New approaches for synthesis and analysis of adducts to N-terminal valine in hemoglobin from isocyanates, aldehydes, methyl vinyl ketone and diepoxybutane

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Abstract

Human exposure to harmful compounds in the environment, from intake via food, occupational exposures or other sources, could have health implications. Exposure to reactive compounds/metabolites can be identified and quantified as hemoglobin (Hb) adducts by mass spectrometry. This thesis aimed at improved synthetic pathways for reference standards, and improved analytical methods for adducts to N-terminal valine in Hb from a range of reactive compounds; isocyanates, aldehydes, methyl vinyl ketone (MVK), and diepoxybutane (DEB).

Isocyanates form urea adducts with N-terminal valine by carbamoylation, which are detachable as hydantoins by hydrolysis. A new synthetic pathway for reference standards of adducts from isocyanates and a method for their analysis by liquid chromatography/mass spectrometry (LC/MS) were developed.

Aldehydes form reversible imines (Schiff bases) with N-termini in Hb. After stabilisation by reduction and detachment by isothiocyanates using modified Edman methods, these adducts could be analysed by gas chromatography/mass spectrometry (GC/MS) or LC/MS. 5-Hydroxymethylfurfural, its metabolites, and other aldehydes related to exposure via food, were studied with regard to analysis by these methods with synthesised standard references. A considerably improved analytical method for imines was developed. Many of the studied adducts are too short-lived *in vivo* or *in vitro* to be used for long-term biomonitoring. However, different approaches for the analysis were evaluated.

Through synthesised reference standards, an observed unknown adduct in blood was verified as the adduct from MVK. There exist both natural and anthropogenic sources for MVK.

DEB, metabolite of butadiene, forms a cyclic adduct to valine-N. A new approach using hydrazinolysis of protein and enrichment by molecularly imprinted solid-phase extraction was tested on synthesised reference DEB-adduct and gave promising results.

Synthesised standards were characterized by NMR, LC/MS and GC/MS.

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Praise is well, compliment is well, but affection….that is the last and final and most precious reward that any man can win, whether by character or achievement.

Mark Twain, 1907

To My Family and Mates

Table of contents

Abbreviations

AUC	Area under the curve concentration
AGE	Advanced glycation end products
ALE	Advanced lipidation end products
DEB	1,2:3,4-Diepoxybutane
FITC	Fluorescein isothiocyanate
FTH	Fluorescein thiohydantoin
FI*R*E procedure	Fluorescein isothiocyanate *R* (stands a covalent bound N-terminal adduct) Edman procedure
FDA	2,5-Furandialdehyde
GC/MS	Gas chromatography - mass spectrometry
GC/ECNI	Gas chromatography - electron capture negative ionisation
GC/NPD	Gas chromatography - nitrogen phosphorous detection
GO	Glyoxal
HMF	5-Hydroxymethylfurfural
LC-MS	Liquid chromatography - mass spectrometry
MDA	Malondialdehyde
MISPE	Molecularly imprinted solid-phase extraction
MDI	Methylenediphenyl diisocyanate
MGO	Methyl glyoxal
MIC	Methyl isocyanate
MVK	Methyl vinyl ketone
MIP	Molecular imprinted polymer
MRM	Multiple reaction monitoring
NPCVMA	N-[(4-nitophenyl)carbamate]-valin methylamide
PFPITC	Pentafluorophenyl isothiocyanate
PFPTH	Pentafluorophenylthiohydantoin
PIC	Phenyl isocyanate
PUR	Polyurethanes
SIM	Selective ion monitoring
SMF	5-Sulfooxymethylfurfural
SRM	Selective reaction monitoring
$NaBH_3CN$	Sodium cyanoborohydride
TDI	2,4-Toluene diisocyanate
VMA	Valine methylamide

List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals I-IV. The published articles are reproduced here with the permission of the publisher.

I **Davies R**., Rydberg P., Motwani H., Westberg E., Johnstone E., Törnqvist M. (2009)

Simplified synthetic routes for preparation of reference compounds for N-carbamoylated terminal valines in Hb for monitoring isocyanates and urea, *Chem. Res. Toxicol.,* (submitted).

II **Davies R**., Hedebrant U., Athanassiadis I., Rydberg P., Törnqvist M. (2009)

Improved method to measure aldehyde adducts to N-terminal valine in hemoglobin using 5-hydroxymethylfurfural and 2,5-furandialdehyde as model compounds, *Food Chem. Tox.*, **47**, 1950-1957.

III von Stedingk H., **Davies R**., Rydberg P., Törnqvist M. (2009)

Methyl vinyl ketone – identification and quantification of adduct to N-terminal valine in human haemoglobin, *J. Chrom. B.,* (submitted).

IV Möller K., **Davies R**., Fred C., Törnqvist M., Nilsson U. (2009)

Evaluation of molecularly imprinted solid-phase extraction for a 1,2:3,4-diepoxybutane adduct to valine in haemoglobin, *J. Chrom. B*., (submitted).

1 General background

1.1 Biomonitoring of reactive compounds

Electrophilic compounds/metabolites can react with proteins and DNA [1]. Such reactions with biomacromolecules could result in medical disorders for instance allergies and cancer. It is important therefore to identify and determine the truest level of exposure which should then be used to estimate human health risk. This awareness is necessary in order to take steps, if possible, to decrease the exposure of such compounds and ultimately improve the quality of life.

Biomonitoring could be defined as the screening of body fluids or exhaled air for potential biomarkers of exposure or effects from harmful compounds. Biomarkers could thus be used to measure exposure *in vivo* [2]. Electrophilically reactive compounds are short-lived *in vivo* and usually cannot be measured as such as they undergo chemical reactions and enzymatic reactions (metabolism). However, their stable adducts, covalently bound to nucleophilic atoms in macromolecules, could be used as biomarkers [2].

Reactions with biomacromolecules in body fluids depend on the structure and physical properties of the reactive compound and the nucleophilic site. Samples of urine, DNA or proteins are usually used for biomonitoring of reactive compounds [2-4]. The choice of biomarker generally depends on the analytical method available and the time since exposure, as biomarkers have different life-span.

Urine biomarkers of electrophiles are normally excreted within 2 days and could be used to measure exposure within short term since termination. The analysis of biomarkers in urine reflects the free concentration of the parent compound or metabolite in the plasma. For instance degradation products of glutathione conjugates, mercapturic acids, have been used extensively as biomarkers, analysed by mass spectrometric methods [4]. Another example is 1-hydroxypyrene, metabolite of pyrene, used as a biomarker of exposure to polycyclic aromatic hydrocarbons (PAH) [5].

DNA adducts are used as biomarkers for genotoxic compounds. It is not only considered to be a biomarker of exposure but also of the reactivity of a parent compound or the metabolite to critical nucleophilic sites [2]. DNA adducts usually have a half life of a few days due to DNA repair. A common method for the determination of adducts (particularly bulky adducts) to DNA has been ^{32}P-postlabelling [6]. New advances in analysis methodology by LC/MS are ongoing [7, 8]. For instance, LC/MS analysis has been used for adducts from glycidamide, the genotoxic metabolite of acrylamide, in samples from acrylamide exposed animals [7].

Proteins used for biomonitoring are primarily the blood proteins hemoglobin (Hb) and serum albumin (SA) [9]. These proteins have well defined life spans where Hb follows the life span of the erythrocytes which are replaced after ca. 125 days and SA has a half life of ca. 20 days in humans. This means that covalently bound stable adducts will have the same well defined life spans as the protein itself, as they are not repaired.

The binding sites of electrophilic compounds to proteins which are principally used as biomarkers in Hb can be N-terminal valine, the thiol group of cysteine, the imidazole nitrogens of histidine and the nitrogen of lysine. Reactive sites in SA particularly used in biochemistry are cysteine and lysine [10]. For the analysis of protein adducts, GC/MS and LC/MS are commonly used techniques.

In the analysis of electrophilic compounds, adducts (as a specific biomarker) can be detached from the protein. It is advantageous if a part of the macromolecule is included in the structure of the biomarker, which gives better specificity in the analysis. To obtain structural information of the biomarker, analysis by mass spectrometric methods is advantageous.

1.2 Hemoglobin for biomonitoring and risk assessment

The use of hemoglobin as a biomarker was initiated in the 1970s and first suggested by Groth and Neumann for measurement of bioavailability of aromatic amines [11]. Ehrenberg and Osterman-Golkar initiated somewhat later the use of Hb as an *in vivo* dose monitor for electrophilic compounds/metabolites [12-14]. It was established that the Hb adduct level could be used to measure the area under the concentration curve (AUC) of the electrophile *in vivo* after an acute exposure. The long life span of Hb gives an accumulation of stable adducts during chronic exposure. The adduct level will reach a “steady state” when old red blood cells are replaced by new. The dose AUC can then be calculated from this steady state condition with the consideration of the life span of Hb [15]. This gives an estimation of the average exposure during the last months. The concept of AUC is useful as a basis in health risk assessment of exposure from electrophilic compounds/metabolites [16, 17].

Quantification of alkylated histidine residues in Hb as a biomarker was initially achieved (in 1978) by total hydrolysis of the protein followed by ion-exchange chromatography of the hydrolysate by Calleman *et al.* [18]. This method was however very time consuming and the risk of artefact formation during the harsh hydrolytic conditions was problematic, and there was a need for a more sensitive and accurate method. A new specific and sensitive method for measurements of N-terminal adducts to Hb was then developed in the following years (see Chapter 2.1) [19, 20]. This method, the N-alkyl Edman method has been a break-through for Hb adducts as biomarkers for many simple alkylating agents [9].

1.3 General aims

Adducts to N-terminal valine in Hb chains have been shown to be useful biomarkers for many electrophilic compounds. Limitations of synthetic routes of reference standards and in the methods for isolation of the formed adducts of potentially harmful compounds however, call for improvements and new developments. The basis of identification and quantification of adducts is the comparison of retention times and fragmentation patterns in mass spectrometric analysis of synthesised reference standards and the data acquired from *in vitro/vivo* samples.

Development of new and simpler synthetic pathways of reference standards and also evaluation and improvements of various analytical methods to isolate and measure adducts to N-termini in Hb was the aim of this thesis. Adducts from isocyanates, aldehydes, diepoxybutan, as well as an otherwise unidentified adduct from methyl vinyl ketone, are focus in this thesis. Specific aims are given in the different chapters.

2 Analysis of adducts to N-terminal valine in Hb

This chapter gives the basis for the methods used in this thesis for the analysis of adducts to N-termini in Hb. The principles of the methods are detachment of the adducted N-termini from the rest of the protein, enrichment by different procedures and analysis by GC/MS or LC/MS. The instrumentation used in the analysis of samples and for characterisation of standards is described at the end of this chapter.

2.1 N-terminal valine in Hb as nucleophilic site

Adult human Hb is made up of double α- and β-peptide chains. On these chains, the amino acid valine is situated at the N-terminal sites with a primary amine group. The pK_a of the α- and β-chains are 7.8 and 6.8, respectively. Valine methyl amide (VMA) which often is used as a model of N-terminal valines, has been allocated 4.2 in nucleophilic strength in the Swain Scott scale [21].

Adducts to N-terminal valine can be formed through nucleophilic additions e.g. 1,4-Michael additions, substitutions or imine (Schiff base) formation. Depending on the adduct structure, the adducts could further react or rearrange [22]. When a stabilised adduct is formed it could be detached and isolated by various methods and then analysed by LC/MS/MS or GC/MS/MS.

2.2 The N-alkyl Edman method

The Edman degradation method was developed by the Swedish biochemist Per Edman for the sequencing of proteins by stepwise detachment of N-terminal amino acids after coupling with phenyl isothiocyanate followed by acidification [23]. The method was tested for detachment of N-terminal valine adducts from isolated globin by the research team at Stockholm University. In initial tests, Hb treated with radioactively labelled ethylene oxide was used. Jensen observed that radioactivity was released already during coupling with the reagent [19]. This means that N-terminal valines with the adduct (R) detach without acidification to form a thiohydantoin derivative (see Figure 2.1). This formed the basis for a successful development of the N-alkyl method using pentafluorophenyl isothiocyanate (PFPITC) for detachment, enrichment and analysis of adducts to N-termini by GC/MS/MS [20].

The mechanism of the detachment of amino acids in protein sequencing is initiated by a nucleophilic attack by the N-terminal nitrogen on the electrophilic carbon of the isothiocyanate group of PFPITC. The alkyl groups on either side of the reacting centres, as in N-substituted valine, hasten the cyclisation by the so called *gem*-dialkyl effect [24]. A hydantoin is formed at

about neutral pH and the N-terminal valine is incorporated into the PFPTH structure together with the R adduct.

Ethylene oxide and other simple alkylating agents have been monitored with the N-alkyl Edman method [9]. One example of application is that acrylamide was discovered as a potential food carcinogen using this method [25]. The method is adapted for GC/MS analysis which means that the possibility to analyse adducts with hydrophilic groups are limited. Furthermore, for instance tertiary amine adducts to N-termini, where the N-terminal nitrogen is blocked for reaction with the Edman reagent, cannot be detached and analysed with this method. The N-alkyl Edman method is applied in chapter 4.

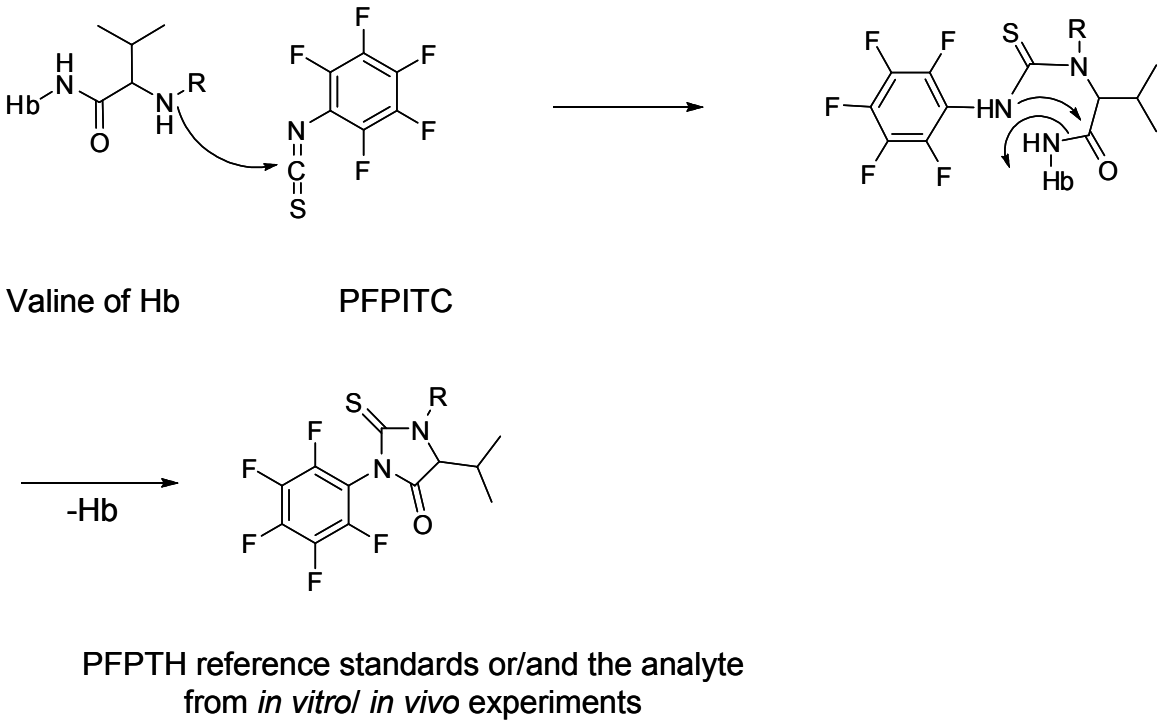


Figure 2.1. Mechanism of the N-alkyl Edman method using PFPITC for the formation and detachment of the PFPTH derivative of N-substituted N-terminal valine in Hb.

2.3 The adduct FI*R*E procedure

A sensitive LC/MS method for the analysis of polar as well as nonpolar compounds using a modified Edman procedure has now been developed in our research group (see Figure 2.2) [26, 27]. An important factor in the workup is that no isolation of the globin is necessary unlike detachment using PFPITC as in section 2.2.

When fluorescein isothiocyanate (FITC) is added to hemolysed human blood samples it becomes ionised and reacts whereafter it dissolves completely. The fluorescein thiohydantoin (FTH) analytes are detached from the samples.

After removal of the cell membranes by centrifugation, the analyte is separated from the protein residual by an SPE ion exchange column. An adjustment of the pH allows for the FTH analyte to cyclise to its spiro form and then be analysed by LC/MS/MS. This method opens for analysis of a broader range of adducts than that could be analysed by GC/MS by the N-alkyl Edman method. This method is applied in chapter 4 for analysis of adducts from glyoxal and in Paper III and chapter 5 for identification of an earlier unidentified adduct.

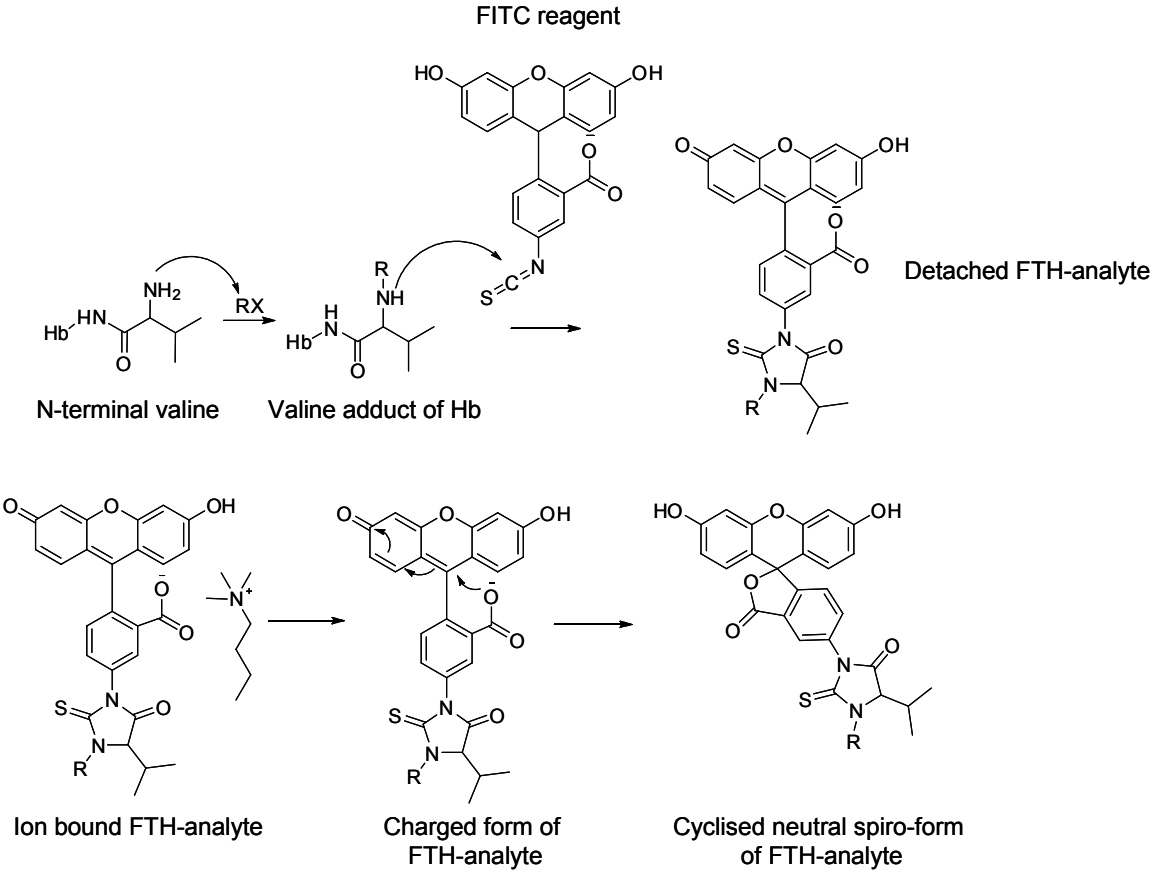


Figure 2.2. A step by step description of the adduct FI*R*E^{TM} procedure.

2.4 Carbamoylation of N-terminal valine in Hb

Already in 1930, a method of peptide sequencing using the carbamoylation of N-terminal amino acids with phenyl isocyanates, were detached under acidic conditions to form corresponding hydantoins of the amino acids [28].

Primary amino nucleophilic groups in macromolecules undergo carbamoylation with isocyanates as seen in Figure 2.3. The isocyanate specific adduct to N-terminal valine can be hydrolysed at low pH to form a detached hydantoin (Figure 2.3). After an extraction procedure, the hydantoin can be analysed by either LC/MS or GC/MS.

The carbamoylation of N-terminal valine by an isocyanate and isolation of the corresponding hydantoin in Hb was first used by Manning *et al.* as a

biomarker in the treatment of sickle-cell disease [29]. The method has been adapted to measure exposure of isocyanates [30, 31]. This method is in focus in Paper I and chapter 3.

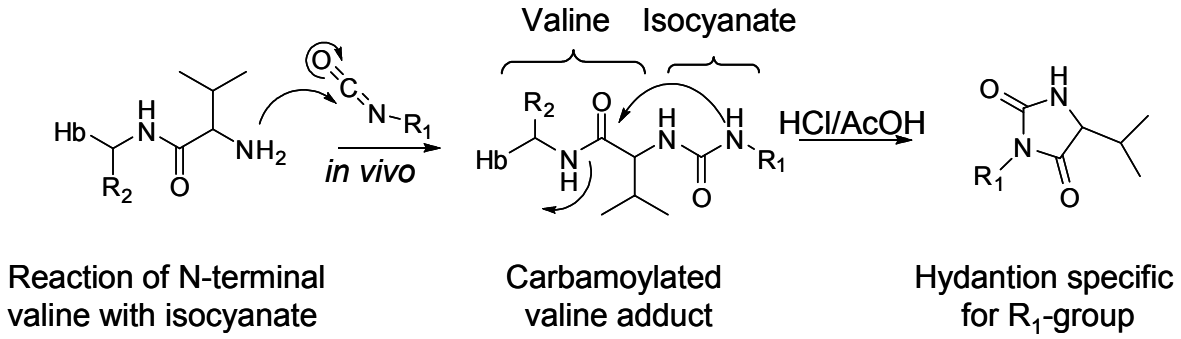


Figure 2.3. Carbamoylation of N-terminal valine followed by hydrolysis and ring closure.

2.5 Schiff base formation to N-termini in Hb/isolation of adducts

When aldehydes or ketones react with primary amines, reversible imines (Schiff bases) are formed. These formed Schiff bases could be stabilised by a reducing agent, usually $NaBH_4$ or $NaBH_3CN$ (see Figure 2.4). N-terminal valine in Hb can form imines with aldehydes at physiological pH, and it has been observed that for instance acetaldehyde preferably reacts with N-terminal β-chains of Hb [32].

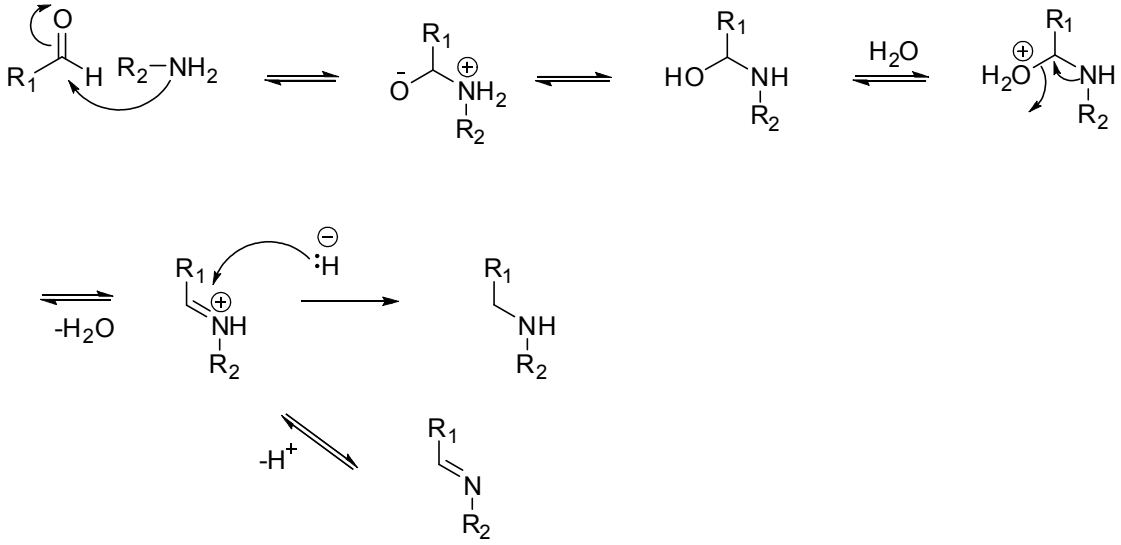


Figure 2.4. Formation of the unstable Schiff base (imine).

In synthetic reactions imines are formed quickly at pH 4-6 when primary amines react with aldehydes or ketones [33]. The formed aminoalcohol in the first step is protonated and an iminium ion is formed with water as the leaving group. The imine is formed after a proton transfer to water. The speed of the reaction depends on the pH. A medium with a too low pH would protonate

the primary amino group and a too high pH would slow down the protonation of the aminoalcohol in the first step.

In earlier studies of Schiff bases from malondialdehyde to N-termini in Hb, $NaBH_4$ was used as the reducing agent before globin was precipitated followed by detachment by PFPITC by the N-alkyl Edman method and analysis by GC-MS [34, 35]. This approach is improved and applied in Paper II and chapter 4.

2.6 Cleavage of N-terminal valine adducts in Hb by other methods

Cyclisation of compounds bound to N-terminal Hb can form stable adducts which block the reaction with an Edman reagent (isothiocyanate). This means that other cleavage and enrichment methods must be utilised for such detachment of the tertiary bound valine nitrogen.

One example is the difunctional metabolite of butadiene, diepoxybutane (DEB), which forms a cyclic adduct to N-terminal valine in Hb (pyr-Val). A method using cleavage by trypsin, and enrichment and analysis of DEB-modified heptapeptides, has been developed by Fred *et al.* [36]. In this thesis a new method for cleavage and purification of the stable cyclic DEB-adduct to N-terminal valine is evaluated. This method is based on hydrazinolysis of the protein to amino acids, followed by enrichment of pyr-Val adducts. This work is presented in Paper IV and chapter 5.

2.7 Instrumentation

An NMR Varian Mercury 400 MHz spectrometer and a Buchi 353 melting point apparatus was used for the characterisation of reference standards. The GC/MS instruments used in this study for identification and quantification studies were an ion trap GC/MS (GCQ Finnigan MAT instrument), and a triple quadruple Finnigan TSQ700 MS coupled to a Varian 3400 GC. The LC/MS used is a Q-trap triple quadrupole Applied Biosystems MS coupled to an LC system. Both electron impact (EI) and electron capture negative ionisation (ECNI) modes were used in GC/MS analysis. Electron spray (ESI) technique was used for the LC/MS analysis.

By comparing the analyte obtained in analysis of *in vivo* samples, with synthesised compounds (reference standards) evidence of the identity can be established. The comparison involves the retention times and MS-fragments. If possible the full scan fragmentation spectra are matched with the standard. Usually selective reaction monitoring (SRM)/multiple reaction monitoring (MRM) of specific fragments are used in the analysis of analytes from biological samples. Selective ion monitoring (SIM) was also used.

3 Isocyanates (Paper I)

3.1 General background

3.1.1 History and usage

Organic isocyanates consist of mono-, di- or poly-isocyanates depending on the number of isocyanate groups per molecule. These compounds have many uses in the chemical industry. Isocyanates have been used in organic chemistry and biochemistry since the early 19th century. The German chemist, Friedrich Wöhler, discovered in 1828 that the inorganic salt potassium cyanate and ammonium chloride can be converted into urea [37]. This reaction is considered by some to be the beginning of the modern age of organic chemistry where inorganic compounds were used for synthesis of organic compounds.

The reaction between an isocyanate and a hydroxyl compound was first reported by Wurtz and Hoffman (1848-1849) and isocyanate chemistry was mainly used in laboratory research until the late 1930s [38, 39]. It was then, in Germany, Otto Bayer discovered the diisocyanate poly addition process. Together with the shortage of rubber in Germany, the discovery spurned on the developments of new products throughout the Second World War. After its end, urethane technology was available and was used in the production of a number of commercial products. From the second half of the 20th century, isocyanates have been important intermediates for a wide range of products e.g. raw materials for the polyurethane and pesticide industry [38].

Chemical companies, for instance Union Carbide, developed and increased production of new carbamate pesticides for pest control in the 1950s, in tangent with a need for more efficient production techniques in agriculture using amongst other starting materials, isocyanates.

In the beginning of the commercial usage of isocyanates, there was not much understanding of the aspect of toxicology. However, the toxicological significance was observed with scientific papers being produced on new products such as herbicides [40]. The functional group name “isocyanate” has been highlighted more negatively over the last 30 years because of the Bhopal gas tragedy in 1984 when the pesticide carbaryl was in production using methyl isocyanate (MIC) as a starting material [41]. The production of carbaryl is still ongoing in certain countries by this method, although it is difficult to assess today’s production. The reactivity of mono- and diisocyanates, which make them favourable for industrial use, stems from the highly electrophilic carbon atom in the isocyanate group which forms urea and carbamate linkages (see Figure 3.1).

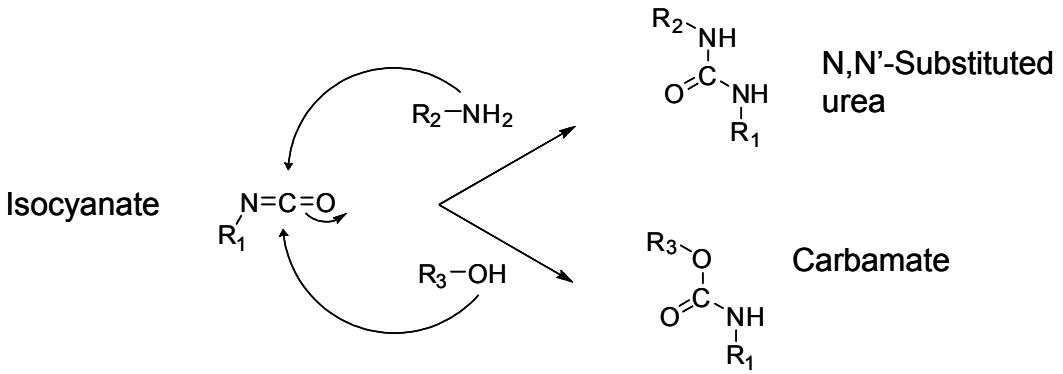


Figure 3.1. Reaction of isocyanate with amino or alcohol group.

Exposure to isocyanates occurs both exogenously from industry, and endogenously through the breakdown of urea [42, 43]. Isocyanates can be divided through their usage into two main groups, mono- and diisocyanates where monoisocyanates contribute to the production of pesticides and diisocyanates are used in the polymer industry.

3.1.2 Monoisocyanates

Methyl isocyanate (MIC)

Monoisocyanates such as MIC are predominately used as synthetic intermediates in the production of pesticides such as carbaryl. By reacting MIC with 1-naphthol a carbamate product can be formed in large amounts. MIC can be synthesised industrially in various ways. Methyl amine and phosgene react to form MIC plus two equivalents of HCl [44]. MIC can also be produced from methylformamide by oxididation at high temperatures.

Low exposure to MIC can cause eye and throat irritation and from higher levels of exposure people have experienced severe lung and eye damage and asthmatic and long term respiratory effects [45, 46]. Although it is unlikely that exposure of MIC can reach the general public through occupational sources, cigarette smoking, the burning of wood and other combustion processes can be sources for people not working industrially with this compound [46, 47]. MIC can be found in cigarette smoke where it is produced through incomplete combustion and has been detected in levels of 0.55 µg per cigarette [47].

Pesticides produced from MIC

MIC is commonly utilised as a starting material in the synthesis of carbamate pesticides such as carbaryl, aldicarb, carbofuran and methomyl utilis [48] (see Figure 3.2) but other more expensive synthetic routes without the use of MIC are available. The most popular of these pesticides is carbaryl. Carbamates owe their insecticidal properties to inhibition of the enzyme acetylcholinesterase thus preventing effective nervous transmission across the synapse through acetylcholine build up. Carbaryl has low mammalian toxicity and is biodegradable. On the other hand aldicarb has very high mammalian toxicity. This difference depends on the ease of penetration to the target

enzyme and speed of metabolism of the carbamate [49]. These types of pesticides are generally toxic to humans and are strictly controlled or banned in many developed countries although in third world countries with a lack of regulation, they can be in widespread distribution and use [50].

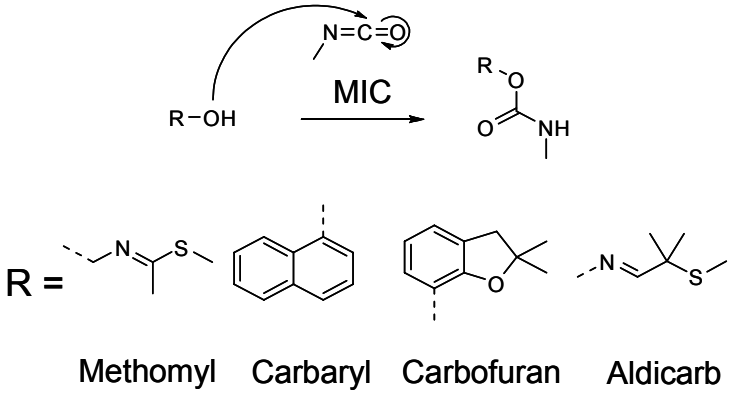


Figure 3.2. The synthesis of carbamate pesticides.

Hopefully a phasing out of these compounds with a more green approach to pest control could be an aswer to the problems in food production concerning damage to the surrounding environment and harm to workers and wildlife. Green pesticides are considered to be safer than synthetic pesticides as they are generally less harmful to mammals, very organism-selective and cause less harm to the ecosystem [51].

Phenyl isocyanate (PIC)

Phenyl isocyanate is used in the chemical industry and was utilised already in early isocyanate research during the first half of the 20th century. No natural sources of PIC have been described in the literature. Phenyl isocyanate has been tested for its acute toxic, cytogenetic and embryotoxic activity in mice and rats. Phenyl isocyanate has an acute oral LD_{50} value for male mice of 196 mg/kg. The molecular mechanism causing the toxic effect is not known although it is thought that an interaction with macromolecules causing changes in activity of enzymes or cell death can be a factor. No significant increase in chromosome aberration was seen with doses up to 204 mg/kg [52].

Isocyanic acid

Isocyanic acid is a natural compound originating from cyanate which is spontaneously produced from urea under physiological conditions (see Figure 3.3). In sufferers of chronic renal failure and uremia, levels of urea increases [53]. This may have implications in uremic toxicity where the increase possibly causes complications such as atherosclerosis, immune abnormalities and cataracts. Furthermore, in renal failure, near complete carbamoylation of human SA results in a two-third reduction of the binding capacity of the protein for small anionic molecules [53].

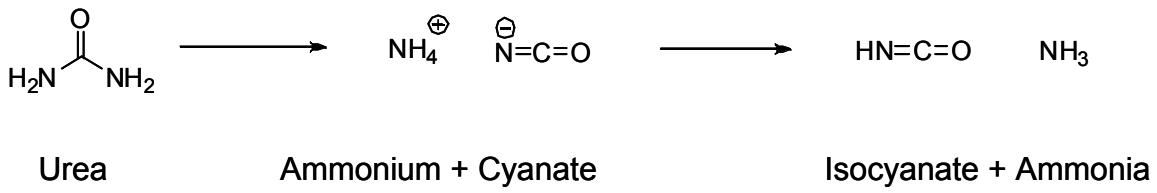


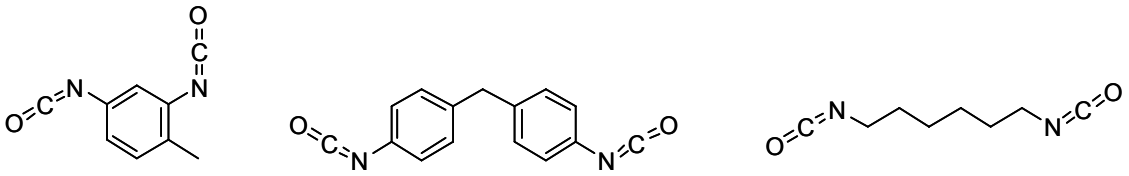
Figure 3.3. Cyanate formation from urea.

The carbamoylation of N-terminal valine (carbHb) has the potential to be a biomarker for chronic renal failure and uremia [54]. In the treatment of uremic patients with hemodialysis an assessment of carbHb adduct levels would possibly serve as an index of the adequacy of the patients dialysis therapy through the estimation of the mean blood urea nitrogen concentration [54, 55].

3.1.3 Diisocyanates

Diisocyanates have two functional isocyanate groups which give the compound the possibility of polymerisation by covalent bonds together with a chosen polyol, a compound with more than one hydroxyl functional group. Polymerisation occurs when the diisocyanate groups react with hydroxyl groups to form a urethane linkage. The second hydroxyl group can react with another diisocyanate and so on until a selected polymer is built.

Diisocyanates (see Figure 3.4) have a wide range of applications in the polymer industry producing such items as flexible and rigid foams, elastomers and coatings to textiles and are known under the collective name polyurethane (PUR). Production of PURs has risen steadily from 1 million tons in 1970 to about 12.3 million metric tons in 2007 [56]. Two main diisocyanates produced are 2,4-toluenediisocyanate (2,4-TDI) and 4,4'-Methylenediphenyl diisocyanate (4,4'- MDI). The 2,4-TDI commercial product comprises of two isomers, 20 % 2,6-TDI and 80 % 2,4-TDI. Its uses in the polymer industry include products such as foams, coatings and elastomers [57]. 4,4'- MDI is considered to be the most important diisocyanate [58]. It is used extensively as thermal isolators in refrigerators to the insulation of buildings. Hexamethylenediisocyanate (HDI) is an aliphatic diisocyanate and has a much smaller share of the world market 2007 [30]. The worldwide annual production of of diisocyanates is estimated to be more than 6 million tons (2007) [58].The structure of PURs varies depending on their commercial products and also implicates their toxicity. The polymers are deemed to be stable and undergo strict regulation during production in western countries but it has been observed that PUR products emit isocyanates up to 30 years post production [59].



2,4-Toluene diisocyanate (TDI)

Methylenediphenyl diisocyanate (MDI)

Hexamethylene diisocyanate (HDI)

Figure 3.4. Commercially important diisocyanates.

Toxicity

Isocyanates have been tested in recent years for toxic effects. Animal experiments have been conducted to test and compare aromatic mono- and diisocyanates and aliphatic mono- and diisocyanates with regard to sensory irritation. It was found that the aromatic TDI and aliphatic diisocyanate HDI had comparable potency. The aromatic monoisocyanate PIC was slightly less potent and the aliphatic monoisocyanate, hexylisocyanate was the least potent [60]. TDI was tested positive for pulmonary sensitisation and allergic skin reactions in guinea pigs [61]. Certain commercial diisocyanates are thought to be the main cause of occupational asthma [62-64]. In general MDI is the least hazardous of the diisocyanates on the market in regard to occupational asthma because of its low vapour pressure. TDI was tested in isocyanate-induced asthma tests where exposed mice showed marked allergic response evidence together with many symptoms including an increase in airway inflammation in subchronic exposure. Mice that received acute TDI exposure showed pathology in the lung consistent with asthma [65]. In the Ames test, the mutagenic activity was ascribed to the hydrolytically formed arylamines for TDI and MDI [66].

3.1.4 Biomonitoring

A method for the cleavage and quantification of the detached valyl-hydantoin of N-terminal valines in Hb as biomarkers deriving from isocyanic acid was first introduced 1973 by Manning *et al.* for the monitoring of patients undergoing sickle-cell anemia treatment [29]. Cyanate therapy, which prevents the gelling of deoxygenated hemoglobin and the sickling of the erythrocytes, was monitored by the hydrolysis of isolated globin and analysis by GC/MS.

Kwan (1990) developed a workup and analysis method by HPLC for carbamoylated N-terminal valine deriving from isocyanic acid [67]. The method involved hydrolysis of washed blood cells by concentrated HCl:HOAc (1:1). After pH adjustment to 4 with NaOH the analyte is extracted with EtOAc and washed with aqueous Na_2CO_3. The workup method has not varied much since its development and is used in uremia research.

The method was first utilised for isocyanates in the 1990s for the analysis of stored blood samples from Bhopal disaster victims exposed to methyl isocyanate (MIC). MIC exposure could be measured as the 3-methyl-5-isopropylhydantoin **(**MVH) analyte formed from carbamoylated N-terminal valine in Hb. The samples were analysed by gas chromatography/nitrogen phosphorous detection [41].

In the analysis of MIC valyl adducts Mraz (1999) dissolved globin in a mixture of HCl:HOAc (2:1) followed by heating at 100 ^{o}C for 1 h [30]. The detached hydantoin, MVH, is then isolated by extraction and analysed by GC/MS. Sabbioni adapted the method for analysing 2,4-toluenediisocyanate (2,4-TDI) by GC/MS or LC/MS. For the analysis by GC/MS, the second amine group is derivatised with pentaflouropropionic anhydride [68].

Another method for the biomonitoring of isocyanates is the unspecific hydrolysis to amines in human plasma or urine [69]. Diisocyanates such as TDI and MDI hydrolyse, decarboxylate and form 2,4-diaminotoluene (TDA) and 4,4´-methylenedianiline (4,4'-MDA) respectively. GC/MS has been used for analysis of extracted amines from acidified urine or plasma after derivatisation of the amines with pentafluoropropionic acid anhydride (PFPA) [69, 70]. However, this method is not specific for adducts formed from isocyanates.

3.2 Experimental/Results

Specific aims

The synthesis of an adequate compound to be used as general precursor for the synthesis of reference standards of valine carbamoylated by mono-, di- or poly-isocyanates. Synthesis of carbamoylated valine and corresponding hydantoin from a few isocyanates and test of the method for measurement of carbamoylated N-terminal valine in Hb by LC/MS/MS.

3.2.1 Synthesis

3.2.1.1 Earlier synthetic routes of reference compounds

The synthetic pathway to produce reference standards of model compounds to N-carbamoylated valine in Hb can be performed by using isocyanates direct in the reaction. Besides the use of different solvents and conditions etc. this reaction resembles N-carbamoylation of proteins *in vivo*.

The adducts to N-termini from monoisocyanates such as MIC and PIC can be synthesised in this way. By adapting dry ACN as the solvent instead of using aqueous condition we achieved a satisfactory yield (over 50 %). The dryness of the reaction is important as water leads to hydrolytic side-reactions generating amines which can react further with isocyanates by the formation of symmetrical urea compounds (see Figure 3.5 and Scheme 4 in Paper I).

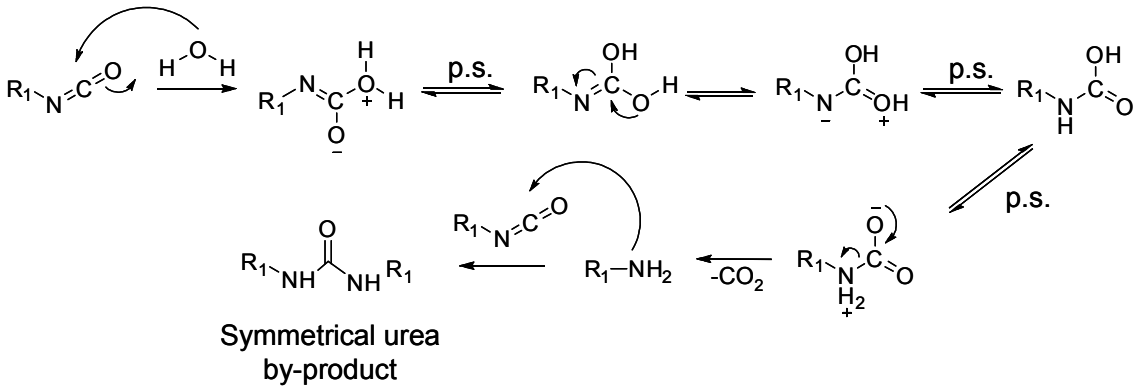


Figure 3.5. The hydrolysis and formation of urea by-products.

The synthesis of diisocyanate reference standards was even more challenging when diisocyanates were used as starting materials. Due to uncontrolled side-reactions leading to formation of polymers when water was used as solvent the obtained yields were below 1 % of the desired products [71].

This difficulty with low overall yields for diisocyanates was attacked by Sabbioni who presented an alternative pathway to synthesize model compounds of carbamoylated amino acids [68]. The idea was to avoid diisocyanates and instead use aromatic nitro monoisocyanates to produce precursors to TDI-carbamoylated valine. In the first step 2-nitro-toluene-4-isocyanate was carbamoylated with a wide range of amino acids. The nitro groups in the obtained products were then reduced and the formed products corresponded to carbamoylated diisocyanates where the second isocyanate group has been hydrolysed (see Figure 3.6).

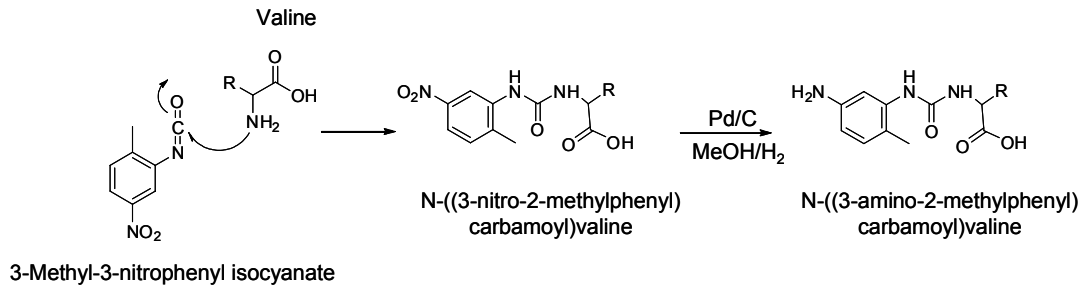


Figure 3.6. Pathway for the synthesis of TDI reference standard (by Sabbioni).

3.2.1.2 New synthetic route of reference compounds

A new synthetic pathway for the synthesis of mono- and diisocyanates reference compounds from a common precursor is presented in Paper I. By utilising the carbamate precursor N-[(4-nitrophenyl)carbamate]-valinmethylamide (NPCVMA) the use of isocyanate starting materials was completely excluded in the synthesis of N-carbamoylated valines (see Scheme 3 in Paper I). This method enables the use of just one precursor to synthesize a chemical library of carbamoylated model compounds. The precursor has been tested for reaction with both mono- and diamines to produce the corresponding valine adducts of mono- or diisocyanates (see Figure 3.7 and Scheme 4 in Paper I). The reference standards synthesised correspond to the carbamoylated valine and the valine-hydantoin from isocyanic acid, MIC, PIC and TDI.

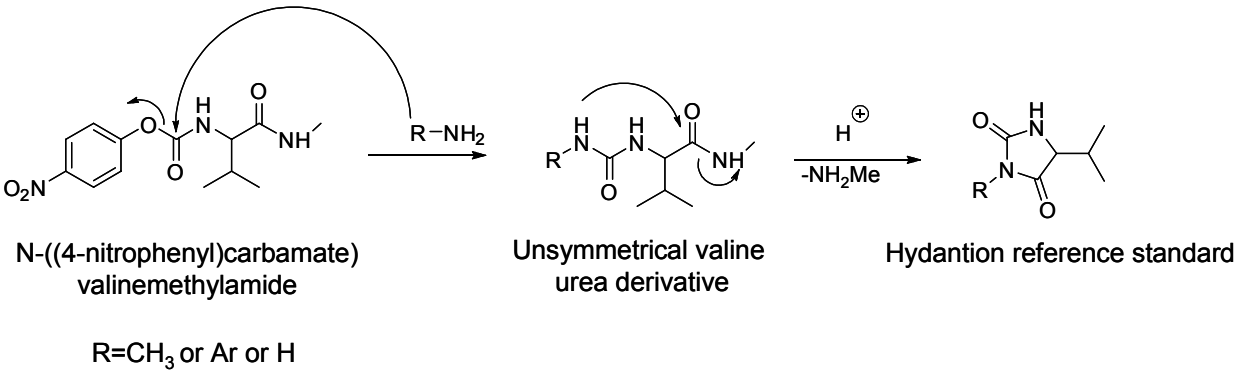


Figure 3.7. New synthesis pathway of reference standards of adducts from isocyanates to valine using a common precursor.

3.2.2 Analysis

The adducts from isocyanic acid and PIC were used in the analytical studies on *in vitro* samples. The concentration of 3-phenyl-5-isopropylhydantoin (PVH) and isopropylhydantoin (VH) formed after hydrolysis, was measured from the addition of different concentrations of the synthesised carbamoylated methyl valine amide to blood. This gave a linear regression and showed a yield of nearly 100 % PVH, which was determined from a calibration curve using the hydantoin standard (see Figure 1 in Paper I). This established a possibility for quantitative measurements of adducts from isocyanates to N-terminal valine in Hb as the corresponding hydantoin, using compounds synthesised by the new route as standards (see Figure 3.7).

For the analysis of yield from blood samples the Kwan method for work-up was chosen and analysis was done with LC/MS method. Anonymous blood donors were used. Isocyanic acid is known to be an endogenously produced isocyanate to which the general populous is exposed. Therefore VH, which is formed from the isocyanic acid adduct to valine (see Figure 3.3), was chosen

as a suitable model compound for the analytical development. VH was as expectedly found as a background level in globin samples (see Figure 2 in Paper I).

3.3 Conclusions

A method to synthesise standards of carbamoylated valines and corresponding hydantoins in good yield and avoiding the use of isocyanates in the synthesis was developed. Using this core synthesis route many other, less well studied, isocyanate reference standards can be synthesised, and allow standards to be available to facilitate biomonitoring of isocyanates used in industry at the present. The work-up in the analytical method is relatively simple.

Isocyanates have been in use for over one hundred years and are presently used in such diverse fields as medical research, to monomers of foam in many household, industrial and agricultural goods. Medical disorders have been attributed to isocyanates, one of the most serious being asthma. Occupational exposure to isocyanates in industry in developed countries has decreased as a consequence of more stringent safety regulations, which in part is due to the hard work of researchers highlighting the medical implications and levels of exposure. However, a need for methods for biomonitoring of exposure in tangent with increased production of PUR particularly in developing countries can be required. The described approach will offer a tool in this context.

4 Aldehydes

4.1 General background

Introduction

Aldehydes account for a large group of organic compounds. Exposure sources to the general population include both exogenous and endogenously produced aldehydes. Aldehydes are formed through incomplete combustion processes including smoking of cigarettes, and are ubiquitous in air pollution [72]. Aldehydes are also formed in foods during processing and storage. Endogenous aldehydes are formed in lipid peroxidation and through metabolism. In addition there occurs exposure both in the home and occupationally to certain aldehydes, for example exposure to acrolein during cooking [73, 74]. This thesis is focused on aldehyde exposure from natural sources.

The Maillard reaction

The Maillard reaction is named after the French physician and chemist Louis-Camille Maillard and can occur in the heat-processing and storage of food [75]. It also occurs in biological systems and in humic substances in the soil and sea. In his initial research Maillard used glycerol and sugars as starting materials in the synthesis of peptides and he discovered that reducing sugars showed extra reactivity. It has been observed that equimolar amounts of a specific amino acid and a reducing sugar can produce scores of different organic compounds. With that in mind the resulting reactions in environments containing reducing sugars and amino acids are considered to be extremely complex. However, the complexity has been broken down into three main subdivision reactions [75].

The Maillard reaction initially begins with reversible reactions between reducing sugars and free amino acids or proteins. A second advanced stage involves sugar dehydration and fragmentation, and amino acid degradation. The final stage produces highly coloured products from aldol and aldehyde-amine condensations resulting in the formation of heterocyclic nitrogenous polymers and copolymer compounds. The whole reaction is described as “nonenzymic browning” due to the gradual colouration of the reaction products through the three stages. Various aldehydes including 5-hydroxymethylfurfural (HMF) and aliphatic-chained aldehydes are produced in the second stage [75]. HMF is discussed in chapter 4.2.

Lipid peroxidation

A wide range of reactive compounds are formed by oxidative degradation of lipids (lipid peroxidation). This includes the dialdehyde malondialdehyde

(MDA), the dicarbonyl compounds glyoxal (GO) and methylglyoxal (MGO), aliphatic-chained and side-chained aldehydes and α,β-unsaturated carbonyl compounds such as 4-hydroxy-2-nonenal (HNE), and acrolein (ACR) [76] [77, 78] (see Figure 4.1). HNE is the most abundant α,β-unsaturated aldehyde produced by lipid peroxidation. It has also been considered the most toxic product of the process [76, 78].

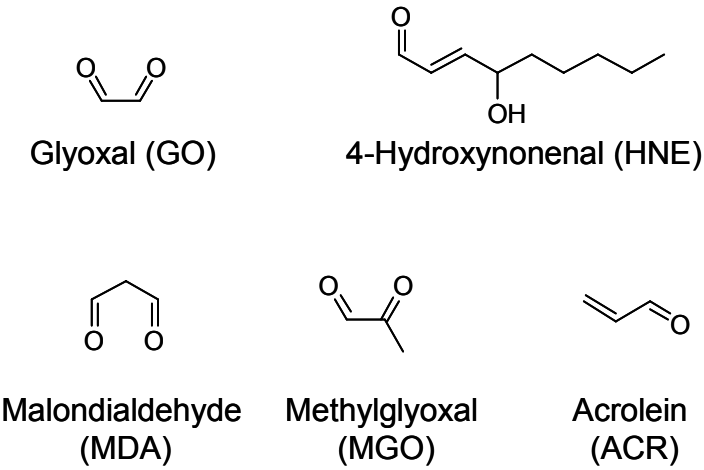


Figure 4.1. Aldehydes produced by lipid peroxidation.

Lipid peroxidation occurs in biological systems when a reactive oxygen species (ROS), usually a hydroxyl radical, initiates a chain reaction with unsaturated hydrocarbon chains [79]. This reaction produces lipid radicals which in turn can react with oxygen, and lipid peroxyl radicals will be formed. These radicals can then react with other unsaturated hydrocarbon chains producing more peroxyl radicals and lipid hydroperoxides, and a chain reaction is then propagated. MA is specifically formed if the lipid peroxyl radical reacts with itself [76]. In this study MGO, GO and MA (Figure 4.1) have been investigated with regard to the possibilities of *in vivo* measurement as adducts to N-terminal valines in Hb by the N-alkyl Edman method or the adduct FI*R*E procedure (described in chapter 2), and are discussed in chapter 4.5 and 4.6.

AGE/ALE

Simple sugars such as glucose and ribose and their degradation products such as GO can react with nucleophilic sites in proteins to form so called advanced glycation end products (AGE). The formation of AGE is often called "browning of proteins" by glucose. Reactive lipid peroxidation products can react with proteins or free amino acids resulting in the formation of advanced lipoxidation end products (ALE). While formation of ALE requires oxygen in its initial stage, the formation conditions of AGE does not [78].

Aldehydes from metabolism

Ethanol, which can be consumed in varying amounts by humans, produces acetaldehyde by alcohol dehydrogenase as a main metabolite. Metabolism produces an array of other aldehydes from parent compounds occurring in biological systems.

Biological activity of aldehydes

Aldehydes are reactive electrophilic compounds and a great deal of their toxicity rests on the aldehyde structure where dialdehydes are generally more potent as cross binding can occur with nucleophilic atoms in macromolecules. Reactions by aldehydes can lead to the formation of AGE/ALE. Generally AGE/ALE are associated with diabetic, vascular and neurodegenerative diseases in humans [80, 81]. However, aldehydes in biological systems can also inhibit the energy metabolism of biosynthesis and cell division. These properties have been shown to give inhibition of the growth of malignant tumors and antibacterial effects [77].

4.2 5-Hydroxymethylfurfural (Paper II and unpublished)

4.2.1 Background

Exposure and metabolism

HMF is one of the major products in the Maillard reaction and can be formed in various foodstuffs including bakery products, jams and fruit-based foods through the degradation of glucose [82]. HMF has been investigated for decades in such diverse areas as toxicology, food flavouring and sickle-cell treatment [83]. In the field of toxicology HMF draws interest because of the high intake per day (30-150 mg) together with that HMF possibly metabolises to other electrophilic compounds (see Figure 4.2) [72, 82, 84, 85].

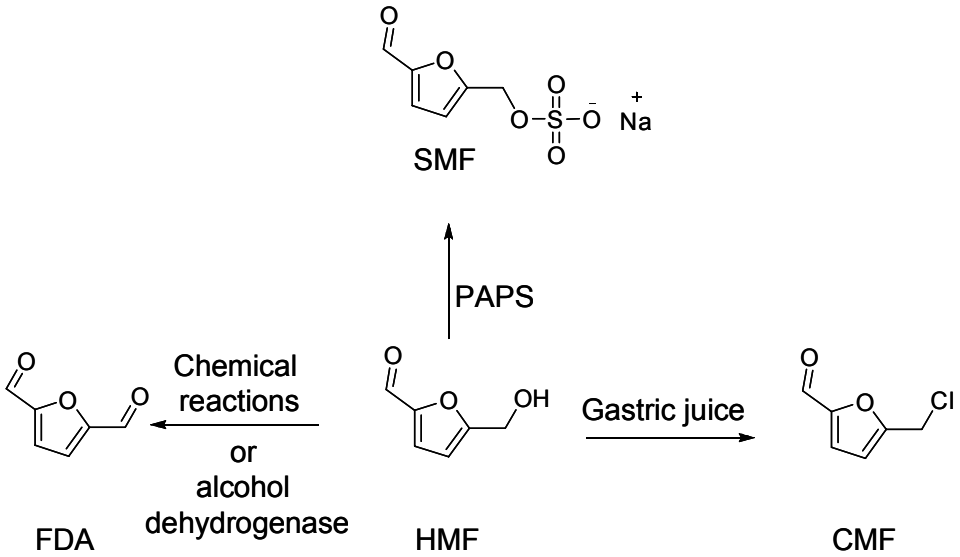


Figure 4.2. Hypothetical metabolism of HMF to reactive metabolites (PAPS = 3^{I}-phosphoadenosine-5^{I}-phosphosulphate).

One of the electrophilic compounds discussed is 5-sulfooxymethylfurfural (SMF), which has recently been identified as a HMF metabolite *in vivo* in mice (by trapping in plasma by dinitrophenylhydrazine) [86]. It was first observed as a metabolite *in vitro* by a sulphotransferase assay [87]. The hypothetical metabolite 5-(chloromethyl)furfural (CMF), possibly formed in gastric juice, has so far not been shown to be formed *in vitro* or *in vivo*. Bacterial mutagenicity assay has established CMF as being strongly mutagenic and toxic [87]. 2,5-Furandialdehyde (FDA), another theoretical bifunctional metabolite has not yet been identified as a metabolite of HMF, but it has been detected as a component in stored honey (see Figure 4.2) [88].

It has been shown that the food contaminant furan can be metabolised to an epoxide intermediate which in turn rearranges to a dialdehyde [89, 90]. A possible route for the metabolism of HMF (which has not been explored or discussed in the literature) is its possible epoxidation (see Figure 4.3).

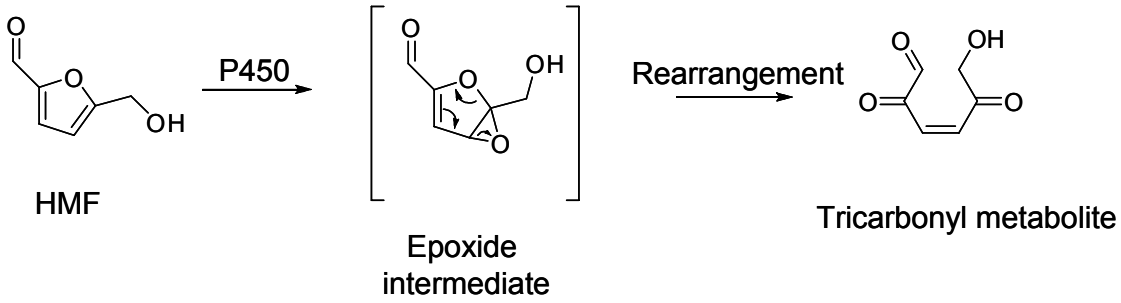


Figure 4.3. Possible enzymatic oxidation of HMF.

Biomonitoring of HMF

The two main ways to monitor HMF exposure have been either by directly analysing food or analysing excreted urine and faeces for HMF and metabolites. Monitoring of HMF in various foodstuffs from bakery products to fruits has been performed using HPLC with UV detection [91, 92].

A number of studies of HMF metabolism have been conducted analysing biomarkers. When HMF was administered to rat, 5-hydroxymethyl-2-furic acid (HMFA) and *N*-(5-hydroxymethyl-2-furoyl)glycine were the main metabolites [93]. In exposed mice as well as in the urine of humans (infants and adults), furan-2,5-dicarboxylic acid has been observed [82, 84]. Recently, HMFA was monitored in urine from human volunteers and the daily intake of HMF was estimated to 32 mg [94]. In this study HMFA, although in small amounts, was also found in food.

4.2.2 Experimental/Results

Specific aims

To evaluate the possibilities to utilise the stabilised Schiff base adducts with N-terminal valine in Hb from HMF and its reactive metabolites as a biomarker, using the N-alkyl Edman method for analysis by GC/MS.

4.2.2.1 Synthesis

HMF- and FDA-adduct reference standards (Paper II)

The N-alkyl Edman method can be used for analysis of Schiff base adducts from aldehydes to N-terminal valine in Hb after stabilisation of the Schiff base to a secondary amine (Figure 4.4). Both HMF and FDA could be expected to have the ability to form Schiff base adducts to N-termini in Hb [83]. To be able to study the formation of adducts and to perform the analysis with the N-alkyl Edman method the corresponding standards of the Edman derivatives (PFPTHs; see Figure 2.1) of HMF and FDA were synthesised.

Synthesis of N-substituted valine derivatives of aldehydes can be performed using a model of N-terminal valine (valine amide in this study) and selected aldehydes as starting materials. To form a stable adduct, the reducing agent

$NaBH_3CN$ was used [33] (see Figure 4.4). The reference standards of the Edman derivatives, the PFPTHs, are formed by the addition of PFPITC in the second step of the synthesis, after the reduction. The PFPTHs (see Figure 2.1) can then be purified on silica gel columns.

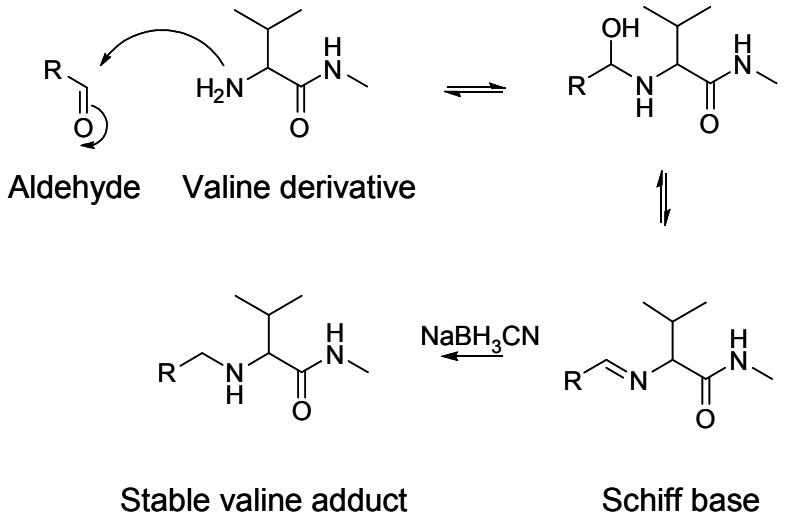


Figure 4.4. Nucleophilic addition followed by reduction of Schiff base.

This pathway was used in the synthesis of HMF-Val-PFPTH (d_0 and d_7). The reference standards of FDA adducts, FDA-Val-PFPTH (d_0 and d_7) were synthesised by oxidising the HMF-Val-PFPTH (d_0 and d_7) reference standards with MnO_2. In the synthesis of the precursor FDA, Dess-Martin periodinane was utilised for the oxidation of the hydroxyl group of HMF to form FDA.

Synthesis of SMF- and CMF- adduct reference standards (unpublished)

The reported metabolite of HMF, 5-sulfooxymethylfurfural (SMF), and the corresponding Edman derivative SMF-Val-PFPTH were synthesised. This was done by activating the respective hydroxyl groups of HMF or HMF-Val-PFPTH using the condensing agent *N*,*N*'-dicyclohexylcarbodiimide (DCCI) followed by a nucleophilic substitution with sulphuric acid in accordance to Surh *et al.* [87] (see ^{1}H NMR and LC/MS data in Appendix 1, Figure 1).

5-(Chloromethyl)furfural (CMF), the putative metabolite of HMF, and its PFPTH-derivative were also synthesised. The synthesis of CMF was successfully performed by converting HMF by thionyl chloride (3 equiv) and $ZnCl_2$ (0.1 equiv.) in dioxane as described by Surh *et al.* [87]. The reference standard CMF-Val-PFPTH was synthesised using the same method but with HMF-Val-PFPTH as the starting material (see ^{1}H NMR and GC/MS data in Appendix 1, Figure 2).

4.2.2.2 Analysis

Materials

In the development of the analytical method human blood samples incubated *in vitro* with the studied aldehyde were used. Experiments were also conducted using human blood samples (*in vivo* samples) for studies of the possible presence of background adduct levels from these aldehydes from intake of HMF (or FDA) from food. The synthesised standards were used for identification and quantification of adducts in blood samples, after reduction and work-up of the blood according to the N-alkyl Edman method.

HMF and FDA (Paper II)

Schiff base adducts *in vivo* have in the past been stabilised with $NaBH_4$ before derivatisation [34]. This earlier procedure first removed the cell membranes from the hemolysate by centrifugation before reduction with high concentrations of $NaBH_4$. A time consuming dialysis step for the removal of salts before precipitation of globin was required before the final detachment of adducted N-terminal by PFPITC. The reduction with $NaBH_4$ is considered as a rather harsh procedure. The method has not been used much due to its drawbacks, and primarily the reduction step needed improvements. The reduction step was therefore the primary focus for development in the present work.

In our study in Paper II we used $NaBH_3CN$ (instead of $NaBH_4$) for the reduction of Schiff bases. According to literature $NaBH_3CN$ is specific for the reduction of the imine functional group in biological samples [33]. Paper II describes various studies of the specificity of the reduction of the Schiff bases from HMF and FDA.

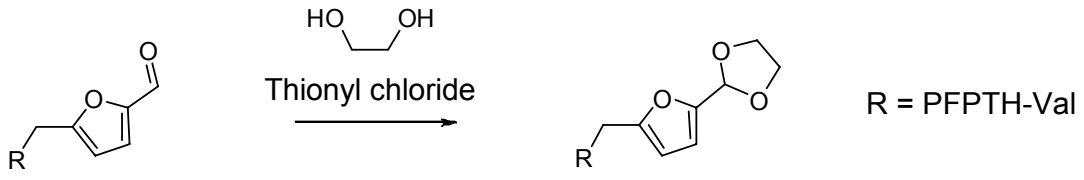
Samples of hemolysate incubated with the aldehydes (HMF and FDA) were used for the tests. $NaBH_3CN$ in amounts of 0.1 – 150 mg/mL hemolysate were tested for the reduction (see Figure 3 in Paper II). It was shown that even high concentrations had only a small influence on pH in the hemolysate during the reduction (see Figure 5 in Paper II). It was established that the reduction with 2 mg $NaBH_3CN$/mL hemolysate was optimal to stabilise the Schiff base of HMF to N-termini in Hb (see Figure 4b in Paper II). This relatively low concentration of $NaBH_3CN$ gives mild conditions during the reduction and makes it possible to exclude the dialysis step in the work-up procedure. Also the ultracentrifugation step for removal of cell membranes was excluded. This resulted in a mild, sufficiently reproducible and considerably simplified procedure for measurement of the Schiff base to N-termini in Hb (see Figure 8 in Paper II). This improved, fast work-up procedure then made it possible to perform studies in more detail.

Using the developed method the stability of the Schiff base of HMF in incubated hemolysate was studied. It was found to have a short half life of 3.4 h at 37 ˚C in human blood (see Figure 6 in Paper II). Despite the short half-life, a low background level of the HMF adduct (10-35 pmol/g Hb) was found in human blood samples, in accordance with an intake of HMF from food (see Figure 7 in Paper II).

The specificity of $NaBH_3CN$ for the reduction of imines was tested for the FDA Schiff base adduct. In experiments with FDA-incubated hemolysate, it was shown that the free aldehyde group in the FDA adduct was also easily reduced, thus giving an analyte equivalent to the HMF adduct (see Figure 3 in Paper II). However, it was possible to isolate the analyte of the stabilised Schiff base adduct from FDA to N-termini in Hb from FDA-incubated hemolysate after stabilising the Schiff base with ca 0.1 mg $NaBH_3CN$/mL hemolysate. The yield, however, of the FDA-Val-PFPTH analyte was very low (see Figure 4a in Paper II). Analysis was done on GC/MS in the electron impact (EI, SRM) mode.

It was studied whether background adduct levels from FDA could be detected in control human blood samples. The FDA-Val-PFPTH analyte was searched for using different GC/MS modes. It was not possible to obtain a specific fragment for FDA-Val-PFPTH when using the more sensitive ECNI mode. The use of the synthesised deuterium-substituted analogue as internal standard for retention time determination showed no background level detection of FDA-Val-PFPTH using GC/MS/MS (EI). The limit of detection was in the low pmol/g globin (See Paper II for details.).

Acetalation of the FDA-Val-PFPTH standard with ethylene glycol was also tested (Figure 4.5) in an attempt to obtain a specific ion in MS analysis. The AcFDA-Val-PFPTH in the analysis by GC/MS/MS (ECNI), however, gave no specific ions (data not shown), and in selective ion monitoring (SIM) the analyte could not be detected in human blood. Unfortunately, LC/MS/MS analysis could not be tested (see Figure 4.5)



FDA-Val-PFPTH reference standard for FDA

AcFDA-Val-PFPTH acetalated reference standard for FDA

Figure 4.5. Acetalated FDA standard.

SMF and CMF (unpublished results)

For SMF, experiments were conducted to test the suitability of using the N-alkyl Edman method for the measurement of N-terminal valine adducts in Hb as a biomarker. At room temperature SMF has a half life of 3.6 h in 0.1 M phosphate buffer (pH = 7.4) and is hydrolysed back to HMF [95] (see Figure 4.6). Consequently, water should be avoided as far as possible in the analytical cleanup procedure.

It was important to assess if the SMF molecule, in spite of its negative charge (see Figure 4.6), could enter the red blood cells, e.g by diffusion. SMF was incubated with human whole blood overnight and the globin was isolated. The samples were reduced with $NaBH_3CN$ and derivatized with PFPITC in formamide to detach adducts. Formamide was then evaporated and dimethyl formamide was added, the globin pelleted and the protein-free supernatant was analysed (see Appendix 1, Figure 3). This experiment showed that SMF could enter red blood cells and form a Schiff base with N-terminal valines in Hb. The control blood sample, which was incubated with SMF and not reduced, gave no signal in the analysis.

As alternative enrichment methods for SMF-Val-PFPTH, from globin samples spiked with the analyte, different SPE columns were then tested. When using ion exchange chromatography the SMF-Val-PFPTH hydrolysed at high rate (see Figure 4.5) to form HMF-Val-PFPTH. The best separation was obtained on an Oasis Max SPE-colum (mix mode anion exchanger), when using 1 % formic acid in methanol for elution. It was observed that the analyte was methanolysed to 5-metoxymethyl-furfural-Val-PFPTH (MeOMF-Val-PFPTH) in the elution step. Also a small part of the SMF-Val-PFPTH analyte reacted with formic acid in a S_N2 type reaction. In principle, the obtained MeOMF-Val-PFPTH analyte could be used as a stable marker from SMF-Val-PFPTH (see analytical data on MeOMF-Val-PFPTH in Appendix 1, Figure 4).

In preliminary experiments with CMF-Val-PFPTH at physiological conditions (pH = 7.4) the standard hydrolysed fast (half life of a few minutes) (see Figure 4.6 and Appendix 1, Figure 5), to HMF-Val-PFPTH. Due to the rapid hydrolysis of CMF-Val-PFPTH no further studies on CMF with incubated blood were pursued.

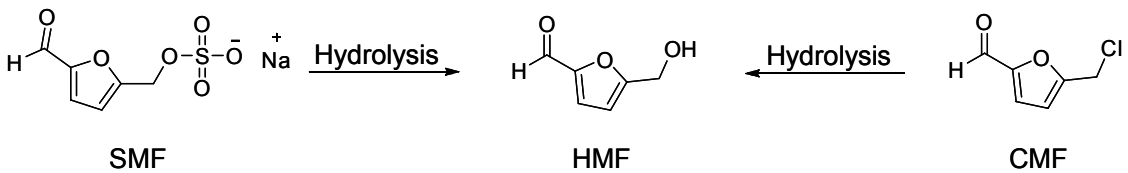


Figure 4.6. Hydrolysis of metabolites of HMF will produce HMF.

4.2.3 Conclusions

PFPTH standards of HMF and its possible metabolites FDA, SMF, and CMF were synthesised. An improved milder method for the analysis of Schiff bases to N-termini in Hb was developed using low concentrations of $NaBH_3CN$ for stabilisation of the Schiff base. The new procedure was ca 10 times less time-consuming than the earlier $NaBH_4$-method. This method should be explored for studies of Schiff bases from other aldehydes.

Due to the short half life of the Schiff base adducts of HMF to N-termini in Hb this HMF adduct will not be useful for measurement of long term exposure of HMF. The new procedure could, though, be used for identification of HMF exposure in humans and in experimental studies, where analysis could be done short time after exposure.

The discussed metabolites of HMF, that is SMF and possibly FDA and CMF, has in addition to the aldehyde function an electrophilic group. This fact complicates the possibilities to measure these adducts as they are short lived. It was found that the reducing agent also reduced the free aldehyde in the FDA-Schiff base adduct. However, at very low concentrations of the reducing agent the FDA analyte was isolated from FDA-incubated blood (although in low yields).

It was shown *in vitro* that SMF in spite of its charge can enter red blood cells and bind to N-terminal valine and then be isolated by the N-alkyl Edman procedure. The PFPTH analyte of the Schiff base from the metabolite SMF and CMF were shown to undergo fast hydrolysis to the corresponding HMF analyte. Although the SMF-Val-PFPTH could be isolated in low amounts, from blood samples incubated with SMF, the hydrolysis of the analyte caused very low yields. A PFPTH analyte which only can originate from the SMF by S_N2 nucleophilic substitution before or during purification by a SPE column has been identified as MeOMF-Val-PFPTH. This can be useful for specific detection of SMF. Earlier an approach has been tested for detection of the SMF metabolite in the form of a mercapturic acid metabolite after conjugation to the sulphate of glutathione [96].

Possibly the new approaches used here could be applied to study the formation of FDA and SMF as Schiff base adducts in *in vivo* experiments, but not for biomonitoring in humans. A further development should be exploration of the remaining electrophilic reactivity in the adduct for further derivatisation of the analyte *in vitro* or for search of cross-linked adducts formed *in vivo*.

Analytical problems of several kinds were encountered, when PFPITC is used for derivatisation of N-terminal valine adducts in Hb. The corresponding fluorinated thiohydantoin analytes normally give high sensitivity when measured by GC-MS in the negative chemical ionisation (ECNI) mode, as

expected for a fluorinated compound. GC-separation normally works well for unpolar analytes which elute nicely from the GC column. Although if no specific ion is observed in GC-MS alternative methods for analysis, e.g., different ion scanning methods or additional derivatsation should be tested (example given with FDA-Val-PFPTH, see Figure 4.5). For more polar adducts, the N-alkyl Edman method has to be modified as with SMF, and the workup be further developed.

4.3 Other aldehydes (unpublished)

4.3.1 Background

Endogenous malondialdehyde (MDA) is a major product of lipid peroxidation. It is genotoxic and can also react rapidly with SA to form dimers, although at physiological pH, it occurs mainly in its enolate anion form, stabilised by a conjugated π-bond which reduces its reactivity [78]. The main focus in this part of the work was MDA, but as it is known from our earlier work that adducts from acetaldehyde (AcA), formaldehyde (FA), and benzaldehyde (BA) occur as background levels in Hb, these aldehydes were also included in the studies of background Hb adduct levels in blood from humans. Acetaldehyde (AcA) is the main metabolite from ethanol and is also found in many foodstuffs. Formaldehyde (FA) is formed in large amounts through combustion processes. Benzaldehyde (BA) can be found in foodstuffs as a flavouring compound [97]. AcA, FA and BA all are formed in incomplete combustion processes [98] and occur as air pollutants [72]. AcA, FA, and BA are also all high volume production (HVP) chemicals (more than 1000 tons per year) in the chemical industry.

Biomonitoring of free MDA in biological samples is usually measured by thiobarbituric acid as a coloured product (see for instance [76]). This measurement is not specific for MDA. A method which has generally been used for biomonitoring of aldehydes is the determination by 2,4-dinitrophenyl hydrazine and analysis by HPLC with UV detector [76]. This method measures both free and released aldehydes from Schiff bases and is therefore less useful for the quantitative long-term *in vivo* dose measurements, though adapted to LC/MS it would be specific and useful for short-term exposure. Hb adducts of AcA as well as urine metabolites have, in the past, been tested as biomarkers for the sufferers of alcohol abuse [99, 100]. Hb adducts from AcA have been measured by different methods, but there has been little progress regarding measurement of alcohol abuse [99]. Using the earlier method for measurement of Schiff base adducts to N-termini by the N-alkyl Edman method, after reduction with $NaBH_4$, a range of background adducts could be measured at high levels in human Hb, though with insufficient reproducibility [34]. These were assumed to be formed from different aldehydes [35, 101].

4.3.2 Imidazolidinone formation

AcA is well-studied with regard to adduct formation to Hb, and several binding sites have been shown [102]. It has been reported that AcA can react with N-terminal valine and undergo internal cyclisation to form a stable imidazolidinone product (see Figure 4.6). The formation of adducts from AcA that is "stable" during dialysis (non Schiff bases), but non-stable during acid hydrolytic conditions was first observed by Stevens *et al.* [102]. The

imidazolidinone structure was suggested by San George and Hobermann [103]. Farmer *et al.* synthesised and characterised an imidazolidinone from acetaldehyde and a dipeptide [99]. Stevens *et al.* also showed that it was possible to at least partially convert the dialyse-stable adduct, assumed to be imidazolidinone adducts, to adducts also stable to adduct hydrolysis, by the mild reducing agent $NaBH_3CN$. It was discussed if this was due to slow conversion of imidazolidinone to a reducible Schiff base. Although it is not absolutely clear if the reducing conditions as such could enhance the conversion (see Figure 4.7). Simple aliphatic aldehydes is a problem to measure as observed by Lucas *et al.* who showed large artifactual production of AcA during the degradation of proteins under acidic conditions [104]. The formed imidazolidinone from AcA has been discussed as a possible biomarker for the monitoring of alcohol abuse [99, 100], and would be a better long-term biomarker than the Schiff base adducts. The formation of imidazolidinone could be expected to occur from a range of aldehydes, however it is unlikely to be formed with HMF.

We have shown that even when large amounts of $NaBH_3CN$ is added to hemolysate the pH of the hemolysate remains around 7-8 (see Paper II, cf Borch [33]). Therefore the cyclised imidazolidinone is in equilibrium with its protonated form under these conditions thereby the adduct can undergo ring opening (path a) and revert to its imine form. Reduction of the Schiff base produces a stable adduct (path b) that can be detached by the N-alkyl Edman method.

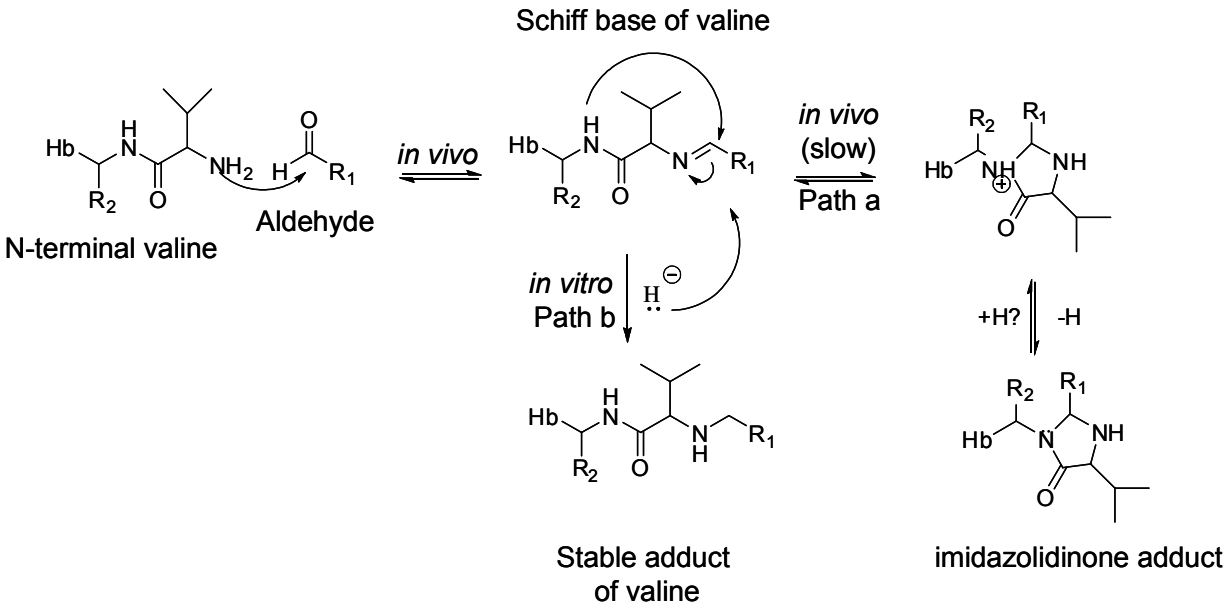


Figure 4.7. Proposed formation of stable aldehyde adducts and ring opening *in vitro*.

The ring-closed imidazolidinone adduct of acetaldehyde is formed from the reversibly bound Schiff base. The mechanism for the reversal of the

imidazolidinone ring is used in the pharmaceutical industry for "pro-drugs" in order to protect the N-terminus against aminopeptidase-catalysed hydrolysis [105]. Reversibility of the imidazolidinone has been measured with half times ranging from 0.7 to 31 h at 37 °C depending on the nature of the studied imidazolidinone compound [105].

4.3.3 Experimental/Results

Specific aim

Evaluate the possibilities of measuring adducts to N-terminal Hb from certain aldehydes (MDA, AcA, FA and BA) occurring in humans at background levels.

4.3.3.1 Synthesis

In the synthesis of the reduced MDA valine adduct, 3-iodo-1-propanol can be used as starting material. The reason for this alternative pathway is that MDA rapidly undergoes aldol condensations leading to dimers and other polymers. Other advantages by using electrophiles such as halogenated alcohols is that the desired product corresponding to the reduced Schiff base adduct, is obtained directly (see Figure 4.8).

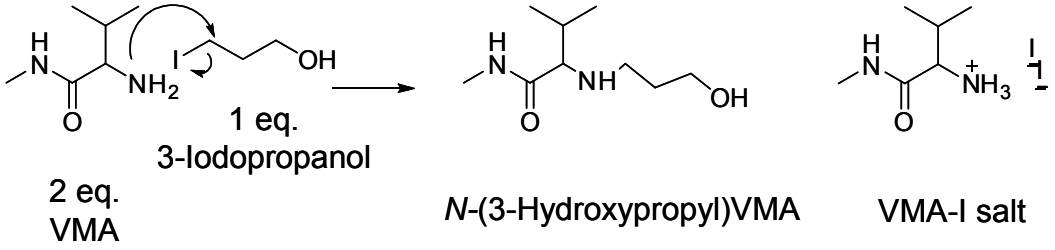


Figure 4.8. Nucleophilic substitution using 2 equvilents of VMA.

Instead of using valine for the synthesis of N-substituted valines alternative routes such the RS-2-bromoisovaleric acid pathway can be used. The 2-bromoisovaleric acid is reacted directly with the selected amines (normally also used as solvent in molecular excess) with substitution of the bromine by the amine resulting in an N-substituted valine in racemic form (see Figure 4.9). This strategy was used in this work for the synthesis of aliphatic valine-PFPTH standards corresponding to reduced Schiff base adducts from FA, AcA and BA. This is an old synthetic route which has previously been used for synthesis of polypeptides [106] and later for the synthesis of N-substituted valine standards [107, 108].

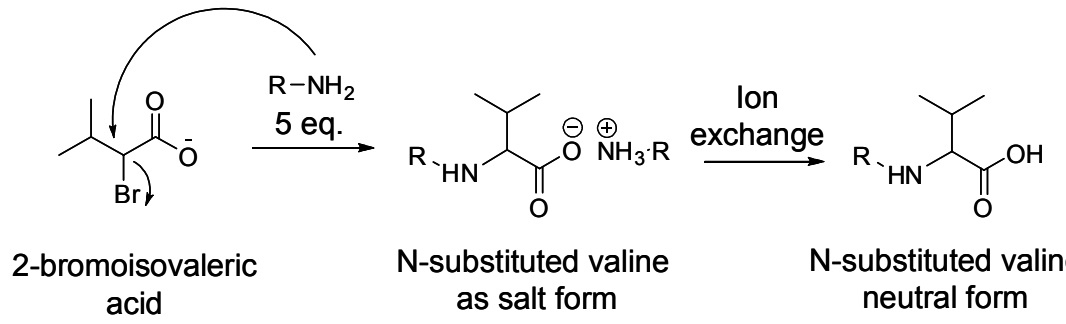


Figure 4.9. The utilisation of 2-bromoisovaleric acid and primary amines as start materials for the synthesis of valine adducts.

4.3.3.2 Analysis of adducts from aldehydes to N-termini and model compound (unpublished results)

Untreated blood samples were analysed for the Schiff base adducts to N-termini formed from AcA, FA, MDA and BA after reduction. To hemolysate various amounts of $NaBH_3CN$/mL hemolysate (1-40 mg) was added for reduction for one hour at room temperature. The globin was precipitated and the reduced Schiff base adducts were detached by PFPITC and the formed PFPTHs were analysed by GC/MS/MS (EI^+). For all studied aldehydes (as shown in Figure 4.10), except HMF (see Figure 4a, in Paper II) the adduct levels increased with added amounts of $NaBH_3CN$. A study was then conducted where samples with 40 mg $NaBH_3CN$ /mL hemolysate were reduced during set times (20 minutes to 3 hours). It was found that the reduction was complete already after 20 minutes (data not shown).

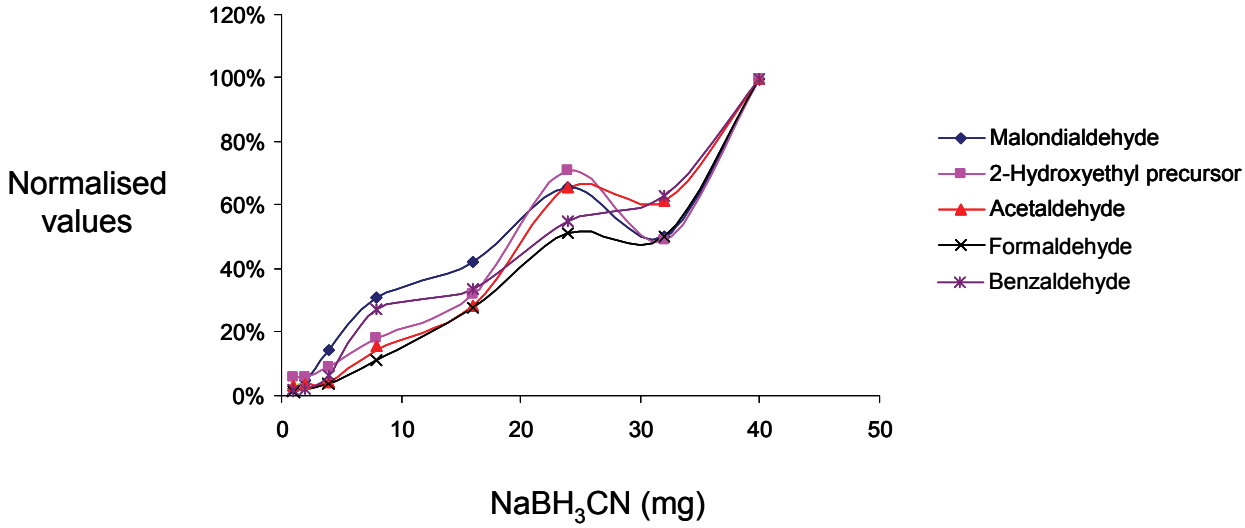


Figure 4.10. Relative adduct levels of various aldehydes isolated as PFPTH analytes.

The maximum levels of the adduct from AcA was preliminary estimated to be ca. 700 nmol/g Hb, and from FA at about the same level. The obtained maximum adduct levels seemed high, and in total will correspond to about 2 % of the N-termini in Hb. This raised the question whether adducts are formed as artefacts during reduction. Another question raised is whether the

reduction was able to convert stable imidazolidinone adducts to reducible Schiff base adducts, which could be measured with the N-alkyl Edman method.

Therefore, an investigation was initiated to study if $NaBH_3CN$ could produce ring-opening of imidazolidinone adducts from aldehydes. A commercially available substance, (2S,5S)-5-benzyl-2-*tert*-butyl-3-methyl-4-imidazolidinone (BBMI), was selected as model for the studies. BBMI in varying amounts was incubated with $NaBH_3CN$ (10-40 mg)/mL in aqueous methanol solution for 1 h. However, no ring-opening was observed by HPLC, GC/MS or LC/MS.

In an attempt to study whether adducts are formed as artefacts tests were done with reduction of isolated globin samples, without the cell membranes, and analysis with the N-alkyl Edman method by GC/MS (EI, SRM mode). An increase of adduct levels with added amounts of $NaBH_3CN$ was observed, though maximum level was preliminary ca 100 times lower than when sample was reduced as hemolysate. Another test was to block the N-termini (with PFPITC) for further Schiff base formation (from possible artefacts) during reduction, which also showed lower adduct levels. This type of precaution to reduce artefact formation has to be further investigated.

4.3.4 Conclusions

We measure high levels of Schiff base adducts from MDA, BA, AcA and FA with the improved method for measurement of aldehyde adducts according to the N-alkyl Edman method. It was observed that adduct levels increased with the addition of $NaBH_3CN$/mL hemolysate. It seems as this level of adducts is not originating from the ring-opened imidazolidinone adducts, but this should be investigated in more detail. Today it cannot be excluded that observed adduct levels partially are produced as artefacts during reduction of samples. The improved method in the present state is probably useful for identification of Schiff base adducts to N-terminal valines in Hb from more complex aldehydes, like HMF which are not expected and have not seen to be formed as artefacts. According to literature ring-closed imidazolidinone adducts are formed to N-terminal valines in Hb from aldehydes, as shown for AcA. If ring-opening of imidazolidinone adducts to N-terminal valine could be done, these N-terminal adducts would be a suitable long-term biomarker for aldehydes, through analysis with any of the methods for specific detachment of N-terminal adducts with isothiocyanates.

4.4 Glyoxal and methylglyoxal (unpublished)

4.4.1 Background

Apart from lipid peroxidation, the formation of GO and MGO have various other sources (see Figure 4.11). GO and MGO are for instance formed through the oxidation of glucose where GO has been found in stored honey and during food processing [75]. MGO is also formed during the anaerobic glycolysis of phosphates and amino acid metabolism. These aldehydes occur as air pollutants [72].

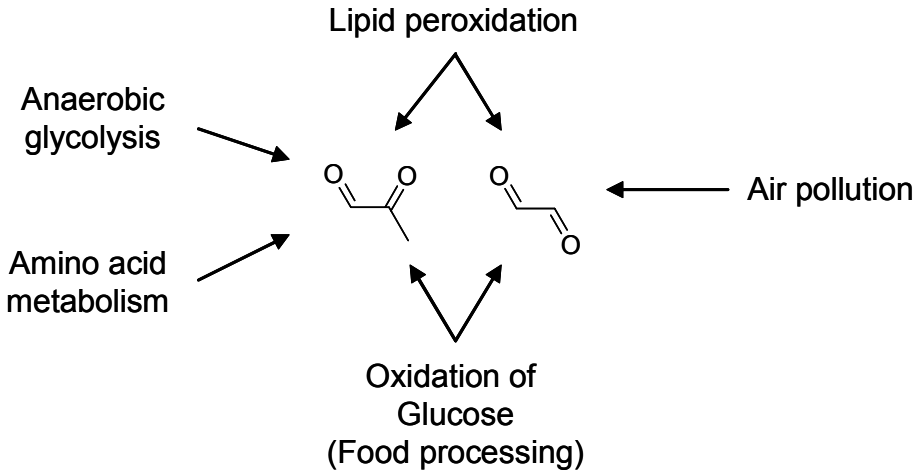


Figure 4.11. Sources of GO and MGO.

When GO or MGO reacts with free amino groups of proteins *in vivo* a Schiff base is first formed. This reversibly bound adduct can then undergo rearrangement with the loss of water to form stable so called Amadori products in proteins followed by further dehydration, cyclisation, oxidation and rearrangement to produce AGEs [78]. GO has also been shown to form an adduct in calf thymus DNA [109].

One biomarker of AGE that has been proposed is adducts initially formed from GO when bound to the thiol group of cysteine or to N-terminal valines in Hb. These Schiff base adducts have been shown to undergo Cannizzaro rearrangement to form irreversibly bound S-(carboxymethyl)cysteine adducts [110, 111] or when bound to N-terminal valines in Hb rearrange to N-(carboxymethyl)valine adducts [112]. The initial reaction forms an aminoalcohol which in turn undergoes a rearrangement producing a stable carboxylic acid adduct (see Figure 4.12).

Carboxylic acid adducts deriving from GO of N-terminal valine in Hb has been identified and quantified in human blood samples by other researchers using the N-alkyl Edman method [112]. A further step with derivatisation of the carboxyl acid group by diazomethane to form the corresponding methyl ester was necessary before the samples were analysed by GC/MS.

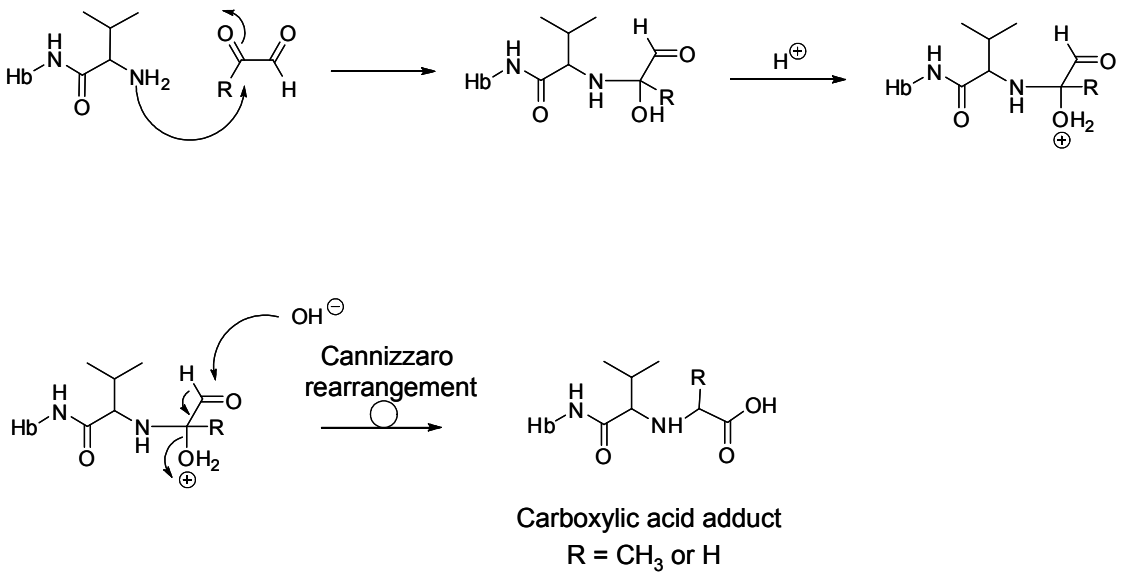


Figure 4.12. Rearrangement of Schiff base adducts of MGO (R= CH_3) and GO (R=H) to stable carboxylic acid adducts.

4.4.2 Experimental/Results

Specific aim

Development of a high throughput analysis method for the quantification of MGO and GO *in vivo* using the adduct FI*R*E procedure.

4.4.2.1 Synthesis

MGO and GO form dioxan dimers when stored at high concentrations and are therefore stored at +4 ˚C as 40 % water solutions. In the synthesis of the Cannizzaro rearrangement products of GO and MGO to valine, 2-bromopropionic acid was reacted with valine to form a carboxylic acid corresponding to MGO (see Figure 4.13). Bromoacetic acid was used to form the corresponding carboxylic acid of GO to valine, 2-carboxymethylvaline, at pH 11 (see Figure 4.13) [113]. 2-Carboxymethylvaline-FTH (CM-Val-FTH) was synthesised by addition of FITC (see MS spectrum in Appendix 2). 2-Carboxyethylvaline-FTH (CE-Val-FTH) was also synthesised in this manner from 2-carboxyethylvaline (see ^{I}H NMR spectrum in Appendix 3).

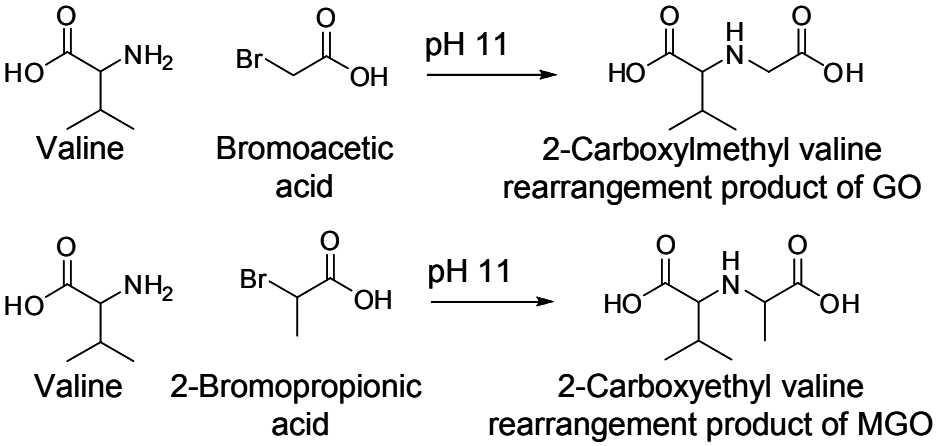


Figure 4.13. Synthesis of GO and MGO Cannizzaro rearrangement products to valine.

For the synthesis of the Schiff base analogues of MGO and GO, valine adducts, *N*-(2-hydroxypropyl)valine and *N*-(2-hydroxyethyl)valine were first synthesised (see Figure 4.9). *N*-(2-Hydroxypropyl)valine was oxidised with Dess-Martin periodinane and after derivatisation with PFPITC formed *N*-(propyl-2-one)valine-PFPTH corresponding to the MGO adduct (see Figure 4.14.). After derivatisation with FITC, the FTH-analyte of *N*-(2-hydroxyethyl)valine corresponds to the GO adduct.

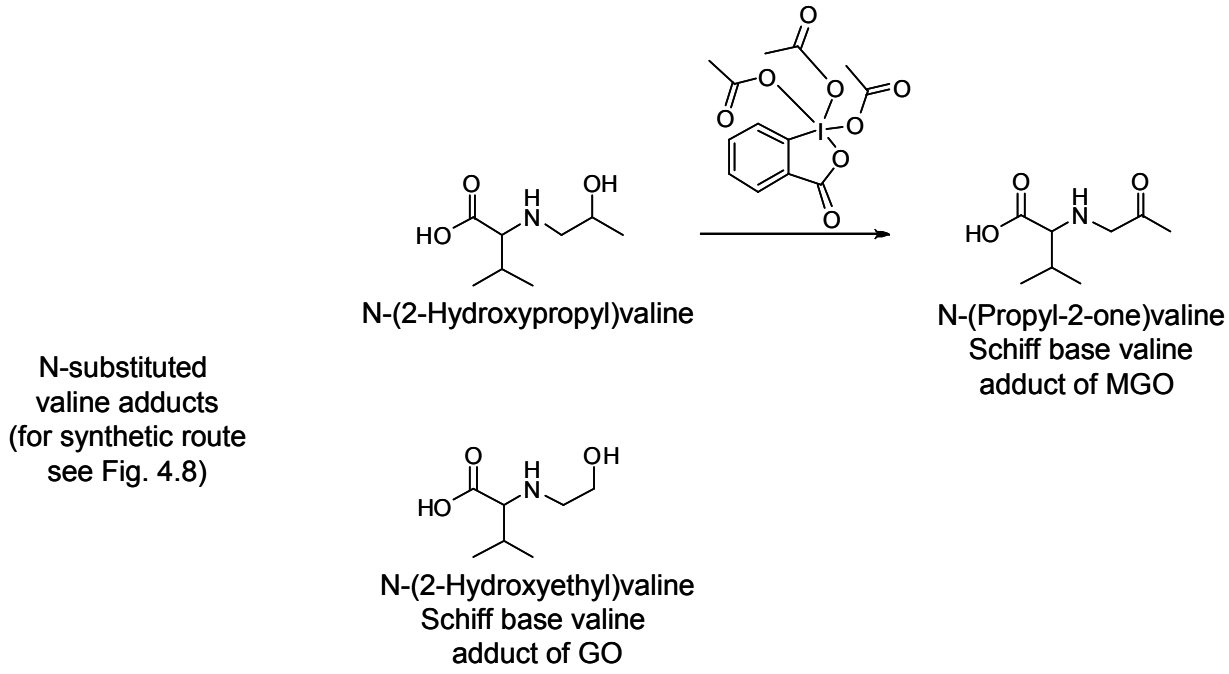


Figure 4.14. Synthesis routes of Schiff base product to GO and MGO with valine.

4.2.2.2 Analysis of GO and MGO using the adduct FIRE procedure

The adduct FI*R*E procedure (see Figure 2.2) was tested for the analysis of Cannizzaro rearrangement product of GO adducts (see Figure 4.12) to N-terminal valine in human blood. Blood was incubated with GO (0.5 M) and the rearrangement product, N-(2-carboxymethyl)valine-FTH (CM-Val-FTH)

was identified after derivatisation. The identification of this adduct was confirmed by a synthesised reference standard. CM-Val-FTH adduct in untreated control blood was then also observed (see spectrum in Appendix 3).

Samples were also prepared by reducing with $NaBH_3CN$ (2 mg/mL hemolysate) after incubation of blood with GO. The CM-Val-FTH level was halved in the reduced samples in parallel with a formation of the expected reduced Schiff base product, 2-hydroxyethylvaline (data not shown) (cf. Figure 4.13 and 4.14).

Samples and reference standards were stored at -20 ˚C for some months before work continued. Freshly worked up samples identified again CM-Val-FTH in control human blood. It was found however that the GO reference standards of valine FTH derivatives had decomposed. Thus the method was deemed unreliable (see Figure 4.15). This could have been overcome by derivatisation, such as for instance methylation, inactivating the free electron pair in the carboxylic acid group. Unfortunately the FITC reagent did not allow for the simplest derivatisation as methylation by diazomethane.

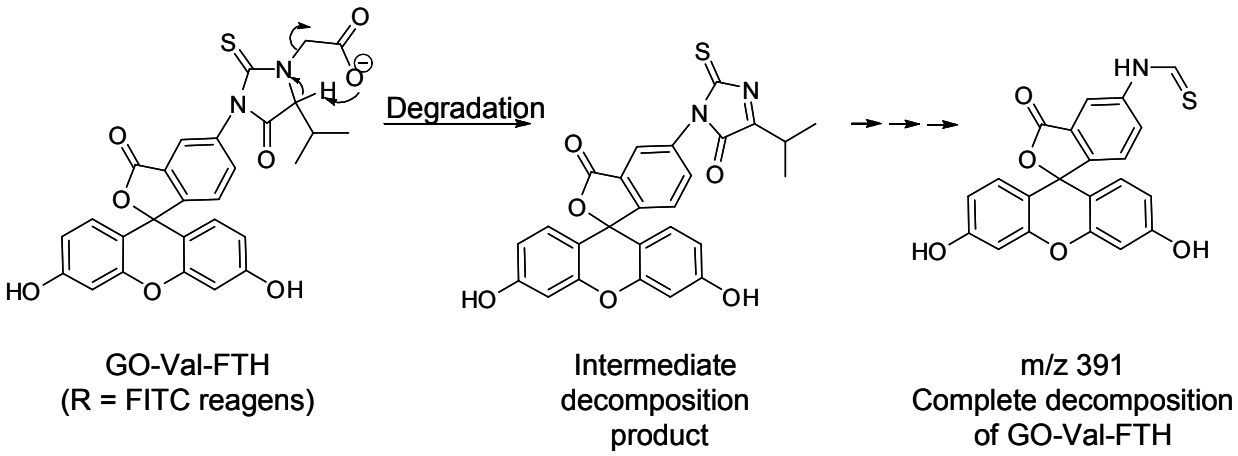


Figure 4.15. Proposed hydrolyse degradation of the reference standards of the CMVal-FTH and MGO.

4.4.3 Conclusions

Reference standards of FTH-analytes of GO and MGO were successfully synthesised, but the reference standards proved non-stable during storage. With the adduct FI*R*E procedure the GO adduct CM-Val-FTH was observed at background levels in human blood. The development of a new analytical procedure using the adduct FI*R*E procedure demonstrated the complexity of protein bound adducts with further reaction of the adduct with the hydantion ring and decomposition. In the analysis of these compounds by the N-alkyl Edman method, a methylation of the carboxylic acid must be performed to make GC/MS analysis possible.

4.5 Furan (unpublished)

4.5.1 Background

Furan is an intermediate in the Maillard reaction and is found in food. Furan also can be found in cigarette smoke and air pollution. The median intake via food in Europe was recently estimated to be 0.78 μg per kg body weight per day [114]. European Food Safety Authority (EFSA) has prioritised investigations of furan due to the high daily intake and carcinogenicity of the compound [114]. *cis*-2-Butene-1,4-dial was identified as a microsomal metabolite of furan [90] (see Figure 4.16). *cis*-2-Butene-1,4-dial cyclises and dehydrates to form stable adducts with nitrogen groups in proteins [112] and has also been detected as an adduct to DNA [115].

4.5.2 Experimental/Results

Specific aim

Clarify whether valine adducts can be formed from the dialdehyde metabolite of furan.

4.5.2.1 Synthesis and analysis

cis-2-Butene-1,4-dial was synthesised by oxidising furan with dimethyldioxirane (DMDO). In initial experiments it was observed that valine ester reacted with the *cis*-2-butene-1,4-dial. The formed cyclised adduct (see Figure 4.16) was characterised with NMR (data not shown). It was observed that the newly synthesised *cis*-2-butene-1,4-dial isomerised readily to its *trans*-analogue, *trans*-2-butene-1,4-dial [116] (see ^{1}H NMR spectrum in Appendix 4). *trans*-2-Butene-1,4-dial is considered to be the most stable of the two isomers.

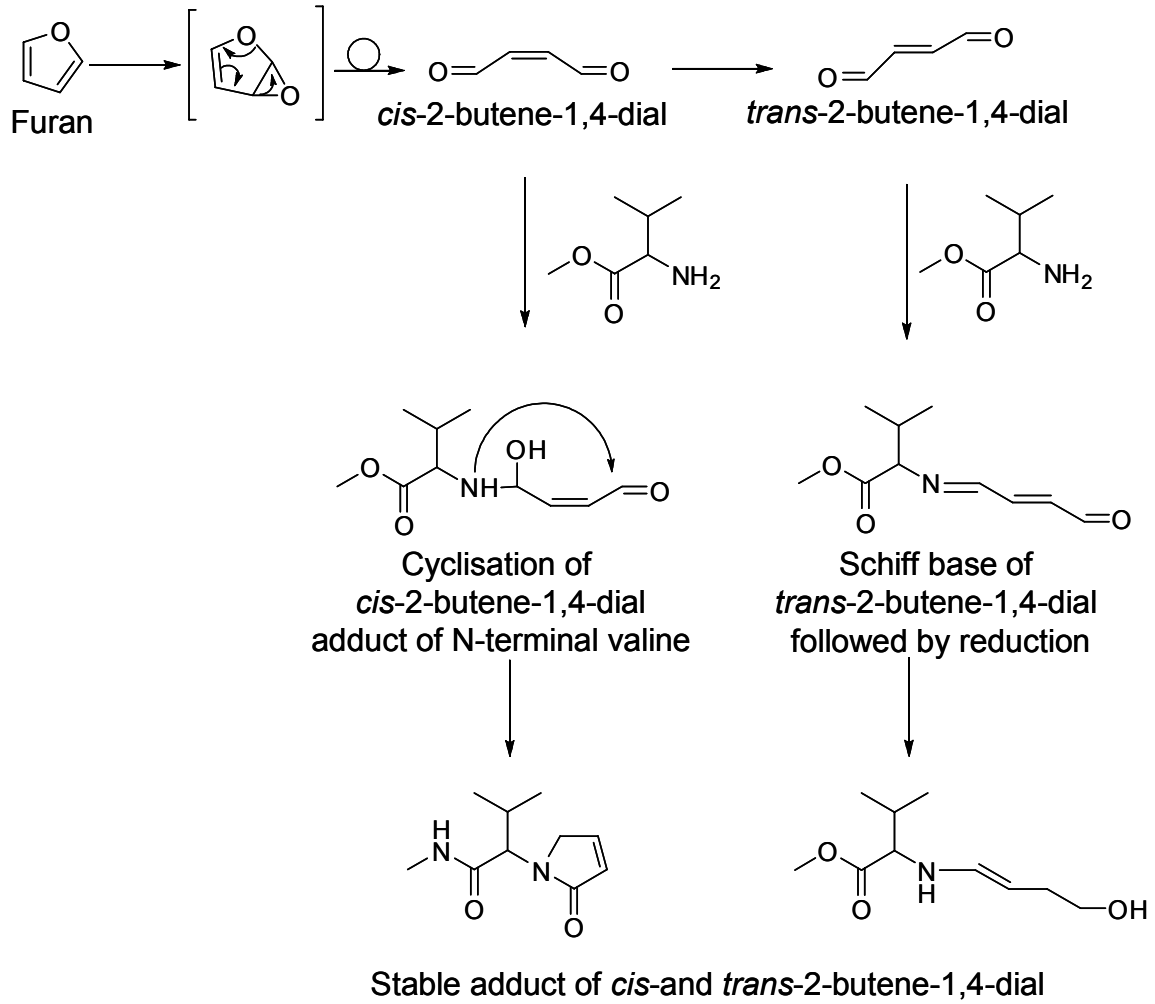


Figure 4.16. Oxidation of furan and reaction with N-terminal valine.

4.5.3 Conclusions

The furan metabolite *cis*-2-butene-1,4-dial was observed to form covalently bound adducts through a cyclisation to a 5-ring compound when reacted to valine ester. The cyclisation, however, blocks free N-terminals in proteins towards derivatisation with Edman reagents. It could be possible to develop a method to analyse *cis*-2-butene-1,4-dial by LC/MS after cleavage of protein by another technique, possibly with trypsin digestion, and enrichment.

The *cis*-2-butene-1,4-dial was observed to readily transform into *trans*-2-butene-1,4-dial *in vitro.* The *trans*-2-butene-1,4-dial however cannot cyclise and with high probability forms Schiff base adducts to N-terminals in proteins. This however depends on the rate of *cis* to *trans* conversion *in vivo*. Furthermore *trans*-2-butene-1,4-dial could form a similar relatively stable enamine as malondialdehyde in N-terminal valine. This biomarker should be a prioritised candidate for future work.

5 Methyl vinyl ketone (Paper III)

5.1 Background

Recently the adduct FI*R*E procedure has been developed for the analysis of adducts to N-terminal valine in Hb as FTH-derivatives by LC/MS/MS (see chapter 2.3) [26, 27]. This method is used for the biomonitoring of ethylene oxide, acrylamide and glycidamide in human blood samples. In initial studies the method has been utilised for detection of unknown adducts. On the basis on known fragmentation patterns of various FTH-analytes an observed unknown peak was hypothesised to be an adduct to N-terminal valine originating from methyl vinyl ketone (MVK).

5.2 Experimental/Results

Specific aims

To synthesise reference compounds for verification of the identity of an unknown valine adduct suspected to originate from MVK observed with the FI*R*E procedure.

5.2.1 Synthesis

5.2.1.1 Unsubstituted reference standard (MVK-Val-FTH)

The synthesis of the starting material, valine methyl amide (VMA), was performed as described by Helleberg *et al.* [117] with minor changes. Helleberg aminolysed the hydrochloride of valine methyl ester with an aqueous solution of methylamine (40 % $MeNH_2$ by weight). Then the solvent was evaporated and the white syrup obtained was suspended in THF where valine, formed by hydrolysis of valine methyl ester, precipitated and could be filtered off. Helleberg then precipitated the obtained VMA by addition of aqueous HCl and purified the VMA-HCl salt by ion-exchanger (cat-ion exchanger) in the H^+ form. In this thesis, this was also tested but the procedure was found to be too time consuming. Instead, an alternative and quicker method was developed where the obtained product formed in the aminolysis step was purified by addition of THF where the desired product was soluble. This solution was left at +4˚C overnight where upon by-products (valine and $MeNH_3{}^+Cl^-$) precipitated and could be filtered off.

Primary amines, such as N-terminal valine in Hb, can react with α, β-unsaturated ketones such as with MVK via a 1,4-Michael addition reaction. For the synthesis of a model compound to N-terminal valine in Hb, VMA was used. VMA was mainly selected as, in contrast to valine, it is soluble also in aprotic solvents such as THF. This fact is sometimes beneficial as hydrolysis

side-reactions can be avoided, which leads to improved yields. Furthermore, the rate of reaction is sometimes also enhanced in aprotic solvents as the primary nitrogen in VMA is more naked, it is not involved in hydrogen bonding with water.

N-(Butyl-3-one)valine-Hb is formed in the reaction between MVK and N-terminal valine via a nucleophilic 1,4-addition reaction (Michael addition). The synthesis of the reference standard, *N*-(butyl-3-one)valine-FTH, started by a reaction between MVK and VMA to form *N*-(butyl-3-one)valine methyl amide (MVK-VMA) (see Figure 5.1). MVK-VMA is then derivatised with FITC to form *N*-(butyl-3-one)valine-FTH (MVK-Val-FTH) (cf. chapter 2.3 and Figure 1 in Paper III).

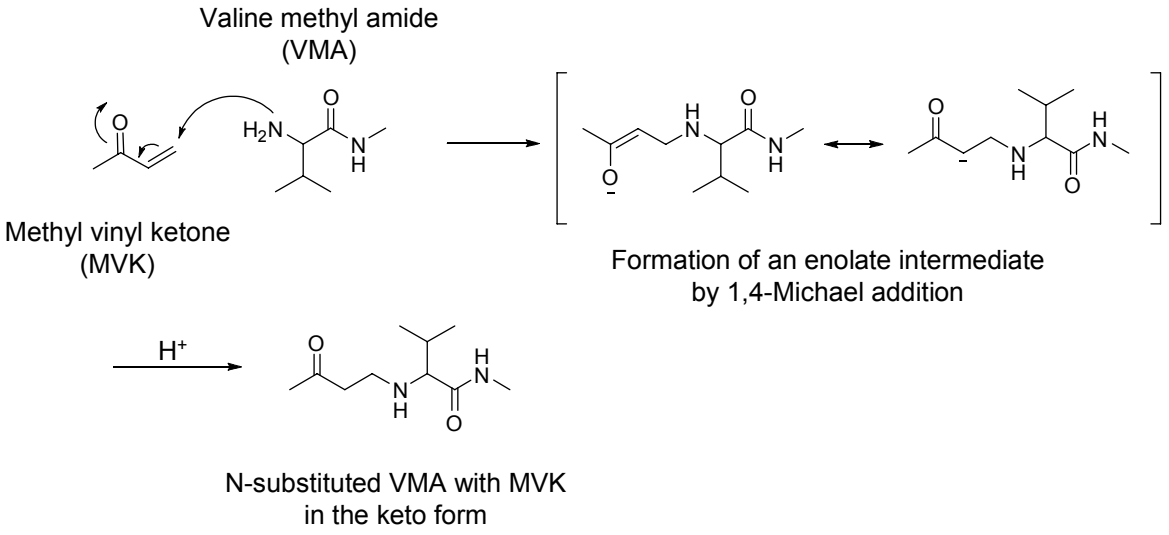


Figure 5.1. Formation of MVK adduct with valine by Michael addition.

5.2.1.2 Deuterium-substituted reference standard MVK-(d_7)-Val-FTH

Deuterium-substituted valine (d_8Val) has been used as a starting material together with different electrophiles to form N-modified-$d_8$valine compounds to be used as standards for the N-alkyl Edman method and the FI*R*E procedure (see chapter 2). These compounds have been used for further synthesis of deuterated internal reference standards through reaction with the isothiocyanate reagents for adducts from acrylamide (data not shown), HMF and FDA to N-terminal valine (see Paper II and Figure 5.2).

The use of dipeptides in the synthesis of deuterium substituted internal reference standards for N-terminals in Hb was developed by Belov [118] and this route was tested for the synthesis of the internal reference standard for the valine adduct of MVK. The formed analyte, MVK-(d_7)Val-FTH, was then used in the adduct FI*R*E procedure as internal standard (see Figure 5.2 and Paper III).

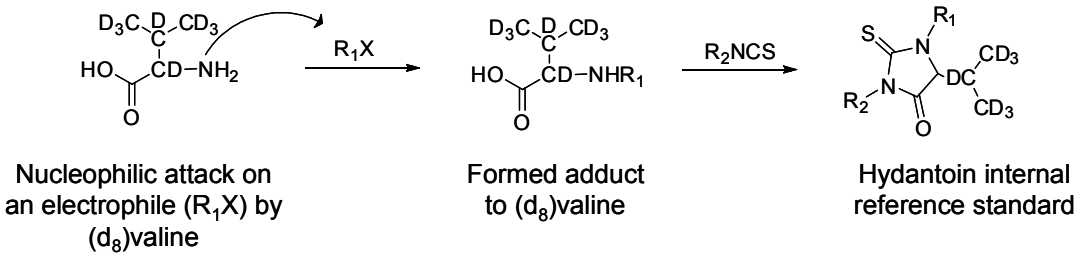


Figure 5.2. Synthesis of deuterium substituted reference standard using valine as starting material.

The synthesis of the dipeptide requires a six step pathway (see Figure 5.3). In the construction of the dipeptide (or longer peptide) initially the amino group is protected by a Boc group (*tert*(but)OCO) whilst the carboxylic group of the amino acid is reacted with another amino acid or another amine compound. The protecting Boc group can be hydrolysed and the formed N-terminal of the compound is free to react with another C-terminal of a protected amino acid.

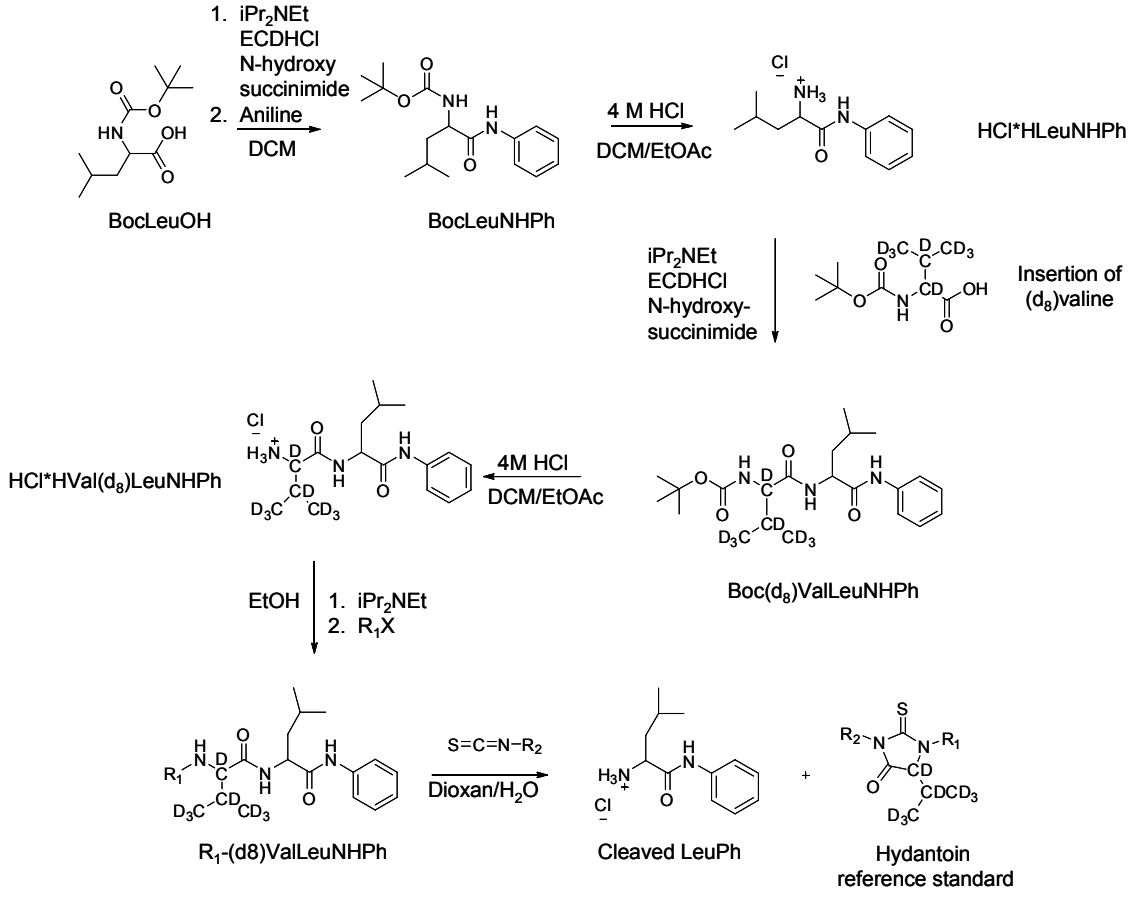


Figure 5.3. Synthesis of deuterium substituted reference standard using a dipeptide.

In the first step in the synthesis of MVK-(d_8)Val-LeuNHPH, aniline is added to the C-termini of BocLeuOH by firstly condensing the carboxylic group of

BocLeuOH by *N*-(3-dimethylamino)propyl-*N′*-ethylcarbodiimide hydrochloride (ECD HCl) followed by the formation of a more stable intermediate using N-hydroxysuccinimide. Aniline is attached forming a stable amide BocLueNHPh. The hydrolysis of the protecting Boc group is acheived by HCl (g) in ethyl acetate by reacting acetylchloride with ethanol in an ethyl acetate solution.

The deuterium-substituted compound, Boc(d_8)Val was prepared from (d_8)Val and DiBoc in a basic medium. The Boc(d_8)Val could then be inserted into HLeuNHPh to form a Boc-dipeptide. After elimination of the Boc-group by hydrolysis, the deuterium substituted dipeptide was reacted with MVK. The formed dipeptide adduct was derivatised by FITC and the formed FTH-derivative was purified by HPLC.

5.2.2 Analysis

The unknown investigated adduct observed in human blood using the adduct FI*R*E procedure was by comparison with the synthesised standards in LC-MS/MS verified to be the MVK-adduct. The adduct level was quantified using the MVK-(d_7)Val-FTH and shown to be about 100 pmol/g globin (see Figure 2 in Paper III).

5.3 Conclusions

The synthesis of d_8ValLeuNHPh (d8-dipeptide) was to be used as a deuterium labelled precursor instead of (d_8)valine and possibly as a precursor for synthesis of various N-modified-d_8-peptide references. By following the same synthetic route as Belov [118] high yields were obtained for all steps with exception for the substitution of the dipeptide with MVK. However, this step was not optimized and if the yields could be increased in the step for N-modification, this dipeptide could be used as a general precursor for synthesis of this type of peptide standards. Such peptides could be added to blood samples as internal standards instead of cyclised N-substituted-d_7-Val-FTHs which would also would undergo coupling and cyclisation.

The identification and quantification of the MVK adduct in human blood was done with the aid of standards synthesised in this work. MVK is a highly toxic compound. There are several anthropogenic and natural sources to MVK in the environment (see references given in Paper III). *In vivo* formation is indicated as well from analysis of urine in different animal species (see refs in Paper III). The data on the occurrence of MVK together with the verification of the identity of the adduct provides evidence that MVK is the origin of the observed adduct *in vivo*.

6 Diepoxybutane (Paper IV)

6.1 Background

1,2:3,4-Diepoxybutane (DEB) is a potent genotoxic metabolite of 1,3-butadiene (BD) and plays an important role in carcinogenesis of BD in animal tests [16, 119]. BD originates from incomplete combustion processes and is used in polymer production. BD has been classified as a human carcinogen by the U.S. Environmental Protection Agency [119] and the International Agency for Research on Cancer [120]

Simple epoxides such as DEB reacts in a nucleophilic substitution reaction with N-terminal valine in Hb. The second epoxy group in DEB can either undergo hydrolysis or further react in an intermolecular cyclisation reaction forming a 5-ring pyrrolidine adduct (see Figure 6.1). The cyclisation of DEB in N-terminal valine to *N,N′*-(2,3-dihydroxybuta-1,4-diyl)valine (pyr-Val) was first observed by Rydberg *et al.* with a model substance [121]. However, the N-alkyl Edman method cannot be used for detachment of the adduct as the tertiary amine structure formed cannot undergo nucleophilic attack on the isothiocyanate. Therefore primary attempts, including acidic conditions by dealkylation were made to convert the tertiary amine to a secondary amine [122].

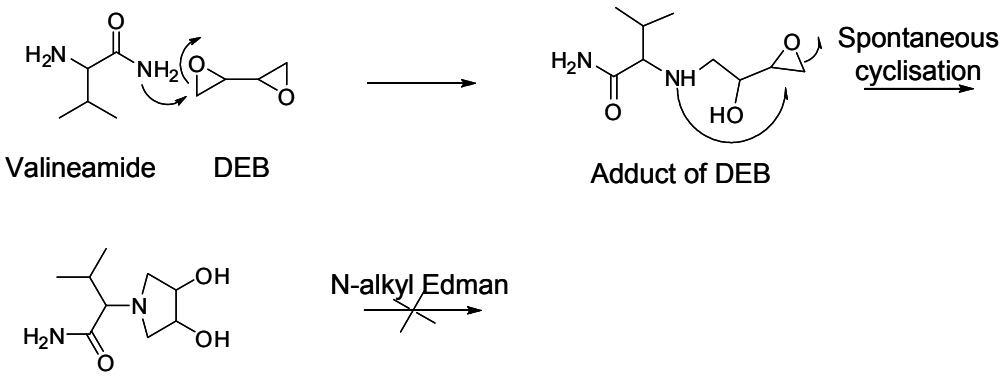


Figure 6.1. Cyclisation of DEB to a stable ring closed adduct (Pyr-Val).

An alternative cleavage and analysis method for the detachment of the tertiary amine structure was developed using tryptic digestion and enrichment of pyr-modified heptapeptides by HPLC/UV and analysis by LC/MS/MS [36]. The sensitivity of the method was considerably improved by immunoaffinity enrichment by Boysen *et al.*, who were capable of detecting levels of DEB-modified heptapeptides in rats and mice exposed to 3 ppm BD [123]. The

sensitivity so far has not been sufficient to detect the very low adduct levels from DEB expected in BD-exposed humans.

We decided to test an alternative method for analysis based on cleavage of proteins by hydrazinolysis and enrichment of pyr-Val adducts by molecularly imprinted solid-phase extraction (MISPE), both as a general test for protein adduct analysis and as a test for this specific problem concerning DEB. MISPE non covalent enrichment method was first introduced by Sellergren [124]. For a review of the latest developments of MISPE, see Tamayo [125]. Hydrazinolysis was first used as a cleavage method of proteins for amino acid isolation by Akabori *et al.* in the 1950s [126]. An approach for the isolation of protein adducts by hydrazinolysis was used by Helleberg in studies of adducts from diolepoxides of PAH to Hb and SA [117]. A nucleophilic attack by hydrazine on the peptide chain produces hydrazide products comprising of different amino acid side chains. The N-terminal valine is cleaved with the adduct and amino acid side chain, the "tag" from the protein, intact.

6.2 Experimental/Results

Specific aims

Synthesis and characterisation of standard compounds to be used for the evaluation of molecularly imprinted solid-phase extraction (MISPE) to enrich hydrazinolysed pyr-Val adducts from DEB.

6.2.1 Synthesis

The synthesis of the DEB reference standards used DEB and valine ester HCl as starting materials in a nuceophilic addition reaction followed by spontaneous cyclisation to the pyr-Val model. Synthesis of the deuterated valine methyl ester used deuterated valine which was esterified with methanol by an acid catalyst, in this case $SOCl_2$, before the nucleophilic addition reaction (see Figure 6.2). The imprint compound pyr-Leu-hydrazide was synthesised by the same route as the pyr-Val-hydrazide compound (see Figure 1 in Paper IV).

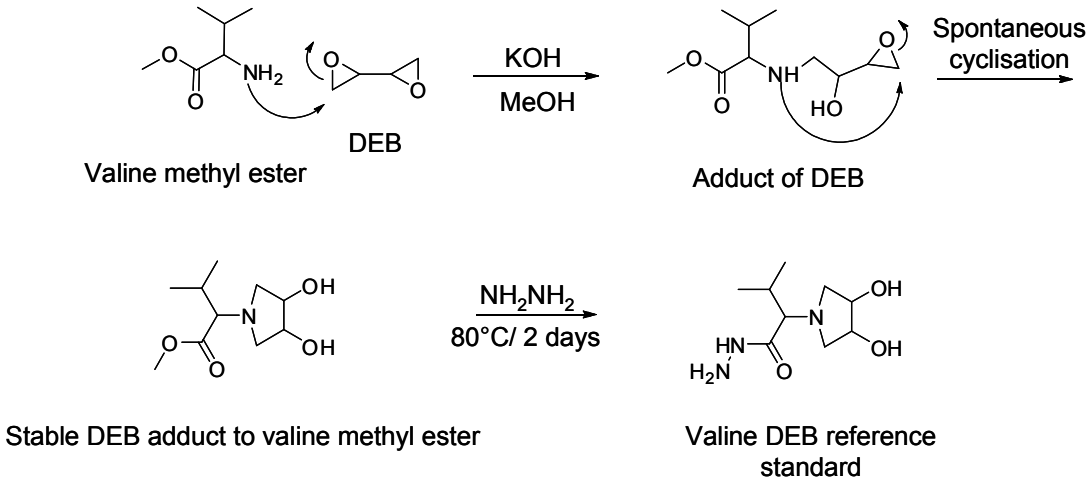


Figure 6.2. Synthesis pathway of DEB analytes.

6.2.2 Analysis

The isolation of pyr-Val-hydrazide from hydrazinolysed Hb on a molecularly imprinted polymer (MIP) requires selected washing steps before the analyte can be eluted with ethanol. The efficiency of the imprinted polymer was compared to a non-imprinted polymer (NIP) by LC/MS (see Figure 2 in Paper IV).

It was found that the total recovery of the pyr-Val-hydrazide analyte from NIP and MIP columns from the loading, washing and elution steps were both similar, about 70-80 %. However, MIP obtained a 60 % recovery in the elution step compared to about 5 % for NIP where 70 % of the pyr-Val-hydrazide analyte was extracted at the loading and washing stages (see Figure 2 in Paper IV).

6.3 Conclusions

The enrichment of the pyr-Val-hydrazide from hydrolysed globin in order to enhance the sensitivity of the analytical method has been achieved by Boysen *et al.* using enrichment by immuno affinity columns. Such technology requires antibodies prepared from animals. Although this method achieved considerable sensitivity, an alternative for the enrichment process could be desired. The solution presented in Paper IV is an approach using a selective sorbent material for enrichment of the analyte from hydrazinolysed globin. The work in Paper IV was an initial test which demonstrated this enrichment procedure was successful for the synthesised reference compound, pyr-Val-hydrazide. The approach could be further developed for the specific enrichment of pyr-Val adduct from DEB for analysis of blood samples. Furthermore the results indicate that by MISPE could be a tool for enrichment of protein adducts in general. MISPE has to our knowledge not earlier been used for protein adducts.

7 General conclusions and future perspectives

Analytical methods are forever pushing the boundaries in respect to isolation, identification and quantification of yet unknown and known harmful compounds in the environment. Identified compounds can be assessed for health risks and if possible excluded from the general environment.

The measurement of N-terminal valine adducts by MS techniques is a specific method for the identification and measurement of electrophiles derived from exposure of endogenous as well as exogenous sources. New synthesised reference compounds and analytical developments in this field create new methodologies for quicker procedures and more accurate measurements.

Electrophilic compounds derived from food include aldehydes which form imines with biomacromolecules. One such compound is HMF, a product of the Maillard reaction, and also its metabolite SMF, and FDA. An improved and less time consuming method for the analysis Schiff base adducts from aldehydes to N-termini was developed. One problem, not yet solved, is that adducts from simple aldehydes (but not HMF) were indicated to be formed as artefacts during the reduction in the work-up procedure. At present, this simple method could at least be used for analysis of complex aldehydes, not being formed as artefacts. The results obtained in this thesis open for further development.

Certain compounds have dual electrophilic functionalities, and a formed adduct would show cross binding properties *in vivo*. The same reactivity would make isolation of an analyte difficult. This was encountered for instance as the observed hydrolysis of the SMF analyte or the reduction of the FDA adduct to the HMF adduct during isolation. The isolation and identification of such compounds are challenging and require modifications of already established sensitive analytical methods, e.g. further derivatization steps. More development has to be done in this field possibly using radioactive isotopic labelling of aldehydes. Another difficulty is the reversibility of the Schiff base, both *in vivo* and *in vitro*.

Another group of studied reactive compounds are isocyanates which could be analysed as adduct to N-terminal valines in Hb. A new synthetic route for the synthesis of hydantoin reference standards of mono- and diisocyanates and their analysis by LC/MS have been presented in chapter 3. A particular success has been the synthesis of hydantoin reference standards from diisocyanates in high yields, previously hampered by low yields due to polymerisation. Furthermore, isolation of carbamoylated urea derivatives of valine methyl amide has the added bonus of measuring the yield of cyclisation

during the (detachment) hydrolysis of the ureas for a more precise quantification. A further development would be the synthesis of internal reference standards using deuterium-substituted VMA, improving quantification in analysis. This versatile synthetic procedure will probably aid in the biomonitoring of diisocyanate exposure also from suspected and less well known sources.

The enrichment of the hydrazinolysed pyrrolidine adduct (pyr-Val-hydrazide) of DEB by MISPE gives a high rate of recovery of the analyte. It is a step forward in regard to using this type of chemical purification technique for enrichment of protein adducts, which has not been used earlier.

The attempt to synthesis a dipeptide-adduct for quantifying purposes of MVK gave insufficient yield in the reaction between the dipeptide and the electrophilic compound. Although a pure deuterium-substituted FTH reference standard was synthesised for MVK quantification, the method needs development to better yields in respect to the overall cost of chemicals. However, the identification and quantification of the stable MVK adduct of N-terminal valine by the FI*R*E adduct procedure is a leap forward in characterisation of the background exposure to electrophilic, and potentially genotoxic and toxic compounds in the general population.

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Appendix 1

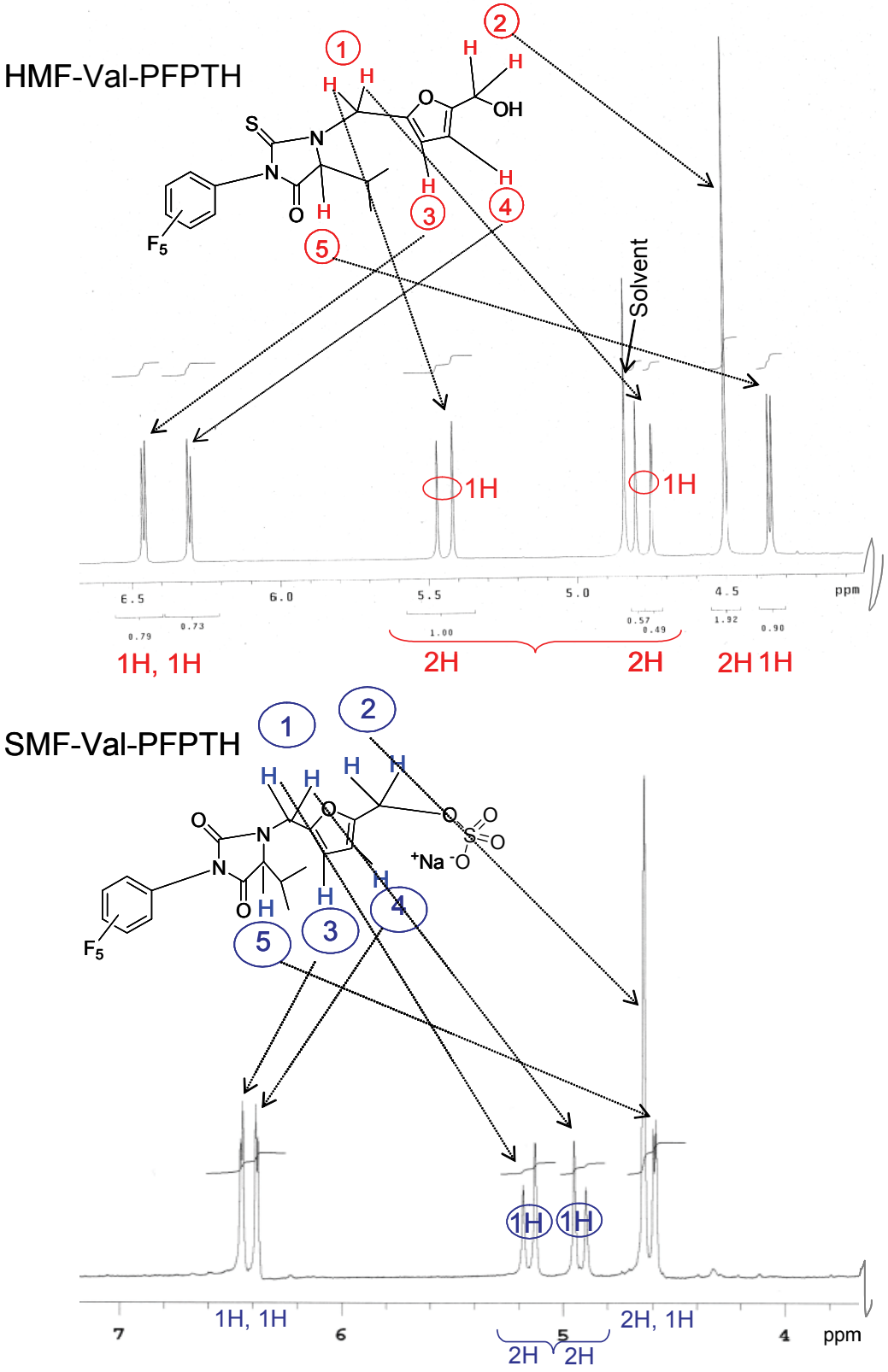

Figure 1. ^{1}H NMR for HMF-Val-PFPTH and SMF-Val-PFPTH.

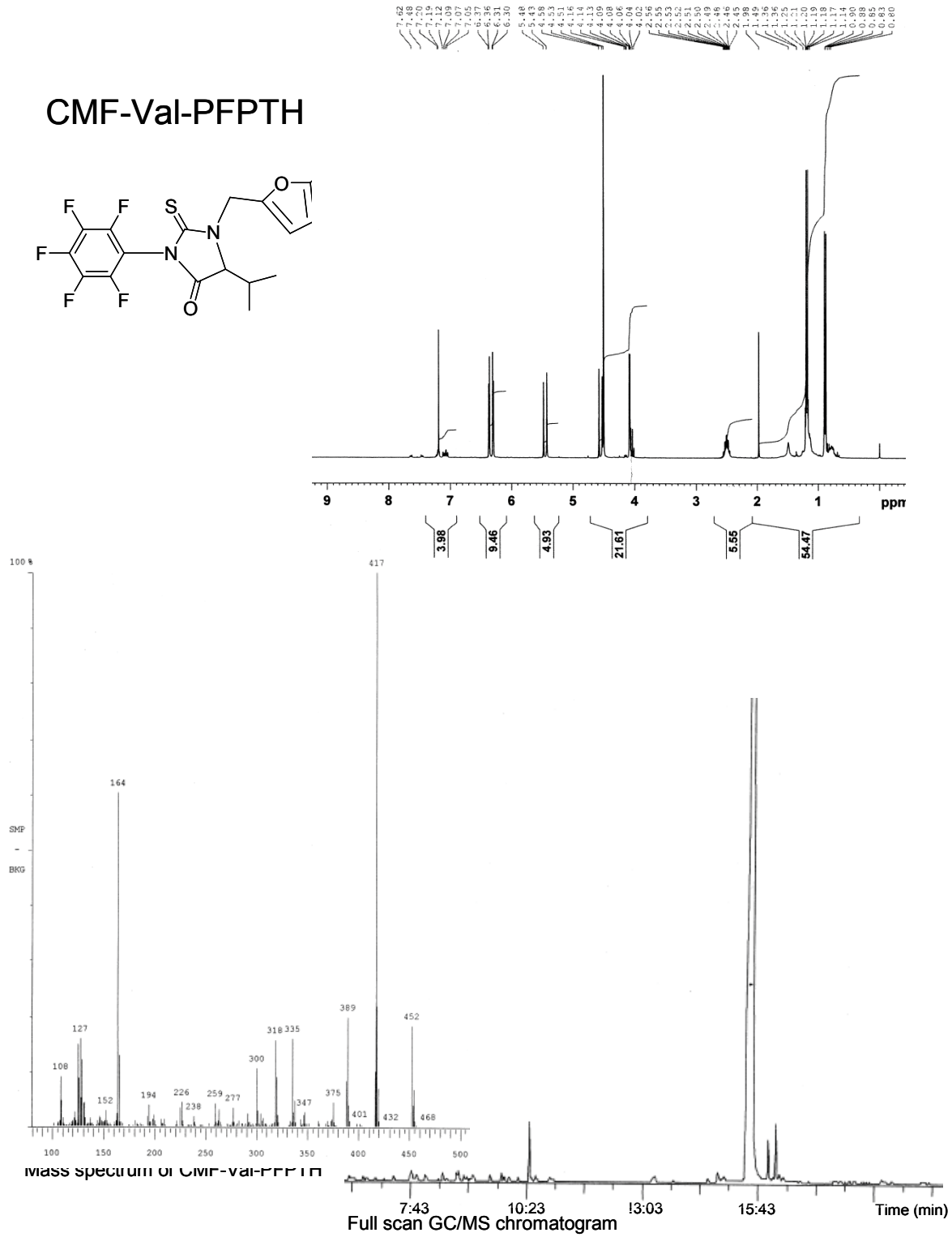

Figure 2. ^{1}H NMR and GC/MS spectrum and chromatogram of CMF-Val-PFPTH.

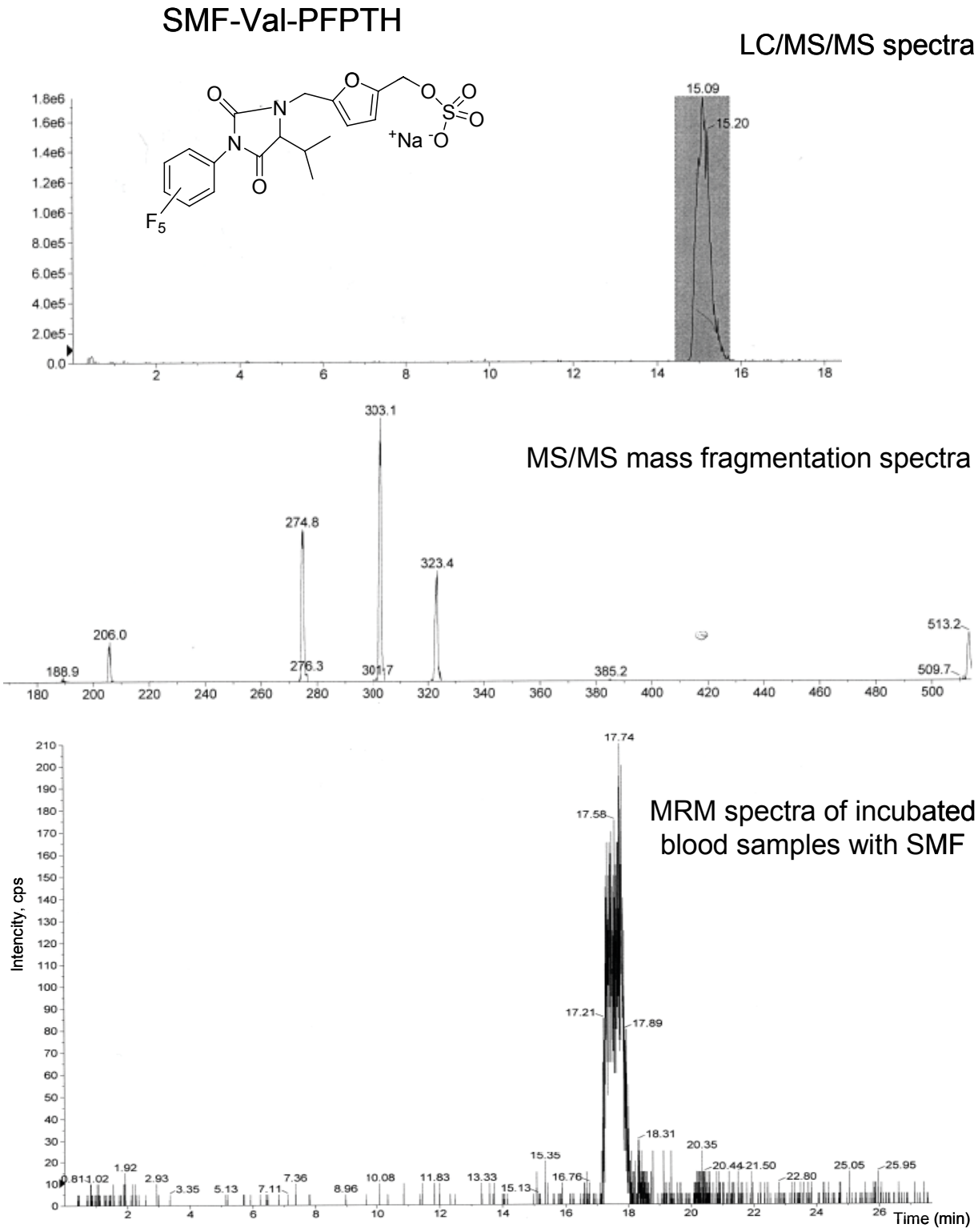

Figure 3. LC/MS/MS chromatogram and mass fragmentation spectra of SMF-Val-PFPTH formed *in vitro* from SMF-incubated whole blood. In the LC/MS/MS (ESI^-) analysis in the MRM mode, a peak [m/z = 513, $(M-1)^-$] corresponding to the SMF-Val-PFPTH standard was observed. This ion was collided in MS/MS mode to give the product ion m/z = 303 $[PFPITC - HF]^-$.

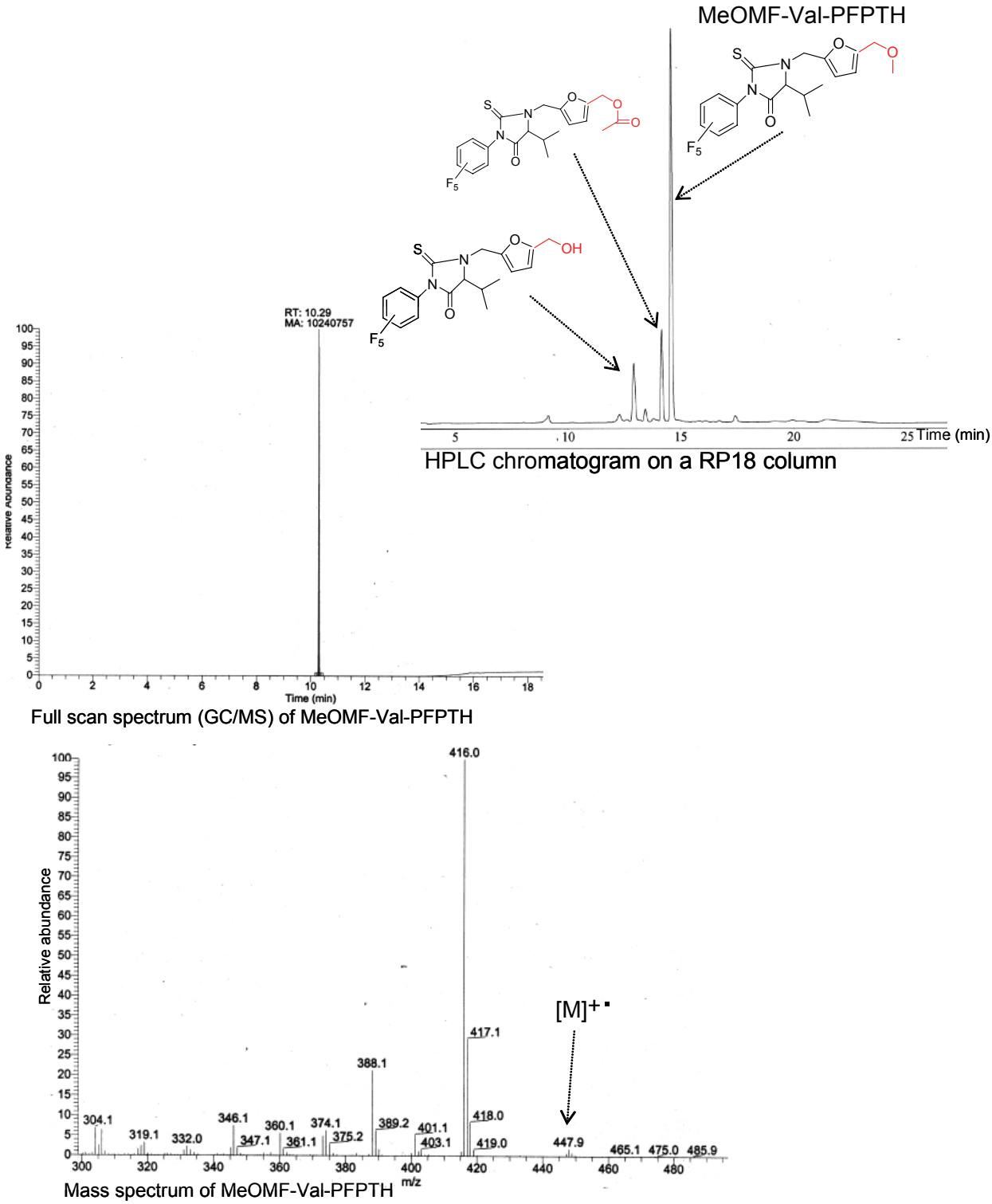

Figure 4. The identity of the MeOMF-Val-PFPTH was determined by GC-MS (EI+) *m/z* = 448 $[M]^+$. This compound was synthesised from CMF-Val-PFPTH reacted in methanol, and identified by ^{1}H NMR. The synthetic MeOMF-Val-PFPTH gave identical data by GC-MS as the substance obtained from the SPE column.

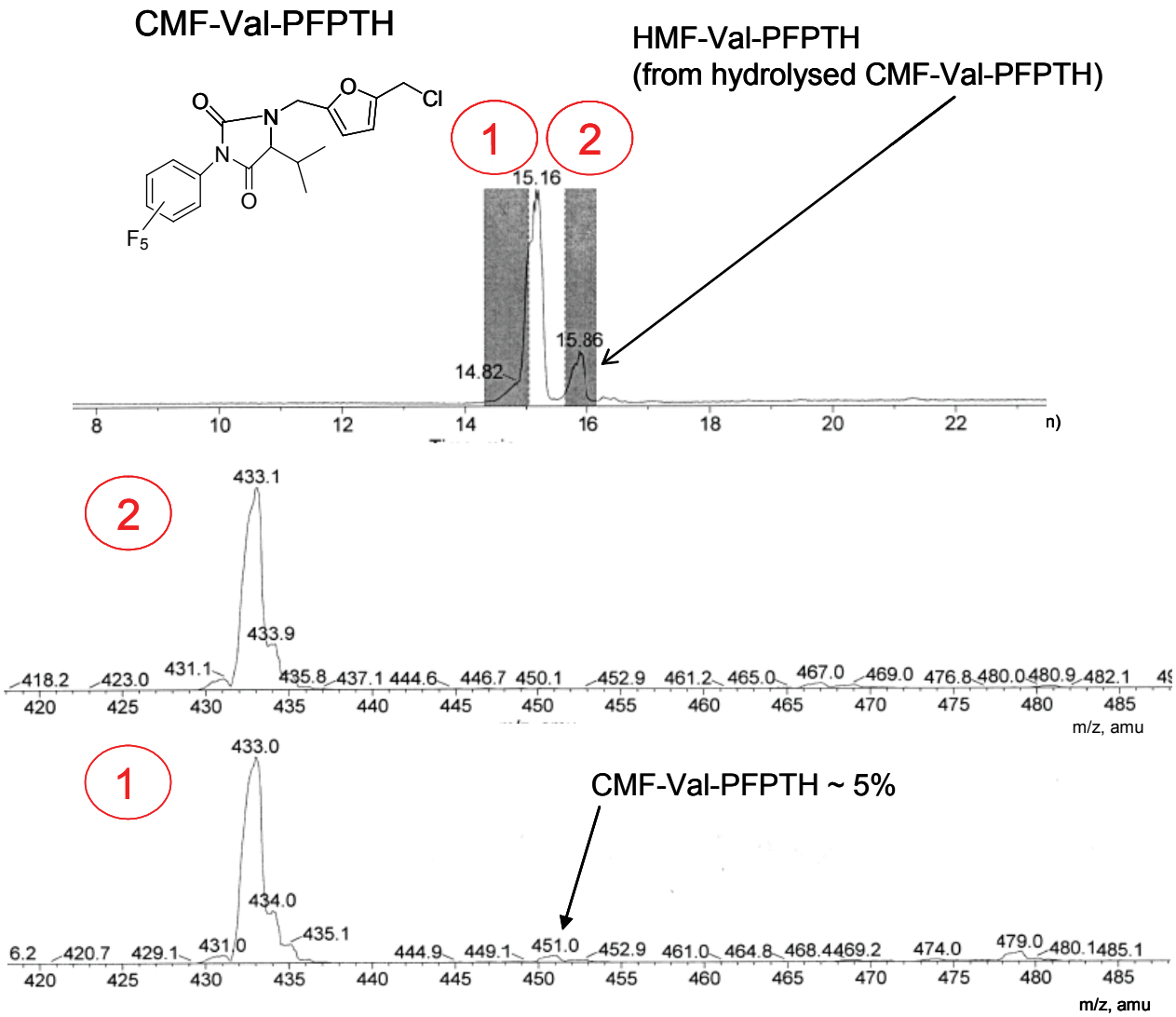

Figure 5. Hydrolysis of CMF-Val-PFPTH during LC/MS/MS analysis. The CMF-Val-PFPTH analyte could be measured by GC/MS (EI+), *m/z* = 452 $[M]^+$.

Appendix 2

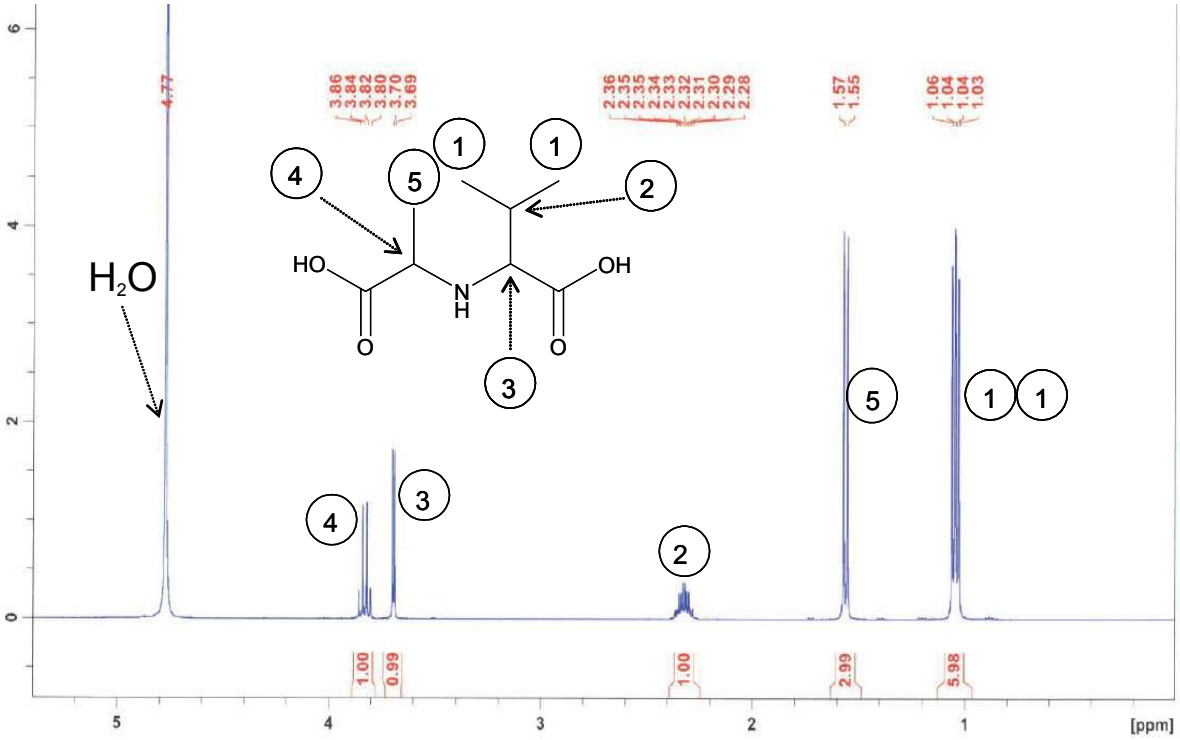

Figure 1. ^{1}H NMR of *N*-(2-carboxyethyl)valine.

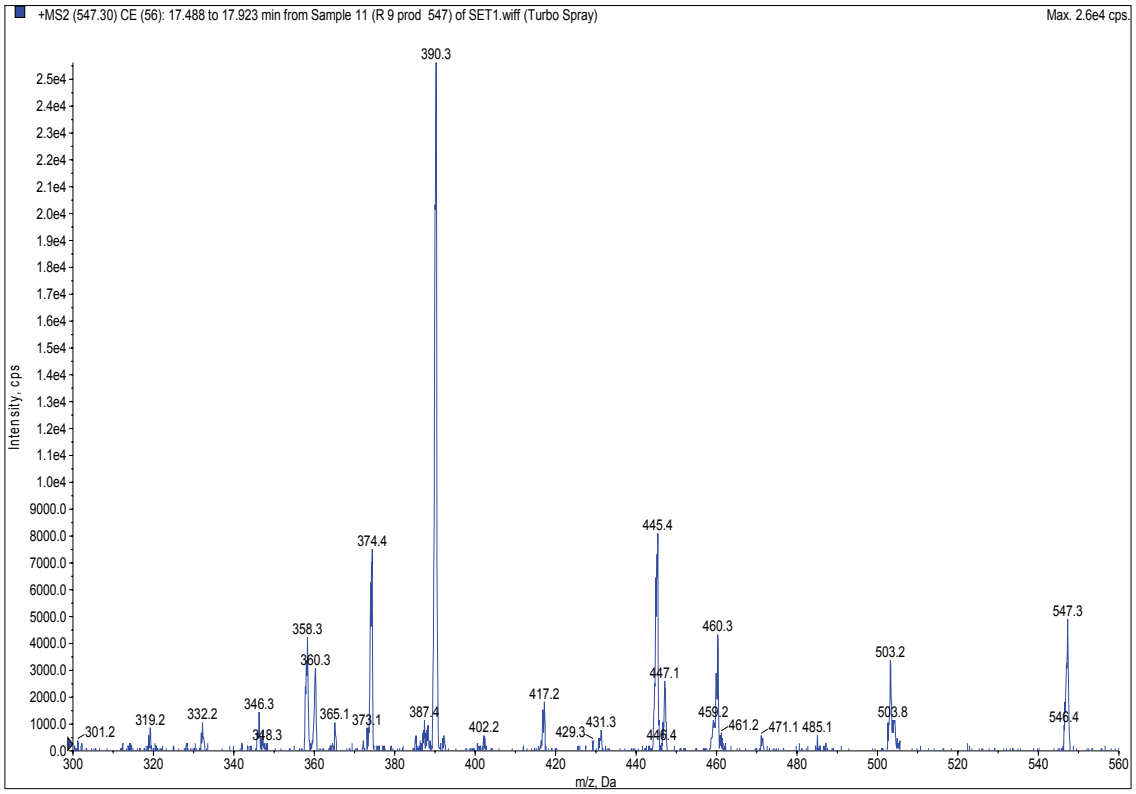

Figure 2. LC/MS/MS spectrum of CM-Val-PFPTH.

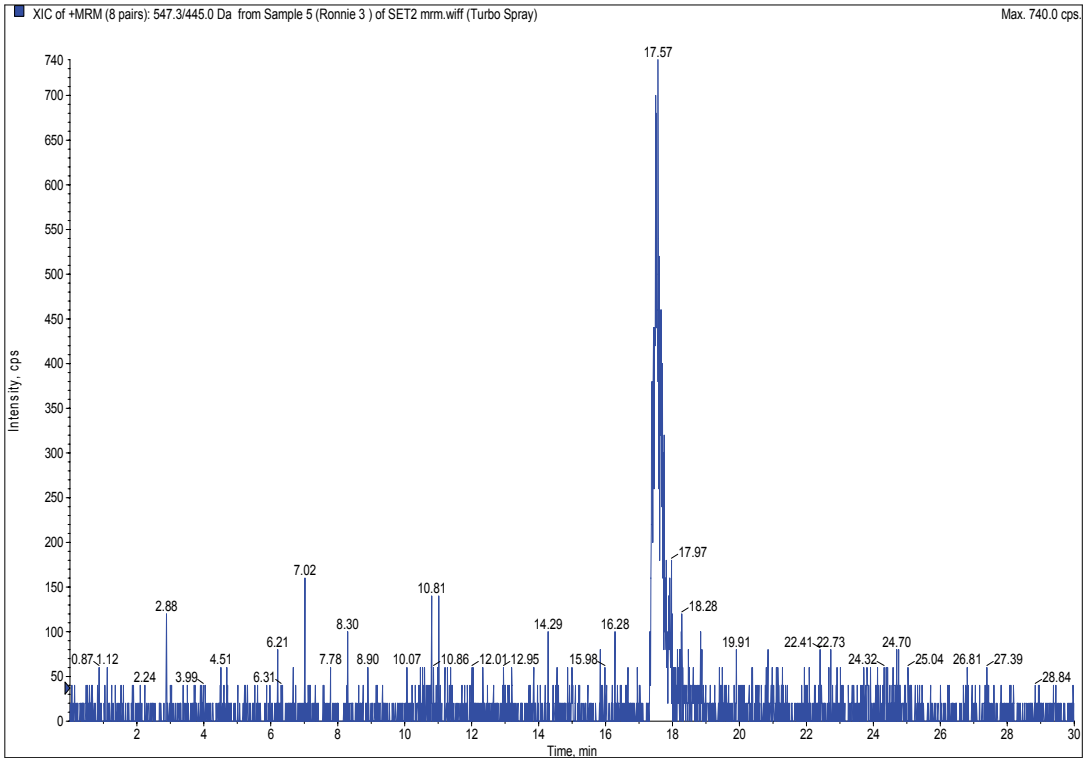

Figure 3. Human blood, untreated with GO nor $NaBH_3CN$. MRM spectrum of 547/445 Da, corresponding to CM-Val-PFPTH.

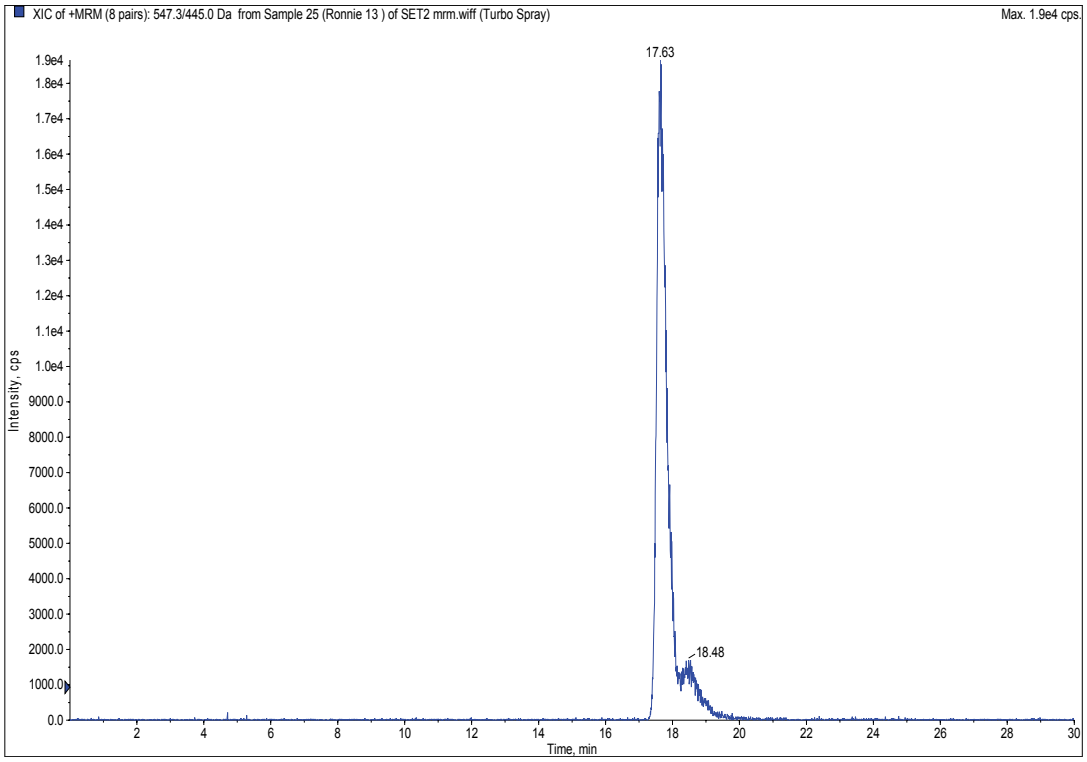

Figure 4. Human blood, treated with GO without reduction with $NaBH_3CN$. MRM spectrum of 547/445 Da, showing a peak corresponding to CM-Val-PFPTH.

Appendix 3

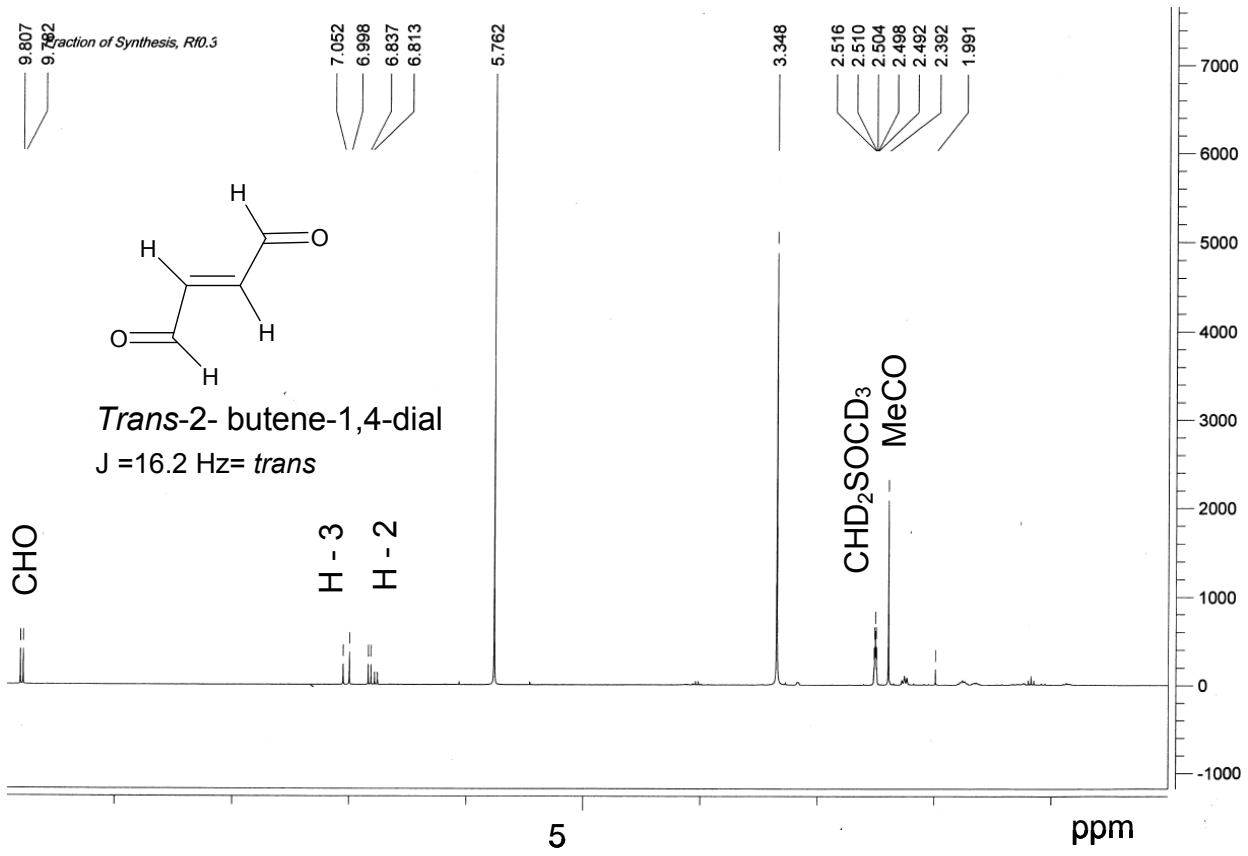

Figure 1. ^{1}H NMR of *trans*-2-butene-1,4-dial.