporated) under N₂ stream. Derivatives were redissolved in 100 μ l chloroform. A 2- μ l aliquot of chloroform solution was injected for analysis. The GC-MS conditions employed were the same as those in the previous experiments.

b) Results and discussion

The TIC responses of most of the derivatives of amino acids were constant when the iBuCF added was 5 to 10 μ l. Their responses dropped significantly when the amount of iBuCF added was more than 10 to 15 μ l. Part of the results are shown in Figure 2.27. The results from these experiments can be explained by the hydrolysis of the large excess of iBuCF. The HCl released from the hydrolysis lowered the pH of the reaction solution. (See discussions in the next section: Effect of concentration of pyridine.)

(4) Effect of pyridine concentration in the reaction medium a) Experimental

A 10- μ l solution of 24 amino acids (each 42 ng/ μ l) was added to each of the six solutions containing 70 μ l H₂O, 30 μ l iBuOH, and various amounts of pyridine: 0, 2, 5, 10, 15, and 20 μ l. To each solution, 10 μ l of iBuCF were added and the reaction mixtures were vortexed for 30 s. The derivatives were extracted by chloroform twice, 200 μ l and 100 μ l for each time; the solvent was removed (evaporated) under N₂ stream. Derivatives were redissolved in 25 μ l chloroform. A 2- μ l aliquot of chloroform solution was injected for analysis. The GC-MS conditions were the same as those used in the previous experiments.



Figure 2.27. Effect of iBuCF concentration for the derivatization of amino acids by iBuCF-iBuOH.

Without pyridine, there were no derivatives detected. When the amount of pyridine was 2 μ l, weak signals for some amino acid derivatives (such as Gly, Val, and Phe) were detected. When the amount of pyridine increased up to 5 μ l, the responses of all the amino acid derivatives increased. When the amount of pyridine was from 10 to 20 μ l, there were no significant changes for the TIC responses for all the amino acid derivatives. (His, Trp, and Cys-Cys derivatives were not detected in these experiments.)

The pHs of the reaction solutions before and after the addition of iBuCF in each experiment with pyridine from 0 to 20 μ l are listed in Table 2.8.

Pyridine (μl)	0	2	5	10	15	20
pH (before)	1-2	5-6	6	6-7	7	7
pH (after)	1-2	1-2	2	4-5	5	5

Table 2.8. pH vs. pyridine volume in the reaction medium for amino acid derivatization by iBuCF-iBuOH

Pyridine did not only act as a catalyst, but also as a buffer to control the pH of the reaction solution for the chloroformate derivatization reaction. When the amount of pyridine added was between 10 to 20 μ l, the pHs before the addition of pyridine were the same (~7) for each solution, and the pHs after the addition of iBuCF were also the same (~5) for each solution. The constant pH changes of the reaction solutions paralleled the constant TIC responses of the amino acid derivatives in the range of addition of 10 to 20 μ l of pyridine . With a lesser amount of pyridine added in the reaction solution, the pHs of the reaction solutions after the addition of iBuCF were low (~1-2). The carboxyl and amino groups were exclusively protonated at these low pH which might be the reason why there were less or no derivatives formed. The amount of pyridine should be no less than 8 to 10 μ l in the reaction solution containing 80 μ l H₂O for the amino acid derivatization with iBuCF.

(5) Effect of other bases and buffer solutions

a) Experimental

A solution of 50 µl of 24 amino acids (2 µg/each) was added to each of the two solutions containing 30 µl H₂O, 30 µl iBuOH. Instead of pyridine, 10 µl of 2,4,6-trimethylpyridine or 100 µl of DMAP (10 µg/µl in chloroform) were added. To each solution, 10 µl of iBuCF were added and reaction mixtures were vortexed for 30 s. The derivatives were extracted by chloroform twice, 200 µl and 100 µl for each time; the solvent was removed (evaporated) under N₂ stream. Derivatives were redissolved in 100 µl chloroform. A 2-µl aliquot of chloroform solution was injected for analysis. The GC-MS conditions were the same as those in the previous experiments.

Instead of any organic base, 50 μ l of buffer: (i) 0.2 M sodium phosphate (pH=7.68), (ii) 0.1 M sodium borate (pH=9.5), (iii) 0.1% HAc/NH4Cl (pH 4.0) were used in the same experiments as above.

b) Results and discussion

Although DMAP was a stronger base (pKa~10), no derivatives of amino acids were detected in GC-MS analysis. The pH of the reaction solution before and after the addition of iBuCF was ~8 and ~5 respectively. Derivatives of amino acids were formed when using 2,4,6trimethylpyridine. The results were about the same as what was observed when pyridine was added. The pHs of the reaction solution before and after the addition of iBuCF were both ~7-8. These bases did not provide any advantages over pyridine.

No derivatives of amino acids were detected in each of the cases of using buffer solutions. This further confirmed the importance of pyridine as a catalyst in the chloroformate derivatization of amino acids.

(6) Evaluation of acid / base wash of the chloroform phase during the extraction of derivatives

a) Experimental

A mixture of amino acids were derivatized by 10 μ l iBuCF in a reaction solution containing 80 μ l H₂O, 30 μ l of iBuOH, and 10 μ l pyridine. Afterwards, different procedures were followed:

i) "Normal": The derivatives were extracted by 200 µl chloroform.

ii) "Acid wash": A 100 μ l of 1 M HCl was added in the reaction solution at the time of chloroform extraction.

iii) "Acid wash + base wash": The chloroform solution from ii) was washed by 100 μ l of 0.5 M NaHCO₃.

The "acid wash" experiment was repeated for the derivatization of the amino acid mixture with 10 μ l of EtCF. The solution contained 60 μ l H₂O, 30 μ l EtOH, and 10 μ l pyridine.
b) Results and discussion

During the chloroform extraction, HCl solution can transfer the remaining pyridine into the upper aqueous phase. For iBuCF derivatives of amino acids, the TIC of the amino acid derivatives prepared with i), ii), and iii) were compared. No significant difference in responses and separations were found. The same result was found for the EtCF derivatives of amino acids.

C. Reproducibility of the derivatization and analyses of amino acids with chloroformate-alcohol reagents

(1) Experimental

a) iBuCF-iBuOH

Five samples were made following the procedure below. A mixture of 24 amino acids (2 μ g/each) were derivatized by 10 μ l iBuCF in a solution containing 60 μ l H₂O, 30 μ l iBuOH, and 20 μ l of pyridine. The derivatives were extracted by chloroform three times, 200 μ l, 100 μ l, and 100 μ l for each time; the solvent was removed (evaporated) under N₂ stream. Derivatives were redissolved in 100 μ l chloroform. A 2- μ l aliquot of chloroform solution was injected for analysis. The GC-MS conditions were the same as those in the previous experiments.

b) EtCF-EtOH

Five samples were made following the procedure in a), but iBuCF was replaced by EtCF.

c) iBuCF-HFBuOH

Three samples were made following the procedure in a), but iBuOH was replaced by HFBuOH.

(2) Results and discussion

The reproducibility of the whole process of chloroformate derivatization and GC-MS analysis of amino acids was evaluated for three chloroformate-alcohol reagents. The results are listed in Table 2.9 which includes the mean relative weight responses (RWR) of peak area and height in TIC (relative to the internal standard, nLeu), and relative standarddeviations (RSD%). The RSDs for EtCF derivatives were around 10-20%. Ser did not show a good peak resulting in a high RSD. Most of the RSD's were around 10% for iBuCF-iBuOH derivatives and were below 10% for iBuCF-HFBuOH derivatives. The reproducibilities were not very good because each step of the whole derivatization procedure could contribute some variations. The reaction proceeded in a small narrow glass tube which was about 4 cm long and 0.3 to 0.4 mm in the diameter. The starting materials and derivatives may attach on to the wall of the glass tube although vortexing has been performed to mix the solution during the reaction. During the extraction-separation step, technically, it was very hard to exactly separate the entire chloroform layer from the aqueous layer. It also may result in losing part of the most volatile derivatives to remove the chloroform solvent under N2 stream.

Table 2.10 compares the TIC responses of the other two derivatives relative to those prepared from EtCF-EtOH. For each amino acid, the TIC response of iBuCF-HFBuOH derivative seems to be higher than those from EtCF-EtOH. The factors of the difference can be up to 3 to 4 for Ala, Gly, Val, Leu, and Ile. Most derivatives of iBuCF-iBuOH show higher TIC responses than those from EtCF-EtOH except for Met, Orn, Lys, Tyr, and Trp. In these experiments, Hyp, Gln derivatives were not detected from these three derivatization reagents. (Hyp was detected in the earlier experiments in section B when the amino acid solution was newly made.) His was only detected from iBuCF-HFBuOH derivatization reagents. Cys was not detected from iBuCF-iBuOH reagents.

The analyses of ten times diluted solution showed that the iBuCF-HFBuOH derivatives had much better signal to background ratios than those of EtCF-EtOH derivatives.

V. Comparisons of EI, PCI, and ECNI mass spectrometry responses of fluorinated derivatives of amino acids

A. Introduction

Introducing fluorinated moieties into amino acid derivatives from fluorinated alcohols through our modified chloroformate derivatization procedure is one of the most important applications of our investigation of the mechanism of the one-step chloroformate derivatization for amino acids. The fluorinated derivatives prepared by this simple method have the potential to facilitate analyses by electron capture negative ionization mass spectrometry. Characterization of EtCF-TFEtOH amino acid derivatives with positive chemical ionization (PCI) and electron capture negative ionization (ECNI) GC-MS based on our modified reaction mechanism was reported earlier this year by Moini [43] paralleling our research.

Table 2.9. Effect of reagent composition on derivatization formation and response. Mean relative weight responses of peak area of TIC (relative to internal standard nLeu) (RWR) and relative standard deviation (RSD) for the indicated amino acid derivatives (Five individual samples for EtCF-EtOH, iBuCF-iBuCF derivatization, and three individual samples for iBuCF-HFBuOH derivatization).(* Leu and Ile coelute.; ** Lys peak contains His.)

Amino	EtCF-	EtOH	iBuCF-	iBuOH	iBuCF-	HFBuOH
Acid	RWR	RSD%	RWR	RSD%	RWR	RSD%
Ala	0.393	20	0.505	20	0.676	6.8
Gly	0.882	17	0.983	7.6	1.17	2.0
Val	0.641	15	0.996	10	1.17	4.4
Leu	0.725	6.1	0.951	4.3	0.830*	0.48
Ile	0.646	11	0.830	3.6	0.830	0.48
Pro	1.21	16	1.08	3.6	1.00	2.3
Thr	0.959	20	0.461	10	0.545	0.65
Ser	0.395	62	0.195	20	0.246	2.7
Asn	1.19	16	0.707	4.8	0.749	4.6
Asp	1.40	16	1.03	7.1	.873	4.1
Met	1.83	15	0.268	28	1.07	4.3
Glu	0.384	15	0.254	12	0.316	12
Phe	2.35	17	1.20	7.2	1.55	2.8
Cys	0.734	18	-	-	0.390	5.2
4-Cl-Phe	2.15	20	0.962	9.0	1.16	6.9
Orn	0.984	21	0.275	15	0.538	17
Lys	1.46	23	0.359	17	0.686**	16*
Tyr	1.93	21	0.505	18	0.950	11
Trp	0.656	38	0.165	30	0.646	11
Cys-Cys					0.421	28

	iBuCF-iBuC	iBuCF-iBuOH/EtCF-EtOH		iBuCF-HFB/EtCF-EtOH	
	Area	Height	Area	Height	
Ala	3.0	3.2	4.1	4.4	
Gly	2.6	2.9	3.1	3.6	
Val	3.5	4.5	4.2	5.2	
Leu	2.9	3.2	2.6	3.1	
Ile	2.9	3.3	3.0	3.8	
nLeu	2.3	2.2	2.3	2.2	
Pro	2.0	2.8	2.0	2.6	
Thr	1.1	1.0	1.4	2.1	
Asn	1.4	1.7	1.5	1.9	
Met	0.30	0.49	1.4	1.7	
Asp	1.7	2.4	1.5	1.5	
Phe	1.2	1.5	1.6	1.4	
Glu	1.5	2.0	1.9	2.4	
Cl-Phe	1.0	1.3	1.3	1.8	
Orn	0.64	0.84	1.4	2.0	
Lys	1.8	0.68	1.1	1.5	
Tyr	0.60	0.55	1.2	1.4	
Trp	0.59	0.58	2.4	3.6	
Cys	-	-	1.3	2.1	

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Table 2.10. Ratio of both peak area and peak height of reconstructed TIC corresponding to the indicated derivatives relative to those made with EtCF-EtOH from analyses by GC-MS (EI) (results from experiments for reproducibility).

B. EtCF-HFBuOH and iBuCF-HFBuOH derivatives of amino acids in EL PCL and ECNI modes of GC-MS analyses

EtCF-HFBuOH amino acid derivatives have been compared in EI, PCI, and ECNI modes. EI analyses were performed under conditions of 70 eV and 100 μ A ionization current. The PCI and ECNI conditions were ~100 eV and 300 μ A.

Comparing the EI and PCI modes for the EtCF-HFBuOH derivatives of amino acids showed similar TIC and mass spectra except for the higher abundance of [M+H]⁺ in PCI spectra (Figure 2.28a and 2.28b). In the ECNI mode the maximum intensity (base peak) of TIC was higher than that in the PCI mode. Ala, Gly, Val, Leu, Ile, Pro, and nLeu derivatives that eluted at a lower temperature, showed relatively low intensities (Figure 2.28c). The reason for this was not clear, however; the same situation was found for iBuCF-HFBuOH derivatives in ECNI mode as well. The ions, [M-HFB]⁻, ([M-183]⁻), and [M-28]⁻ ions, were observed in relatively high abundance for some amino acid derivatives in the ECNI spectra. But the spectra of the EtCF-HFBuOH amino acid derivatives in the ECNI mode were not simplified as expected and the base peak in each spectrum was not from one specific fragmentation pathway common to all the spectra of these derivatives. The spectra did not have simple, clear patterns.

No dominating characteristic ions, formed from a common specific fragmentation pathway for each of the EtCF-HFBuOH amino acid derivatives, can be used for quantitation in ECNI mode to increase the sensitivity of detection for amino acids. The intensities of each $[M+1]^+$ ion in PCI mode and each $[M-1]^-$ ion in ECNI mode from their mass chromatograms for the amino acid EtCF-HFBuOH derivatives are listed in Table 2.11. It can be noted that regarding the $[M+1]^+$ ion in PCI and the



Figure 2.28. TICs of EtCF-HFBuOH derivatives of amino acids in (a) EI, (b) PCI, and (3) ECNI modes of GC-MS analysis.

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	EtCF-HFBuOH	PCI	ECNI
Amino acid	MW	I [M+1] ⁺	I [M-1] ⁻
Ala	343	22.2	13.1
Gly	329	54.1	13.4
Val	371	17.4	17.9
Leu + Ile	385	27.9	24.2
nLeu	385	~26	~17
Pro	370	33.4	12.5
Thr	373	7.9	4.3
Asp	569	14.7	12.0
p-Glu	311	46.2	4.4
Ser	359	5.4	2.5
Asn	368 (-H ₂ O)	29.2	119.5
Glu	583	2.3	0.8
Met	403	17.9 (M+)	21.6
Phe	419	20.7	25.9
Суз	447	25.6	8.2
Cl-Phe	453	20.9	50 / 469.4 (by M)
Orn	458	~8 (M+)	~8
His	481	53.3	18.4
Lys	472	10.4	21.4
Trp	458	31.2 (M+)	34.0
Gln	400	-	4.8
Cys-Cys	748	2.3	28.7

Table 2.11. Comparison of absolute intensities of [M+1]⁺ in PCI and [M-1]⁻ in ECNI of amino acid EtCF-HFBuOH derivatives.

[M-1]⁻ ion in ECNI modes, the negative ion mode does not show any advantage for most of the N-ethoxycarbonyl amino acid heptafluorobutyl esters. A similar result was reported for EtCF-TFEtOH amino acid derivatives [43].

The same chromatographic and spectral results were found for iBuCF-HFBuOH derivatives of amino acids when they were compared in EI, PCI, and ECNI modes of analyses by GC-MS.

C. EtCF-pentafluorobenzyl alcohol (PFBzlOH) derivatives of amino acids in PCI and ECNI modes of GC-MS analyses

EtCF-PFBzlOH amino acid derivatives have been compared in PCI and ECNI modes of GC-MS analyses. The PCI spectra of these derivatives followed general fragmentation pathways as described in section I of this chapter and in Ref. 4. The major fragmentation pathway resulted from $[MH-PFBCO_2H]+([MH-226]+)$ plus an additional PFB+ (m/z 181) ion. (Thr, Ser, Gln, Glu, His, Trp, Cys-Cys peaks were not detected or not identified in this experiment). In the ECNI mode, the [M-PFB]⁻ ([M-181]⁻) ion dominated each spectrum of the derivative, and the spectra were simple with only a few ions. (Ser, Gln, Trp, and Cys-Cys peaks were not detected or not identified). Representative mass spectra of each ionization mode are shown in Figure 2.29 for the Val derivative and in Figure 2.30 for the Phe derivative. It can be seen that both Val and Phe EtCF-PFBzlOH derivatives show only two ions in their ECNI spectra while their PCI mass spectral peaks represent more fragmentation. The ECNI spectrum of Val is dominated by ion of m/z 188, the [M-181]⁻ ion, and the ECNI spectrum of Phe is also dominated by the [M-181]⁻ ion (m/z 236). The ECNI spectra of EtCF-PFBzlOH derivatives of amino acids are summarized in Table 2.12. The

intensities of the base peaks, [M-PFB]⁻, in ECNI and the intensities of [MH-PFBCO₂H]⁺ ions (the base peaks in most spectra) in PCI of amino acid EtCF-PFBzlOH derivatives are listed in Table 2.13. These intensities are from the mass chromatogram for each ion. It is probable that most of the base peaks in the ECNI spectra are offscale since they all reach a count of 1600 which might be the maximum for the instrument utilized. The different intensities of these two ions indicate that sensitivity is increased by at least one to two order of magnitude in the ECNI mode over PCI ion detection for most of the amino acid derivatives detected in both modes. The [M-181]⁻ ions can be used as the trace ions for profiling and quantitation of amino acids in the ECNI mode of GC-MS analysis after derivatization with EtCF-PFBzlOH reagents.

Although an increase in sensitivity of detection of amino acid derivatives in the ECNI mode compared to PCI mode by EtCF-PFBzlOH derivatization was observed, there are still some practical problems for this derivatization scheme: 1) In ECNI, the resolution of TIC was not as good as that in PCI, and there were peaks which were not identified. 2) It was verified from the TIC and spectra in PCI mode, that for some amino acids, the TIC response of the derivatization product in which the ester part was an ethyl group from ethyl chloroformate was half of that of the major product of pentafluorobenzyl ester. These may come from the weak nucleophilic reactivity of pentafluorobenzyl alcohol. 3) PFBzlOH was not volatile and cannot be removed under N₂ stream.



Figure 2.29. (a) PCI and (b) ECNI mass spectra of Val EtCF-PFBzlOH derivative.



Figure 2.30. (a) PCI and (b) ECNI mass spectra of Phe EtCF-PFBzlOH derivative.

Amino acid	[M-181] ⁻	Base peak	Other ions
Ala	m/z 160	m/z 160	m/z 114
Gly	m/z 146	m/z 146	m/z 100
Val	m/z 188	m/z 188	m/z 142
Leu	m/z 202	m/z 202	m/z 156
Ile	m/z 202	m/z 202	m/z 156
nLeu	m/z 202	m/z 202	m/z 156
Pro	m/z 186	m/z 186	
Thr	m/z 190	m/z 190	m/z 163
p-Glu	m/z 128	m/z 128	
Asn	m/z 185	m/z 185	m/z 139, 227
Met	m/z 220	m/z 220	m/z 174
Phe	m/z 236	m/z 236	m/z 190
Cys	m/z 264	m/z 264	m/z 218, 158, 174
Cl-Phe	m/z 270	m/z 270	m/z 224
Asp	m/z 384	m/z 384	m/z 186, 338
Orn	m/z 275	m/z 275	m/z 229
Glu	m/z 398	m/z 398	m/z 200, 218, 352
Lys	m/z 289	m/z 289	m/z 180, 243
His	m/z 298	m/z 298	m/z 180, 252

Table 2.12. Summary of ECNI mass spectra of amino acid EtCF-PFBzlOH derivatives.

Amino	EtCF-	ECNI	Intensity	PCI	Intensity
acid	PFBzlOH			[MH-	
		[M-PFB] ⁻		PFBCO ₂ H]+	
	(MW)	([M-181] ⁻)	[M-181] ⁻	[MH-226]+	[MH-226] ⁺
Ala	341	m/z 160	1600*	m/z 116	64
Gly	327	m/z 146	400	m/z 102	69
Val	369	m/z 188	1600*	m/z 144	100
Leu	383	m/z 202	1600*	m/z 158	~70-80
Ile	383	m/z 202	1600*	m/z 158	126
nLeu	383	m/z 202	1600*	m/z 158	~90-100
Pro	367	m/z 186	1600*	m/z 142	100
Thr	371	m/z 190	350	-	-
p-Glu	309	m/z 128	400	-	-
Asn	366 (-H ₂ O)	m/z 185	1600*	m/z 141	200
Met	401	m/z 220	1600*	m/z 176#	12
Phe	417	m/z 236	1600*	m/z 192#	36
				m/z 328 (bp)	73
Cys	445	m/z 264	1600*	m/z 220#	42
Cl-Phe	451	m/z 270	1600*	m/z 226#	16
				m/z 362 (bp)	48
Asp	565	m/z 384	1600*	m/z 340	18
Orn	456	m/z 275	768	m/z 142	7.0
Glu	579	m/z 398	169	-	-
Lys	470	m/z 289	400	m/z 156	3.0
His	479	m/z 298	152	-	•

Table 2.13. Comparison of intensities of [M-PFB]⁻ in ECNI and [MH-PFBCO₂H]⁺ in PCI of amino acid EtCF-PFBzlOH derivatives.

*. maximum intensity

#. not base peak

VI. Conclusions

Characterization of chloroformate-alcohol derivatives of amino acids has been conducted to facilitate the identification of amino acids in analysis by GC-MS using the chloroformate derivatization method. The one-step chloroformate derivatization of amino acids in an aqueous medium has been extended with the use of a variety of alkyl chloroformate and alcohol reagents. It was discovered that the ester moiety of the amino acid derivatives is directly dependent upon the type of alcohol used in the aqueous reaction medium. Based on these findings, a new mechanism for ester formation is proposed to involve an alcohol exchange reaction with an intermediate mixed anhydride of the carboxyl group. These results have provided new insight into the one-step derivatization reaction and have provided the basis for preparing a variety of derivatives that can be assessed for optimizing the analysis of amino acids by GC with FID or by GC-MS. Discovering the influence of the alcohol on the chloroformate reaction in an aqueous medium opens the possibility for preparing a wide variety of ester derivatives that can be tailored to the analytical needs of a specific problem. The derivatization conditions of amino acids with isobutyl chloroformateisobutanol have been completely investigated. Fluorinated moiety was introduced into the amino acid derivatives through a modified reaction procedure. Comparisons of responses from EI, PCI, and ECNI ionization for these fluorinated derivatives of amino acids in analysis by GC-MS have been conducted. Although the reaction conditions need improvement ethyl chloroformate-pentafluorobenzyl alcohol derivatives of amino acids showed increased sensitivity of detection in ECNI mode than in PCI mode. The one-step aqueous medium chloroformate derivatization method can apply to the gas phase analysis of a variety of compounds having amino, carboxyl

polar functional groups. Its characters of simple and fast show advantages over other derivatization methods for analysis of amino acids by GC or GC-MS. In next two chapters, we will discuss the applications of the method and its further extension to peptide analysis in desorption mass spectrometry.

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Chapter III

Application of chloroformate derivatization in the quantitative assessment of incorporation of stable isotope-labeled amino acids into photosynthetic proteins of *Synechocystis* PCC 6803

I. Introduction

This chapter will describe the work of the author in a collaborative research project with Dr. N. R. Bowlby who worked with Dr. L. McIntosh in the MSU-DOE Plant Research Laboratory and Dr. C. Hoganson from the laboratory of Dr. G.T. Babcock in the Chemistry Department at MSU [1]. The goal of the research project was to develop analytical procedures for quantitative assessment of the incorporation of stable-isotope labeled amino acids into photosynthetic proteins of the cyanobacterium *Synechocystis* 6803. The one-step aqueous medium amino acid derivatization / GC-MS analysis method was applied as part of a quantitative assessments for some aspects the research project.

The conversion of the energy of light into chemical energy is one of the fundamental process of life. In plant and algae photosynthesize, Photosystem II (PS II) centers were found to independently store oxidizing power and catalyze the formation of dioxygen [2,3]. The mechanism of photosynthesis has not been well understood. The introduction of biochemical techniques for the purification of photosynthetic complexes at the end of 70s made it possible to resolve the structure/function relationship in these protein complexes by using spectroscopic techniques.

Isotopic substitution of particular amino acids thought to be important in the catalytic process of the photosynthetic proteins can provide information about events at the atomic level. Aromatic amino acids have distinctive electronic and chemical properties, they may perform important functions within enzymes. Many non-photosynthetic organisms are unable to synthesize aromatic amino acids and require that they be present in their diet or growth medium. For such an organism, providing the appropriately labeled amino acid will ensure that the proteins of interest become labeled, a phenomenon that has been widely exploited in characterizing molecular processes in these systems. Photosynthetic organisms, including cyanobacteria, however, are usually capable of synthesizing *de novo* all of the amino acids, and show little tendency to take up exogenous amino acids from the growth medium. This property has prevented researchers in photosynthesis from achieving quantitative isotopic labeling of specific amino acids.

In Synechocystis, however, an excess of phenylalanine in the medium inhibits the biosynthesis of tyrosine and tryptophan, as well as that of phenylalanine [4]. In this collaborative research project, incorporation of isotope-labeled aromatic amino acids into the photosynthetic proteins of Synechocystis by the addition of these amino acids to the growth medium has been investigated. The analytical procedures that were used to quantify the extent of incorporation of labeled amino acids into proteins of the photosynthetic apparatus will be described.

The experiments in this project were undertaken in different laboratories. The incorporation of labeled amino acids into the proteins and the electron paramagnetic resonance spectroscopy (EPR) experiments were carried out by other collaborators. The quantitative assessment of the incorporation of labeled amino acids was accomplished by the author. Experiments and results from the other collaborators will also be described and discussed briefly in order to give an overall picture of the research accomplished.

II. Experimental

Blue-green cyanobacterium Synechocystis 6803 cells were grown by the standard method. Phenylalanine was added in the growth medium to inhibit the endogenous aromatic amino acid biosynthesis [4]. Cell density was measured at various times following inoculation. Cell harvest was followed by protein isolation, purification and hydrolysis. The amount of amino acids in the culture was monitored by UV spectroscopy. These experiments were carrried out by Dr. N. R. Bowlby. The relative change in the amount of amino acids in the culture was also monitored by the direct derivatziation of the amino acids in the clarified growth medium and GC-MS analysis with the method described in chapter II. Amino acid analysis of the protein hydrolysates was performed by the method of derivatization with isobutyl chloroformate and analysis by GC-MS as discussed in chapter II. GC-MS analysis was performed with a Hewlett-Parkard 5890J gas chromatograph-JOEL AX-505H double-focusing mass spectrometer. The amino acids thus generated were derivatized with isobutyl chloroformate and 0.05 to 0.25 nmol of sample was subjected to GC-MS analysis. Controls were performed to ensure that the isotopic labeled amino acids were not degraded during the acid hydrolysis. For the experimental details see Ref.1.

III. Results and discussion

Techniques have been developed to monitor the flux of exogenous amino acid labels in *Synechocystis* through the use of induced obligate auxotrophy, protein purification, and GC-MS quantification of individual amino acids. Kinetics of growth and amino acid uptake in otherwise photoautotrophic cultures of *Synechocystis* 6803 have been conducted.

Isotope labeled amino acid (¹⁷O Tyr) was used in the detailed study of the properties and environment of the redox active tyrosines Y_D and Y_Z by EPR [5]. To use the isotope labeling approach effectively, however, it is necessary to have the capability of quantifying the extent of incorporation of the labeled amino acids into the photosynthetic apparatus. Amino acid analysis with the simple derivatization method played a significant role in this part of the project.

A. Evidence of incorporation of labeled amino acids into cells from cell growth curves

The standard procedure for monitoring the growth of Synechocystis cells is to measure the apparent optical density at 730 nm. Typical growth curves for Synechocystis cells grown in the absence and presence of the amino acids are shown in Figure 3.1. The growth curve in the absence of amino acids (panel a) has a normal shape characterized by a lag phase followed by logarithmic growth. In the presence of the amino acids (panel b), the growth rate is slower than fully autotrophic cells, as observed by Barry and Babcock [4]. A stationary phase is reached at about 0.6 OD₇₃₀ when the cells grow in the presence of the isotope label. If the cells are allowed to grow beyond the plateau at 160 hours, the growth again becomes logarithmic, and a second plateau is reached corresponding to the normal cell density at stationary phase (i.e. 1.2 to 1.5 OD₇₃₀).

Thus, when cells are grown in the presence of exogenous amino acids it is useful to quantify amino acids present in the growth medium to

ensure that functional auxotrophy is maintained. Figure 3.1b also shows the quantity of the aromatic amino acids present in the growth medium at the times when cell density was determined. UV absorption spectra, at selected times, of the growth medium after separation from the cells are presented in Figure 3.2. The spectra show a strong absorbance at 276 nm arising from aromatic amino acids. (While the absorption at 276 nm is a combination of absorbances from all three amino acids, it is dominated by tryptophan). As the cells enter the log phase of growth, the quantity of exogenous amino acids in the growth medium declines rapidly, with nearly complete depletion of these amino acids as the cells reach the first stationary phase. After remaining in stationary phase for several days, the cells will again enter a logarithmic growth phase and reach the true stationary phase, as noted above. During the second growth 'spurt' the cells synthesize the needed amino acids since the growth medium has been depleted of the exogenous phenylalanine to a level that does not maintain auxotrophy. Continuation of cell growth beyond the first stationary phase thus results in the accumulation of non-labeled amino acid in proteins. It was found that harvesting the cells within 24 hours of amino acid depletion is optimal. This generally corresponds to an OD_{730} of between 0.45 and 0.65. Samples collected at the second stationary phase and subjected to GC-MS analysis show a nearly complete loss of the labeled amino acid (see below).

B. Quantitative assessment of incorporation of isotope labeled amino acids into the photosynthetic proteins

As mentioned in the introduction, derivatization / GC-MS analysis of amino acids played an important role in this research project. In order to quantify the extent of incorporation of isotopic label, and to follow the time



Figure 3.1. Cell density and abundance of aromatic amino acids in the growth medium of *Synechocystis* cells. Panel A shows cell density as measured by OD_{730} of a culture grown in the absence of exogenously added amino acids. In panel B is shown the cell density (O, \odot open and filled symbols show data from two different cultures) and growth characteristic of cells grown in the presence of phenylalanine (0.50 mM), tryptophan (0.25 mM) and ¹⁷O-tyrosine (0.25 mM). The aromatic amino acid absorbance (\blacksquare) was monitored at 276 nm (see Figure 3.2) in samples represented by the filled circles on the growth curve (Reprinted from Ref. 1).



Figure 3.2. Absorption spectra of culture medium obtained after growth for the number of hours indicated. Cells and other solids were removed by centrifugation before recording the spectra. The reference cuvette contained BG-11 medium with no added amino acids (Reprinted from Ref.1).

course of incorporation, the one-step aqueous medium amino acid derivatization and GC-MS analysis method has been applied. The amino acid analysis by GC-MS functioned to: 1) quantify the incorporation (uptake) of isotope labeled amino acids into the photosynthetic-proteins by analyzing the photosynthetic protein hydrolysates, monitor the time course of the amino acid incorporation by analyzing the hydrolysates of proteins from cells harvested at the different time of growth; 2) monitor the quantities of amino acids present in the growth medium to verify those results obtained from the UV absorption experiments; 3) verify if there is a discrimination of uptake between the different isotopes of the same amino acid (i.e., ¹⁶O Tyr and ¹⁷O Tyr); 4) ensure no exchange of hydrogens and deuteriums between different amino acids during the acid hydrolysis. The EPR experiments from the project partner failed to answer these questions because of the lack of sensitivity of the technique to low levels of unlabeled amino acid.

Derivatization of amino acids in protein hydrolysates with iBuCF leads to the formation of N(O,S)-isobutoxycarbonyl amino acid isobutyl ester, which is amenable to GC-MS analysis.

We will discuss the GC-MS amino acid analysis results for the representative photosynthetic protein fractions obtained from cells grown on different labeled amino acids.





Figure 3.3 shows the mass spectra of isobutyl chloroformate erivatives of unlabeled phenylalanine (a), and d₈-phenylalanine (b) from tock solutions. For phenylalanine iBuCF derivative, one series of ragmentation reactions is shown in Scheme 3.1. Elimination of NH_2CO_2iBu from M^{+.} gives the conjugated ion m/z 204. Subsequent loss of C_4H_8 leads to the formation of the high abundance ion m/z 148, which can be used for the quantification. Per-deuterated phenylalanine $(d_8$ phenylalanine) shows a shift of seven mass units for m/z 148 and m/z 204 (one ²H is lost during cleavage of the NH₂CO₂iBu moiety). Figure 3c shows the TIC and mass chromatograms of the derivatives of the mixture of phenylalanine and d_8 -phenylalanine (1:1) from the stock solutions. These two derivatives eluted as one peak in the TIC, but m/z 148 and m/z 155 reached their maximum at a different scan number. Figure 3.4 shows the mass spectra of the derivative of phenylalanine in the hydrolysates of phycobiliproteins recovered from control cells (a) and from cells that were grown in the presence of d_8 -phenylalanine (b). Figure 3.4c shows the TIC / mass chromatograms for the case with d_8 -phenylalanine in the growth medium. In the d_8 -phenylalanine experiment, the phycobiliproteins used for analysis were isolated from cells that had been allowed to grow well beyond the initial plateau at ~0.6 OD₇₃₀ and had reached the second stationary phase at about 1.2 OD₇₃₀. The results clearly show the absence of d_8 -phenylalanine in these proteins. This observation cannot be explained by simple dilution of label, as the cell number increased by only about 3-4-fold (from the first stationary phase to beyond the second stationary phase). Rather, these results suggest that, when endogenous aromatic amino acid



Figure 3.3. EI mass spectra of isobutyl chloroformate derivatives of unlabeled phenylalanine (a) and d_8 -phenylalanine (b) from stock solutions. TIC/mass chromatograms of iBuCF derivatives of Phe/d₈-Phe mixture (1:1) (c). (50 nmol derivatization, 250 pmol injection for GC-MS analysis.)



Figure 3.4. Analysis of protein hydrolysates from Synechocystis by GC-MS after derivatization with isobutyl chloroformate. EI mass spectra of the phenylalanines in protein hydrolysates from unlabeled (a) and d_8 -labeled (b) cells. TIC/mass chromatograms for Phe in protein hydrolysates from cells that were grown in the presence of d_8 -Phe (c). (2 nmol protein derivatization, 50 pmol injection for GC-MS analysis.)

biosynthesis resumes in the cells, the labeled phenylalanine is broken down and recycled for use in other biosynthetic processes.



(Scheme 3.2)

Incorporation of labeled tyrosines into photosynthetic proteins has also been carried out. Similar fragmentation as described for the phenylalanine derivative produces the high abundant ions of m/z 220 and m/z 164; m/z 107 provides a third specific fragmentation ion (see Scheme 3.2). These fragmentation ions are easily observed in the mass spectrum of the iBuCF derivative of ¹⁶O-tyrosine (unlabeled) (Figure 3.5a). Figure 3.5b and 3.5c show the mass spectra of ¹⁷O-tyrosine (40% ¹⁷O) and 3,5, d₂tyrosine (99% ²H) derivatives, respectively. ¹⁶O-Tyr and ¹⁷O-Tyr coeluted and reached their maximum at the same scan number (Figure 3.5d). Figure 3.6 show the TIC / mass chromatograms (a) and mass spectrum (b) of the iBuCF derivative of tyrosine in a Photosystem I preparation that was isolated from cells grown on 17O-Tyr to about 0.6 OD₇₃₀. After correction for the contribution from natural isotope abundance, it was estimated approximately 14% incorporation of ¹⁷O-Tyr into the Photosystem I proteins. Interestingly, phycobilisomes isolated from these cells showed a somewhat lower abundance of the ¹⁷O-label (approximately 10% label incorporation), supporting the idea that these nitrogen-rich proteins additionally serve as a transient repository for amino acids that are rapidly taken up from the medium (Figure 3.7). In these experiments, the incorporation of ¹⁷O-Tyr is significantly less than the extent of labeling of the 17 O-Tyr stock solution (40% 17 O) that was supplied to the cells. It may reflect isotope discrimination against the ¹⁷O-hydroxyl isotope by one or more of the enzymes involved in the import and incorporation of the exogenous amino acids. Degradation of the label during growth can be excluded by the observation that ¹⁷O-Tyr incubated in an alkaline (pH 11) aqueous solution remains stable for at least seven days. Since the cells were harvested at about 0.6 OD_{730} , they were expected to have a high extent of incorporation. Other contamination in the growth medium may be part of the reason for low incorporation of label. The quantitative analysis by GC-MS for the incorporation of isotope labeled amino acids helped to evaluate the successfulness of the individual cell growth experiments.

> Cell growth was repeated for ¹⁷O-Tyr incorporation into photosynthetic proteins under well controlled conditions. Figure 3.8-3.10 show the TICs / mass chromatograms and mass spectra of tyrosine iBuCF derivatives from hydrolysates of Photosystem I, Photosystem II, and phycobiliprotein, respectively, that were isolated from cells grown on ¹⁷O-Tyr to about 0.8 OD₇₃₀. The ratios of ¹⁶O-Tyr/¹⁷O-Tyr/¹⁸O-Tyr, after



Figure 3.5. EI mass spectra of isobutyl chloroformate derivatives of unlabeled tyrosine (a), ¹⁷O-tyrosine (40% ¹⁷O) (b), and 3,5-²H-tyrosine (c) from stock solutions. TIC/mass chromatograms of iBuCF derivatives of Tyr and ¹⁷O-Tyr mixture (d). (10 nmol derivatization, 200 pmol injection for GC-MS analysis.)



Figure 3.6. Analysis of protein hydrolysates from Synechocystis by GC-MS after derivatization with isobutyl chloroformate. TIC/mass chromatograms (a) and EI mass spectrum (b) of the tyrosines in Photosystem I proteins isolated from cells grown in the presence of 1^{7} O-tyrosine (40% 1^{7} O). (25 pmol PS I center derivatization, 1 pmol injection for GC-MS analysis.)



Figure 3.7. Analysis of protein hydrolysates from Synechocystis by GC-MS after derivatization with isobutyl chloroformate. TIC/mass chromatograms (a) and EI mass spectrum (b) of the tyrosines in phycobiliproteins isolated from cells grown in the presence of 17 O-tyrosine (40% 17 O). (2 nmol protein derivatization, 100 pmol injection for GC-MS analysis.)

correction of natural isotope abundance, in the growth medium and in each proteins are listed in Table 3.1. These data were calculated from the peak areas and peak heights of the mass chromatograms for each characteristic abundant ion of 16 O-Tyr, 17 O-Tyr, and 18 O-Tyr (m/z 107/108/109, m/z 164/165/166, m/z 220/221/222, and m/z 320/321/322). The results from these experiments showed that although the isotope discrimination against 17 O-Tyr in the incorporation still exits, the extent of incorporation of labeled tyrosines into photosynthetic proteins (PS I, PS II, and phycobilisomes) are much higher than those obtained from the previous experiment. In PS I and PS II, the ratio of 17 O-Tyr to 16 O-Tyr is about 65% which is close to that in the starting growth medium (~80%). For these two photoproteins, if 16 O-Tyr is fully incorporated, the percentage for 17 O-Tyr incorporation is ~80%. Again, phycobiliprotein showed a lower abundance of 17 O-label. PS I and PS II took 2.5-fold more 17 O-Tyr than phycobiliprotein did.

In the cell growth experiment, UV absorption spectra used to monitor the concentration of the amino acids in the growth medium (as in Figure 3.2) did not show consistent results. In the second experiment just described above, the UV spectra did not show a significant decrease of the response from the aromatic amino acids in the growth medium as the growth time increased. Derivatization / GC-MS analyses of the growth medium for aromatic amino acids, phenylalanine, tyrosine, and tryptophan were carried out to clarify the UV absorption result. Growth medium of 40 μ l (20 nmol Phe, 10 nmol Tyr, and 10 nmol Trp for the starting medium) separated from the cells at different time: "0 day", "8 days", "16 days" were directly treated with 10 μ l of iBuCF with the addition of 40 μ l water, 30 μ l isobutanol, and 10 μ l pyridine. 10 nmol of 4-Clphenylalanine were added as an internal standard. A 2- μ l aliquot of the



Figure 3.8. Analysis of protein hydrolysates from Synechocystis by GC-MS after derivatization with isobutyl chloroformate. TIC/mass chromatograms (a) and EI mass spectrum (b) of the tyrosines in Photosystem I proteins isolated from cells grown in the presence of 17 O-tyrosine (40% 17 O). (20 pmol PS I center derivatization, 2 pmol injection for GC-MS analysis.)


Figure 3.9. Analysis of protein hydrolysates from Synechocystis by GC-MS after derivatization with isobutyl chloroformate. TIC/mass chromatograms (a) and EI mass spectrum (b) of the tyrosines in Photosystem II proteins isolated from cells grown in the presence of 17 O-tyrosine (40% 17 O). (30 pmol PS II center derivatization, 3 pmol injection for GC-MS analysis.)



Figure 3.10. Analysis of protein hydrolysates from *Synechocystis* by GC-MS after derivatization with isobutyl chloroformate. TIC/mass chromatograms (a) and EI mass spectrum (b) of the tyrosines in phycobiliproteins isolated from cells grown in the presence of ¹⁷O-tyrosine (40% ¹⁷O). (2 nmol protein derivatization, 200 pmol injection for GC-MS analysis.)

	16O-/17O-/18O- Tyr	16O-/17O-/18O- Tyr
	(by peak area)	(by peak height)
Growth medium (start)	100/80/78	100 / 79 /77
Photosystem I	100/64/62	100 /62 / 60
Photosystem II	100/66/66	100/65/65
Phycobilisomes	100 / 25/ 24	100/25/22

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Table 3.1. Quantitation of the extend of incorporation of ¹⁷O-tyrosines into the indicated proteins in *Synechocystis* cells grown in the presence of ¹⁷O-tyrosine (40% ¹⁷O) (after correction of natural isotope abundance)

final 20-µl of chloroform solution was analyzed by GC-MS. The relative responses (relative to internal standard 4-Cl-phenylalanine) of peak areas and peak heights of TICs and relative responses of peak areas and peak heights of the characteristic ions from the mass chromatograms, for each amino acid in the growth medium at different growth times were calculated. No significant changes of the relative responses were found (Figure 3.11), which supported the results from the UV absorption for the second experiment. The inconsistency of the change of the concentration of the aromatic amino acids in the growth medium may be explained by the presence of another organism besides the cyanobacteria in the cell growth medium which consumed the amino acids to form other proteins.

Amino acid analysis also confirmed that no exchange of label between different amino acids, i.e., $3,5-d_2$ -tyrosine in the cell growth medium did not result in $3,5-d_2$ -phenylalanine in the proteins.

IV. Conclusions

With the rise of popularity of Synechocystis as an experimental organism for the study of PS I and PS II, and its ease of manipulation, both in terms of site-directed mutagenesis and inducible auxotrophy, it has become more important to study the kinetics and extent of amino acid labeling. The procedure described in this chapter, and the results show that protein isolation, hydrolysis, and GC-MS analysis is an analytical methodology that is capable of providing quantitative insight into these issues. The convenient one-step aqueous medium chloroformate derivatization / GC-MS analysis of amino acids contribute significantly to the procedure to quantitate the extent of incorporation of isotope labels. Amino acid derivatization has been conducted directly in the growth



Figure 3.11. Analysis of the growth medium separated from *Synechocystis* cells at different times as indicated for Phe, Tyr, and Trp by GC-MS after derivatization with isobutyl chloroformate.

medium separated from the cells. The conditions necessary for successful incorporation of isotopically labeled aromatic amino acids *in vivo*, and the limit in cell density necessary to achieve the labeling have been verified by GC-MS amino acid analysis. While the induced auxotrophy described in this chapter is limited to the aromatic amino acids phenylalanine, tyrosine and tryptophan, these amino acids have been implicated as participants in important reactions in PS II [6,7]. This labeling approach should prove valuable in the elucidation of the structure / function relationships of these amino acids. In addition, although the focus of this study is on the roles of aromatic amino acids in PS II and PS I, the techniques developed are applicable to the study of any enzyme or protein complex that contains these amino acids as important components.

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Chapter IV

Investigation of the one-step chloroformate derivatization of small peptides prior to analysis by FAB-MS

I. Introduction

Fast atom bombardment mass spectrometry of peptides is often used for molecular weight determinations and sometimes for amino acid sequencing. However, experimental factors, such as the poor ionization efficiency of some hydrophilic peptides, can prevent successful analysis. The ionization efficiency can be improved by preparing derivatives of the peptides prior to analysis by FAB-MS. Derivatization methods involve a variety of peptide modifications to increase peptide hydrophobicity, ranging from esterification to the attachment of a hydrophobic moiety, or a moiety containing a fixed charge, to a specific site on the peptide as were reviewed in chapter I of this dissertation. However, none of the previously reported methods simultaneously derivatizes both amino and carboxyl groups in a single reaction, and most of the methods are time-consuming.

Prior to this investigation, the one-step chloroformate derivatization reaction in an aqueous medium developed [6] and refined [7-9] for analysis of amino acids has not been applied to peptide derivatization. In this chapter, the results of an investigation of the one-step chloroformate derivatization procedure for preparing N(O,S)-ethoxycarbonyl ethyl ester derivatives of peptides to enhance the FAB response and to generate sequencing ions by FAB-MS/MS during analysis of low-level samples will be reported. A detailed investigation of the reaction conditions will be described. A preliminary investigation of forming precharged derivatives of peptides using the chloroformate derivatization procedure will be discussed.

II. Experimental

<u>Materials</u>

The peptides were purchased from SIGMA Chemical Company (St. Louis, MO), and the alkyl chloroformates and alcohols were purchased from Aldrich Chemical Company (Milwaukee, WI).

Derivatization

Typically, 1-10 nmol of peptide were added to a 100- μ l volume of the reaction medium (H₂O/EtOH/pyridine (Py)=60/30/5-10). Ethyl chloroformate (5-10 μ l) were added and the reaction mixture was vortexed for 5-40 seconds. Chloroform (100-300 μ l) was added to extract the derivative; the chloroform was removed by vacuum (Speed-Vac, Savant Instruments Inc., Farmingdale, NY).

HPLC

The HPLC-UV experiments were carried out on a Beckman HPLC (a 112 Solvent Delivery Module, a 420 Controller, a 340 Organizer) and a Spectroflow 757 absorbance (λ =214 nm) detector (Kratos). The HPLC column (length = 25 cm, I.D. = 4.6 µm) was packed with 5-µm particles (Econosphere) with a chemically bonded C₁₈ stationary phase. The mobile phase was a gradient of CH₃CN and H₂O (0.1% TFA); CH₃CN concentration increased from 0 to 100% in 30 minutes.

Mass spectrometry

Secondary ions were produced with a 15-keV primary beam of Xe atom in a JEOL HX-110 double-focusing mass spectrometer operated in the positive ion mode. The accelerating voltage was 10 kV and the resolution was set to 1000 (10% valley). For CID MS/MS, helium was used as the collision gas in a cell located in the first field-free region. The helium pressure was adjusted to reduce the abundance of the precursor ions by 50%. A JEOL DA-5000 data system generated linked scans at constant B/E. The derivative was dissolved in 1/1 CH₃CN/H₂O, and 1 µl of this solution (100 pmol to 1nmol) was added to 1 µl of glycerol /thioglycerol /methanol (1/1/1) matrix.

III. Enhancement of FAB signal of model peptides by derivatization

The FAB response of most of the ethyl chloroformate derivatives, as N(O,S)-ethoxycarbonyl and ethyl esters, of the model compounds (di-peptides to penta-peptides) increased five- to fifty-fold relative to that of the underivatized analytes (see Table 4.1). In Figure 4.1, comparison of the FAB mass spectra of derivatized and underivatized glutathione (y-ECG) shows an approximately 40-fold signal enhancement after derivatization by ethyl chloroformate. Furthermore, the mass shift of the MH⁺ ion shows that the terminal amino and carboxyl groups as well as the thiol and carboxylic side chain groups are derivatized. A penta-peptide, RKDVY, also shows enhancement of FAB response by a factor of about 50 after derivatization by ethyl chloroformate (see Figure 4.2). From the mass of the protonated molecule (MH⁺=952) and the CID-MS/MS spectrum (Figure 4.3) of the ethyl chloroformate-ethanol derivative of RKDVY, it is apparent that the Nterminal, the C-terminal, the amino group on the side chain of Lys (K), the carboxyl group on the side chain of Asp (D), and the phenolic group on the side chain of Tyr (Y) are derivatized during the one-step reaction with ethyl chloroformate. Blockage of the hydrophilic functional groups (at terminals and on side chains) by the derivatization reagent is likely the reason for the enhancement of the FAB signal. The derivatization increases the hydrophobicity of the small peptide and hence its surface concentration in

Table 4.1. Estimation of FAB signal enhancement from small peptides as their ethyl chloroformate derivatives relative to the FAB response of underivatized peptides.

Peptide	Enhancement				
RKDVY	50 / 80 *(one free -COOH)				
γ-ECG	35**				
GHK	50* ¹				
YGG	20*				
DG	30*				
VGDE	10 (one free -COOH)				
КҮК	25 / 40 (one free -COOH)				
LGG	5*				
GGF	5**				
SY	20***				

* Average from analyses of 3 samples, each done in duplicate. ** Average from analyses of 4 samples, each done in duplicate. *** Average from analyses of 2 samples.

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1. side chain of His is not derivatized



Figure 4.1. Comparison of FAB mass spectra of γ -ECG (glutathione) underivatized and derivatized with ethyl chloroformate. Top panel is mass spectrum obtained from 20 nmol of underivatized γ -ECG; bottom panel is the FAB mass spectrum obtained from 1 nmol of N,S,O-ethoxycarbonyl/ethyl ester of γ -ECG.



Figure 4.2. Comparison of FAB-MS spectra of RKDVY, underivatized (upper panel, 1nmol) and derivatized (lower panel, 100 pmol) with ethyl chloroformate.



Figure 4.3. MS/MS spectrum of fully derivatized RKDVY (MH⁺ 952). A complete series of the N-terminal ions are present.

the matrix on the probe tip [2]. RKDVY derivatives prepared by other chloroformate-alcohol reagents, such as EtCF-HFBuOH, iBuCF-iBuCF, iBuCF-HFBuOH, iBuCF-(3-pyridinemethanol) (PyCH₂OH), and iBuCF-PyCH₂OH have been synthesized. However, the FAB response of derivatives formed by chloroformate reagents that introduce a larger alkyl group (e.g., isobutyl) did not show greater enhancement of the FAB signal over that from an analogous derivative containing an ethyl group.

IV. Evaluation derivatization efficiency

A. Introduction

In Figure 4.2b, the FAB spectrum of ethyl chloroformate-ethanol derivatized RKDVY, a peak at m/z 924 has a relatively high abundance and it appears 28 u lower than the peak at m/z 952 for MH⁺. Similarly, in the mass spectrum of derivatized γ -ECG (Figure 4.1b), there is a significant peak at m/z 480, which is 28 u lower than that at m/z 508 for MH⁺. These ions could be fragments of the MH⁺ or could be individual components resulting from the derivatization reactions. The product ion spectrum (B/E linked scan) of MH⁺ 508, the ethyl chloroformate derivatized γ -ECG, showed no fragment ion peak at m/z 480. The m/z 480 ion must represent an individual product from derivatization. Although the ion of m/z 924 was found in the product ion spectrum of the derivatized RKDVY ($MH^+ = 952$), one component in the RKDVY ethyl chloroformate derivatization product mixture was separated by HPLC and confirmed by FAB-MS to have a peak at m/z 924 for the MH⁺. Figure 4.4 is the HPLC chromatogram of the RKDVY ethyl chloroformate reaction mixture (products). Analysis by FAB-MS indicated that the two larger, late-eluting components have peaks at m/z 924 and m/z 952, respectively, representing two different MH⁺ species. These results indicated



Figure 4.4. HPLC chromatogram of derivatized RKDVY with CH_3CN/H_2O as the solvent. The reaction medium was $H_2O/EtOH/Py$ (70/30/5). The two major peaks represent the fully derivatized compound and the species containing one free carboxyl group.

that the derivatization of RKDVY by ethyl chloroformate does not form a single product, and peptides are not quantitatively derivatized under the conditions that were assumed to achieve full derivatization of amino acids in a simple one-step reaction [6]. The two components with peaks at m/z 924 and m/z 952 as their protonated molecules, represent the derivative with one free carboxyl group and the derivative with both carboxyl groups fully derivatized, respectively. Another small peak in the chromatogram represents the derivative with two free carboxyl groups. None of the peaks correspond to a species with a free amino or a free phenolic group. A similar result was found for γ ECG derivatization by ethyl chloroformate; the peak at m/z 480 in the FAB-MS spectrum corresponds to the derivative with one free carboxyl group. Using the same procedure, HPLC separation and FAB-MS analysis, other small peptides, such as GGF, KYK, and GHK, were also confirmed to be incompletely derivatized under conditions for amino acid derivatization in a one-step reaction. In the case of GHK, the reaction on the side chain of histidine was also incomplete. All these results indicated that derivatization of amino (N-terminal and side chain), thiol, and phenolic groups is complete, but that conversion of carboxyl groups (C-terminal and side chain) is not complete.

Results of other experiments demonstrated that the size of the peptide is not a major factor in causing incomplete reaction of the carboxyl group. When three peptides of different sizes (SY, RKDVY, RPKPQQFFG) were derivatized by ethyl chloroformate, subsequent analysis by FAB-MS indicated that even the di-peptide, SY, had two derivatization products: one with the carboxyl group derivatized and one with a free carboxyl group. Similar results were obtained for the penta-peptide, RKDVY, and the ninemer, RPKPQQFFG. Figure 4.5 shows the FAB-MS spectrum of the two ethyl





Figure 4.5. FAB-MS spectrum of RPKPQQFFG derivatized with ethyl chloroformate.

chloroformate derivatization products of RPKPQQFFG (derivatized at the 1nmol level, with 200 pmol transferred to the probe tip). The peak at m/z 1276 corresponds to MH^+ for the fully derivatized species; the peak at m/z 1248 (28 u lower) corresponds to a species in which one of the carboxyl groups has not be converted to an ethyl ester. Thus, the incomplete reaction of carboxyl groups is a problem, regardless of the size of the peptide (from dipeptide to ninemers in our model compounds).

A thorough investigation to evaluate the derivatization efficiency and to find the reaction conditions for complete reaction for both amino and carboxyl groups of small peptides in the one-step chloroformate derivatization has been carried out.

B. Location of the underivatized carboxyl group

To determine whether the free carboxyl group in an incomplete derivatized peptide is on a residue side chain or at the C-terminal, MS/MS spectra of the MH⁺ of γ -ECG (glutathione) derivatives were obtained. Figure 4.6a shows the MS/MS spectrum of the fully derivatized product (MH⁺=508.5 u). For the derivative with carboxyl groups partially derivatized there are two possibilities: (a) free C-terminal; derivatized γ -glutamate side chain. (b) derivatized C-terminal; free γ -glutamate side chain, or a mixture of both cases. The structures are shown in Figure 4.7 together with m/z values for possible fragment ions. If the C-terminal is not derivatized (structure 1 in Figure 4.7), N-terminal ions (**a**, **b**, **c**, **d**) will be the same as those in the spectrum of the fully derivatized case, but C-terminal ions (**x**, **y**, **z**) will be shifted 28 u lower. If γ -Glu side chain -COOH is not derivatized (structure 2 in Figure 4.7), C-terminal ions will be the same as those for the fully derivatized species, but N-terminal ions will be shifted 28 u lower in mass. Peaks for both cases were found in the FAB-MS/MS spectrum (Figure 4.6b), so the reaction mixture contained two products, each having one free carboxyl group, either at the C-terminal or on the side chain (or vice versa). For example, part of the y₂ ion current (m/z 279) in Figure 4.6a (fully derivatized) is shifted 28 u to m/z 251 (\underline{y}_2) in Figure 4.6b (incomplete reaction), but the same $\mathbf{b_1}$ ion (m/z 230) and the $\mathbf{b_2}$ ion (m/z 405) were found in both spectra ($\mathbf{b_1}$ and b_2 in Figure 4.6b). These peaks provide evidence for a free C-terminal in the incomplete reaction product. Meanwhile, peaks at m/z 279 (y'2) and m/z 305 (x'_2) found in Figure 4.6b were the same as those for the y_2 and the x_2 ions in Figure 4.6a, while the a₂ ion (m/z 377) in Figure 4.6a is shifted 28 u to m/z 349 (a'_{2}) in Figure 4.6b, which is consistent with the free γ -Glu side chain -COOH. It should be noted in Figure 4.6b that the \underline{a}_n ions (corresponding to the free C-terminal) cannot be distinguished from the b'_n ions (corresponding to the free γ -Glu side chain -COOH), because the mass difference of 28 u between corresponding **b** and **a** ions is the same as that between an ethyl ester and a free carboxyl group. Fortunately, by combining information from other sequence ion series, we can draw the above conclusion that for the reaction product with one free carboxyl group, the underivatized position was both at the C-terminal and on the γ -Glu side chain.

C. Effect of reaction medium composition

The effect of solvent composition on the efficiency of derivatization was examined. Three reaction media were studied: $H_2O/EtOH/Py$ (60/30/10), $H_2O/EtOH/Py/CH_3CN$ (60/30/10/10), and $H_2O/EtOH/Py/THF$ (60/30/10/10). The suitability of the derivatization was assessed with the model compound, RKDVY, by comparing the FAB-MS response of the derivatives produced in the different media with 5 µl of ethyl chloroformate. Two series of



Figure 4.6. Product ion spectra (B/E linked scan) of fully derivatized γ -ECG (glutathione) (upper panel, MH⁺ 508.5) and partially derivatized γ -ECG (lower panel, MH⁺ 480.5) Underlined ions correspond to the product with the free C-terminal and the derivatized γ -Glu side chain carboxyl group (structure 1 in Figure 4.7); *italized* ions correspond to the product with the free γ -Glu side chain carboxyl group and the derivatized C-terminal (structure 2 in Figure 4.7).



Structure 1: free C-terminal

Structure 2: free side chain -COOH

Figure 4.7. Structures and fragment ions in FAB-CID-MS/MS spectrum (Figure 4.6b) of partially derivatized **Y-ECG** (glutathione).

experiments for each reaction medium composition were carried out. In one series, the FAB spectrum was taken after the derivatives were extracted by 100 μ l chloroform, then dried and redissolved in CH₃CN. In the other series, the derivatives were not extracted with chloroform. Instead, the reaction mixture was dried under vacuum and then redissolved in CH₃CN. The results are listed in Table 4.2. Within each series for the three reaction media, no significant difference in the yield of derivatives or the ratio of FAB response for the different derivatives was found. Adding acetonitrile or tetrahydrofuran did not affect the reaction much. In the first series, the ratio of the response of the fully derivatized product to that with one free -COOH is similar for each reaction medium composition (about 1:3). This ratio for the second series is about 1:6. The difference between these two ratios might be because of the chloroform extraction of the derivatives. In this case. extraction by 100 µl of chloroform might not be sufficient to efficiently partition the derivatives into the chloroform layer, especially the more polar derivatives, e.g., those with one or two free carboxyl groups. That is, the derivative with one free -COOH may be less soluble in the chloroform layer than the fully derivatized one. The ratio of the two species in the sample with no chloroform extraction should reflect more accurately the ratio after the derivatization reaction. (No experimental results were obtained to explain the absolute response of the fully derivatized product with no chloroform extraction being lower than that from the series with extraction by chloroform. Both series were done on the same day with same instrument conditions.)

	W/E/P		W/E	W/E/P/A		W/E/P/T	
	AI	NI	AI	NI	AI	NI	
		I (with c	hloroforn	n extractio	n)		
fully derivatized	27	34	25	28	34	34	
1 free -COOH	80	100	87	100	100	100	
2 free -COOH	15	18	25	29	24	24	
		II (no ch	II (no chloroform extraction)				
fully derivatized	13	18	11	15	9.3	17	
1 free -COOH	70	100	74	100	56	100	
2 free -COOH	23	33	28	38	24	43	

Table 4.2. FAB-MS response for indicated species as function of reaction medium (model compound: RKDVY).

*W=water; E=ethanol; P=pyridine; A=acetonitrile; T=tetrahydrofuran

AI=absolute intensity, NI=normalized intensity.

D. Extraction efficiency

During the experiment described above, it was noticed that variations in the mode of chloroform extraction affected the relative and also the absolute FAB response of the samples. Experiments were designed to evaluate the extraction efficiency as a function of the volume of chloroform used. Table 4.3 shows the results of using different volumes of chloroform for the model compound RKDVY ethyl chloroformate derivatives. The ratios of the FAB responses (of MH⁺ ion) in the water layer (after extraction) to that in the chloroform layer were listed. The efficiencies of single extractions by 100 μ l, 200 μ l, 300 μ l, and 500 μ l were compared with those of multiple extractions by 200 μ l/100 μ l, 300 μ l/100 μ l, and 100 μ l/100 μ l/100 μ l. The multiple extraction protocol made the extraction quite complete.

For the ethyl chloroformate derivative of the penta-peptide RKDVY, no detectable underivatized species were found by FAB-MS and HPLC-UV, in either the chloroform extract layer or the aqueous layer after the extraction. The same result was found for the case with no chloroform extraction when the reaction mixture was dried directly in vacuum.

E. Effect of solvent ratio in the reaction medium

The solvent ratio in the reaction medium was adapted from the procedure for amino acid analysis [6-9] which is about 60/30/10 (v/v/v) for H₂O/EtOH/Py. To find more suitable conditions for small peptides, experiments were carried out with different solvent ratios, and the results are listed in Figure 4.8 for the ethyl chloroformate derivatives of the model compound RKDVY. From the point of view of absolute response or relative response of different derivatives or the reaction yield, a solvent ratio of 60/30/10 was optimal for small peptide derivatization. A more detailed and

	(Single	Extraction)		
Volume (µl)	100	200	300	500
fully derivatized	25%	5.4%	2.4%	2.0%
1 free -COOH	34%	11%	5.9%	8.6%
2 free -COOH	22%	14%	18%	13%

Table 4.3. Extraction efficiency vs volume of CHCl3 for RKDVY (EtCF)(MH+ intensity in H_2O layer / MH+ intensity in CHCl3 layer)

Volume (µl)	200/100	300/100	100/100/100
fully derivatized	0%	0%	0%
1 free -COOH	2.6%	2.2%	1.2%
2 free -COOH	21%	14%	11%

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Figure 4.8. FAB-MS spectra (partial) of 0.5 nmol of RKDVY derivatized in the indicated ratio of solvents. The peak at m/z 952 represents the fully derivatized derivative; peaks at m/z 924 and m/z 896 represent the products with one or two free carboxyl groups, respectively; peaks at m/z 880 and m/z 852 and others represent the fragmentation at the derivatization sites and \prime or other incomplete derivatized products.

systematic evaluation of the effect of each component in the reaction medium on the efficiency of the derivatization reaction has been carried out.

(1) Effect of pyridine concentration

Based on the results of the derivatization of amino acids by chloroformates in chapter II, pyridine functions as a catalyst and as a buffer for the chloroformate derivatization reaction. The concentration or volume of pyridine has an important effect on the reaction. The effect of pyridine concentration on the efficiency of ethyl chloroformate derivatization of peptides was examined. The experiment was carried out for different volumes of pyridine in a solution of $H_2O/EtOH$ (70/30; v/v). The HPLC-UV $(\lambda = 214 \text{ nm})$ responses of the different derivatization products of RKDVY derivatized by ethyl chloroformate are shown in Figure 4.9, and the corresponding pH values of the reaction mixture before and after the addition of ethyl chloroformate as a function of the volume of the pyridine added are listed in Table 4.4. First of all, these data demonstrated that varying the volume of pyridine does not make the reaction go to completion to form a single product. When the volume of pyridine in the mixture was less than about 3.5 μ l, the recovery of the fully derivatized product decreased significantly. The fully derivatized product reached the highest recovery when 3 to 5 μ l of pyridine were added to the reaction mixture. When the amount of pyridine added to the reaction mixture increased from 5 to 10 μ l, the recovery of the fully derivatized product decreased. The recovery of the product with one free -COOH increased as the amount of pyridine added increased from 0 to 10 μ l. When 10 to 20 μ l of pyridine were added to the reaction mixture, recoveries of the two products described above were almost constant although the recovery of the fully derivatized product decreased



Figure 4.9. The HPLC-UV (λ =214 nm) responses of the different derivatization products of RKDVY prepared with ethyl chloroformate vs. the volume of pyridine in the reaction medium.

Table 4.4. pH vs. pyridine volume in the reaction mixture

Before: before the addition of ethyl chloroformate.

After: after the addition of ethyl chloroformate.

H ₂ O/EtOH/Py		(H ₂ O/EtOH=70/30)						
Ру(μl)	0.0	1.0	2.5	3.5	5.0	10.0	15.0	20.0
pH(before)	6	6-7	7	7	7	7	7-8	7-8
pH(after)	3	0-1	1	3-4	4-5	5-6	5-6	5-6

slightly. In Table 4.4, as $V_{pyridine}$ increased from 1 to 5 µl, the pH (after) increased from 1-2 to about 4-5, and when $V_{pyridine}$ increased from 10 to 20 µl, the pH (after) was stable at about 5-6 which paralleled the constant recoveries for both derivatization products in this range of pyridine volume. Recovery of the product with two free -COOH groups was less sensitive to the quantity of pyridine used than was that described above for the other two reaction products.

The same trend in recovery as above was found when a similar experiment was repeated on FAB-MS for RKDVY ethyl chloroformate derivatization. The experiment was carried out with different volumes of pyridine (5, 10, 15, 20 μ l) added in a solution of H₂O/EtOH (60/30; v/v). The FAB-MS responses of different derivatization products of RKDVY derivatized by ethyl chloroformate are shown in Figure 4.10. Each data point was the average of duplicate or triplicate analyses. The only difference between the HPLC and the FAB results was that the decrease of the recovery of the fully derivatized product was more significant when V_{pyridine} increased from 10 to 20 μ l for the FAB result in Figure 4.10. The results from both HPLC and FAB-MS indicated that the addition of about 5-10 μ l of pyridine is more appropriate for the derivatization of RKDVY by 5 μ l of ethyl chloroformate although varying the volume of pyridine did not result in a complete reaction that forms only one product. The amount of pyridine required to achieve a higher absolute and relative yield for the derivatization of RKDVY is similar to what was found in the derivatization of amino acids.

A result similar to that of RKDVY derivatization from FAB-MS was found for the derivatization of γ -ECG by ethyl chloroformate (Figure 4.11). The results from the experiments on FAB-MS for GHK (Figure 4.12) and GGF (Figure 4.13) ethyl chloroformate derivatives showed more constant



Figure 4.10. The FAB-MS responses of the different derivatization products of RKDVY prepared with ethyl chloroformate vs. the volume of pyridine in the reaction medium.



Figure 4.11. The FAB-MS responses of the different derivatization products of γ -ECG prepared with ethyl chloroformate vs. the volume of pyridine in the reaction medium.



Figure 4.12. The FAB-MS responses of the different derivatization products of GHK prepared with ethyl chloroformate vs. the volume of pyridine in the reaction medium.



Figure 4.13. The FAB-MS responses of the different derivatization products of GGF prepared with ethyl chloroformate vs. the volume of pyridine in the reaction medium.

recoveries over the entire range of 5 to 20 μ l of pyridine added to the reaction mixtures. Each data point in these figures are the average of duplicate or triplicate analyses.

(2) Effect of different bases (catalyst and buffer)

The effect of 4-dimethylaminopyridine (4-DMAP) on the completion of the derivatization of RKDVY by ethyl chloroformate has been evaluated. The results are summarized in Table 4.5. In A of Table 4.5, the experiment was carried out for different volumes of 4-DMAP and pyridine in a solution of H₂O/EtOH (60/30; v/v); 10 nmol RKDVY were derivatized by 10 μ l ethyl chloroformate. The derivatization products were separated by HPLC, and confirmed by FAB-MS. The m/z values of MH⁺ for each product are listed. In B, 4-DMAP solution 10, 30, and 50 μ l replaced the 4-DMAP / pyridine mixture in the derivatization reactions in A. The m/z values of the ions related to the derivatives found in each FAB-MS spectrum are listed. These results indicated that 4-DMAP in the reaction medium reduces the derivatization efficiency. No fully derivatized product was detected with only 4-DMAP (not combined with pyridine) in the reaction medium.

The derivatization of RKDVY by 10 μ l of ethyl chloroformate was also carried out for 5, 10, and 20 ml of 2,4,6-trimethylpyridine in a solution of H₂O/EtOH (60/30; v/v). The separation of the derivatization products by HPLC and molecular weight confirmation by FAB-MS indicated that the reactions were incomplete although the fully derivatized product was formed. In the presence of 2,4,6-trimethylpyridine more peaks were detected in the HPLC chromatograms and in the FAB spectra of the derivatization products. Additional peaks at m/z 906 and m/z 878 were present and had not been seen in the spectra of derivatives formed with pyridine in the reaction medium.

A .	(4-DMAP) (10 μg	/ µl CHCl ₃) /	Pyridine			
4-DM	DMAP/Py (µl) m/z value of MH+ of peaks in HPLC					
		chromatogram				
	0/10	952	924	8 9 6		
	2/8	952	924	8 9 6	824	
	5/5	952	924	896	824	
	10/0				824	752
B.	4-DMAP					
4-DMAP (μl)		m/z of ions in	n FAB spectr	a		
	10		924	896	824	
	30			896	824	
	50		924	896	824	

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Table 4.5. Effect of different bases for RKDVY EtCF derivatization (catalyst and buffer)
(3) Effect of ethanol concentration

The derivatization of 10 nmol of RKDVY by 10 μ l of ethyl chloroformate was carried out for different volumes of ethanol (0, 10, 30, 50, 70 μ l) in a solution containing 60 μ l H₂O and 10 μ l pyridine. The HPLC-UV responses (peak height) of the different derivatization products of RKDVY and the yield of the fully derivatized product as a function of the volume of ethanol added are shown in Figure 4.14. Figure 4.14 shows that no complete derivatization has been achieved for the amount of ethanol added. The response for each derivatization product is quite constant when 10 to 50 μ l of ethanol are added. The yield of the fully derivatized product is evaluated as the ratio of the response of the fully derivatized product over the sum of the responses of the fully derivatized product over the sum of the responses of the different products. Each data point in the figures is an average of duplicate analyses.

(4) Effect of H_2O concentration

Following the same strategy of the experiment above, the effect of the amount of water in the reaction medium on the derivatization efficiency was evaluated. Similarly, the derivatization of 10 nmol of RKDVY by 10 μ l of ethyl chloroformate was carried out for different volumes of water (20, 40, 60, 80, 100 μ l) in a solution containing 30 μ l ethanol and 10 μ l pyridine. The HPLC-UV responses (peak height) of the different derivatization products of RKDVY and the yield of the fully derivatized product as a function of the volume of ethanol added are shown in Figure 4.15. Again, it shows that complete derivatization has not been achieved for the amount of water added. The response for each derivatization product is quite constant when 60 to 80



Figure 4.14. The HPLC-UV (λ =214 nm) responses of the different derivatization products (a) and the yield of fully derivatized product (b) of RKDVY prepared with ethyl chloroformate vs. the volume of ethanol in the reaction medium.



Figure 4.15. The HPLC-UV (λ =214 nm) responses of the different derivatization products (a) and the yield of fully derivatized product (b) of RKDVY prepared with ethyl chloroformate vs. the volume of water in the reaction medium.

 μ l of water are added. The yield of the fully derivatized product is about 20% when 40 to 100 μ l of water are added.

F. Effect of ethyl chloroformate concentration on the derivatization

Does the addition of more ethyl chloroformate reagent drive the reaction to completion? In the derivatization of amino acids, it was noticed that adding more chloroformate reagents would lower the TIC responses of the amino acid derivatives which probably resulted from the pH conditions of the reaction medium. The effect of the amount of ethyl chloroformate added on the derivatization efficiency was assessed for RKDVY. Figure 4.16 shows the HPLC-UV responses (peak height) of the different derivatization products of RKDVY as a function of the volume of ethyl chloroformate added. These results were from the derivatization of 10 nmol of RKDVY by different amounts of ethyl chloroformate (5, 10, 20, 30 μ) in a solution containing 60 μ l H₂O, 30 μ l ethanol and 10 μ l pyridine. The conclusion of the effect of the concentration of ethyl chloroformate is the same as those for the water and ethanol: complete derivatization has not been achieved.

G. Effect of reaction time

The kinetics of the derivatization reaction were investigated. For RKDVY and γ -ECG (glutathione), the FAB responses of the derivatives as a function of the vortexing time for the reaction are shown in Figure 4.17. (The reaction is fast and therefore the vortexing time was considered as the reaction time). Vortexing times ranging from 2 seconds to 3 minutes gave a constant yield of each product indicating that the reaction was essentially instantaneous although incomplete. In the case of GHK and GGF



Figure 4.16. The HPLC-UV (λ =214 nm) responses of the different derivatization products of RKDVY prepared with ethyl chloroformate vs. the volume of ethyl chloroformate added in the reaction.

derivatization by ethyl chloroformate, the FAB responses of the derivatives decreased slightly when the vortexing time increased (Figure 4.18), but the yield of the fully derivatized products were approximately constant when the vortexing time varied. Each experiment above was carried out by derivatizing 10 to 20 nmol peptide with 5 μ l of ethyl chloroformate in a reaction medium containing 60 μ l H₂O, 30 μ l ethanol and 10 μ l pyridine. All the data points in the figures are averages of duplicate or triplicate analyses.

H. "Reverse" vs. "normal" - different procedure for the derivatization

It was suggested that reversing the sequence of the addition of pyridine and chloroformate reagents might achieve better yields for the derivatization of hydroxy fatty acids by chloroformates [10]. The same strategy was tested for the derivatization of the peptide RKDVY by ethyl chloroformate. The derivatization of 10 nmol RKDVY by 10 μ l ethyl chloroformate in a solution containing 60 μ l H₂O, 30 μ l ethanol and 10 μ l pyridine was carried out in both "normal" and "reverse" modes. "Normal" is defined as the addition of ethyl chloroformate after the addition of pyridine as all of the experiments described earlier; "reverse" refers to changing the sequence of the addition of these two reagents. The HPLC-UV responses of the derivatization products from the experiments of both modes are listed in Table 4.6. Two samples for each mode were prepared and each was analyzed in duplicate . The results indicated that regarding both the yield of fully derivatized product and the absolute response for each derivatization product, both "normal" and "reverse" modes provided the same results.



Figure 4.17. FAB-MS responses of the derivatization products of RKDVY (a) and γ -ECG (glutathione) (b) prepared with ethyl chloroformate vs. vortexing (reaction) time.



Figure 4.18. FAB-MS responses of the derivatization products of GHK (a) and GGF (b) prepared with ethyl chloroformate vs. vortexing (reaction) time.

	2 free	1 free	fully	yield
	-СООН	-СООН	derivatized	H ₃ /
	H ₁	H ₂	H ₃	$H_1+H_2+H_3$
N ₁	42	172	40	
N ₁	42	220	50	
Ave. N ₁	42	196	45	
N ₂	36	160	37	
N ₂	40	210	48	
Ave. N ₂	38	185	43	
Ave. N	40	190	44	16%
R ₁	30	134	38	
R ₁	32	142	33	
Ave. R ₁	31	138	35	
R ₂	34	184	37	
R ₂	34	184	37	
Ave. R ₂	34	184	37	
Ave. R	33	161	36	16%

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Table 4.6. "Reverse" vs. "normal" - effect of different reaction procedure on RKDVY EtCF derivatization

I. Effect of FAB matrix on the responses of the derivatives

It was found from experiments that glycerol and thioglycerol were a better matrix than nitrobenzyl alcohol (NBA) for the ethyl chloroformate derivatives of the small model peptides. To quantitatively transfer the matrix to the probe tip, a mixture of glycerol, thioglycerol, and methanol with 1:1:1 ratio of volume was prepared.

The effect of the volume of the matrix mixture on the FAB responses of both derivatized and underivatized peptides were evaluated with a dipeptide Asp-Gly (DG) as the model compound. Twenty nmol of DG were derivatized by 5 μ l ethyl chloroformate in a solution of 60 μ l H₂O, 30 μ l ethanol, and 10 μ l pyridine. After extraction by chloroform and evaporation of the chloroform under vacuum, the derivatives were dissolved in 20 μ l acetonitrile / water (1:1). A 1-µl aliquot of this solution was transferred to the probe tip on which different amount of the matrix, 0.5, 1.0, 1.5, 2.0, 2.5 µl, were added. The FAB responses of DG ethyl chloroformate derivatives as a function of the different volume of the matrix are shown in Figure 4.19a. For each volume of matrix, the sample was analyzed in duplicate. The results indicated that when the volume of the matrix was less than 1.5 μ l, the FAB responses of the analytes (DG ethyl chloroformate derivatives) were relative higher, but the reproducibility of FAB responses of the analytes were more dependent on the accuracy of the matrix volume on the probe tip. When the volume of the matrix was between 1.5 and 2.5 μ l, the FAB responses of the analytes were relatively lower, but were less sensitive to the volume of the matrix; that is to say the responses were about constant for the different volumes of matrix added in this range. There was a compromise between sensitivity and reproducibility from the results of these experiments. The experimental

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Figure 4.19. FAB-MS responses of ethyl chloroformate derivatized (a) and underivatized (b) di-peptide DG vs. volume of matrix (glycerol/thioglycerol/ methanol; 1/1/1).

results for the underivatized DG is shown in Figure 4.19b which shows the same trend as in Figure 4.19a.

J. Effect of multi-cycle derivatization

To improve the efficiency of the ethyl chloroformate derivatization reaction with peptides, multi-cycle derivatization was assessed with RKDVY as a model compound. After removal of the chloroform in the final step of the derivatization described in the experimental section, the residues of the reaction mixture were exposed to the derivatization procedure one to three additional times. The relative responses of the different products following one to four cycles of derivatization are compared in Figure 4.20 and 4.21 as obtained from analysis by HPLC-UV. Two samples were prepared for each cycle and each was analyzed in duplicate. As shown in these figures, multicycle derivatization does not drive the derivatization reaction to completion, but each additional cycle of derivatization does increase the yield of the fully derivatized product. Furthermore, the product with two free carboxyls is absent or in relatively low abundance after the second exposure to EtCF reagents. The ratio of A_3/A_2 (peak area of the fully derivatized product/peak area of the product with one free -COOH) increases from about 0.3 (single cycle) to about 4 after four cycles of derivatization (an increase of more than 10-fold). The ratio of $A_3/A_3+A_2+A_1$ (peak area of the fully derivatized product/sum of the peak areas of all the products) increases from about 0.2 (single cycle) to about 0.8, which means that the total yield of the reaction reaches about 80% after four cycles of derivatization (an increase of about 4fold) assuming the molar absorbance for the different derivatization products of RKDVY are the same. Because of the very short time for the reaction in each cycle, the total time for all multi-cycles is still considerably short. It also



exposure of analyte to reagents

peak 3: fully derivatized

peak 2: 1 free -COOH,

peak 1: 2 free -COOH,



Figure 4.21. The HPLC-UV (λ =214 nm) responses of the different derivatization products (a) and the yield of fully derivatized product (b) of

can be noticed from Figure 4.21 a that with the completion of the second cycle, the absolute response of the fully derivatized product doubles compared to that from the first cycle. Comparing the result after the fourth cycle with that after three cycles, the response of the fully derivatized product does not increase, but the response of the derivative with one free -COOH still decreases. Thus, the yield of the fully derivatized product which is the ratio of A_3 over the sum of $A_1+A_2+A_3$ is increasing. If this increase of the yield is real after the fourth cycle of derivatization, the low absolute responses for both products (after the 4th cycle) may be explained by the losses during the extraction and / or the variations of the individual samples. It is also possible that the increase of the yield $(A_3 / A_1 + A_2 + A_3)$ after the fourth cycle comes from the relatively more severe loss of the derivative with one free -COOH during the extraction, but not the real increase of the fully derivatized Regarding the efficiency of the extraction, more than four product. derivatization cycles are not recommended.

V. Preliminary investigation of forming precharged derivatives of peptides using the chloroformate derivatization procedure

As reviewed in chapter I, precharged peptide derivatives can increase the ionization efficiency in analyses of peptides by FAB. Furthermore, precharged peptide derivatives may influence the fragmentation in CID-MS/MS experiments to form one or several complete series of structurally informative fragment ions to assist in peptide sequencing [3-5]. With our modified one-step chloroformate derivatization method, a charged moiety can be introduced into the peptide derivatives by applying a charged alcohol, such as choline, in the reaction medium. See Reaction 4-1.



First, Phe was chosen as the model compound to react with ethyl chloroformate-choline chloride reagents to test the proposed idea. A $10-\mu$ l volume of Phe solution (100 µg) was added to a solution containing 80 µl of choline chloride / water solution (1:1; w/w) and 10 µl pyridine. Ethyl chloroformate of 10-µl volume was added to the solution. The solution was vortexed for 10 s, and 100 µl of chloroform were added. Both the chloroform and the aqueous layers were analyzed by FAB. The expected derivatization product, N-ethoxycarbonyl-Phe choline ester (M⁺ at m/z 323 in Figure 4.22) was detected in the aqueous layer.

An ion of m/z 238 was also detected in the chloroform layer which might be N-ethoxycarbonyl-Phe (the species with a free carboxyl group). No or very low signal was detected for N-ethoxycarbonyl-Phe ethyl ester in the chloroform layer. It can be concluded that almost all the ester derivatization product came from choline. It also can be noticed in Figure 4.22 there were some overscaled ions, m/z 387, m/z 315, m/z 243. m/z 176, and m/z 104, which did not come from the FAB matrix glycerol. Product spectra (B/E linked scan) (Figure 4.23) of these ions revealed that they may come from the reaction between the derivatization reagents, ethyl chloroformate and choline chloride, or may come from the adducts of the reagents and their reaction products. The possible structures of these ions are in Figure 4.24. If not separated from the amino acid or the peptide derivatization products, these ions which have high concentration may suppress the signal from the analytes. 100 nmol of tri-peptide GGF were derivatized following the procedure described above, and 1μ l of the aqueous layer was analyzed by FAB. No corresponding N-ethoxycarbonyl-GGF choline ester was detected. No further experiment has been attempted.

The preliminary conclusions are, precharged choline moiety can be attached on the carboxyl group of Phe by the simple ethyl chloroformate derivatization reaction based on the mechanism described in chapter II. Nethoxycarbonyl Phe was also detected. The efficiency of the derivatization with peptides needs further investigation. The enhancement of FAB signal of peptides by this derivatization procedure needs to be assessed. The conditions to separate the peptide derivatization products from the reaction products derived from the reagents need to be investigated. Furthermore, the choline may attach to the carboxyl group on side chains of aspartic acid and glutamic acid which results in doubly or even more highly charged products when more carboxyl groups exist on side chains of peptides. This aspect of the derivatization needs investigation.



Figure 4.22. FAB mass spectrum of Phe ethyl chloroformate-choline derivative (M⁺ 323).



Figure 4.23. Product ion spectra (B/E linked scan) of ions from Figure 4.22: (a) m/z 387, (b) m/z 315, (c) m/z 243.





VI. Conclusions

The results of this study indicate that preparation of the ethyl ester, N,O,S-ethoxycarbonyl derivative of small peptides prior to analysis by FAB-MS increases the detectability of the derivatized peptide by 5- to 50-fold relative to that of the underivatized peptide. The chloroformate reagent can be used in an aqueous medium to react with both the amino and carboxyl groups as well as phenolic and sulfhydryl groups, but not aliphatic hydroxyl groups. Amino, phenolic and sulfhydryl groups are completely derivatized, but carboxyl groups are only partially esterified regardless of location on side chains or at the C-terminus. From a thorough investigation of the derivatization conditions, a multi-cycle derivatization improves the efficiency of esterification reaction; four cycles of derivatization drives the reaction to 80% completion while other variations do not make the reaction complete. The one-step chloroformate derivatization method has the possibility of bringing charged moieties into the peptide structure based on our modified derivatization procedure. Preliminary results from reacting the amino acid Phe with ethyl chloroformate-choline reagents proved the idea. More investigations need to be performed.

The results from small peptides indicate that carboxyl group derivatization with ethyl chloroformate does not go to completion in the onestep aqueous medium chloroformate derivatization. This suggests that the reaction efficiency of ethyl chloroformate with carboxyl groups of amino acids should be examined. This work will be described in chapter V.

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Chapter V

Further investigations on the quantitative aspect of the one-step chloroformate derivatization in an aqueous medium for amino acids and small peptides

I. Introduction

In chapter IV, we saw that even with all the variations of reaction conditions, the derivatization of RKDVY, more specifically, the esterification of its carboxyl groups with ethyl chloroformate, was incomplete. These results initiated a further investigation to evaluate the esterification efficiency of ethyl chloroformate with amino acids. No reaction yields for the ethyl chloroformate derivatization with amino acids were reported in the original method development by Husek [1-3]. Also in our earlier investigations for derivatization of amino acids by chloroformate-alcohol reagents, the underivatized amino acids were not detected. The incompletely derivatized amino acids may remain in the aqueous phase during the chloroform extraction, or they may not be volatile enough to elute from the GC column during the GC-MS analyses.

In this chapter, two reaction schemes will be used to evaluate the esterification efficiency of ethyl chloroformate with amino acids in order to have a better knowledge of the advantages and the limitations of the onestep aqueous medium chloroformate derivatization method. Further investigations to improve the reaction yield for amino acid and peptide derivatization with ethyl chloroformate were pursued.

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II. Evaluation of the reaction efficiency of ethyl and isobutyl chloroformate with carboxyl groups of amino acids

A. Method I

In this method, a two-step reaction procedure was applied to evaluate the reaction efficiency of carboxyl groups of amino acids in the one-step ethyl chloroformate and isobutyl chloroformate derivatization method. The first reaction followed the general chloroformate derivatization procedure outlined in section II of chapter II of this dissertation. One variation was that no chloroform extraction of the derivatization products was performed. Instead, the solvent and the reagent in the reaction mixture were removed under vacuum. By doing this, all the amino acid derivatization products (carboxyl groups derivatized and underivatized) by the chloroformate reagents remained in the reaction vial; thus, partial or total loss of the underivatized species during the chloroform extraction was avoided. In the following step, conventional methylation of carboxyl groups by diazomethane (CH_2N_2) was performed. The reaction and the condition of methylation of carboxyl groups by diazomethane were elucidated in Reaction 5-1. Any remaining free acid from the first reaction was converted to its methyl ester. The overall reaction scheme is outlined in Figure 5.1. The derivatization products after the two-step reaction were analyzed by GC-MS.



(5-1)



Figure 5.1. Reaction scheme of method I to evaluate esterification efficiency of ethyl (isobutyl) chloroformate with amino acids.

The efficiencies of esterification of Phe, Tyr, Asp, Glu, and Lys by reaction with ethyl chloroformate have been evaluated by the method described above. The results indicated that reaction of ethyl chloroformate with the carboxyl groups of these amino acids by the one-step aqueous medium reaction is incomplete to different extents dependent on the types of amino acid. The results are summarized in Table 5.1. The completion of the -COOH reaction (yield) was evaluated as the ratio of the peak area for the ethoxycarbonyl amino acid ethyl ester over the sum of the peak areas for the ethoxycarbonyl amino acid ethyl ester and methyl ester (equation below).

$$Yield = \frac{A_a}{A_a + A_b} \times 100\%$$

A: peak area in TIC or mass chromatogram of GC-MS analysis

a: ethyl ester; b: methyl ester

The peak areas were from either the TICs or the mass chromatograms of the characteristic ions of these derivatives. Representative TIC and mass spectra are shown in Figure 5.2 for Phe and in Figure 5.3 for Tyr. The esterification efficiencies for Phe, Tyr, and Lys were fairly good (70-90%) while the esterification of the acidic amino acids, Asp and Glu, were far from complete based on our experimental results under optimal reaction conditions.

The esterification efficiency of isobutyl chloroformate with carboxyl groups of Phe, Asp, Glu, Gln, and Ser was also examined by method I described above. The results are summarized in Table 5.2. The results again indicated that the esterification efficiency for Asp and Glu were

Amino	Sample	Injection	Yield (%)	
Acid	(µg)	(µg)		
Phe	2.0	0.200	~70	(by TIC)
			~85	(by m/z 192)
			~70	(by m/z 176
				and m/z 162)
Tyr	20.0	0.900	~90-95*	(by TIC and
				mass chrom)
Lys	2.0	0.200	~90*	
Asp	2.0	0.200	~10	(by TIC)
			~30	side chain:
				free COOH
			~20	C-terminus:
				free COOH
			~40	2 free -COOH
Glu	2.0	0.200	4	(by TIC)
			~20	side chain:
				free COOH
			~20	C-terminus:
				free COOH
			~60	2 free -COOH

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Table 5.1. Results from method I - esterification efficiency with ethyl chloroformate.

* by both TIC and mass chromatogram



Figure 5.2. TIC and EI mass spectra of Phe derivatized with (1) EtCF and (2) CH_2N_2 (method I).

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Figure 5.3. TIC and EI mass spectra of Tyr derivatized with (1) EtCF and (2) CH_2N_2 (method I).

poor relative to those for the other amino acids tested. Figure 5.4 shows the TIC, mass chromatograms, and mass spectra of different Asp derivatization products with isobutyl chloroformate and diazomethane.

It should be pointed out that method I may under-estimate the esterification efficiency of amino acids with chloroformate reagents. Partial loss of the ester products from the first step may occur during the relatively long process of removing the solvents and reagents under vacuum. Experiments indicated that these losses may have occurred, resulting in inconsistent recovery of the derivatives.

B. Method II

A different experimental scheme, outlined in Figure 5.5, was designed to avoid the potential problem in method I created by the vacuum during the drying process. The procedures are as follows, commercially available amino acid ethyl and methyl esters were used as standards to evaluate the esterification efficiency of ethyl and methyl chloroformate with Phe, Leu, Asp, and Glu. 100 nmol of Phe, Leu, Glu, and their ethyl esters were derivatized individually with ethyl chloroformate following the standard procedure. Methyl stearate (25 or 50 nmol) was added to each reaction medium as an internal standard for quantitation. A 2- μ l aliquot of the final chloroform solution of the derivatives was injected for analysis by GC-MS (0.2 nmol of amino acid derivative, 0.1 or 0.05 nmol of methyl stearate). Asp was examined by methyl chloroformate reaction.

Amino Acid	Yield (%)	
Phe	~90	
Gln	~95	
Ser	~95	
Asp	~10	
	~45	side chain: free COOH
	~20-25	C-terminus: free COOH
	~20-25	2 free COOH
Glu	~5	
	~35	side chain: free COOH
	~20	C-terminus: free COOH
	~40	2 free COOH

Table 5.2. Results from method I - esterification efficiency with isobutyl chloroformate.

* 10 μ g for each derivatization and 500 ng for each injection

* yield is calculated from peak areas of TIC





(continued to next page)



Figure 5.4. TIC and EI mass spectra of Asp derivatized with (1) iBuCF and (2) CH_2N_2 (method I).

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A: peak area in TIC or mass chromatogram of GC-MS analysis

I.S.: internal standard (methyl stearate)

As outlined in Figure 5.5 and the equation above, the responses of the TIC and the characteristic ion of the ethyl chloroformate derivative from each amino acid were compared with the responses of those from the corresponding derivative derived from its ethyl ester. The TICs of EtCF derivatives of Phe and Phe ethyl ester are shown in Figure 5.6 as a representative example. The results are summarized in Table 5.3. Each data was from the average of analysis of two individually prepared samples, each analyzed in duplicate. The esterification efficiency of Phe derived from method II was the same as that calculated from method I. The efficiency of methylation of Asp by methyl chloroformate (~40%) derived from method II was higher than the efficiency of ethylation of Asp by ethyl chloroformate (~10%) derived from method I. This may result from the different efficiencies of methylation and ethylation of amino acids by chloroformate reagents, or from loss of the derivative during the vacuum drying process as we discussed earlier.

C. Conclusions

The results from both method I and method II proved that although the one-step aqueous medium chloroformate derivatization for the analysis of amino acids is a simple derivatization procedure, the reaction is not complete for each amino acid. The esterification efficiency for acidic amino



Figure 5.5. Reaction scheme of method II to evaluate esterification efficiency of ethyl chloroformate with amino acids





Figure 5.6. TICs of Leu (a) and Leu ethyl ester (b) derivatized with EtCF (method II).
Amino Acid	Yield (%)	
Phe	76	(by TIC)
	73	(by m/z 192)
	72	(by m/z 176)
Leu	43	(by TIC)
	47	(by m/z 158)
Asp*	37	(by TIC)
	40-45	(by m/z 188)
Glu	<10	(by TIC)

Table 5.3. Results from method I - esterification efficiency with ethyl chloroformate.

* Asp was examined by methyl chloroformate reaction.

Each datum is the average of two individually prepared samples, each analyzed in duplicate.

10 nmol for each derivatization and 0.2 nmol for each injection for GC-MS analyses.

acids are low by the chloroformate derivatization. These experimental results also answered the questions raised in the derivatization of small peptides by ethyl chloroformate. Both amino acid and peptide esterification by the chloroformate reagents under the current derivatization procedure are incomplete.

Although not totally identical, results from other researchers agree with our findings. Esterification yield of some dicarboxylic acids, namely those containing 4 and 5 carbon atoms (succinic, glutamic) was very low. Correspondingly, the yield of their substituents, Asp and Glu, were low [4]. The yields of esterification of amino acids by methanol or ethanol in chiral menthyl chloroformate derivatization were reported as 95% [5].

III. "Non-aqueous" vs. "aqueous" reaction medium for derivatization of amino acids and peptides with ethyl chloroformate reagent

A. Introduction

Since incomplete esterification is a common result for the one-step aqueous medium chloroformate derivatization of amino acids and peptides, experiments to investigate and improve the yield of esterification have been attempted.

The one-step aqueous medium chloroformate derivatization method for amino acids in analytical scale developed by Husek was partially based on the method of esterification of fatty acids by the chloroformate reaction on organic macro scale [6,7]. In the original method, the reaction proceeded in a non-aqueous medium. It was also noticed that for esterification of short chain fatty acids, higher yields were easier to achieve in a non-aqueous reaction medium (such as, CH_3CN , CH_2Cl_2 , and hexane in the presence of alcohol). Even with a higher concentration of EtOH, the esterification of fatty acids by ethyl chloroformate in the reaction medium containing water reached around 90%. Lowering the EtOH concentration drastically decreased the reaction yield [8].

These results might be explained by the esterification mechanism discussed in the chapter II (Figure 2.5). In that mechanism, the ester is formed by nucleophilic attack of the alcohol in the reaction medium on the acylpyridinium species 2a. If water exists in the reaction medium, nucleophilic attack of water on 2a (hydrolysis of 2a) would compete with the attack of alcohol. This process drives the intermediate 2a back to the free acid. The esterification may reach a certain level of equilibrium which depends on the concentration and the nucleophilic reactivity of the alcohol in the reaction medium. If this explanation is true, the yield of esterification of amino acids by chloroformate reagents may be improved in the non-aqueous reaction medium although this would complicate the method somewhat.

Experiments were designed to verify this idea by comparing the results from aqueous and non-aqueous reaction media for derivatization of amino acids and the penta-peptide RKDVY.

B. Amino acid derivatization

Leu (50 nmol) with 25 nmol of methyl stearate as an internal standard was derivatized by 10 μ l of ethyl chloroformate in each of three reaction media: (1) a solution of 60 μ l H₂O, 30 μ l EtOH, and 10 μ l pyridine (the conventional aqueous medium); (2) a solution of 60 μ l CH₃CN, 30 μ l EtOH, and 10 μ l pyridine; (3) a solution of 90 μ l CH₃CN and 10 μ l pyridine. The derivatives from (1) were extracted with 200 μ l chloroform, and the chloroform was removed under N₂ stream. The derivatives were redissolved in 110 μ l of chloroform. No extraction was made for derivatives from reaction media (2) and (3). A 2- μ l aliquot of each solution was injected for analysis by GC-MS.

Ratios of the peak areas (Leu relative to methyl stearate) from both the TICs and mass chromatograms (m/z 158 for Leu, m/z 74 for methyl stearate) are listed in Table 5.4 for the results from the analysis of three reaction media. Comparing the results from (1) and (3), it can be seen that the relative response of Leu ethyl chloroformate derivative increased (0.25 to 0.50) when water was absent from the reaction medium. Among the three reaction media, the relative response of Leu ethyl chloroformate derivative was the highest in the non-aqueous reaction medium (2) which also contained EtOH. If the amino group reaction yield did not change in the three reaction media, we can say that the yield of esterification of amino acid is higher in a non-aqueous medium. The Leu derivative from reaction medium (2) was rerun one hour after the first run. Its relative response from TIC and the characteristic ion did not change. This indicated that the reaction did not proceed further, and the reaction yield did not increase with time.

Similar experiments with amino acids, Leu, Phe, Tyr, Lys, each 50 nmol, and 5 nmol methyl stearate, in the three reaction media were carried out. The derivatives were extracted by 200 μ l chloroform with 100 μ l water as a counter phase in each case. A 2- μ l aliquot of the chloroform layer was injected for analysis by GC-MS.

The relative responses of peak areas of TIC for these amino acid ethyl chloroformate derivatives are listed in Table 5.5. These results are similar to that in Table 5.4. For Leu and Phe derivatives, the relative responses increased in a non-aqueous media compared with those from an aqueous

Leu (EtCF)	H ₂ O/EtOH/Py	CH ₃ CN/EtOH/Py	CH ₃ CN/Py
from TIC	0.25	0.75	0.50
from mass			
chromatogram	0.30	0.94	0.60

Table 5.5. Relative responses of peak area of TIC (relative to internal standard) of amino acids derivatized with ethyl chloroformate in different reaction medium as indicated.

Amino Acid	H ₂ O/EtOH/Py	CH ₃ CN/EtOH/Py	CH ₃ CN/Py
Leu	~2.2	~3.6	~3.9
Phe	~2.7	~4.2	~4.7
Tyr	~0.94	~0.23	~0.20
Lys*	~0.15	~0.07	~0.09

...

* ratio of peak height

Table 5.4. Relative responses of peak area from TIC and mass chromatogram (relative to internal standard) of Leu derivatized with ethyl chloroformate in different reaction medium as indicated.

medium. But, for Tyr and Lys, with a side chain amino or phenolic group, the relative responses were higher when the reaction was conducted in an aqueous medium. This may indicate that amino and phenolic group derivatization by ethyl chloroformate achieves a better yield in an aqueous medium while the carboxyl group reaction gives a better yield in a nonaqueous medium. Similar results were observed when RKDVY derivatization by ethyl chloroformate was compared in aqueous and nonaqueous reaction media.

C. Peptide derivatization

The problem of incomplete esterification in the one-step chloroformate derivatization was raised when the derivatization method was applied to the analyses of small peptides by FAB-MS. Reaction conditions have been systematically reported in chapter IV; no reaction condition for complete esterification has been found. In a series of experiments to evaluate the effect of water concentration in the reaction medium, 20 μ l water in a solution containing 30 μ l EtOH and 10 μ l pyridine has been tested. In these experiments, the response of the fully derivatized RKDVY product (Figure 4.15) was the same as, while the response of the product with one free -COOH was lower than those obtained from the reaction media containing more water. Other experiments also indicated as seen in Figure 4.8 (FAB-MS spectra) that when the water volume was 10 μ l in the reaction medium, the absolute responses of the derivatization products were lower than those from other reaction medium compositions with more water. All these results suggest that less water did not favor the **RKDVY** derivatization with ethyl chloroformate.

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Further investigation of the reaction conditions were undertaken by partially and totally replacing the water, and even the ethanol, in the reaction medium by CH_3CN to compare the effect of a non-aqueous medium with other reaction media. The experiments were carried out as: 20 nmol of RKDVY were derivatized by 10 µl (210 µmol) EtCF in four different reaction media which are listed in Table 5.6. Two individual samples were prepared for each reaction medium composition.

	H ₂ O	CH ₃ CN	EtOH	Pyridine
	(μl)	(μl)	(µl)	(µl)
1	60	0	30	10
2	30	30	30	10
3	0	60	30	10
4	0	90	0	10

Table 5.6. Reaction medium compositions for RKDVY derivatization with EtCF.

The reaction mixtures were separated by HPLC (with the similar conditions as in the chapter IV), fractions corresponding to the derivatives were collected and analyzed by FAB-MS. Partial HPLC chromatograms of the reaction products from the four reaction media are shown in Figure 5.7.

These results showed that the total reaction yield of ethyl chloroformate derivatization of RKDVY decreased when water in the reaction medium was partially replaced by CH_3CN (a to b in Figure 5.7, V_{H_2O} : 60 to 30 µl). The yield kept decreasing as water was totally replaced by CH_3CN , and the chromatographic pattern changed indicating the change of the structures of the major derivatization products (b to c in

Figure 5.7). In chromatogram c ($V_{H2O} = 0$), m/z values of MH⁺ of the major components were identified to be 880 and 852 (peak 2+3 and 1 in c). Ion of m/z 880 was confirmed by MS/MS spectra to be the derivative with phenolic group not derivatized, and the ion of m/z 852 corresponded to the derivative with phenolic and C-terminus carboxyl groups not derivatized. Also in chromatogram c, component with MH⁺ at m/z 896 was not identified and component with MH⁺ at m/z 924 was insignificant which was part of peak 3. These may indicate the same conclusion as that from the amino acid experiments in last section: in a non-aqueous reaction medium, esterification efficiency with ethyl chloroformate increased, but phenolic group derivatization efficiency decreased. The overall effect was that the total derivatization efficiency decreased. From chromatogram c to d in Figure 5.7 (V_{EtOH} : 30 to 0 µl), the peak with MH⁺ at m/z 852 increased (peak 1 in c and d) which may indicate that without EtOH in the reaction medium (d) the esterification efficiency will be lower than that with EtOH (c).

D. Conclusions

The overall conclusion is that the water in the reaction medium lowers the esterification efficiency in the one-step chloroformate derivatization. However, elimination of water in the reaction medium results in overall lower yield of derivatization for amino acids and small peptides with multi-functional groups.

IV. Conclusions

The reaction efficiency of ethyl and isobutyl chloroformate with carboxyl groups of amino acids has been examined. It was found that the esterification of amino acids by the one-step aqueous medium ethyl



Figure 5.7. Partial HPLC chromatograms of RKDVY derivatized with ethyl chloroformate in different reaction media: (a) 60 µl H₂O, 30 µl EtOH, 10 µl pyridine; (b) 30 µl H₂O, 30 µl CH₃CN, 30 µl EtOH, 10 µl pyridine; (c) 60 μ l CH₃CN, 30 μ l EtOH, 10 μ l pyridine; (d) 90 μ l CH₃CN, 10 μ l pyridine. (isobutyl) chloroformate reaction is also incomplete as found for peptide derivatization in the chapter IV with the current reaction conditions. The esterification efficiency varies with different types of amino acid, typically 40-95% for the amino acids examined. Acidic amino acids have lower esterification efficiency. Derivatization of amino acids and peptide RKDVY in a non-aqueous reaction medium has been investigated. The results indicated that esterification efficiency by ethyl chloroformate may increase, but amino and phenolic group derivatization may decrease in a nonaqueous medium. For overall reaction yield, RKDVY derivatization with ethyl chloroformate needs water in the reaction medium although the esterification reaction is incomplete.

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