DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
109

DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
109

SÄDE VIIRLAID

Novel glutathione analogues and their antioxidant activity

TARTU UNIVERSITY PRESS

Institute of Chemistry, Faculty of Science and Technology, University of Tartu, Estonia

Dissertation was accepted for the commencement of the degree of *doctor philosophiae* in Chemistry at the University of Tartu on June 17th, 2011 by the Council of Institute of Chemistry, Faculty of Science and Technology, University of Tartu.

Supervisors: Prof. Ursel Soomets, prof. Jaak Järv, prof. Ülo Langel, University of Tartu, Estonia

Opponent: Armin Sepp (PhD), Targeted Biopharm Discovery, Biopharm R&D, UK

Commencement: Room 1021, Chemicum, 14A Ravila Street, Tartu, on August 30th in 2011, at 11.00

ISSN 1406–0299
ISBN 978–9949–19–789–7 (trükis)
ISBN 978–9949–19–790–3 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr. 436

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

I. **Säde Viirlaid**, Riina Mahlapuu, Kalle Kilk, Aleksei Kuznetsov, Ursel Soomets, Jaak Järv. Mechanism and stoichiometry of 2,2-diphenyl-1-picrylhydrazyl radical scavenging by glutathione and its novel alpha-glutamyl derivative. Bioorganic Chemistry, 2009, 37(4), 126–132.

II. Kersti Ehrlich*****, **Säde Viirlaid***, Riina Mahlapuu, Külliki Saar, Tiiu Kullisaar, Mihkel Zilmer, Ülo Langel, Ursel Soomets. Design, synthesis and properties of novel powerful antioxidants, glutathione analogues. Free Radical Research, 2007, 41 (7), 779–787.

III. Vaher, Merike; **Viirlaid, Säde**; Ehrlich, Kersti; Mahlapuu, Riina; Jarvet, Jüri; Soomets, Ursel; Kaljurand, Mihkel. Characterization of the antioxidative activity of novel nontoxic neuropeptides by using capillary electrophoresis. Electrophoresis, 2006, 13, 2582–2589.

Author's contribution:
***** These 2 authors have equally contributed to the manuscript

Paper I: The author synthesized and purified alpha-glutamyl GSH derivatives, performed DPPH radical scavenging assays and data analysis. The author participated in interpretation and writing of the manuscript.

Paper II: The author participated in design of novel antioxidants and synthesized and purified studied UPF peptides. The author participated in data analysis, interpretation and writing of the manuscript.

Paper III. The author participated in design of novel antioxidants, synthesized and analyzed these compounds, participated in kinetic experiments and in the writing of the manuscript.

ABBREVIATIONS

Acm	acetamidomethyl
AcN	acetonitrile
1-adom	1-adamantyloxymethyl
AE	antiradical efficiency
Boc	*tert*-butyloxycarbonyl
BOP	benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate
2-BrZ	2-bromobenzyloxycarbonyl
Bzl	benzyl
Bum	*tert*-butyloxymethyl
cDo	cyclododecyl
CE	capillary electrophoresis
cHex	cyclohexyl
Che	cyclohex-2-enyl
Chp	cycloheptyl
Chx	cyclohexyl
Coc	cyclooctyl
Cpe	cyclopentyl
DCC	*N,N'*-dicyclohexylcarbodiimide
DCM	dichloromethane
2,6-di-Cl-Bzl	2,6-dichlorobenzyl
DCV	dichlorovinyl
DIEA	*N,N*-diisopropylethylamine
DIPCDI	diisopropylcarbodiimide
DMAP	4-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulphoxide
DMTMM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium
DMT-MM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
Doc	2,4-dimethylpent-3-yloxycarbonyl
DPPH*	α,α-diphenylpicrylhydrazyl radical
EC_{50}	half maximal effective concentration
EDT	1,2-ethanedithiol
EMS	ethylmethylsulphide
Fmoc	9-fluorenylmethyloxycarbonyl
For	formyl
GGT	γ-glutamyl transpeptidase
GOH	γ-glutamylserinyglycine
GR	glutathione reductase
GSH	glutathione
α-GSH	α-L-glutamyl-L-cysteinyl-glycine

$GSHP_x$	glutathione peroxidase
GSSG	glutathione disulphide
GST	glutathione *S*-transferase
HATU	2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	2-(1*H*-7-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HCTU	1*H*-benzotriazolium 1-[*bis*(dimethylamino)methylene]-5-chlorohexafluorophosphate (1-),3-oxide
Hmb	2-hydroxy-4-methoxybenzyl
Hoc	cyclohexyloxycarbonyl
HNTU	2-(5-norbornene-2,3-dicarboximido)-1,1,3,3-tetramethyluronium tetrafluoroborate
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	hydroxybenzotriazole
HODhbt	3-hydroxy-3,4-dihydro-1,2,3-benzotriazin-4-one
HOSu	*N*-hydroxysuccinimide
HPLC	high performance liquid chromatography
K562 cells	erythroleukemia cells
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
MALDI-TOF MS	matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MAP	model amphipathic peptide
MBHA	*p*-methylbenzhydrylamine
4-MeBzl	4-methylbenzyl
MIS	1,2-dimethylindole-3-sulphonyl
Mtr	4-methoxy-2,3,6-trimethylbenzene sulphonyl
Mts	mesitylene-2-sulphonyl
MTX	methotrexate
NAC	*N*-acetylcysteine
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
Nsc	2-(4-nitrophenyl)sulphonylethoxycarbonyl
OxS	oxidative stress
PAM-resin	4-(hydroxymethyl)phenylacetamidomethyl-resin
PBS	phosphate buffered saline
PD	Parkinson's disease
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulphonyl
Pmc	2,2,5,7,8-pentamethylchroman
PyBrOP	bromo-tris(pyrrolidino)phosphonium hexafluorophosphate
PyBOP	benzotriazol-1-yloxy-tris(pyrrolidino)phosphonium hexafluorophosphate
RBCs	red blood cells

Rink Amide MBHA	Rink Amide *p*-methylbenzhydrylamine
ROS	reactive oxygen species
RP-HPLC	reverse-phase high performance liquid chromatography
SDS	sodium dodecyl sulphate
SPPS	solid phase peptide synthesis
t-Boc	*tert*-butyloxycarbonyl
TBTU	2-(1*H*-benzotriazole-1yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
tBu	*tert*-butyl
tButhio	*tert*-butylthio
TFA	trifluoroacetic acid
TFFH	tetramethylfluoroformamidinium hexafluorophosphate
TFMSA	trifluoromethanesulphonic acid
THA	terephthalic acid
TIS	triisopropylsilane
TMP	2,4,6-trimethylpyridine
Tos	*p*-toluenesulphonyl
Trt	triphenylmethyl
UV	ultraviolet

I. INTRODUCTION

The thiol group containing compounds are central actors in many biochemical and pharmacological reactions in living cells and their response to any stress involves changes in cell thiol content. The tripeptide glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) (Figure 1) is the prevalent low-molecular weight thiol compound in eukaryotic cells. It has two characteristic structural features: a γ-glutamyl linkage and a sulphydryl group.

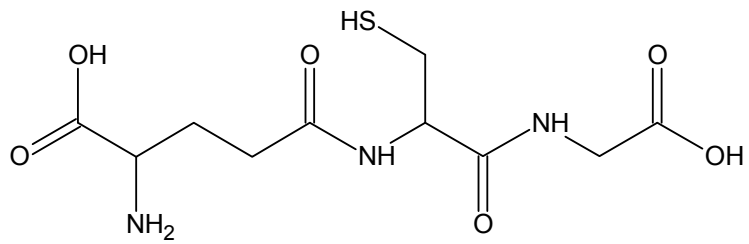


Figure 1. Structure of GSH

GSH is a nucleophile and this property plays critical role in the cellular protection against potentially harmful electrophiles generated by endogenous oxidative processes or formed from xenobiotic sources (Anderson 1998). The glutathione system is very important for the cellular defence against reactive oxygen species (ROS). At high intracellular concentration glutathione protects against variety of different ROS (Dringen 2000). Thiols, including glutathione, have ability to scavenge the superoxide radical anions, nitric oxide radicals, hydroxyl radicals (Saez et al. 1990; Clancy et al. 1994; Winterbourn et al. 1994; Singh et al. 1996), hydrogen peroxide, and also alkoxyl and peroxyl radicals generated from various organic molecules (Chance et al. 1979). GSH is converted into an oxidized disulphide (GSSG) form during elimination of these reactive species. It has been shown, that GSH can protect the cell against oxidative injury (Shan et al. 1990). Therefore glutathione analogues with similar or even better cell-protecting activity might have clinical perspectives and many researchers have explored synthesis and bioactivity of these compounds (Mates et al. 2000; Locigno et al. 2001; Paolicchi et al. 2002).

In this work we have designed and synthesized a new series of tetrapeptidic glutathione analogues called UPF peptides, and antioxidant activity, toxicity and stability of these compounds were studied in *in vitro* assays. These peptides were characterized by ability to scavenge hydroxyl and DPPH radicals and the mechanism of the latter reaction, often used as a non-biological model for evaluation of antioxidant properties, was investigated. The most promising analogues of the UPF series were determined on the basis of the results of *in vivo* experiments, and the structural features of these tetrapeptides, which govern their bioactivity, were discussed.

2. LITERATURE OVERVIEW

2.1. Solid Phase Peptide Synthesis (SPPS)

Peptides and proteins form a class of biomolecules, which are linear polymers of amino acids, linked by peptide bonds. Peptides are generally distinguished from proteins by their length – peptides are shorter polymers of less than approximately 50 amino acids, lacking stable three-dimensional structure in water. Synthetic peptides have been widely used in biochemical and biomedical research and analogues of the natural peptides have been invaluable tools in the study of peptide-receptor interactions.

2.1.1. Peptide synthesis

In 1963, Bruce Merrifield introduced the concept of solid phase peptide synthesis (SPPS) (Merrifield 1963). Merrifield demonstrated that peptides could be assembled by stepwise acylation of the α-amino groups of amino acid residues attached by their C-terminal carboxylic acid to a solid support, consisting of cross-linked polystyrene (resin). The synthesis of compounds on solid phase has subsequently become an extremely popular technique not only for obtaining peptides, but also peptide analogues (Desai et al. 1994; Obrecht and Villalgordo 1998). The basis of the SPPS is that a growing chain composed of monomers is covalently attached *via* one terminus to the insoluble solid resin, so that each next monomer is incorporated only to the free terminus of the chain (Chan and White 2000; Benoiton 2005).

Advantage of SPPS if compared to peptide synthesis in solution is that attachment to the solid support reduces aggregation and there is no need for separation of other products formed in the reactions. However, the main disadvantage of SPPS is that the peptides with errors in their sequence are accumulating during the synthesis.

SPPS is widely used for routine synthesis of peptides and presently the methodology of this process has been tremendously improved. The procedures of activation, coupling, deprotection, final release of product into solution (cleavage) and purification have been subjects of number of studies (Kaminski et al. 2005; Amblard, et al. 2005; Coin et al. 2007; Loidl et al. 2009; Crich et al. 2009; Schneggenburger et al. 2010). More recently, application of microwave radiation to SPPS has been found to reduce reaction time and/or increase the initial purity of synthetic peptide products. In peptide synthesis, microwave irradiation has been used to complete long peptide sequences with high degrees of yield and low degrees of racemization. There is however no clear evidence that microwave is better than simple heating and some peptide laboratories regard microwave just as a convenient method for rapid heating of the peptidyl resin. Heating to above 50-55 ^{o}C also prevents aggregation and accelerates the coupling. Despite the main advantages of microwave irradiation of peptide synthesis, the main disadvantage is the racemization which may occur with the

coupling of cysteine and histidine (Palasek et al. 2007; Sabatino et al. 2008; Loffredo et al. 2009; Pedersen, et al. 2010; Murray et al. 2011; Friligou et al. 2011). SPPS requires large amounts of organic solvents. Peptide synthesis, if performed in water and using less or nontoxic reagents, may circumvent the disposal problem (Montanari et al. 2004; Hojo et al. 2006). Using water as a solvent (coupling and deprotection) for the solid-phase synthesis of peptides using the most common Boc-amino acid derivatives. Key aspects of this methodology are the use of a PEG-based resin, EDC-HONB (*N*-ethyl-*N*'-(3-dimethylaminopropyl)carbodiimide hydrochloride in combination with *N*-hydroxy-5-norbornene-2,3-dicarboxylic acid imide) as a coupling method, and microwave irradiation as an energy source. (Galanis et al. 2009). Nowadays, peptide synthesizers are usually used for routine preparation of standard peptides.

Still two main strategies, usually called Boc-chemistry (Merrifield 1963) and Fmoc-chemistry (Atherton et al. 1978a) are used for peptide synthesis. These methods use different N-terminal protecting groups. In the Boc-method the acid-labile *tert*-bytyloxycarbonyl group (*t*-Boc-group) is used for protection, while in the Fmoc-method the base-labile 9-fluorenylmethoxycarbonyl group (Fmoc-group) is used. The Boc-group is removed by treatment with trifluoroacetic acid, whereas the cleavage of the product is performed by HF. Fmoc-group is removed by weak base, usually piperidine, whereas the cleavage of the product is performed by treatment with trifluoroacetic acid (Greene and Wuts 1999). The reactive functional groups of the amino acid side chains are protected with permanent protecting groups that are not affected under the reactions conditions employed during peptide chain assembly, and which are cleaved out together with the release of the peptide from resin, after the synthesis is completed.

The Fmoc-strategy utilizes milder conditions, but is also more susceptible to side reactions due to involvement of a base (*i.e.,* racemization of chiral amino acids caused by deprotonation of Cα during the Fmoc-deprotection steps) (Benoiton 2005).

Both methods have specific disadvantages, but the major problem in both protocols seems to be connected with difficult couplings, caused by inter-molecular aggregation of growing peptide chains (Kent 1985; Due Larsen et al. 1998; Coin et al. 2007). In most cases coupling efficiency was improved by use of different methods, like reversible amide bond protection (Johnson et al. 1993), *in situ* neutralization coupling protocol (Schnölzer et al. 1992; Yoshizawa-Kumagaye et al. 2005), use of lower degree of resin substitution (Pugh et al. 1992; Moss 2005; Amblard et al. 2006; Deng et al. 2010), more polar solvents (Hyde et al. 1992) as well as adding chaotropic salts to solvents (Stewart and Klis 1990; Thaler et al. 1991).

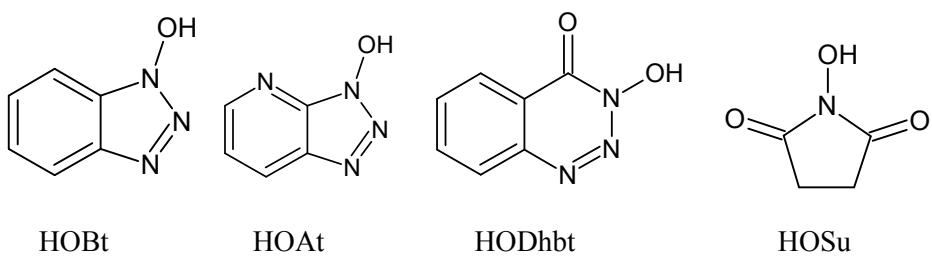
The main disadvantage of SPPS, if compared to solution synthesis of peptides, is that every step of the synthesis has to go to completion, as only in this case the correct peptide sequence is obtained with high yield. The improved synthetic methods include development of chemically well-defined resins,

introduction of better coupling reagents for forming peptide bonds under conditions where racemization is efficiently suppressed, and introduction of new protecting groups to suppress side reactions (Steinauer et al. 1989; Benoiton et al. 1992; Han et al. 1997; Thieriet et al. 2000; Mergler et al. 2001; Angell et al. 2002; Palasek et al. 2007; Deng et al. 2010; Ni et al. 2010).

2.1.2. Activation and coupling of amino acids

Most methods of amide bond formation involve chemical activation of the carboxyl component and is based in most cases on formation of active esters, pre-formed or generated *in situ*. In order to drive the acylation reaction to completion, excess of the activated amino acid derivative is utilized. This excess is typically 2-10 times if compared with the resin functionality. The most important consideration is to maintain as high as possible excess of reagents (Chan and White 2000; Kaminski et al. 2005; Coin et al. 2007; Mitchell 2008).

In order to form a peptide bond, the α-carboxyl group of the amino acid is activated to facilitate nucleophilic attack by the α-amino group of the previously coupled amino acid. Therefore the presence of good leaving group is critical for this reaction. In earlier works, this good leaving group was generated by activation of carboxyl group by *N,N'*-dicyclohexylcarbodiimide (DCC) (Sheenan and Hess 1955). But several unwanted side reactions and formation of by-products accompanied this procedure. To avoid these side reactions, active esters of the protected amino acids with alcohols of low pKa were introduced, and among these alcohols HOBt, HOAt, HODhbt, HOSu, *p*-nitrophenol or pentachlorophenol have central importance (Figure 2) (König 1970a; König 1970b). The active esters formed in these reactions are less reactive and produce much less side reactions if compared with *O*-acylurea (Bodanszky 1984).

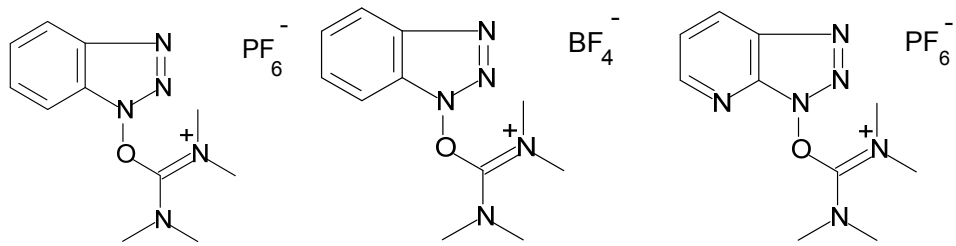


HOBt HOAt HODhbt HOSu

Figure 2. Some common alcohols used for the formation of active esters.

Presently a new class of coupling reagents, based on uronium and phosphonium salts, has become increasingly popular. The use of these reagents has been reported to be more convenient and superior to the use of DCC-mediated couplings (Hudson 1988). Some of the most commonly used coupling reagents are BOP (Castro et al. 1975), PyBOP (Coste et al. 1990), TBTU and HBTU

(Knorr et al. 1989), which generate HOBt esters; HATU (Carpino 1993), which generates HOAt esters (Figure 3); TFFH (Carpino and El-Faham 1995), which generates acyl fluorides; and PyBrOP (Castro et al. 1990), which generates acyl bromides. For fast Fmoc solid-phase peptide synthesis efficient coupling reagent 1*H*-Benzotriazolium 1-[*bis*(dimethyl-amino)methylene]-5-chloro-hexa-fluorophosphate (1-),3-oxide (HCTU) can be used (Hood et al. 2008). To perform SPPS in water, the coupling reagent must be water-soluble and maintain its reactivity in water. For this the efficacy of the water-soluble coupling reagents, 2-(5-norbornene-2,3-dicarboximido)-1,1,3,3-tetramethyl-uronium tetrafluoroborate (TNTU) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) was tested (Hojo et al. 2006). A new generation of triazine-based coupling reagents, designed according to the concept of "superactive esters", was obtained by treatment of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium (DMTMM) chloride with lithium or silver tetrafluoroborate (Kaminski et al. 2005).



HBTU TBTU HATU

Figure 3. Most common coupling reagents used in peptide synthesis.

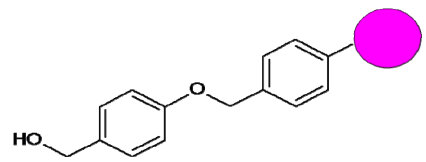
2.1.3. Protecting strategies in SPPS

One of the demanding parts in peptide synthesis is the necessity to block those functional groups that must not participate in the peptide bond formation. In peptide synthesis the α-amino groups are protected with temporary protecting groups, which are cleaved after each coupling reaction, and the functional groups of amino acid side chains are protected with permanent protecting groups, which are cleaved after the synthesis is completed. The solid phase strategy described in the first papers by Merrifield is still widely used, but it has undergone several methodological improvements. The Boc-group is cleaved by acid, e.g. TFA in DCM or by other strong acids, e.g. HBr in TFA (Merrifield 1964), liquid HF (Sakakibara and Shimonishi 1965, Lenard and Robinson 1967) or TFMSA (Yajima et al. 1974).

Sheppard and co-workers (Atherton et al. 1978a; Atherton et al. 1978b) developed other strategy based on the 9-fluorenylmethyloxycarbonyl (Fmoc)

group as N^{α}-protecting group. The Fmoc group is stable to acid, but is cleaved by base, e.g. piperidine in DMF. Most of functional groups of side chains are protected with *tert*-butyl groups, which are stable to base, are cleaved by moderately strong acid, e.g. TFA. The peptide is linked to a TFA-stable resin, such as p-benzyloxybenzyl alcohol (Wang/HMP) resin (Figure 4) (Wang 1973).

A.



B.

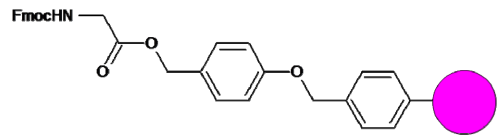


Figure 4. A. Wang resin and B. Fmoc-Gly Wang resin used in Fmoc chemistry

The chloromethyl resin (Merrifield 1963) is still used although the benzyl ester linkage is more acid-labile than it would be ideal for the use in Boc protocol. The 4-(hydroxymethyl)phenylacetamidomethyl (PAM) resin (Mitchell et al. 1976) is a more acid-stabile resin. For the synthesis of C-terminally amidated peptides the 4-methylbenzhydrylamine (MBHA) resin (Matsueda and Stewart 1981)) is commonly used (Figure 5).

A. B.

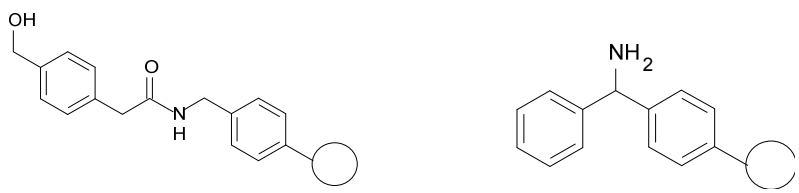


Figure 5. A. PAM resin and B. MBHA resin used in Boc chemistry

Presently both the Boc/benzyl-base strategy and the Fmoc/*tert*-butyl-based strategy are widely used.

In the synthesis of complex peptides it is sometimes desirable to cleave selectively one protective group in the presence of other protected functional

groups. Ideally, the remaining protection should be inert to the reaction conditions used for cleavage of the orthogonal protecting group. One of the most commonly used types of orthogonal protecting groups and linkers are allyl-based structures, which are cleaved by palladium catalysis. Allyl protection for most amino acid side chains (Loffet and Zhang 1993) as well as allyl-based linkers and resins (Kunz and Dombo 1988; Guibe et al. 1989; Kaljuste et al. 1996) are available. The allyl-based structures are reported to be stable under conditions, used in both Boc and Fmoc protocols and are cleaved under mild conditions. In the synthesis of peptides, which contain disulfide bonds, the selective cleavage of orthogonal protecting groups is often a prerequisite for achieving the correct pairing of disulfides, and for this purpose several different protecting groups for cysteine have been developed (Andreu et al., 1994; Barlos et al. 1996; Ni et al. 2010).

2.1.4. Protecting groups

The selection of protecting groups for trifunctional amino acids in peptide synthesis is an important issue since a number of side reactions are highly dependent on how the side chain is protected. Most protecting groups used in Boc chemistry are based on the benzyl group and most protecting groups used in Fmoc chemistry are based on the *tert*-butyl group.

2-(4-Nitrophenyl)sulphonylethoxycarbonyl (Nsc) is an alternative base-labile N(alpha)-protecting group to 9-fluorenylmethoxycarbonyl (Fmoc) for amino acids (Ramage et al. 1999; Balse et al. 2000; Carreno et al. 2000). A new strategy for SPPS in the reverse direction based on the use of 2-Cl-trityl resin, an allyl ester as the temporary protecting group, and $Cu(OBt)_2$/DIPCDI or HATU/DIEA as the coupling method is used (Thierirt 2000).

Aspartic and glutamic acid

In Boc chemistry aspartic acid was protected as a β-benzyl (Bzl) ester. To prevent acid- and base-catalyzed aspartimide formation, β-cyclohexyl (cHex) ester of aspartic acid is used (Tam et al. 1979). These compounds are sterically hindered if compared to benzyl group and markedly decrease aspartimide formation (Tam et al. 1979; Tam et al. 1988; Nicolas et al. 1989). Presently the β-cyclopentyl ester (Cpe) (Blake 1979), the β-cycloheptyl ester (Chp) (Yajima et al. 1985; Fujii et al. 1986), the β-cyclooctyl ester (Coc) (Fujii et al. 1986) and β-cyclododecyl ester (cDo) (Kawasaki et al. 1994) of aspartic acid are in use.

In Fmoc chemistry, the β-*tert*-butyl ester was usually used for protection of aspartic acid (Chang et al. 1980), and this was in most cases efficient for prevention of the base-catalyzed aspartimide formation (Schön et al. 1979; Tam et al. 1988). However, the base-catalyzed aspartimide formation has been reported to be the major side reaction in the synthesis of certain sensitive sequences with Fmoc chemistry, if *tert*-butyl group has been used (Dölling et al. 1994; Lauer et al. 1994; Yang et al. 1994). In some cases a new base-labile

N(alpha)-protecting group 2-(4-nitrophenyl)sulphonylethoxycarbonyl (Nsc) was introduced for Fmoc amino acids Ramage et al. 1999; Balse et al. 2000; Carreno et al. 2000). To solve aspartimide problem some Fmoc-Asp-OH derivatives were made: the beta-(4-pyridyl-diphenylmethyl) and beta-(9-phenyl-fluoren-9-yl) esters and also the orhoester Fmoc-beta-(4-methyl-2,6,7-trioxabicyclo[2.2.2]-oct-1-yl)-alanine. 3-Methylpent-3-yl protection of the Asp side chain resulted in significant improvements with respect to aspartimide formation. Complete suppression was achieved using the combination OtBu side chain protection and Hmb backbone protection for preceding Gly residue (Mergler et al. 2003; Mergler et al. 2005).

Arginine

The trifunctional guanidine side group (three nucleophilic nitrogens: N^{δ}, N^{ω} and $N^{\omega'}$) is easily acetylated if it is not protected during SPPS. Usually not all three but only N^{ω} is protected. Most often used protective groups are the nitro, urethane (acyl), aryl sulphonyl and trityl group. In Boc chemistry arginine is usually protected with several arylsulphonyl groups – tosyl (Tos), 2,4,6-trimethylbenzenesulphonyl (Mts) and 4-methoxy-2,3,6-trimethylbenzene sulphonyl (Mtr) (Yajiama et al. 1978; Ramachandran et al. 1962; Ramage et al. 1987; Carpino et al. 1993). These protecting groups were efficient in synthesis of arginine-containing peptides analogues efficiently introduced of protected guanidines. Further, 1,2-dimethylindole-3-sulphonyl group (MIS) is more acid-labile than Pmc and Pbf and can therefore be a better option for Arg side chain protection in Arg-rich sequences, acid-sensitive peptides and large-scale syntheses (Isidro et al. 2009). In Fmoc chemistry the same groups are used for protection of Arg side chain.

Cysteine

The synthesis of cysteine containing peptides is complicated because some products require that the Cys-residue is involved in the intramolecular disulfide bond, while in some cases the free sulphydryl group is important in the structure of peptides. Among the protecting groups used for cysteine coupling, 4-methylbenzyl (4-MeBzl) group (Erikson et al. 1973) and the trityl (Trt) group (Amiard et al. 1956) are cleaved by acid, whereas the acetamidomethyl (Acm) group (Veber et al. 1968) is cleaved by I_2, Hg^{2+} or Tl^{3+} and the *tert*-butylthio (tButhio) group (Atherton et al. 1985) is cleaved by Bu_3P or thiols. In addition to these groups, a wide selection of protecting groups for cysteine is available, which can be selectively deprotected to allow the control of formation of disulfide bridges (Andreu et al. 1994). To reduce racemization of cysteine containing peptides the following methods were recommended: O-penta-fluorophenyl (O-Pfp) ester in DMF; O-Pfp ester/1-hydroxybenzotriazole (HOBt) in DMF; N,N'-diisopropylcarbodiimide (DIPCDI)/HOBt in DMF; HBTU/HOBt/2,4,6-trimethylpyridine (TMP) in DMF (preactivation time 3.5-7.0 min in all of these cases); and HBTU/HOBt/TMP in CH_2Cl_2/DMF (1:1) with no preactivation (Han et al. 1997; Angell et al. 2002).

Histidine

In Boc synthesis the imidazole side chain of histidine has been left unprotected (Loffet 1967; Gutte et al. 1971) but it leads to racemization of the residue (Jones et al. 1980) and a number of other potential side reactions may occur e.g. cyclization of the activated histidine to a lactam (Sheehan et al. 1959).

In Fmoc chemistry the side chain of histidine has been protected with the N^{τ}-*tert*-butyloxycarbonyl (Boc) group and the N^{τ}-9-fluorenylmethyloxycarbonyl (Fmoc) group (Atherton et al. 1985), which are both electron-withdrawing urethane-protecting groups. Both of these prevent racemization but they are not sufficiently stable to piperidine. The N^{τ}-trityl (Trt) group introduced as protection for histidine in Fmoc chemistry (Sieber et al. 1987) is stable to piperidine but can result in unacceptably high levels of racemization of histidine during activation (Harding et al. 1995). The N^{π}-*tert*-butyloxymethyl (Bum) group (Colombo et al. 1984) and the N^{π}-1-adamantyloxymethyl (1-adom) group (Okada et al. 1996a; Okada et al. 1996b) have been introduced as N^{π}-protecting groups for use in Fmoc chemistry. To minimize racemization of histidine residues during coupling and esterification reactions applicated Fmoc-His(3-Bum)-OH (Mergler et al. 2001).

Serine and threonine

In Boc chemistry serine and threonine may be used without protective groups on the side chain. If needed, the most common protective group for Ser and Thr is benzyl group. In Fmoc chemistry O-*tert*-butyl ethers of serine and threonine are also used for protection (Chan and White 2000).

Tryptophan

Due to sensitivity of the indole nucleus against oxidation and alkylation under acidic conditions, the side chain of tryptophan is usually protected in Boc chemistry (Fontana and Toniolo 1976; Geiger and König 1981). The most commonly used protecting group is the N^{in}-formyl group (For) (Yamashiro et al. 1973; Ohno et al. 1973) but it is stable to acid and has to be removed by nucleophiles in separate deprotection step (Barany and Merrifield 1979). Recently the N^{in}-cyclohexyloxycarbonyl (Hoc) group has been introduced for tryptophan for use in Boc chemistry (Nishiuchi et al. 1996), and this protecting group is cleaved by HF.

In Fmoc chemistry unprotected tryptophan has been usually used. But under the acidic conditions used for the final deprotection of the peptides, the indole nucleus may be alkylated or re-attached to the resin. The N^{in}-*tert*-butyloxycarbonyl (Boc) group has found to be useful as protecting group for tryptophan in Fmoc chemistry (Grehn et al. 1984; Franzen et al. 1984; White 1994) as it prevents modifications of the indole ring during the synthesis and the final deprotection of the peptide.

The application of the following Nin-protecting groups, mesitylene-2-sulphonyl (Mts), cyclohexyloxycarbonyl (Hoc), and 2,4-dimethylpent-3-

yloxycarbonyl (Doc) for an efficient synthesis of the Trp-containing cystide peptide by the silyl chloride method was examined. In order to find a feasible scheme of the successive treatment with CH_3SiCl_3-PhS(O)Ph/TFA and TFMSA-thianisole in TFA, somatostatin was synthesized using Trp(Mts), Trp(Hoc) or Trp(Doc) derivatives (Fujiwara 2000).

Tyrosine

The phenolic side chain of tyrosine has been left unprotected in the Boc chemistry, but since the phenolate ion formed under basic conditions is a potent nucleophile, it is acylated during coupling (Ramachandran and Li 1963). The first protecting group introduced for side chain of tyrosine was the benzyl ether (Bzl) (Marshall et al. 1965). Several protecting groups have been suggested for protection of the hydroxyl group of tyrosine in Boc chemistry (Stewart 1981). Now the most commonly used protecting group for tyrosine in Boc chemistry are the 2,6-dichlorobenzyl (2,6-di-Cl-Bzl**)** (Erikson 1973) and the 2-bromo-benzyloxycarbonyl group (2-BrZ) (Yamashiro et al. 1973). These protecting groups, however, are not entirely satisfactory because of two side reactions, *i.e*., migration of the *O*-protecting group to the 3-position of the benzene ring of Tyr, and partial removal of the *O*-protecting group under basic conditions. For the hydroxy-protection of Tyr use of the cyclohexyl (Chx) and cyclohex-2-enyl (Che) groups was described (Nishiyama et al. 2001).

In Fmoc chemistry tyrosine is unprotected or protected with the O-*tert*-butyl group (Chang et al. 1980). Recently was described an efficient strategy for Fmoc-based solid phase synthesis of sTyr peptides in which the sulphate group in the sTyr residue is protected with a DCV group (FmocTyr(SO_3DCV)OH). After cleavage of the peptide from the support the DCV group is removed by hydrogenolysis (Ali et al. 2010).

2.1.5. Side reactions in peptide synthesis

The chemical synthesis of peptides on solid phase is limited by side reactions, which decrease the yield and make the purification of the correct product more difficult. If side reactions could be eliminated or significantly reduced, it is likely that longer peptides and even small proteins could be synthesized on a more or less routine basis.

Common side reactions are:

- Incomplete acylation/deprotection due to aggregation of the peptide chains
- Alkylation of sensitive residues by cations derived from the acidolytic cleavage of protecting groups.
- Aspartimide (succinimide) formation.
- Side reactions due to insufficient protection of the functional groups of the amino acid side chains.
- Racemization.

Incomplete acylation is a serious problem in SPPS, since deletion peptides lacking one or more amino acid residues are produced. A convenient way to prevent the formation of deletion peptides is to cap the remaining free amino groups after acylation (Merrifield 1963). For the capping acetic anhydride (Merrifield 1963) is often used. Reasons for incomplete acylation are slow couplings with bulky, sterically hindered amino acids (i.e. Thr, Leu and Val) (Ragnarsson et al. 1971) and aggregation of the growing peptide chain (Kent 1985; Kent et al. 1992). Among the methods that are most frequently used to disrupt the hydrogen bonds between the aggregating peptide chains is the use of more polar solvents (Kent 1985; Hyde et al. 1992), addition of chaotropic salts or detergents (Stewart and Klis 1990; Pugh et al. 1992), use of low resin loadings (Kent and Merrifield 1981) and use of elevated temperatures (Tam 1985; Tam and Merrifield 1987).

An analogous problem is the incomplete deprotection of the α-amino group due to intermolecular aggregation of the peptide chains. This side reaction is known to occur in Fmoc chemistry (Larsen and Holm 1994) and remains one of the principal objections against Fmoc synthesis of peptides. In Boc chemistry deprotection is generally quantitative, since TFA, which is used for cleavage of the Boc group, is excellent solvent for the peptide-resin (Kent 1985).

A well-known side reaction which occurs in Boc chemistry is alkylation of sensitive amino acid residues (Erickson et al. 1973; Engelhard et al. 1978; Bodansky et al. 1981), N → O-acyl schift (Sakakibara et al. 1962), aspartimide formation (Merrifield 1967; Baba et al. 1973) and acylation of the γ-carboxyl group of glutamic acid (Freinberg et al. 1975). Cleavage of the benzylic protecting groups used in Boc chemistry by anhydrous HF or TFMSA proceeds by an S_N1 mechanism and benzylic cations are generated. The carbocations can alkylate the nucleofilic side chains of Cys, Tyr, Met and Trp. The addition of scavengers such as *p*-cresol suppresses this side reaction, but does not always provide sufficient protection.

Cleavage of protecting groups and peptides from the solid phase is often performed by using TFMSA-thioanisole in TFA, which has been shown to be the most efficient mixture among different combinations of sulphides and methanesulphonic acids, used for cleavage of protecting groups (Fujii et al. 1977; Kiso et al. 1979a; Kiso et al. 1979b; Kiso et al. 1980). Cleavage by this reagent is proposed to proceed by an S_N2 mechanism, where the acid protonates the oxygen of the protecting group and thioanisole functions as a nucleophile and cleaves the protecting group (Kiso et al. 1979b).

In Fmoc chemistry, where TFA is used for the final deprotection, some of the side reactions caused by the considerably stronger acid HF are suppressed, such as acid-catalyzed aspartimide formation (Tam et al. 1988). However, TFA cleavage of the side chain protecting groups produces relatively stable carbocations, which sometimes are difficult to trap efficiently by scavengers and which can alkylate the side chains of Cys, Tyr, Trp and Met (Noble at al. 1976; Lundt et al. 1978).

One of the side reactions, which affected significantly synthesis of the UPF series, was aspartimide (succinimide) formation. This was sequence-dependent side reaction, catalyzed by base or by acid (Bodansky et al. 1978a; Bodansky et al. 1978b). The nucleophilic nitrogen attacks the side chain carbonyl carbon of the protected aspartyl residue and a 5-membered succinimide ring is formed (Bodansky et al. 1978c). A proposed mechanism for acid-catalysed aspartimide formation is that the carbonyl oxygen is protonated, which enhances the electrophilicity of the carbonyl carbon and facilitates attack from the peptide bond nitrogen (Ondetti et al. 1968; Tam et al. 1988). Another possible mechanism discussed in the literature (Sheppard 1977, Schön et al. 1979; Tam et al. 1988) assumes that after cleavage of the protecting group protonation of the carboxyl group by strong acid results in the loss of water and formation of highly reactive acylium cation, which can react with the peptide bond nitrogen.

In the design of amino acid protecting groups several factors have to be taken into account. The protecting group has to be stable during the synthesis under the reaction conditions used, and must be quantitatively removed at the end of the synthesis. It is also convenient if different amino acid side chain protecting groups used in peptide synthesis have similar chemical properties and can be removed by a single procedure. Several common side reactions in SPPS have been shown to be highly dependent on choice of protecting group (Barany and Merrifield 1979) and it is likely that with de development of better protecting groups these reactions can be suppressed.

2.2. Glutathione

Glutathione (GSH) is a water-soluble tripeptide of MW 307, composed of amino acids glutamine, cysteine and glycine and having the following primary structure L-gamma-Glu-L-Cys-Gly (see Figure 1). In reduced form GSH is the major low-molecular weight thiol compound in animals and plants, and it is present in millimolar concentration range in various mammalian cells (Meister and Anderson 1983; Anderson 1997). GSH is synthesized in a number of tissues, but liver is the central organ in its metabolism and GSH level in RBC and blood reflect basically the synthetic power of the liver. GSH is oxidized to glutathione disulfide (GSSG). Protein molecules containing cysteine residues readily participate in thiol-disulfide exchange reactions with GSH.

GSH can be oxidized to glutathione disulfide (GSSG). The latter is maintained at less than 1% of the total glutathione pool (Brigelius et al. 1983; Dickinson and Forman 2002), as rapid reduction to GSH occurs by a concerted action of NADPH and flavoenzyme glutathione reductase (GR;EC 1.8.1.7). Therefore, increases in GSSG during oxidative stress are usually transient.

The breakdown of GSH, GSSG and GSH-conjugates is mediated by the enzyme γ-glutamyl transpeptidase (GG; EC 2.3.2.2), located in the cell membrane. GGT removes the γ-glutamyl moiety from the glutathione and produces dipeptide Gly-Cys or Gly-Cys-conjugate, respectively. Removed glutamate is

transported to an acceptor, which is an amino acid or other dipeptide. Extracellular dipeptidases hydrolyse the bond between Cys and Gly, resulting in free amino acids. In the case of Gly-Cys conjugates, the conjugated part remains bound with cysteine. All formed compounds are further taken up by specific transporters and used for glutathione re-synthesis (Franco et al. 2007).

2.2.1. Biological functions of glutathione and oxidative stress

The isopeptidic nature of the γ-glutamyl linkage renders GSH resistant to most peptidases. The electronic structure of sulphur atom (available *d*-orbitals, permitting overlap during the formation of transition states and dissipation of electrons transferred from radicals) underlies the high reactivity of the thiol group towards nucleophilic addition, redox reactions (e.g. *via* radical mechanism) and metal chelation. Due to these special structural features GSH is able to fulfil an impressive number and variety of biological functions (Zilmer et al. 2005).

- GSH, as the major cellular non-enzymatic antioxidant, eliminates reactive species like hydroxyl radicals, peroxynitrites, peroxides and N_2O_3 and plays a principal role in cellular defence against high-grade oxidative and nitrosative stress, mainly via co-operation with Se-glutathione peroxidase (GSHPx; EC 1.11.1.9). Whereas a rapid reduction of GSSG back to GSH by NADPH and GR is needed (Anderson et al. 1989; Meister 1994; Lucente et al. 1998; Griffith and Mulcahy 1999).
- GSH is involved in the restoration of thiol groups of proteins (maintenance of hemoglobin, other proteins and enzymes in active forms) and coenzyme A. It is required for the stabilization of cell membranes and for the synthesis of proteins, nucleic acids, leukotrienes and prostaglandines (Anderson 1997; Valencia et al. 2001; Pastore et al. 2003).
- GSH is used for detoxification of several xenobiotics by GSTs, involved in the transport of nitric oxide, and in the modulation (glutathionylation) of several key-enzymes and proteins (GAPDH, phosphorylase, creatine kinase, Ras) (Anderson 1997; Karelson et al. 2002; Townsend et al. 2003).
- GSH is the main redox regulator of cell, and the glutathione redox ratio (GSSG/GSH) as the redox buffer, modulates the action of numerous redox-sensitive proteins, including several transcription factors. The glutathionylation of proteins at key cysteine residue is a redox-sensitive post-translational signaling mechanism and redox-sensitive regulator of cellular activities (Schafer and Buettner 2001; Filomeni et al. 2002; Huang and Huang 2002).

This impressive spectrum of biofunctionality of GSH in different pathological conditions explains why pharmaco-clinical strategies have begun to use the glutathione system as drug target.

One promising approach is to use chemically modified GSH analogues. For example, the esters of GSH are readily taken up by cells and are de-esterified inside cells to provide GSH. These esters have protective effect against cerebral brain ischemia in rats (Anderson et al. 2004; Yamoto 1993). Following this strategy, compounds of increased hydrophobicity (to improve penetration through biomembranes) and better stability (weaker substrates for glutathione S-transferase) would be of great interest. Such GSH-like new compounds would expand the possibility to support some parts of the glutathione system and may have an impact, as an adjuvant therapeutic factor, for instance, in the case of high-grade oxidative stress (OxS) when the production of the potent pro-oxidant GSSG is powerful.

2.2.2. Glutathione analogues

Different strategies have been applied to maintain the functionality of the GSH system. Bioavailability of cysteine has been determined as the main limiting factor of the *de novo* synthesis of GSH. To avoid the toxicity problems (Olney et al. 1990) N-acetyl-L-cysteine has been used (Bernard 1991; Ortolani et al. 2000). Several GSH-like substances with extremely different properties have been synthesized (Lucente et al. 1998; Zilmer et al. 2005), and various modifications of GSH molecule have been performed to improve stability and cellular uptake of these drugs.

Firstly, esterification of glycine with ethyl or isopropyl moiety was used to obtain glutathione monoesters, which are effectively taken up by cells and thereafter hydrolysed to GSH. Therefore these monoesters have protective properties in models of stroke and spinal cord injury, Parkinson's disease (PD), diabetic cataract, LDL oxidative modification and liver perfusion injury with rats (Anderson et al. 2004; Grattagliano et al. 1999; Guizar-Sahagun et al. 2005; Rajasekaran et al. 2005; Zeevalk et al. 2007; Zhang et al. 2008). GSH monoethyl ester protects against GSH deficiency due to biological aging and the acetaminophen-induced decrease of GSH pool in old mice (Chen et al. 2000). γ-Glutamylcysteine ethylester was also used for neuroprotection in animal models (Chinta et al. 2006; Reed et al. 2009).

GSH diesters are transported even faster into cells, where they rapidly split to monoesters. However, at the same time monoesters are quickly transported out of cells that reduces effectiveness of this chemical modification (Anderson 1998).

The GSH analogue N-(N-r-L-glutamyl-L-cysteinyl)glycine 1-isopropyl ester sulphate monohydrate (YM737) has been shown to have protective qualities in rat cerebral ischemia by inhibiting lipid peroxidation (Yamamoto et al. 1993; Schulz et al. 2000; Zilmer et al. 2005). Substitution of the amino group at the GSH molecule N-terminus with a pyrrole ring has given new antioxidants, which do not inhibit the glutathione reductase nor the glutathione peroxidase due to steric hindrances (Gaullier et al. 1994). Replacing the native γ-glutamyl

moiety with the *cis*- or *trans*-4-carboxyl-L-proline residue produced conformationally rigid skeleton and made this GSH analogue resistant to degradation by γ-glutamyl transpeptidase (Paradisi et al. 2003). The outstanding group of GSH analogues are cysteine-substituted S-nitrosoglutathiones that have been designed proceeding from physiological roles of both GSH and nitric oxide (Richardson et al. 2002). An overview about different chemical modifications in the GSH molecule has been summarized (Zilmer et al. 2005).

In cancer therapies the goal could be diminishing of GSH level in cancer tissue. Over-expression of GST has been reported to be one of the biochemical mechanisms of drug resistance in cancer cells. GST plays an important role in deactivation of a number of alkylating agents used in cancer therapies (Wu et al. 2004). For this reason, large number of GSH analogues has been designed to inhibit different GST isoenzymes. Among these compounds were phosphono analogues (Kunze et al. 2000) and the peptidometic analogues of GSH that were stable towards GGT, the main enzyme of GSH breakdown (Burg et al. 2002). One of the latest and more successful GSH analogues in cancer therapy, compound TLK 286, is in clinical trials (Rosen et al. 2003). Some designed GSH analogues act as glyoxalase inhibitors and have shown to possess potent anti-proliferative and anti-tumour activity (Lo et al. 1992). Still, the improvement of GSH analogues stability towards peptidases and proteases stands as general problem. One possible solution to overcome this problem is cyclization of GSH molecule. Such analogues have been tested for antitumor activity (Sheh et al. 1990).

Other strategies of chemical modification of GSH proceed from the ideas of replacement of the amino acids in this tripeptide, addition of more amino acid residues or making peptide bond derivatives. Following these basic principles of peptide modifications the novel glutathione analogue 4-MeO-Tyr-γ-Glu-Cys-Gly (UPF1, assigne Vulpes Ltd., no. 110035500, PCT/SE01/01351) was designed. In the UPF1 sequence additional nonproteinogenic amino acid 4-metoxy-phenylalanine was added to GSH N-terminus to improve antioxidant properties and to increase hydrophobicity of this GSH derivative. This compound was predecessor of the series of compounds designed and studied in this dissertation.

2.3. Reaction of DPPH radical with antioxidants *in vitro*

There are several methods used for estimating antioxidant properties of bioactive substances (Sanchez-Moreno 2002; Schwarz et al. 2001). The most commonly used methods for *in vitro* determination of antioxidant capacity have been reviewed (Magalhães et al. 2008). One popular assay method for estimating antioxidant activity is based upon the use of a stable free radical, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) (Molyneux 2004). This compound reacts with different hydrogen atom radical donors (phenols, thiols) in solution and the

reaction can be followed by change in spectrum of the DPPH$^{\bullet}$: the purple chromogen radical is reduced by reducing compounds to the corresponding pale yellow hydrazine (Figure 6) (Blois 1958; Rusell 1954; Jimenez-Escrig et al. 2000; Fagali et al. 2008).

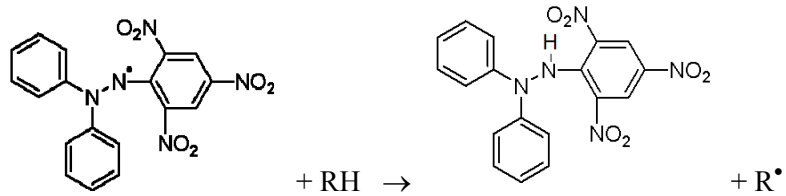


Figure 6. Structure of DPPH radical and the non-radical form. RH is a hydrogen donating antioxidant.

The scavenging capacity of antioxidants is generally evaluated in organic reaction media by monitoring the absorbance decrease at 515-528 nm until the absorbance remains constant. Generally, the results are reported as the efficient concentration (EC_{50}). This parameter denotes the amount of antioxidant that is necessary to decrease the initial DPPH$^{\bullet}$ concentration by 50% (Brand-Williams 1995).

In opposition to what was initially suggested, the reaction mechanism is based on an electron transfer reaction whilst the hydrogen atom abstraction is marginal reaction pathway, because it occurs slowly in strong hydrogen-bond-accepting solvents like methanol and ethanol (Foti 2008). As in other assays based on electron transfer, the scavenging capacity against DPPH$^{\bullet}$ is strongly influenced by solvent and pH of the reaction media (Magalhães et al. 2007). The steric accessibility of DPPH$^{\bullet}$ is the major determinant of the reaction selectivity, since small molecules that have better access to the radical site have relatively higher antioxidant capacity (Huang et al. 2005). However, despite these limitations, application of DPPH$^{\bullet}$ has been widely used and this convenient assay procedure has been applied for screening antioxidant capacity of both pure compounds and complex samples (Magalhães et al. 2008). In addition to the most widely used procedures of antioxidant titration experiments, denominated as "antioxidant assay" in literature, we investigated also kinetics and mechanism of this reaction.

3. OBJECTIVES OF DISSERTATION

The main objectives of this study were:

1. Design of novel tetrapeptidic GSH analogues, proceeding from general understandings about antioxidant properties of other compounds and from structure of the lead compound of the novel UPF series.
2. Synthesis of series of new GSH analogues by using Fmoc and Boc chemistry.
3. Investigation of kinetic mechanism of reaction of DPPH radical scavenging by GSH and its derivatives.
4. Investigation of dimerization and stability of novel UPF peptides.

4. MATERIALS AND METHODS

4.1. Chemicals and materials

Wang resin, 9-fluorenylmethoxycarbonyl (Fmoc)-Gly-Wang resin, Rink Amide MBHA (*p*-methylbenzhydrylamine) resin, Fmoc-protected amino acids, Boc-protected amino acids, dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBt), 2-(1*H*-bensotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) were purchased from Novabiochem, Switzerland; *N,N*-dimethylformamide (DMF), dichloromethane (DCM), dimethylsulphoxide (DMSO), *N,N*-diisopropylethylamine (DIEA), acetonitrile from BDH Laboratory Supplies, England; trifluoroacetic acid (TFA), ethylmethylsulphide (EMS), triisopropylsilane (TIS) from Fluka Switzerland; hydrofluoric acid (HF) from AGA, Sweden; 1,2-ethanedithiol (EDT), glutathione, terephthalic acid (THA), $CuSO_4 \cdot 5H_2O$, disodiumphosphate, hydrogen peroxide 30% (w/w, water solution), α,α-diphenyl-β-picrylhydrazyl (DPPH$^{\bullet}$) from Sigma-Aldrich, Germany. PBS was obtained from Calbiochem, USA.

Aprotic solvents *N, N*-dimethylformamide or dichloroethane were used for synthesis to secure good solubility of reagents (Benoiton 2005, Chan and White 2000). DMF and DCM were stored on molecular sieves (4 Å, Merck, Germany). Isolation of products after every step of SPPS was performed by filtration, whereas the remaining reagents and by-products of the synthesis were flushed away. For synthesis high concentration of reagents was utilized, to increase reaction rate (Benoiton 2005; Chan and White 2000). The orthogonality requirement of protection/depotection conditions was fulfilled for both the Boc- and Fmoc-strategy (Benoiton 2005; Chan and White 2000). In the former case, the Boc-group was removed by treatment with trifluoroacetic acid, whereas the cleavage of the product was performed by HF. In the case of Fmoc chemistry the N-terminal protecting group was removed by piperidine, whereas the cleavage of the product was performed by treatment with trifluoroacetic acid (Greene and Wuts 1999). Thus the Fmoc-strategy utilized milder conditions, but was also more susceptible to side reactions due to involvement of base, as racemization of the chiral amino acids could be caused by deprotonation of Cα during the Fmoc-deprotection steps (Benoiton 2005).

4.2. Synthesis and purification of peptides

The solid phase peptide synthesis may be described by the following general reaction scheme as shown in Figure 7.

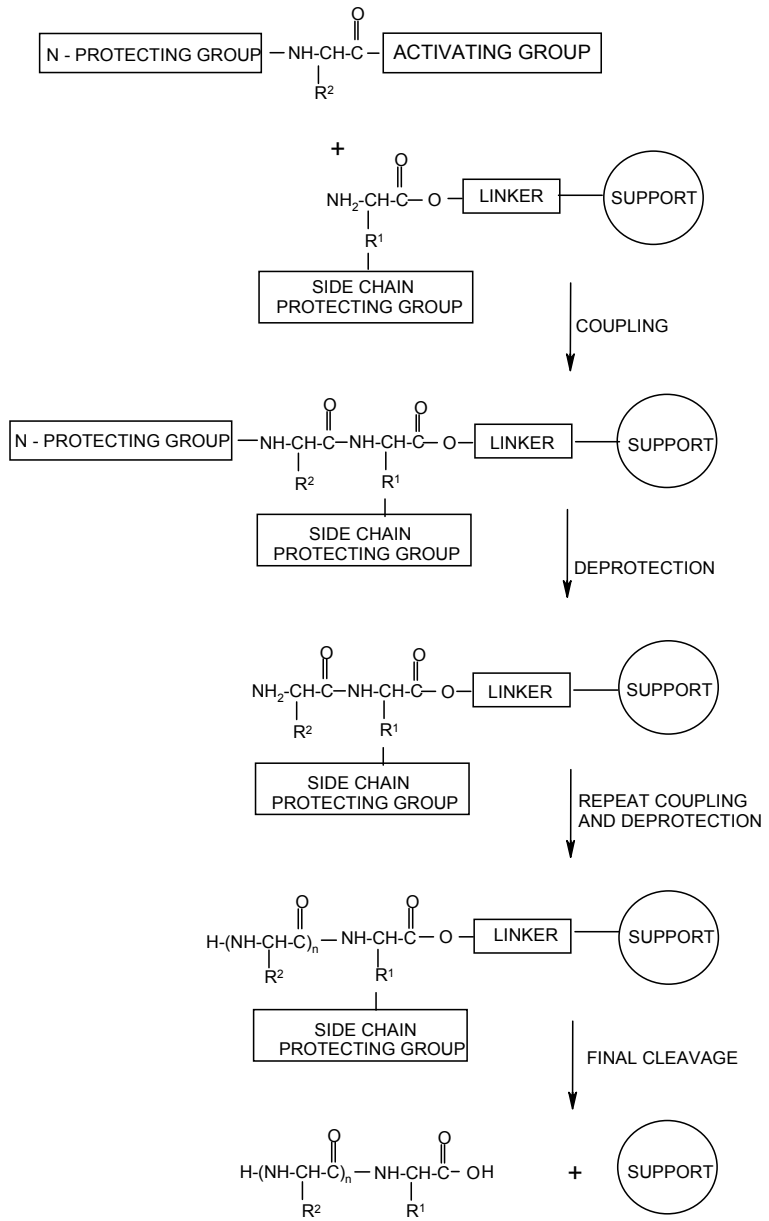


Figure 7. General scheme of solid phase peptide synthesis, SPPS.

Peptides studied in this work were synthesized either manually by using Fmoc-chemistry (Soomets et al. 2005) or by an automatic synthesizer (Applied Biosystem/Perkin-Elmer Model 431A) using Boc-chemistry (Langel et al. 1992).

For the manual peptide synthesis *p*-benzyloxybenzyl alcohol (Wang resin) was usually used. The first amino acid was attached to Wang resin using an activating agent such as dicyclohexylcarbodiimide (DCC) and a catalytic amount of 4-dimethylamino-pyridine (DMAP). These conditions can lead to partial epimerization of the amino acid, so HOBt is normally added to reduce racemization. In our initial experiments the obtained crude products consisted of many impurities, the yield of right product was low and the time of synthesis was long. Amino acid and DCC were dissolved in minimum amount of DCM separately outside of reaction vessel, cooled to 0 ^{o}C, then mixed and allowed to stand for 30 min at 0 ^{o}C. The anhydride solution was added to the peptide resin, then after 15 min the base (DIEA, to neutralize the acid formed by symmetrical anhydride coupling) was added. Coupling was performed for 30 min. To avoid these problems Fmoc-Gly-Wang resin was used as most of designed peptide sequences had glycine as C-terminal amino acid. Latter synthesis resulted in more pure products and better yields were obtained.

MBHA resin is the most widely used resin for preparing C-terminally amidated peptides using Boc chemistry. For the synthesis of C-terminally amidated peptides the 4-methylbenzhydrylamine (Rink Amide MBHA resin) was used (Novabiochem).

Couplings of 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acids were carried out in a stepwise manner using the standard 2-(1*H*-bensotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and *N*-hydroxybenzotriazole (HOBt) activation in dimethylformamide (DMF). *N,N*-diisopropylethylamine (DIEA) was added for *in situ* neutralization. Coupling was performed as shown in Figure 8:

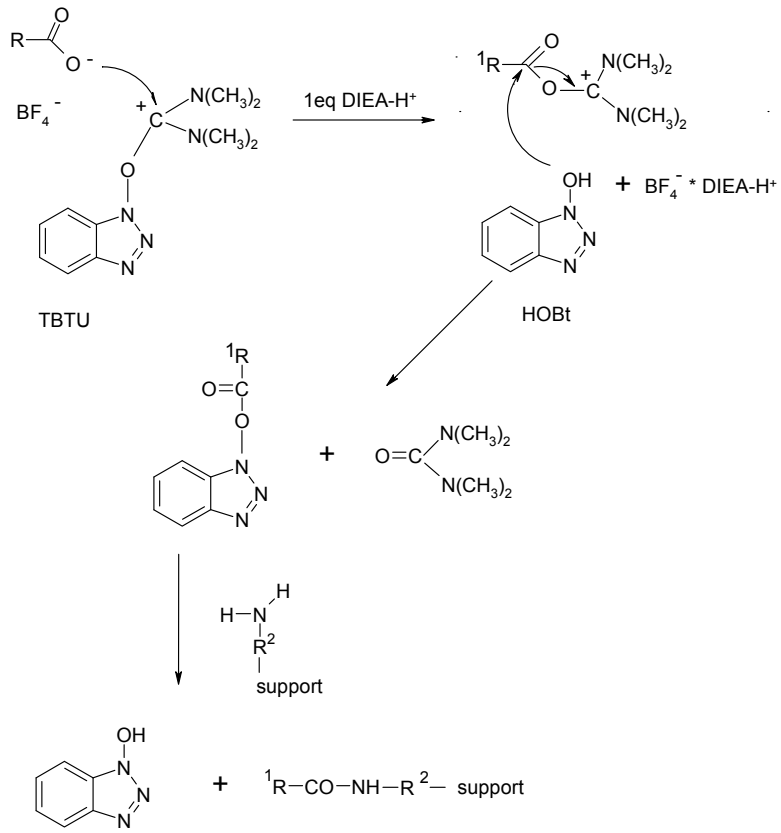


Figure 8. Mechanism of activation of amino acid by TBTU and HOBt reagent. (Chan and White 2000)

20% piperidine in DMF was used for Fmoc group removal. The peptides were removed from the resin and simultaneously deprotected with TFA in the presence of scavengers: water 2% (v/v), EDT 2% (v/v) and TIS 2.5% (v/v) by stirring for 90 minutes at room temperature, or in some cases water 2% (v/v), EDT 2% (v/v) and EMS (ethylmethylsulphide) 2.5% (v/v) for 90 min at room temperature. To collect the synthesis product, the resin was removed by filtration under the reduced pressure. For precipitation the peptide cold diethyl ether was added to obtained solution. The formed suspension was centrifuged, diethyl ether layer was removed and the crude peptide was washed with cold diethyl ether and centrifuged repeatedly (3 times). After final centrifugation (Universal 32R) the pellet was dissolved in water or in 20% acetonitrile/water mixture, frozen and lyophilized.

The automatic synthesis of UPF peptides was carried out in a stepwise manner in a 0.1 mmol scale on an Applied Biosystem Model 431A peptide synthesizer on a solid support using DCC/HOBt activation strategy. *tert*-Butyloxycarbonyl (*tert*-Boc) amino acids were coupled as hydroxybenzotriazole

esters to a phenylacetamidomethyl-resin (PAM) (0.6 mmol/g), to achieve the C-terminal free carboxylic acid, or to a *p*-methylbenzylhydrylamine (MBHA) resin (1.1 mmol/g) to obtain C-terminally amidated peptides. The peptides were finally cleaved from the resin with liquid HF at 0 °C for 30 min. Deprotection of the side chains, cleavage of the peptides and purification on HPLC was achieved.

For monitoring the course of peptide synthesis reaction the Kaiser test was used after removal of the appropriate protecting group.

The peptides were purified by using reverse-phase high-performance liquid chromatography (RP-HPLC). Purity of peptides was >99% as demonstrated by HPLC on an analytical Nucleosil 120-3 C_{18} reversed-phase column (0.4 cm x 10 cm). Routinely the crude peptides were purified by the semi-preparative RP-HPLC column (ZORBAX 300 SB-C18 9,4 mm x 25 cm, a 1100 Hewlett Packard HPLC apparatus) employing an acetonitrile/water mixture (containing 0.1% TFA) as an eluant at a flow rate of 4 ml/min and absorbance of 218 nm. Gradient 20/80 AcN/H_2O to 90/10 AcN/H_2O. Purified compounds were lyophilized.

The molecular masses of the peptides were determined by a matrix-assisted laser desorption ionization-time-of-light mass-spectrometry (MALDI-TOF MS, Voyager DE Pro, Applied Biosystems) and were compared with the calculated values in each case. According to the Applied Biosystems guidelines, saturated solution of α-cyano-4-hydroxycinnamic acid was chosen for the matrix (10 mg/ml in 50:50 acetonitrile/water mixture with 0.1% TFA content).

4.3. Stability studies

Dimerization of UPF1 and UPF17, which are the representatives of UPF peptides with two different backbones containing γ- and α-glutamate residue, respectively, was studied in water and in the physiological solution (0.9% NaCl) at room temperature during 14 days. From 1 mM solution of peptide at certain time points 100 µl sample was taken and analyzed on analytical RP-HPLC column (ZORBAX 300 SB-C18 4.6 mm x 15 cm) using a linear acetonitrile-water gradient from 20 to 90% acetonitrile (v/v) (0.1% TFA) and flow rate of 2 ml/min, monitoring absorbance at 220 nm. Peak areas were used to calculate amounts of reduced and oxidized forms of peptides. Fractions were collected and molecular masses of peptides (monomeric and dimeric forms) were determined by the MALDI-TOF MS (Voyager DE Pro, Applied Biosystems). The quantities of the reduced and oxidized forms of studied analogues in the sample were expressed in percents. Summarized areas under the peaks of monomeric and dimeric forms of UPF peptides were constant and were considered as 100% through all experiments.

Dimerization process and reactivity towards H_2O_2 and Cu^{2+} ions of various compounds of UPF series were analyzed by capillary electrophoresis (CE). These analyses were performed using a CE system built in Tallinn University of

Technology in Chair of Analytical Chemistry. This system was equipped with a fused-silica capillary (Polymicro Technologies, Phoenix AZ, USA), 55 cm (effective length 39 cm) x 50 μm I.D., high-voltage power supply (Spellmann, Hauppauge, NY, USA) and UV detector (PrinCe Technologies). The UV detector was coupled to a personal computer, data acquisition was done by the software written in-house, using a Lab-View program (National Instruments, Austin, TX, USA). The software recorded the detector signal via an ADAM 4018/4060 interface (Advantech, Taipei, Taiwan). Separation of peptides was performed in 25 mM phosphate buffer (pH 7.5) containing 50 mM sodium dodecyl sulphate (SDS), voltage 25 kV at 22 ^{o}C. Under these condition good separation compounds was achieved due to different charge-to-mass ratio and to hydrophobic interaction with SDS micelles. The detection was performed at 215 nm, according to the absorption of peptide bond, and using standard compounds to identify the peaks. Capillary was conditioned prior to use with 1 M NaOH for 20 min and with H_2O for 30 min. After each run capillary surface was regenerated by sequential washing with 0,1 M NaOH, H_2O and separation buffer for 5 min each. Compared to other methods (HPLC; spectroscopic) the capillary electrophoretic (CE) technique offered significant advantages due to the high separation speed, low sample and buffer requirements.

4.4. Reaction with DPPH radical

Reaction of thiol-containing peptides with α,α-diphenyl-β-picrylhydrazyl radical (DPPH$^{\bullet}$) was measured spectrophotometrically in ethanol/water mixture or ethanol/citrate buffer (a Jenway 6405 UV/Vis spectrophotometer, Jenway Ltd., England). Concentration of reagents in the reaction mixture was as follows: thiols from 2.5 to 200 μM, DPPH$^{\bullet}$ 0.05 mM. Absorbance of the reaction mixture was monitored at 517 nm and the readings were taken at time intervals 0.5 min until the steady state was reached. In this assay lower absorbance represented higher DPPH$^{\bullet}$ scavenging activity of the peptide. The percentage of remaining DPPH$^{\bullet}$ against the peptide concentration was plotted to obtain the amount of antioxidant necessary to decrease 50% of the initial DPPH$^{\bullet}$ concentration (EC_{50}). The time needed to reach the steady state at EC_{50} concentration (T_{EC50}) was calculated graphically. The scavenging effect was expressed as antiradical efficiency $AE = 1/(EC_{50}T_{EC50})$, as described in the standard assay protocol (Sanches-Moreno et al. 1998). As the scavenging capacity against DPPH$^{\bullet}$ is strongly influenced by solvent and pH of the reaction media (Magalhães et al. 2007) the citric acid-sodium citrate buffer was used.

The same method for investigation into kinetics of GSH and α-GSH reaction with DPPH$^{\bullet}$ was used. Briefly, DPPH$^{\bullet}$ (100 μM, in 95% ethanol) was mixed with GSH or α-GSH (from 5 μM to 1000 μM) in citric acid-sodium citrate buffer (20 mM, pH 3-6). 0.25 ml solution of GSH or α-GSH (concentration range from 20 to 1000 μM) in citric acid-sodium citrate buffer (20 mM, pH 3 - 6) was added to

0.25 ml solution of 100 μM $DPPH^{\bullet}$ in 95% ethanol. Changes in the absorbance at 517 nm, occurring due to scavenging of the $DPPH^{\bullet}$, were monitored in 1 cm thermostated quartz cells at 25 ºC (UV-VIS spectrophotometer Unicam UV300, ThermoSpectronic, USA). From these time-courses the kinetic curves of the scavenging process were obtained and used for kinetic analysis.

4.5. Oxidation studies

The ability of GSH and UPFs to scavenge reactive oxygen species expresses their antioxidative properties. The peptides were oxidized with H_2O_2 and products of this process were analyzed to determine dimeric and heterodimeric products (if mixture of compounds was used). Reactions were performed at 1 mM concentration of H_2O_2 and 250 μM concentration of peptides and using capillary electrophoresis the time-courses were monitored. Products were identified by MALDI-TOF analysis.

For low molecular mass thiols the reaction occurred as follows

$$RS^- + H_2O_2 \longrightarrow RSOH + H_2O$$
$$RSOH + RSH \longrightarrow RSSR + H_2O$$

The observed rate constants for this reaction system were determined by following dimer formation. As excess of hydrogen peroxide was used the oxidation reaction was described as the pseudo-first-order process and the plots of the natural logarithm of concentration of peptides versus reaction time were linear. From these data the second-order rate constants were calculated.

The measurement of the hydroxyl radical scavenging activity of peptides was carried out by using terephthalic acid (THA, benzene-1,4-dicarboxylic acid) as the chemical dosimeter for hydroxyl radicals (Barreto et al. 1995). The final concentration of THA was 10 mM and hydroxyl radical was generated via Fenton reaction between $CuSO_4$ and H_2O_2 with final concentrations of 10 μM and 1 mM, respectively. All solutions used in this study were prepared in 14.75 mM sodium phosphate buffer at pH 7.5. The hydroxyl radical suppression was detected by measuring fluorescence at 312 nm excitation and at 426 nm emission on a Perkin-Elmer LS50B spectrofluorimeter and hydroxyl radical elimination was expressed in EC_{50} values, determined from sigmoid dose-response plots.

4.6. Toxicity tests

For the most promising analogues UPF1, UPF6, UPF17 and UPF19 toxicity tests were performed. In some studies for the antioxidant toxicity evaluations K562 cells were used (Mohammadi et al. 2009). In viability experiment the effect of 200 μM solution of peptides and 20 μM MTX on K562 cells were compared after 24 h incubation.

Further the effects of the same set of peptides were studied on the integrity of K562 cell membrane in lactate dehydrogenase (LDH) leakage assays. This assay was based on the measurement of LDH release from cells with a damaged membrane (Saar et al. 2005). In these experiments the final concentration 100 μM for peptides, 10 μM for model amphipathic peptide (MAP) and 0.1% for Triton X-100 was used and the fluorescence was recorded.

4.7. pKa studies of thiol group

Ratio of thiol and thiolate group concentration was determined by using their different absorbances at 240 nm (Benesch et al. 1955, Kortemme et al. 1995). Solution of thiol compounds in phosphate buffered saline (50 μM, PBS) was titrated with 1M NaOH solution and plot of the absorbance vs pH was obtained. Absorbance increased due to the increase in thiolate form of compounds and the pKa values were calculated from these titration curves. Absorbance was recorded on a PerkinElmer Lambda 25 spectrometer.

4.8. LC-MS analysis

GSH/DPPH$^{\bullet}$ complex formation was studied by liquid chromatography-mass spectrometry. 50 μl of sample 50 μM (DPPH$^{\bullet}$, GSH or mixture) was injected automatically into Shimadzu Prominence HPLC supplied with Phenomenex Luna $C_{18}(2)$ 3 μm column with 100 × 2 mm measurements. The gradient was built with solvents A: 99.9% H_2O + 0.1% HCOOH and B: 99.9% AcN + 0.1% HCOOH. Gradient started at 100% A for 5 min, followed by a linear increase to 100% B in 20 min and finally 20 min wash at 100% B. Flow rate was 0.75 ml/min. Mass spectrometry detector was Q-Trap 3200 (Applied Biosystems, Inc., USA). Depending on the experiment, either Q1 scan from 50–1500 Da or MS2 scan on specific ions (306.0, 394.5 and 699.0) was used. The ionization mode was negative, voltage –4500 V.

4.9. Computational methods and data processing

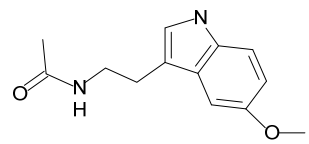
Modelling studies were performed using the Spartan 5.0 software suite (Wavefunction, Inc., USA). Conformational searches were performed by using molecular mechanics with additional conditions of aqueous medium for founding of starting geometry and final geometry were performed by PM3 semi empirical model. The geometry of DPPH$^{\bullet}$ complexes with peptides were optimized. Calculations and statistical analysis of the data were made using the GraphPad Prism (version 4.0, GraphPad Software Inc., USA) and the SigmaPlot software packages (version 8.0, SPSS Inc., USA). The results reported were given with their standard errors.

5. RESULTS AND DISCUSSION

5.1. General background of design of peptides (*Paper II*)

Various low molecular weight antioxidants, including melatonin, N-[2-(5-methoxy-1H-indol-3-yl)ethyl]acetamide, carvedilol, 1-(9H-carbazol-4-yloxy)-3-[2-(2-methoxyphenoxy)ethylamino]propan-2-ol (Figure 9) and its metabolite SB211475 (1-(3-hydroxycarbazolyl-(4)-oxy)-3-((2-methoxyphenoxyethyl) amino)propanol-(2)), have a methoxy moiety in their aromatic structures, and the presence of this structural element was found to increase antioxidant activity of these compounds (Jimenez-Escrig 2000). Noteworthy, carvedilol analogue SB 211475 possessed even higher antioxidant activity than the parent compound, tested with variety of assays, including superoxide release from phorbol ester stimulated human neutrophiles (Yue et al. 1994). However this compound differed from carvedilol by presence of an OH group introduced at position 3 of the carbazole moiety of carvedilol.

A.



B.

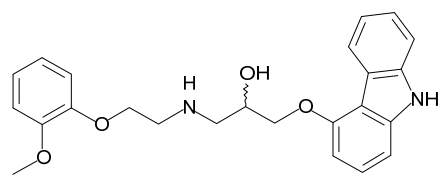


Figure 9. Structures of A. melatonin and B. carvedilol.

Much attention has been devoted to the pharmacology of melatonin in view of its potential applications in various therapeutic areas (Maestroni et al. 1997). Melatonin was shown to act as a free radical scavenger and a general antioxidant (Marshall et al. 1996, Abuja et al. 1997, Chan et al. 1996). Gozzo *et al*. systematically studied of the influence of the amide group, of the methoxy group, and of the aromatic ring on the antioxidant activity of melatonin in the LDL oxidation model (Gozzo et al. 1999). They designed and synthesized series of melatonin analogues and found that modifying the amide group in melatonin can profoundly affect their antioxidant activity. They showed that the presence and the position of a methoxy group both play a role in the antioxidant activity.

The replacement of methoxy moiety with hydroxyl group increased anti-oxidativity even more but some experimental models have proposed the pro-oxidant activity of phenolic compounds. The compounds with aromatic ring showed lower antioxidant properties. Therefore authors concluded that the structure-activity relationship are related not only to the antioxidant potential of the compounds studied but also to the model used (Gozzo et al. 1999).

Proceeding from these data we decided to introduce methylated phenolic OH group into the structure of GSH. By this way UPF1 was designed, where O-methyl-L-tyrosine was added to the N-terminus of glutathione (Table 1). Later, other members of this series of compounds were designed to improve the quality of their protective properties and to investigate into the effects of structural variations in these peptides and peptidomimetics (Paper II).

Table 1. Glutathione analogues and their hydroxyl radical scavenging ability

Nr.	Sequence	MW	EC_{50} ± SEM μM
UPF1	H_2N-Tyr(Me)-(γ-Glu)-Cys-Gly-COOH	484.5	20.5 ± 1.3
UPF2	H_2N-(γ-Glu)-Cys-Gly-Tyr(Me)-COOH	484.5	19.8 ± 0.8
UPF5	H_2N-D-Asp-(γ-Glu)-Cys-Gly-COOH	421.4	34.7 ± 0.9
UPF6	H_2N-D-Ser-(γ-Glu)-Cys-Gly-COOH	394.4	21.2 ± 0.4
UPF7	Biotinyl-Tyr(Me)-(γ-Glu)-Cys-Gly-COOH	710.5	ND*
UPF8	H_2N-Tyr(Me)-(γ-Glu)-Cys-Gly-$CONH_2$	483.5	24.4 ± 0.4
UPF10	H_2N-D-Tyr(Me)-D-(γ-Glu)-D-Cys-Gly-$CONH_2$	483.5	25.5 ± 0.6
UPF14	H_2N-D-Tyr(Me)-D-(γ-Glu)-D-Cys-Gly-COOH	484.5	19.6 ± 0.8
UPF15	H_2N-Tyr-(γ-Glu)-Cys-Gly-COOH	469.4	19.0 ± 1
UPF16	H_2N-Ser-(γ-Glu)-Cys-Gly-COOH	394.4	17.3 ± 1.1
UPF17	H_2N-Tyr(Me)-Glu-Cys-Gly-COOH	484.5	0.038 ± 0.003
UPF18	H_2N-D-Tyr(Me)-Glu-Cys-Gly-COOH	484.5	0.044 ± 0.007
UPF19	H_2N-D-Ser-Glu-Cys-Gly-COOH	394.4	0.031 ± 0,004
UPF24	H_2N-Ser-Glu-Cys-Gly-COOH	394.4	0.046 ± 0.003
UPF25	H_2N-Tyr(Me)-Glu-Cys-Gly-$CONH_2$	483.5	0.032 ± 0.006
UPF26	H_2N-Tyr(Me)-Glu-Ser-Gly-COOH	468.5	21.1 ± 0.9
UPF27	H_2N-D-Ser-(γ-Glu)-Cys-Gly-$CONH_2$	393.4	22.1 ± 0.9
α-GSH	(α-Glu)-Cys-Gly-COOH	307.3	ND*
GSH	(γ-Glu)-Cys-Gly-COOH	307.3	1231.0 ± 311.8

* ND – not determined

Different amino acids were added to glutathione via peptide bond, resulting in tetrapeptide library. Mainly, the additional units were added to the N-terminus, but their positioning in the C-terminus was also investigated (UPF2). Part of the library was synthesized so that α-glutamate was used instead of γ-glutamate (UPF17 – UPF25). In the case of several peptides, all L-amino acids (UPF10, UPF14) or only the first N-terminal L-amino acid were substituted with their D-analogues (UPF5, UPF6, UPF18, UPF19, UPF27) to investigate the

stereoisomeric impact on antioxidant properties and to improve their resistance towards endogenous peptidases. The amidation of the C-terminus (UPF8, UPF10, UPF25 and UPF27) was used for the same purpose. UPF26 was the only peptide where cysteine was replaced with serine to observe the effect, caused by sulphydryl group removal. And finally, whereas significant increase in hydroxyl radical scavenging activity was demonstrated with UPF peptides, where γ-glutamyl moiety was replaced by α-glutamyl moiety, α-congener of GSH was synthesized.

Taking together, the full list of compounds of the UPF series and GSH analogues prepared and studied in this dissertation is given in Table 1.

5.2. Chemical properties of synthesized peptides (*Paper II*)

First, stability of peptides was characterized in water and in a physiological solution by measuring rate of their dimerization. Two peptides UPF1 and UPF17 were selected for these experiments (Figure 10). Formation of the oxidation products was monitored by sensitive RP-HPLC analysis. In both cases reliable amounts of dimers were found on the second day of experiments, while the process was somewhat faster in physiological solution. These results also revealed that UPF1 had lower stability if compared with UPF17.

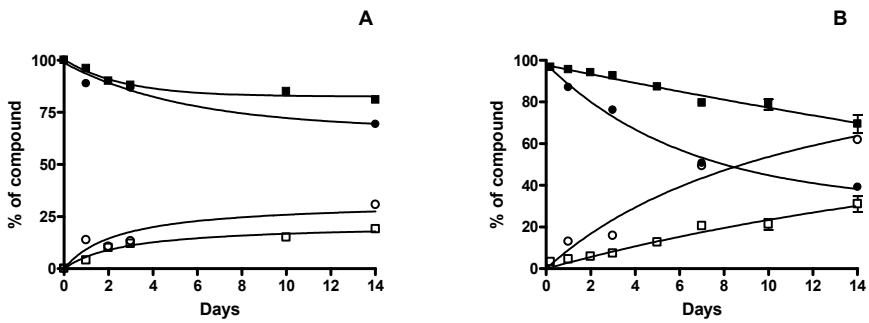


Figure 10. Dimerization of UPF1 and UPF17 in water and in 0.9% NaCl solution at room temperature. Content of monomeric (●) and dimeric (○) form of UPF1 and of monomeric (■) and dimeric (□) form of UPF17 in water (A) and in 0.9% NaCl solution (B).

Second, capillary electrophoresis was used to characterize changes in composition of mixtures of GSH and selected UPF peptides (UPF1, UPF6 UPF17 and UPF19) in the presence of oxidising agent H_2O_2. In this case the oxidation reaction was practically completed after 30 minutes at room temperature for all investigated peptides. Standard compounds were used to identify the peaks of GSH (reduced form) and GSSG (oxidized form). For

additional investigations the reaction mixture was also subjected to mass-spectrometric analysis. Below the results for mixture of GSH and UPF1 are discussed, while in the case of UPF6, UPF17 and UPF19 analogous results were obtained.

Interestingly, besides monomeric and homodimeric forms of GSH and UPF peptides, a remarkable quantity of an additional compound was observed. MALDI-TOF MS analysis confirmed that this compound was the heterodimer between GSH and UPF1. Although in mass spectra all homo-and heterodimeric forms were identified, MALDI-TOF MS is not a quantitative method and therefore CE was used to estimate the explicit amounts of reaction products. The CE analysis showed that the formation of heterodimer was prevailing and the production of mixed dimer was approximately two-fold higher if compared to homodimers.

In this part of work the first- and the second-order rate constants of oxidizing reactions of peptides (UPF1, UPF6 UPF17, UPF19, GSH, GSH+UPF1, GSH+ UPF6) with H_2O_2 were determined. The second-order rate constants for the studied peptides were very close: for GSH $0{,}204 \times 10^3$ $M^{-1}min^{-1}$ and for UPF1 $0{,}208 \times 10^3$ $M^{-1}min^{-1}$, showing that the structural features of peptides do not influence the rate of the oxidizing reaction induced by H_2O_2. In the mixture of the GSH and UPF peptides, the rate constants were also similar, indicating that the oxidation was not affected by the presence of the other peptides (Paper III). The reaction rate for GSH has also been measured previously and the second order rate constant 0.052×10^3 $M^{-1}min^{-1}$ was obtained (Winterboun et al. 1994), although the reaction conditions were rather different.

5.3. Scavenging of radicals (*Paper I, II, III*)

The hydroxyl radical scavenging assay was used to obtain the preliminary estimate of the antioxidative activity of the designed peptides. The EC_{50} values obtained for the studied compounds were listed in Table 1. The results revealed that all designed peptide molecules were stronger hydroxyl radical scavengers than GSH itself (paper II). Peptides with γ-peptide linkage in backbone showed the EC_{50} between 17-35 μM if compared with the same parameter 1231.0 μM for GSH (Figure 11). Substitution of all amino acids or only the N-terminal L-amino acid to respective D-enantiomers did not change the hydroxyl radical scavenging abilities of the compounds. The comparison of radical scavenging properties of compounds with the free acid and with amidated C-terminus revealed tendency that amides were slightly weaker hydroxyl radical scavengers than free acids. This can be observed, if data for the following pairs of compounds are considered: UPF1 and UPF 8; UPF 14 and UPF10; UPF6 and UPF27; UPF 17 and UPF 25. Surprisingly, substitution of cysteine with serine residue in UPF26 sequence did not reduce the hydroxyl radical scavenging ability, suggesting that not only SH group is involved in this radical depletion reaction.

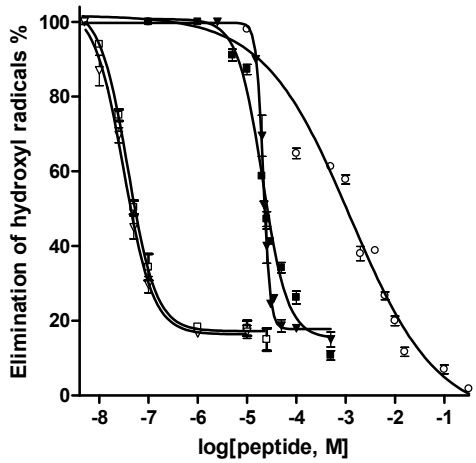


Figure 11. Concentration dependent hydroxyl radical scavenging effects of UPF1 (■), UPF6 (▼), UPF17 (□), UPF19(▽) and GSH (○) *in vitro*

Most powerful antioxidants in the hydroxyl radical scavenging assay were sequences, were γ-glutamate residue was replaced with α-glutamate residue (UPF17 versus UPF25). These analogues showed submicromolar EC_{50} values for the scavenging reaction (EC_{50} between 30–50 nM) and the peptide solution with 1 μM concentration revealed 80% of the maximal inhibiting effect. Exact EC_{50} measurements in the case of peptides with α-glutamyl moiety were complicated by slight elevation of the radical production in the end of the run, if moderately small peptide concentrations remaining below 0.5 μM were used.

Reaction with DPPH radical was used to characterize reactivity of the synthesized compounds. In this case properties of the most promising compounds were studied and the results were listed in Table 2. It can be seen that all these compounds reacted effectively with the DPPH radical. In this table the EC_{50} values together with the T_{EC50} values are listed and from these results antiradical efficiency parameter $AE = 1/EC_{50}T_{EC50}$ was calculated. If the EC_{50} values were compared for GSH and for UPF compounds, it was found that approximately 1.2-fold greater concentration of the latter compounds was needed to scavenge 50% of $DPPH^{\bullet}$. In the other way, however, the designed molecules achieved their steady state with shorter time (T_{EC50}) and thereby showed higher antiradical efficiency than GSH.

Table 2. DPPH$^{\bullet}$ scavenging activity of GSH and several compounds of UPF series.

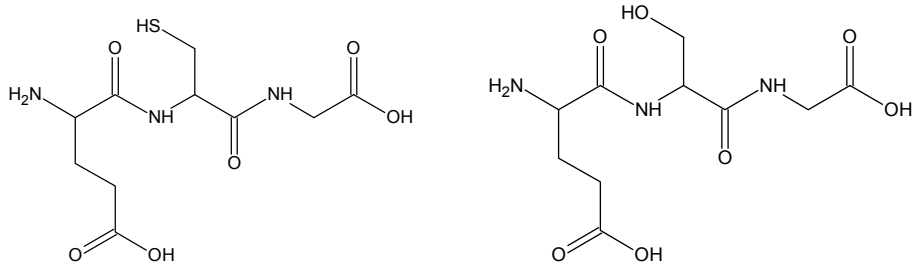
Compounds	EC_{50} (μM)	T_{EC50} (min)	AE ($\times 10^{-3}$)*
GSH	23.6±2.1	40	1.06
UPF1	28.1±1.6	10	3.56
UPF6	29.0±1.5	21	1.64
UPF17	28.3±1.7	4	8.83
UPF19	27.0±0.9	9	4.12
UPF8 (UPF1 amide)	29.3±2.0	20	1.71
UPF25 (UPF17 amide)	36.4	7	3.89

* $AE = 1/EC_{50}T_{EC50.}$

Peptides with α-glutamyl moiety in the backbone, UPF17 and UPF19, have the highest antiradical efficiencies in this assay. The T_{EC50} of GSH is 10-fold higher than this value for UPF17 and the antiradical efficiency of UPF17 was 8.3-fold higher than the same parameter for GSH. A comparison of T_{EC50} in pairs of peptides UPF1 and UPF8, UPF17 and UPF25 revealed that amidated peptides (UPF8 and UPF25) needed about two-fold more time to reach steady state than the similar free acid peptides, as the T_{EC50} values for these compounds were 10 and 20, 4 and 7 min, respectively. However, as some dose-response curves had rather specific form, it was concluded that the mechanism of this assay was not similar in all cases that hampered somewhat comparison these results.

5.4. Comparison of reactivity of α-glutamyl and γ-glutamyl derivatives (*Paper I*)

It can be seen in Table 2 that α-glutamyl (UPF17, UPF19) and γ-glutamyl (UPF1, UPF6) derivatives have different reaction time T_{EC50}, although intrinsic reactivity of SH group in these compounds should be similar. Therefore we synthesized two GSH derivatives: one with α-glutamyl residue instead of γ-glutamyl (α-GSH, shown below in figure 12), and another with serine residue instead of cysteine (α-GOH, shown below in Figure 12) and reactivity of these two compounds and GSH towards DPPH radical were systematically studied.



α-GSH α-GOH

Figure 12. Structures of α-GSH and α-GOH

In parallel, reactivities of cysteine SH groups in these derivatives were characterized by pK_a values, and their accessibilities were studied by molecular modelling.

The titration experiment yielded the pK_a value 9.0±0.2 for GSH that was in good agreement with the pK_a values published in literature: 9.20 (Litwinenko et al. 2005) and 8.75 (Foti et al. 2008). These results also agreed with pKa values for the thiol group in other cystein-containing peptides. The same titration method yielded pK_a = 9.1±0.1 for α-GSH. Good agreement between these results indicated that the thiol groups have similar acidities in GSH and α-GSH, and the way of glutamyl residue coupling has no direct influence on reactivity of the cysteine residue in these compounds. This conclusion was supported by the results of molecular modeling of GSH and α-GSH structure, shown in Figure 13. It can be seen that the thiol groups of these peptides were not involved in hydrogen bonding with other parts of these tripeptides.

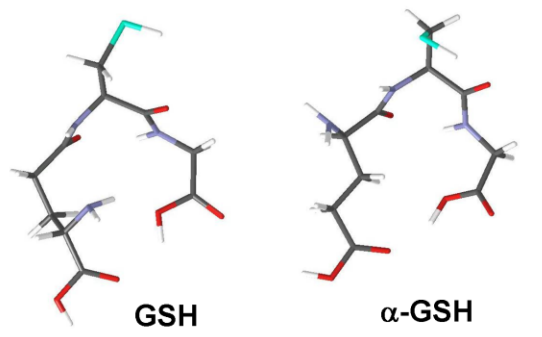


Figure 13. Calculated structures for GSH and α-GSH.

Further, the antioxidant properties of GSH, α-GSH and α-GOH were studied by kinetic analysis of the reaction mechanism between these peptides and DPPH•. The results were unambiguous for the Ser-containing peptide, indicating that no scavenging of the radical occurred in the presence of this compound. This means that the effects observed in spectrum of DPPH• are, indeed, related to the reaction between SH groups and DPPH•.

Firstly, kinetic experiments were carried out at excess of DPPH• over the concentration of thiol-containing peptides, as commonly used for the assay of antioxidant properties of natural and synthetic compounds (Huang et al. 2005). If 50 μM DPPH• was reacted with 5 and 10 μM solution of the scavenger peptides, only part of DPPH• was consumed (Figure 14) and the time-course of the process followed the first-order reaction mechanism.

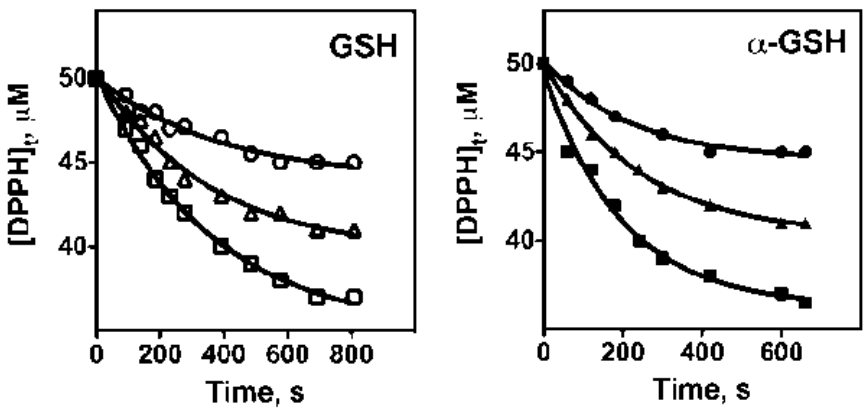


Figure 14. Reaction of 50 μM DPPH• with 5, 10 and 15 μM scavenging peptides (GSH left panel and α-GSH right panel).

Moreover, comparison of the plateau values of DPPH• and amounts of the peptides used in kinetic runs revealed that stoichiometry of the reaction between scavengers and DPPH• was 1:1 (Figure 15).

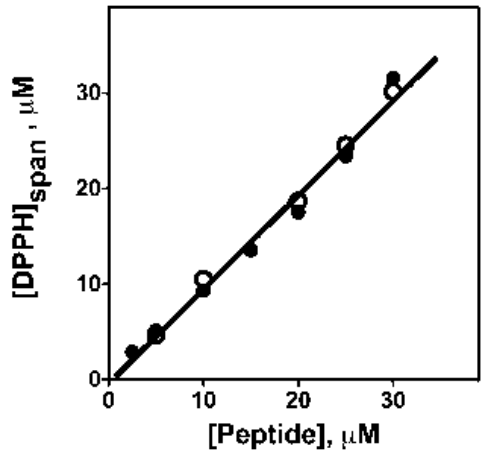


Figure 15. Consumption of DPPH$^{\bullet}$ in reaction with different amounts of scavenging peptides GSH (open symbols) and α-GSH (filled symbols).

To check this conclusion kinetics of the radical scavenging were measured at equal peptide and DPPH$^{\bullet}$ concentrations (50 μM) (Figure 16). These kinetic data also agreed with the 1:1 stoichiometry of the scavenging reaction. Therefore the conclusion can be drawn that at relatively low peptide concentration the process could be presented as a bimolecular process, where one molecule of peptide is needed for scavenging of one radical (DPPH$^{\bullet}$).

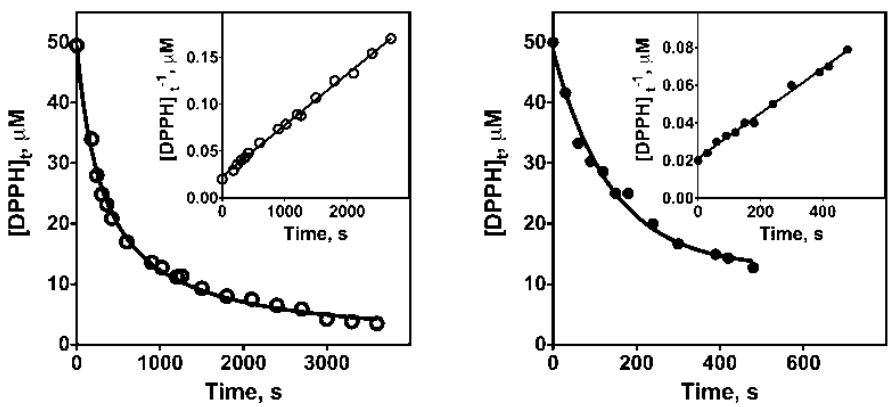


Figure 16. Reaction of 50 μM DPPH$^{\bullet}$ with 50 μM GSH (left panel) and 50 μM α-GSH (right panel). Inserts present the plots of the data following the rate equation for bimolecular reaction.

Further kinetic experiments were made at excess of the radical scavenging peptides over DPPH concentration. Under these conditions again the exponential rate equation (1) was followed, as illustrated for scavenging of 20 μM DPPH$^{\bullet}$ by 500 μM GSH and α-GSH in Figure 17.

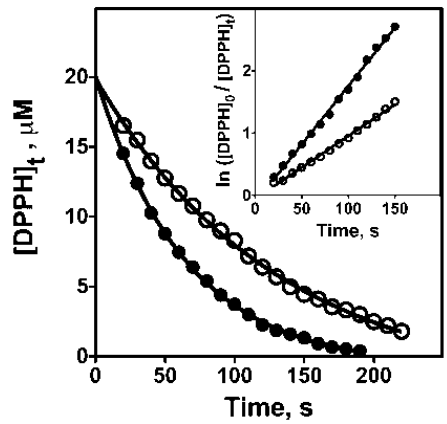


Figure 17. Reaction of 20 μM DPPH$^{\bullet}$ with 500 μM GSH (open symbols) and α-GSH (filled symbols). Inserts present the plots of the data following the rate equation for a pseudo-first order reaction.

In this case, however, the more detailed kinetic mechanism of the DPPH scavenging reaction can be derived proceeding from the hyperbolic k_{obs} vs peptide concentration plot shown in Figure 18.

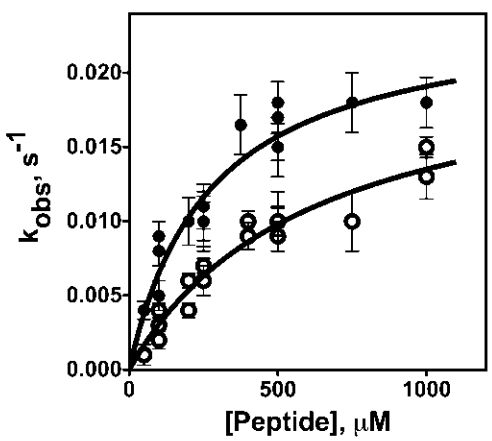


Figure 18. Dependence of the observed rate constants of DPPH$^{\bullet}$ scavenging reaction upon GSH (open symbols) and α-GSH (filled symbols) concentration.

These results demonstrated formation of the non-covalent complexes between DPPH$^{\bullet}$ and GSH or α-GSH molecules, characterized by the dissociation constant K = 0,61 mM and K = 0,27 mM, respectively. These equilibriums were responsible for the apparently different radical scavenging effectiveness of the two peptides, while the genuine reactivity of the thiol groups was practically the same. Therefore, based on these data, it would be impossible to draw significant conclusions about antioxidant properties of the peptides in biological systems, where their physiological activity should also be governed by different binding effectiveness with their target sites. However, the specificity pattern could be much more complex in biological systems.

Proceeding from the plot shown in Figure 18, the minimal reaction scheme for the primary scavenging reaction, where DPPH was consumed, should involve at least two consecutive steps: the fast equilibrium of complex formation between DPPH$^{\bullet}$ and the peptide, followed by a reaction step, producing the reduced DPPH molecule:

$$\text{DPPH} + \text{Peptide} \underset{}{\overset{K}{\rightleftharpoons}} \text{DPPH} * \text{Peptide} \xrightarrow{k} \text{Products}$$

where K stands for the dissociation constant of the DPPH-peptide reversible (non-covalent) complex and k for the rate constant of the chemical reaction. As no meaningful ordinate intercept could be observed within the error limits in Figure 18, this reaction step was treated as an irreversible process. This type of k_{obs} vs ligand concentration plots were analyzed in detail by Strickland et al (Strikland et al. 1975). Taking together, the kinetic analysis revealed formation of a reversible and most probably non-covalent complex prior the covalent step of the process. This reaction mechanism explains different reactivity of α-glutamyl and γ-glutamyl derivatives in this model reaction. On the other hand, these results reveal several complications associated with use of this reaction to model antioxidant activity of compounds, as the complex formation governing reactivity in this model process could have different meaning in biological system.

5.5. Toxicity of UPF peptides (*Paper II*)

To study toxicity effects of UPF peptides on cell culture two different assays were used. Firstly, influence of UPF peptides and MTX on viability of K562 cells was studied. It was found that UPF1, UPF6, UPF17 and UPF19 do not show any toxic effects in this assay system. Secondly, influence of the same peptides on the integrity of K562 cells membrane was determined by comparing their effect with the effect of MAP. None of the UPF peptides studied caused significant LDH leakage from K562 cells (Figure 19). However, interestingly,

UPF17 and UPF19 disturbed membranes more than peptides with the γ-peptide linkage (UPF1 and UPF6). These results were rather promising regarding perspectives of drug design on the basis of UPF-like sequences.

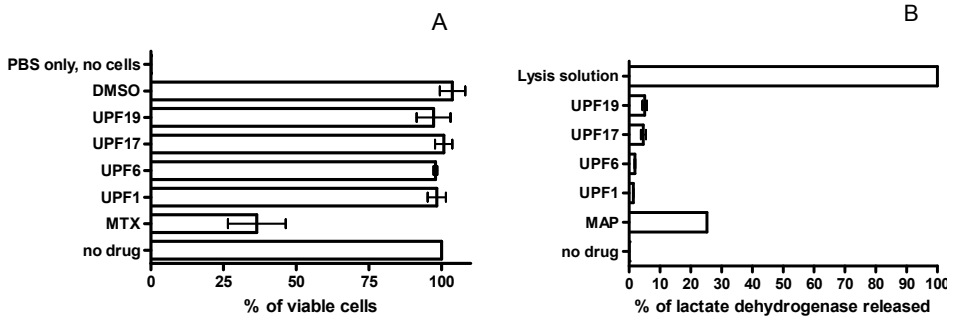


Figure 19. The toxicity test. The influence of UPF1, UPF6, UPF17 and UPF19 to K562 human erythroleucemia cells. **A** – UPF peptides 200 μM, MXT 20 μM; **B** – UPF peptides 100 μM, MAP 10 μM.

6. SUMMARY

In this dissertation a series of novel glutathione-like compounds of antioxidant activity, consisting of four amino acid residues and denominated as UPF peptides were designed, synthesized and characterized by using several chemical and biological assay systems. To analyze structure-activity relationships, different variations in structure of these ligands were made. These variations included: linking of fourth amino acid to GSH backbone, replacing the native γ-glutamyl moiety in the GSH backbone with α-glutamyl moiety, using D-amino acids instead of L-isomers and amidation of the terminal carboxyl group. O-methyl-L-tyrosine was mostly used as the fourth amino acid to increase antioxidant activity of the compounds. In parallel, two tripeptidic derivatives of GSH were synthesized and analyzed: α-GSH, including the α-glutamyl moiety, and GOH, where cysteine residue was replaced with serine.

It can be concluded that the principles used for design of these novel compounds appeared to be sound and yielded products of increased hydroxyl radical scavenging ability if compared with GSH itself. Substitution of the γ-glutamyl moiety by α-glutamyl group drastically improved the hydroxyl radical scavenging properties of UPF peptides; while other structural modifications had somewhat less pronounced effects. In assay of DPPH radical with GSH had slightly lower EC_{50} value than all UPF peptides, but regarding antiradical efficiencies, UPF peptides exceeded GSH, whereas UPF17 was the most effective compound. The kinetic mechanism of scavenging of $DPPH^{\bullet}$ with GSH and its α-glutamyl derivative was kinetically characterized and participation of a non-covalent complex in this process was discovered. This seems to explain higher scavenging rate of α-glutamyl derivatives in model assay of antioxidant properties of compounds.

It was found that UPF peptides were relatively stable, as reliable amounts of their dimeric forms were not detected before two days of incubation in physiological solution, and at least half of the peptides were in monomeric form after one week. These results, together with data that the compounds of UPF series do not reveal toxicity in used model systems, makes the compounds studied in this dissertation rather promising candidates of potential drugs.

REFERENCES

Abuja, P. M., Liebmann, P., Hayn, M., Schauenstein, K., Erstebauer, H. (1997) Antioxidant role of melatonin in lipid peroxidation of human LDL., FEBS Letters, 413, 289–293.

Ali, A. M., Taylor, S. D. (2010) Synthesis of disulfated peptides corresponding to the N-terminus of chemokines receptors CXCR6 (CXCR6 1–20) and DARC (DARC 8–42) using a sulfate-protecting group strategy., J. Pept. Sci., 16(4), 190–199.

Amblard, M., Fehrentz, J. A., Martinez, J., Subra, G. (2005) Fundamentals of modern peptide synthesis., Methods Mol. Biol., 298, 3–24.

Amblard, M., Fehrentz, J. A., Martinez, J., Subra, G. (2006) Methods and protocols of modern solid phase peptide synthesis., Mol. Biotechnol., 33(3), 239–254.

Amiard, G., Heymes, R., Velluz, L. (1956) Nouvelle synthese du glutathion. Bull. Chem. Soc. Fr., 698.

Anderson, M. E., Underwood, M. (1989) Glutathione metabolism at the blood-cerebrospinal fluid barrier. FASEB Journal, 3(13), 2527–2531.

Anderson, M. E. (1997) Glutathione and glutathione delivery compounds. Advances in Pharmacology., 38, 65–78.

Anderson, M. E. (1998) "Glutathione: an overview of biosynthesis and modulation." Chem. Biol. Interact, 111–112, 1–14.

Anderson, M. F., Nilsson, M., Eriksson, P. S., Sims, N. R. (2004) Glutathione monoethyl ester provides neuroprotection in a rat model of stroke. Neurosci. Lett., 354, 163–165.

Andreu, D., Albericio, F., Sole, N. A., Munson, M. C., Ferrer, M., Barany, G. (1994) Formation of disulfide bonds in synthetic peptides and proteins, in Methods in Molecular Biology, Vol. 35: Peptide Synthesis Protocols, Ed. Pennington, M. W., Dunn, B. M., Humana Press Inc., Totowa, NJ., 91.

Angell, Y. M., Alsina, J., Albericio, F., Barany, G. (2002) Practical protocols for stepwise solid-phase synthesis of cysteine-containing peptides., J. Pept. Res., 60(5), 292–299.

Atherton, E., Fox, H., Harkiss, D., Logan, C. J., Sheppard, R. C., Williams, B. J. (1978a) A mild procedure for solid phase peptide synthesis: use of fluorenylmethoxycarbonylamino-acids. J. Chem. Soc. Chem. Commun., 537.

Atherton, E., Fox, H., Harkiss, D., Sheppard, R. C. (1978b) Application of polyamide resins to polypeptide synthesis: an improved synthesis of β-endorphin using fluorenylmethoxycarbonylamino-acids. J. Chem. Soc. Chem. Commun., 539.

Atherton, E., Pinori, M., Sheppard, R. C. (1985) Peptide synthesis. Part 6. Protection of the sulphydryl group of cysteine in solid-phase synthesis using N_{α}-fluorenylmethoxycarbonylamino-acids. Linear oxytoxin derivatives. J. Chem. Soc., Perkin Trans. I., 2057.

Baba, T., Sugiyama, H., Seto, S. (1973) Rearrangement of a to β-aspartyl peptide with anhydrous hydrogen fluoride. Chem. Pharm. Bull., 21, 207.

Balse, P. M., Kim, H. J., Han, G., Hruby, V. J. (2000) Evaluation of new base-labile 2-(4-nitrophenylsulfonyl)ethoxycarbonyl (Nsc)-amino acid for solid-phase peptide synthesis., J. Pept. Res., 56(2), 70–79.

Barany, B., Merrifield, R. B. (1979) Solid-Phase Peptide Synthesis in The Peptides, Vol. 2, Ed. Gross, E., Meienhofer, J., Academic Press, New York, 1–284.

Barlos, K., Gatos, D., Hatzi, O., Koch, N., Koutsogianni, S. (1996) Synthesis of the very acid-sensitive Fmoc-Cys(Mmt)-OH and its application in solid-phase peptide synthesis., Int. J. Pept. Protein Res., 47(3), 148–153.

Barreto, J. C., Smith, G. S., Strobel N. H., McQuillin, P. A., Miller, T. A. (1995) Terephthalic acid: a dosimeter for the detection of hydroxyl radicals in vitro. Life Sci., 56, L89-L96.

Benesch, R. E., Benesch, R. (1955) The acid strength of the –SH group in cysteine and related compounds. J. Am. Chem. Soc., 77, 5877–5881.

Benoiton, N. L., Lee, Y. C., Steiauer, R., Chen, F. M. (1992) Studies on sensitivity to racemization of activated residues in coupling of N-benzyloxycarbonyldipeptides., Int. J. Pept. Protein Res., 40(6), 559–566.

Benoiton, N. L. (2005) Chemistry of peptide synthesis, CRC Press Taylor and Francis Group.

Bernard, G. R. (1991) N-acetylcysteine in experimental and clinical acute lung injury. Am. J. Med., 91, 54S–59S.

Blake, J. (1979) Use of cyclopentyl ester protection for aspartic acid to reduce base catalyzed succinimide formation in solid-phase peptide synthesis. Int. J. Pept. Protein Res., 13, 418.

Blois, M. S. (1958) Antioxidant determinations by the use of a stable free radical., Nature, 181, 1199–1200.

Bodansky, M., Kwei, J. Z. (1978a) Side reactions in peptide synthesis VII. Sequence dependence in the formation of aminosuccinyl derivatives from β-Benzyl-aspartyl peptides. Int. J. Pept. Protein Res., 12, 69–74.

Bodansky, M., Martinez, J. (1978b) Side reactions in peptide synthesis. 8. On the phenacyl group in the protection of the β-carboxylic function of aspartyl residues. J. Org. Chem., 43, 3071.

Bodansky, M., Tolle, J., Deshmane, S. S., Bodansky, A. (1978c) Side reactions in peptide synthesis VI. A reexamination of the benzyl group in the protection of the side chains of tyrosine and aspartic acid., Int. J. Pept. Protein Res., 12, 57–68.

Bodansky, M., Martinez, J. (1981) Side reactions in peptide synthesis. Synthesis, 333.

Bodanszky, M. (1984) Principles of Peptide Synthesis. Springer-Verlag, Berlin Heidelberg.

Brand-Williams, W., Cuvelier, M. E., Berset, C. (1995) Use of a free radical method to evaluate antioxidant activity, Lebensmittel-Wissenschaft und –Technologie/Food Science and Technology, 28, 25–30.

Brigelius, R. C., Muckel, C. (1983) Identification and quantitation of glutathione in hepatitis protein mixed disulfides and its relationship to glutathione disulfide. Biochemical Pharmacology, 32(17), 2529–2534.

Burg, D., Filippov, D. V., Hermanns, R., van der Marel, G. A., van Boom, J. H., Mulder, G. J. (2002) Peptidomimetic glutathione analogues as novel gammaGT stable GST inhibitors. Bioorg. Med. Chem., 10, 195–205.

Carpino, L. A., Shroff, H., Triolo, S. A.,Mansour, E.-S., Wenschuh, H., Albericio, F. (1993b) The 2,2,4,6,7-pentamethyldihydrobenzofuran–5-sulfonyl group (Pbf) as arginine side chain protectant. Tetrahedron Lett., 49, 7829.

Carpino, L. A. (1993a) 1-Hydroxy–7-azabenzotriazole. An effecient peptide coupling additive. J. Am. Chem. Soc., 115, 4397.

Carpino, L. A., El-Faham, A. (1995) Tetramethylfluoroformamidinium hexafluorophosphate: a rapid-acting peptide coupling reagent for solution and solid phase peptide synthesis., J. Am. Chem. Soc., 117, 5401.

Carreno, C., Mendez, M. E., Kim, H. J., Kates, S. A., Andreu, D., Albericio, F. (2000) Nsc and Fmoc Nalpha-amino protection for solid-phase peptide synthesis: a parallel study., J. Pept. Res., 56(2), 63–69.

Castro, B., Dormoy, J. R., Evin, G., Selve, C. (1975) Reactifs de couplage peptidique IV (1) – l'hexafluorophosphate de benzotriazolyl N-oxytrisdimethylamino phosphonium (B.O.P.), Tetrahedron Lett., 14, 1219.

Castro, B., Coste, J., Dufour, M.-N., Pantaloni, A. (1990) BROP: A new coupling reagent for M-methyl amino acids, in Peptides, Chemistry, Structure and Biology, Proc. 11th Am. Pept. Symp., Ed. Rivier, J. E., Marshall, G. R., ESCOM, Leiden, 900.

Chan, T.-Y., Tang, P.-L. (1996) Characterization of the antioxidant effects of melatonin and related indoleamines in vitro., J. Pineal Res., 20, 187–191.

Chan, W. C., White, P. D. (2000) Fmoc Solid Phase Peptide Synthesis, Oxford University Press.

Chance, B., Sies, H., Boveris, A. (1979) Hydroperoxide metabolism in mammalian organs., Physiol. Rev., 59, 527–605.

Chang, C.-D., Waki, M., Ahmad, M., Meienhofer, J., Lundell, E. O., Haug, J. D. (1980) Preparation and properties of Na–9-fluorenylmethyloxycarbonylamino acids bearing tert-butyl side chain protection. Int. J. Pept. Protein Res., 15, 59.

Chen, T. S., Richie, J. P., Nagasawa, H. T., Lang, C. A. (2000) Glutathione monoethyl ester protects against glutathione deficiencis due to aging and acetaminophen in mice. Mech. Ageing Dev., 120, 127–139.

Chinta, DS. J., Rajagopalan, S., Butterfield, D. A., Andersen J. K. (2006) In vitro and in vivo neuroprotection by gamma-glutamylcysteine ethyl ester against MPTP: relevance to the role of glutathione in Parkinson's disease. Neurosci. Lett., 402, 137–141.

Clancy, R. M., Levartovsky, D., Leszcynska-Piziak, J., Yegudin, J., Abramson, S. B. (1994) Nitric oxide reacts with intracellular glutathione and activates the hexose monophosphate shunt in human neutrofils: evidence for S-nitrosoglutathione as a bioactive intermediate., Proc. Natl. Acad. Sci. USA, 91, 3680–3684

Coin, I., Beyermann, M., Bienert, M. (2007) Solid-phase peptide synthesis: from standard procedures to the synthesis of difficult sequences. Nat. Protoc., 2(12), 3247–3256.

Colombo, R., Colombo, F., Jones, J. H. (1984) Acid-labile histidine side chain protection: the N(π)-t-butoxymethyl group., J. Chem. Soc., Chem. Commun., 292.

Coste, J., Le-Nguyen, D., Castro, B. (1990) PyBop: a new peptide coupling reagent devoid of toxic by-product., Tetrahedron Lett., 31, 205.

Crich, D., Sana, K. (2009) Solid-phase synthesis of peptidyl thioacids employing a 9-fluorenylmethyl thioester-based linker in conjunction with Boc chemistry. J. Org. Chem., 74(19), 7383–7388.

Deng, F. K., Mandal, K., Luisier, S., Kent, S.B., (2010) Synthesis and comparative properties of two amide-generating resin linkers for use in solid phase peptide synthesis., J. Pept. Sci., 16(10), 545–50.

Desai, M. C., Zukermann, R. N., Moos, W. H. (1994) Drug Dev. Res., 33, 174–188.

Dickinson D. A., Forman, H. J. (2002) Cellular glutathione and thiols metabolism. Biochem. Pharmacol., 64, 1019–1026.

Dringen, R. (2000) Metabolism and function of glutathione in brain. Progress in Neurobiology, 62, 649–671.

Due Larsen, B., Holm, A. (1998) Sequence-assisted peptide synthesis (SAPS)., J. Pept. Res. 52(6), 470–476.

Dölling, R., Beyermann, M., Haenel, J., Kernchen, F., Krause, E., Franke, P., Brudel, M., Bienert, M. (1994) Piperidine-mediated side product formation for Asp(Obut)-containing peptides., J. Chem. Soc., Ghem. Commun., 853–854.

Engelhard, M., Merrifield, R. B. (1978) Tyrosine protecting groups: minimization of rearrangement to 3-alkyltyrosine dyring acidolysis. J. Am. Chem. Soc., 100, 3559.

Erikson, B. W., Merrifield, R. B. (1973) Acid stability of several benzylic protecting groups used in solid-phase peptide synthesis. Rearrangement of O-benzyltyrosine to 3-benzyltyrosine. J. Amer. Chem. Soc., 95, 3750–3756.

Fagali, N., Catala, A. (2008) Antioxidant activity of conjugated linoleic acid isomers, linoleic acid and its methyl ester determined by photoemission and DPPH techniques. Biophys. Chem., 137, 56–62.

Filomeni, G., Rotilio G., Ciriolo, M. R. (2002) Cell signalling and the glutathione redox system. Biochem. Pharmacol., 64, 1057–1064

Fontana, A., Toniolo, C. (1976) The chemistry of tryptophan in peptides and proteins, in Progress in the chemistry of organic natural products., Vol. 33, Ed. Herz, W., Grisebach, H. And Kirby, G. W., p. 309.

Foti, M. C., Daquino, C., Mackie, I. D., Dilabio, G. A., Ingold, K. U. (2008) Reaction of phenols with the 2,2-diphenyl–1-picrylhydrazyl radical. Kinetics and DFT calculations applied to determine ArO-H bond dissociation enthalpies and reaction mechanism., J. Org. Chem., 73, 9270–9282.

Franco, R., Schoneveld, O. J., Pappa, A., Panayiotidis, M. I. (2007) The central role of glutathione in the pathophysiology of human diseases. Arch. Physiol. Biochem., 113, 234–258.

Franzen, H., Grehn, L., Ragnarsson, U. (1984) Synthesis, properties, and use of N^{in}-Boc-tryptophan derivatives., J. Chem. Soc., Chem. Commun., 1699.

Freinberg, R. S., Merrifield, R. B. (1975) Modification of peptides containing glutamic acid by hydrogen fluoride-anisole mixtures. γ-Acylation of anisole or the glutamyl nitrogen. J. Am. Chem. Soc., 97, 3485.

Friligou, I., Papadimitriou, E., Gatos, D., Matsoukas, J., Tselios. (2011) Microwave-assisted solid-phase peptide synthesis of the 60–110 domain of human pleiotrophin on 2-chlorotrityl resin. Amino Acids, 40(5), 1431–1440.

Fujii, N., Funakoshi, S., Sasaki, T., Yajima, H. (1977) Studies on peptides. LXXII. Examination of the N^{ε}-alkylation of lysine in the methanesulphonic acid procedure for peptide synthesis, 25, 3096.

Fujii, N., Nomizu, M., Futaki, S., Otaka, A., Funakoshi, S., Akaji, K., Watanabe, K., Yajima, H. (1986) Studies on peptides. CXXXII. Evaluation of two β-carboxyl protecting groups of aspartic acid, cycloheptyl and cyclooctyl, for peptide synthesis., Chem. Pharm. Bull., 34, 864.

Fujiwara, Y. (2000) A novel synthetic approach of tryptofan-containing cysteine peptides by regioselective disulfide bond-forming reaction using the silyl chloride-sulfoxide system. Yakugaku Zasshi, 120(2), 197–205.

Galanis, A. S., Albericio, F., Grøtli, M. (2009) Solid-phase peptide synthesis in water using microwave-assisted heating. Org. Lett. 11(20), 4488–91.

Gaullier, J. M., Lafontant, P., Valla, A., Bazin, M., Giraud, M., Santus, R. (1994) Glutathione-Peroxidase and Glutathione-Reductase Activities Towards Glutathione-Derived Antioxidants. Biochemical and Biophysical Research Communications, 203, 1668–1674.

Geiger, R., König, W. Amine protecting groups, in The Peptides, 1981, Vol. 3, Ed. Gross, E. And Meienhofer, J., J. Academic Press, New York, ch. 1.
Gozzo, A., Lesieur, D., Duriez, P., Fruchart J.-C., Teissier, E. (1999) Structure-activity relationships in a series of melatonin analogues with the low-density lipoprotein oxidation model. Free radical Biology & Medicine, 26 (11/12) 1538–1543.
Grattagliano, I., Vendemiale, G., Lauterburg, B. H. (1999) Reperfusion injury of the liver: role of mitochondria and protection by glutathione ester. J. Surg. Res., 86, 2–8.
Greene, T. W., Wuts, P. G. M., Protective Groups in Organic Synthesis, John Wiley & Sons, Inc. (1999), 3rd edition.
Grehn, L., Ragnarsson, U. (1984) A convenient method for the preparation of 1-(tert-butyloxycarbonyl)pyrroles., Angew. Chem. Int. Ed. Engl., 23, 296.
Griffith, O. W., Mulcahy, R. T. (1999) The enzymes of glutathione synthesis: gamma-glutamylcysteine synthetase., Adsvances in Enzymology & Related Areas of Molecular Biology, 73, 209–267.
Guibe, F., Dangles, O., Balavoine, G., Loffet, A. (1989) Use of an allylic anchor group and of its palladium catalyzed hydrostannolytic cleavage in the solid phase synthesis of protected peptide fragments., Tetrahedron Lett., 30, 2641.
Guizar-Sahagun, G., Ibarra, A., Espitia, A., Martinez, A., Madrazo, I., Dranco-Bourland, R. E. (2005) Glutathione monoethyl ester improves functional recovery, enhances neuron survival, and stabilizes spinal cord blood flow after spinal cord injury in rats., 130, 639–649.
Gutte, B., Merrifield, R. B. (1971) The synthesis of Ribonuclease A., J. Biol. Chem., 246, 1922.
Han, Y., Albericio, F., Barany. (1997) Occurrence and Minimization of Cysteine Racemization during Stepwise Solid-Phase Peptide Synthesis (1), (2). J. Org. Chem., 62(13), 4307–4312.
Harding, S. J., Heslop, I., Jones, J. H., Wood, M. E. (1995) The racemization of histidine in peptide synthesis: Further studies, in Peptides 1994, Proc, 23rd Eur. Pept. Symp., Ed. Maia, H. L. S., ESCOM, Kedien, p. 189.
Hojo, K., Maeda, M., Tanakamaru, N., Mochida, K., Kawasaki, K. (2006) Solid phase peptide synthesis in water VI: evaluation of water-soluble coupling reagents for solid phase peptide synthesis in aqueous media.
Hood, C. A., Fuentes, G., Patel, H., Page, K., Menakuru, M., Park, J. H. (2008) Fast conventional Fmoc solid-phase peptide synthesis with HCTU. J. Pept. Sci., 14, 97–101.
Huang, D. J., Ou, B., Prior, R. L. (2005) The chemistry behind antioxidant capacity assays., J. Agri. Food Chem., 53, 1841–1856.
Huang, K. P., Huang, F. L. (2002) Glutathionylation of proteins by glutathione disulfide S-oxide. Biochemical Pharmacology, 64(5–6), 1049–1056.
Hudson, D. (1988) Methodological implications of simultaneous solid-phase peptide synthesis. 1. Comparison of different coupling procedures. J. Org. Chem., 53, 617.
Hyde, C., Johnson, T., Sheppard, R. C. (1992) Internal aggregation during solid phase peptide synthesis. Dimethyl sulfoxide as a powerful dissociating solvent. J. Chem. Soc., Chem. Commun., 1573.
Isidro, A., Latassa, D., Giraud, M., Alvarez, M., Albericio, F. (2009) 1,2-Dimethylindole–3-sulfonyl (MIS) as protecting group for the side chain of arginine., Org. Biomol. Chem., 7(12) 2565–2569.
Jimenez-Escrig A., Jimenez-Jimenez, I., Sanchez-Moreno, C., Saura-Calixto, F. (2000) Evaluation of free radical scavenging of dietary carotenoids by the stable radical

2,2-diphenyl–1-picrylhydrazyl. Journal of the Science of Food and Agriculture, 80, 1686–1690.
Johnson, T., Quibell, M., Owen, D., Sheppard, R. C. (1993) A reversible protecting group for the amide bond in peptides. Use in the synthesis of 'difficult sequences'. J. Chem. Soc., Chem. Commun., 369.
Jones, J. H., Ramage, W, I., Witty, M. J. (1980) Mechanism of racemization of histidine derivatives in peptide synthesis., J. Pept. Protein Res., 15, 301.
Kaljuste, K., Unden, A. (1996) Solid phase synthesis of peptide aminoalkylamides using an allyl linker., Tetrahedron Lett., 37, 3031.
Kaminski, Z. J., Kolesinska, B., Kolesinska, J., Sabatini, G., Chelli, M., Rovero, P., Blaszczuk, M., Glowka, M. L., Papini, A. M. (2005) N-triazinylammonium tetrafluoroborates. A new generation of efficient coupling reagents useful for peptide synthesis. J. Am. Chem. Soc., 127(48), 16912–16920.
Karelson, E., Mahlapuu, R. Zilmer, M., Soomets, U., Bogdanovic, N., Langel, Ü. (2002) Possible signaling by glutathione and its novel analogue through potent stimulation of fontocortical G proteins in normal aging and in Alzheimer's disease. Annals of the New York Academy of Sciences, 973, 537–540.
Kawasaki, K., Murakami, T., Koshino, K., Namikawa, M., Maeda, M., Hama, T., Mayumi, T. (1994) Amino acids and peptides. XX. Preparation of β-cyclododecyl aspartate and its application to synthesis of fibronectin- and laminin-related peptides. Chem. Pharm. Bull., 42, 792.
Kent, S. B. H., Merrifield, R. B. (1981) The role of crosslinked resinsupports in enhancing the solvation and reactivity of self-aggreagating peptides: solid phase synthesis of acyl carrier protein (65–74), in Peptides 1080, Proc. 16th Eur. Pept. Symp., Ed. Brunfeldt, K., Scriptor, Copenhagen, p. 328.
Kent, S. B. H. (1985) Difficult sequences in stepwise peptide synthesis: common molecular origins in solution and solid phase?, In Peptides: Structure and Function, Proc. 9th Am. Pept. Symp., Ed. Deber, C. M., Hruby, V. J., Kopple, K. D., Pierce Chemical Co., Rockford, IL, p 407.
Kent, S. B. H., Alewood, D., Alewood, P., Baca, M., Jones, A., Schnölzer, M. (1992) Total chemical synthesis of proteins: evaluation of solid phase synthetic methods illustrated by the total chemical synthesis of the HUIV–1 protease, In Innovation and Perspectives in Solid Phase synthesis, Ed. Epton, R., Intercept Ldt., Andover, p 1.
Kiso, Y., Ito, K., Nakamura, S., Kitagawa, K., Akita, T., Moritoki, H. (1979a) A deblocking method using thioether-sulfonic acid systems. Application to the synthesis of Met-enkaphalin. Chem. Pharm. Bull., 27, 1472.
Kiso, Y., Nakamura, S., Ito, K., Ukawa, K., Kitagawa, K., Akita, T., Moritoki, H. (1979b) Deprotection of O-methyltyrosine by a „push-ball" mechanism using the thioanisoletrifluoromethanesulfonic acid system. Application to the convenient synthesis of a potent N-methylenkephalin derivative. J. Chem. Soc., Chem. Commun., 971–972.
Kiso, Y., Ukawa, K., Akita, T. (1980) Efficient removal of N-benzyloxycarbonyl group by a „push-ball" mechanism using thioanisoletrifluoromethanesulfonic acid, exemplified by a synthesis of Met-enkephalin., J. Chem. Soc., Chem. Commun., 101.
Knorr, R., Trzeciak, A., Bannwarth, W., Gillessen, D. (1989) New coupling reagents in peptide chemistry. Tetrahedron Lett., 30, 1927.

Kortemme, T., Creighton, T. E. (1995) Ionisation of cysteine residues at the termini of model alpha-helical peptides. Relevance to unusual thiol pKa values in proteins of the thioredoxin family., J. Mol. Biol., 253, 799–812.
Kunze, T., Heps, S. (2000) Phosphono analogs of glutathione: inhibition of glutathione transferases, metabolic stability, and uptake by cancer cells. Biochem. Pharmacol., 59, 973–981.
Kunz, H., Dombo, B. (1988) Solid phase synthesis of peptides and glycopeptides on polymeric supports with allylic anchor groups. Angew. Chem. Int. Ed. Engl., 27, 711.
König, W., Geiger, R. (1970a) Eine neue Methode zur Synthese von Peptiden: Aktivierung der Carboxylgruppe mit Dicyclohexylcarbodiimid unter Zusatz von 1-Hydroxybenzotriazolen. Chem. Ber., 103, 788.
König, W., Geiger, R. (1970b) Racimisierung bei Peptidsynthesen. Chem. Ber., 103, 2024.
Land, T., Langel, Ü., Löw, M., Berthold, M., Undèn, A., Bartfai. (1991) Linear and cyclic N-terminal galanin fragments and analogs as ligands at the hypothalamic galanin receptor. Int. J. Pept. Protein Res., 38, 267–272.
Langel Ü., Land, T., Bartfai, T. (1992) Design of chimeric peptide ligands to galanin receptors and substance P receptors. Int. J. Pept. Protein, 39, 516–522.
Larsen, B. D., Holm, A. (1994) Incomplete Fmoc deprotection in solid phase synthesis of peptides. Int. J. Protein Res., 43, 1.
Lauer, J., L., Fields, C. G., Fields, G. B. (1994) Sequence dependence of aspartimide formation during 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis. Letters in Peptide Science, 1, 197–205.
Lenard, J., Robinson, A. B. (1967) Use of hydrogen fluoride in Merrifield solid-phase peptide synthesis. J. Am. Chem. Soc., 89, 181.
Litwinenko, G., Ingold, K. U. (2005) Abnormal solvent effects on hydrogen atom abstraction. 3. Novel kinetics in sequential proton loss electron transfer chemistry., J. Org. Chem, 70, 8982–8990.
Lo, T. W.; Thornalley, P. J. (1992) Inhibition of proliferation of human leukaemia 60 cells by diethyl esters of glyoxalase inhibitors in vitro. Biochem. Pharmacol., 44, 2357–2363.
Locigno, R., Castronovo, V. (2001) Reduced glutathione system: Role in cancer development, prevention and treatment. Int. J. Oncol., 19, 221–236.
Loffet, A. (1967) Synthesis of a pentapeptide by the Merrifield method. Experientia, 23, 406.
Loffet, A., Zhang, H. X. (1993) Allyl-based groups for side-chain protection of amino-acids. Int. J. Pept. Protein Res., 42, 346–351.
Loffredo, C., Assunção N. A., Gerhardt, J., Miranda, M. T. (2009) Microwave-assisted solid-phase peptide synthesis at 60 degrees C: alternative conditions with low enantiomerization. J. Pept. Sci., 15(12), 808–817.
Loidl, G., Dick, F., Mergler, M., Schoenleber, R. O. (2009) Optimized coupling protocols for the incorporation of Cys derivatives during Fmoc-SPPS. Adv. Exp. Med. Biol., 611, 163–164.
Lucente, G., Luisi, G., Pinnen, F. (1998) Design and synthesis of glutathione analogues. Farmaco, 53, 721–735.
Lundt, B. F., Johansen, N. L., Volund, A., Markussen, J. (1978) Removal of t-butyl and t-butoxycarbonyl protecting groups with trifluoroacetic acid. Int. Pept. Protein Res., 12, 258.

Maestroni, G. J. M., Conti, A., Reiter, R. J. (1997) Therapeutic potential of melatonin. Ed. Karger, Basel.

Magalhães, L. M., Segundo, M. A., Reis, S., Lima, J. L., Toth, I. V., Rangel, A. O. (2007) Automatic flow system for sequential determination of ABTS*+ scavenging capacity and Folin-Ciocalteu index: a comparative study in food products., Anal. Chim. Acta, 592(2), 193–201.

Magalhães, L. M., Segundo, M. A., Reis, S., Lima, J. L. (2008) Methodological aspects about in vitro evaluation of antioxidant properties. Anal. Chim. Acta, 613, 1–19.

Marshall, K.-A., Reiter, R. J., Poeggeler, B., Aruoma, O. I., Halliwell, B. (1996) Evaluation of the antioxidant activity of melatonin in vitro., Free Radic. Biol. Med., 21, 307–315.

Marshall, T. R., Merrifield, R. B. (1965) Synthesis of angiotensins by the solid-phase method. Biochemistry, 4, 2394.

Mates, M. (2000) Effects of antioxidant enzymes in the molecular control og reactive oxygen specific toxicology. Toxicology, 153, 83–104

Matsueda, G. R., Stewart, J. M. (1981) A p-methylbenzhydrylamine resin for improved solid-phase synthesis of peptide amides. Peptides, 2, 45.

Meister, A., Anderson, M. E. (1983) Glutathione. Annu. Rev. Biochem., 52, 711.

Meister, A. (1994) Glutathione-ascorbic acid antioxidant system in animals. J. Biol. Chem., 269, 9397–9400.

Mergler, M., Dick, F., Sax, B., Schwindling, J., Vorherr, T. H. (2001) Synthesis and application of Fmoc-His(3-Bum)-OH., J. Pept. Sci., 7(9), 502–510.

Mergler, M., Dick, F., Sax, B., Weiler, P., Vorherr, T. (2003) The aspartimide problem in Fmoc-based SPPS. Part I., J. Pept. Sci., 9(1), 36–46.

Mergler, M., Dick, F. (2005) The aspartimide problem in Fmoc-based SPPS. Part III., J. Pept. Sci., 11, 650–657.

Merrifield, R. B. (1963) Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J. Am. Chem. Soc., 85(14), 2149.

Merrifield, R. B. (1964) Solid phase peptide synthesis. II. The synthesis of bradykinin. J. Am. Chem. Soc., 86, 304.

Merrifield, R. B. (1967) New approches to the chemical synthesis of peptides. Recent Prog. Horm. Res., 23, 451.

Mitchell, A. R., Erikson, B., Ryabtsev, M. N., Hodges, R. S., Merrifield, R. B. (1976) tert-Butoxycarbonylaminoacyl–4-(oxymethyl)phenylacetamidomethyl-resin, a more acid-resistant support for solid-phase peptide synthesis. J. Am. Chem. Soc., 98, 7357.

Mitchell, A. R. (2008) Studies in solid-phase peptide synthesis: a personal perspective. Biopolymers. 90(3), 215–233.

Mohammadi, M., Yazdanparast, R. (2009) Methoxy VO-salen complex: In vitro anti-oxidant activity, cytotoxicity evaluation and protective effects on CCl(4)-induced oxidative stress in rats. Food. Chem. Toxicol., 47, 716–721.

Molyneux, P. (2004) The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J. Sci. Technol., 26(2), 211–219.

Montanari, V., Kumar, K. (2004) Just add water: a new florous capping reagent for facile purification of peptides synthesized on the solid phase., J. Am. Chem. Soc., 126(31), 9528–9529.

Moss, J. A., (2005) Guide for resin and linker selection in solid-phase peptide synthesis. Curr Protoc Protein Sci. Chapter 18, Unit 18.7.

Murray J. K., Aral, J., Miranda, L. P. (2011) Solid-phase peptide synthesis using microwave irradiation. Methods Mol. Biol., 716, 73–88.

Ni, S., Zhang, H., Huang, W., Zhou, J., Qian, H., Chen, W. (2010) The application of an aryl hydrazine linker prevents beta-elimination side products in the SPPS of C-terminal cysteine peptides. J Pept Sci. 16(6), 309–13.

Nicolas, E., Pedroso, E., Giralt, E. (1989) Formation of aspartimide peptides in Asp-Gly sequences. Tetrahedron Lett., 30, 497–500.

Nishiuchi, Nishio, H., Inui, T., Kimura, T., Sakakibara, S. (1996) N^{in}-Cyclohexyloxy-carbonyl group as a new protecting group for tryptofan. Tetrahedron Lett., 37, 7529.

Nishiyama, Y., Ishizuka, S., Shikama, S., Kurita, K. (2001) An Efficient Synthesis of N-tert-Butoxycarbonyl-O-cyclohexyl-L-tyrosine., Chem. Pharm. Bull. 49(2) 233–235

Noble, R. L., Yamashiro, D., Li, C. H. (1976) Synthesis of a nonadecapeptide corresponding to residue 37–55 of ovine prolactin. Detection and isolation of the sulfonium form of methionine-containing peptides. J. Am. Chem. Soc., 98, 2324.

Obrecht, D., Villalgordo, J. M. (1998) Solid-Supported Combinatorial and Parallel Synthesis of Small-Molecular-Weight Compound Libraries, Elsevier Science.

Ohno, M., Tsukamoto, S., Sato, S., Izumiya, N. (1973) Improved solid-phase synthesis of tryptophan-containing peptides. II. Use of N^{α}-t-butyloxycarbonyl-N^{i}-formyl-tryptophan. Bull. Chem. Soc. Jpn., 46, 3280.

Okada, Y., Wang, J., Yamamoto, T., Mu, Y. (1996a) Development of a new N^{π}-protecting group for histidine, N^{π}-adamantyloxymethylhistidine. Chem. Pharm. Bull., 44, 871.

Okada, Y., Wang, J., Yamamoto, T., Mu, Y. (1996b) Synthesis of a N^{π}-2-adamantyloxymethylhistidine, His(N^{π}-2-Adom), and its evaluation for peptide synthesis. J. Chem. Soc., Perkin Trans. I., 753.

Olney, J. W., Zorumski, C., Price, M. T., Labruyere, J. (1990) L-cysteine, a bicarbonate-sensitive endogenous excitotoxin. Science, 248, 596–599.

Ondetti, M. A., Deer, A., Sheenan, J. T., Pluscer, J., Kocy, O. (1968) Side reactions in the synthesis of peptides containing the aspartylglycyl sequence. Biochemistry, 7, 4069.

Ortolani, O., Conti, A., De Gaudio, A. R., Moraldi, E., Cantini, Q., Novelli, G. (2000) The effect of glutathione and N-acetylcysteine on lipoperoxidative damage in patients with early septic shock. Am. J. Respir. Crit. Care. Med., 161, 1907–1911.

Palasek, S. A., Cox, Z. J., Collins, J. M. (2007) Limiting racemization and aspartimide formation in microwave-enhanced Fmoc solid phase peptide synthesis., J. Pept. Sci., 13(3), 143–148.

Paolicchi, A., Dominici, S., Pieri, L., Maellaro, E., Pompella, A. (2002) Glutathione catabolism as a signaling mechanism. Biochem. Pharmacol., 64, 1027–1035.

Paradisi, M. P., Mollica, A., Cacciatore, I., Di Stefano, A., Pinnen, F., Caccuri, A. M., Ricci, G., Dupre, S., Spirito, A., Lucente, G. (2003) Proline-glutamate chimeras in isopeptides. Synthesis and biological evaluation of conformationally restricted glutathione analogues. Bioorg. Med. Chem., 11, 1677–1683.

Pastore, A., Federici, G., Bertini, E., Piemonte, F. (2003) Analysis of glutathione: implication in redox and detoxification. Clin. Chim. Acta, 333(1), 19–39.

Pedersen, S. L., Sørensen, K. K., Jensen, K. J. (2010) Semi-automated microwave-assisted SPPS: Optimization of protocols and synthesis of difficult sequences. Biopolymers. 94(2), 206–212.

Pugh, K. C., York, E. H., Stewart, J. M. (1992) Effects of resin swelling and substitution on solid phase synthesis., Int. J. Pept. Protein Res., 40, 208

Ragnarsson U., Karlsson, S., Sandberg, B. (1971) Studies on the coupling step in solid phase peptide synthesis. Some preliminary results from competition experiments. Acta Chem. Scand., 25, 1487.

Rajasekaran, N. S., Sathyanarayanan, S., Devaraj, N. S., Devaraj, H. (2005) Chronic depletion of glutathione (GSH) and minimal modification of LDL in vivo: its prevention by glutathione monoester (GME) therapy. Biochim. Biophys. Acta, 1741, 103–112.

Ramachandran, J., Li, C. H. (1962) Preparation of crystalline N^G-tosylarginine derivatives. J. Org. Chem., 27, 4006.

Ramachandran, J., Li, C. H. (1963) The synthesis of L-valyl-L-lysyl-L-valyl-L-tyrosyl-L-proline., J. Org. Chem.,28, 173.

Ramage, R., Green, J. (1987) NG–2,2,5,7,8-pentamethylchroman–6-sulfonyl-L-arginine: a new acid labile derivative for peptide synthesis., Tetrahedron Lett., 28, 2287.

Ramage, R., Jiang, L., Kim, Y. D., Shaw, K., Park, J. L., Kim, H. J. (1999) Comparative studies of Nsc and Fmoc as N(alpha)-protecting group for SPPS., J. Pept. Sci., 5(4), 195–200.

Reed, T. T., Owen, J., Pierce, W. M., Sebastian, A., Sullivan, P. G., Butterfield, D. A. (2009) Proteomic identification of nitrated brain proteins in traumatic brain-injured rats treated postinjury with gamma-glutamylcysteine ethyl ester: insights into the role of elevation of glutathione as a potential therapeutic strategy for traumatic brain injury. J. Neurosci. Res., 87, 408–417.

Richardson, G., Benjamin, N. (2002) Potential therapeutic uses for S-nitrosothiols. Clin. Sci. (Lond), 102, 99–105.

Rosen, L. C., Brown, J., Laxa, B., Boulos, L., Reiswig, L., Henner, W. D., Lum, R. T., Schow, S. R., Maack, C. A., Keck, J. G., Mascavage, J. C., Dombroski, J. A., Gomez, R. F., Brown, G. L. (2003) Phase I study of TLK286 (glutathione S-transferase P1–1 activated glutathione analogue) in qadvanced refractory solid malignancies. Clin. Cancer Res., 9, 1628–1638.

Rusell, K. E. (1954) J. Phys. Chem., 58, 437–439.

Sabatino, G., Papini, A. M., (2008) Advances in automatic, manual and microwave-assisted solid-phase peptide synthesis. Curr. Opin. Drug Discov. Devel., 11(6), 762–770.

Saez, G. T., Bannister, W. H., Bannister, J. V. (1990) Free radical and thiol compounds – the role of glutathione against free radical toxicity. In: Vina, J. (Ed.), Glutathione: Metabolism and Physiological Functions. CRC Press, Boca Raton, FL. USA, 237–254.

Sakakibara, S., Shin, K. H., Hess, G. P. (1962) An approach to the specific cleavage of peptide bonds. I. The acyl migration in dipeptides containing hydroxyamino acids in anhydrous hydrogen fluoride. J. Am. Chem. Soc., 84, 4921.

Sakakibara, S., Shimonishi, Y. (1965) A new method for releasing oxytocin from fully-protected nona-peptides using anhydrous hydrogen fluoride. Bull. Chem. Soc. Jpn., 38, 1412.

Sanches-Moreno, C., Larrauri, J. A., Saura-Calixto, F. (1998) A procedure to measure the antiradical efficiency of polyphenols. J. Sci. Food Agric., 76, 270–276.

Sanchez-Moreno, C. (2002) Review: methods used to evaluate the free radical scavenging activity in foods and biological systems. Food Sci. Tech. Int., 8(3), 121–137.

Schafer, F. Q., Buettner, GT. R., (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic. Biol. Med., 30, 1191–1212.

Schneggenburger, P. E., Worbs, B., Diederichsen, U. (2010) Azide reduction during peptide cleavage from solid support – the choice of thioscavenger? J. Pept. Sci., 16(1), 10–14.

Schnölzer, M., Kent, S. B. H. (1992) Constructing proteins by dovetailing unprotected synthetic peptides: Backbone-engineered HIV protease. Science, 256, 221.

Schulz, J. B., Lindenau, J., Seyfried, J., Dichgans, J. (2000) Glutathione, oxidative stress and neurodegeneration. Eur. J. Biochem., 267, 4904–4911.

Schön, I., Kisfaludy, L. (1979) Formation of aminosuccinyl peptides during acidolytic deprotection followed by their transformation to piperazine–2,5-dione derivatives in neutral media. Int. J. Pept. Protein Res., 14, 485.

Schwarz, K., Bertelson, G., Nissen, L. R., Gardner, P. T., Heinonen, M. I., Hopia, A., Huynh-Ba, T., Lambelet, P., McPhail, D., Skibsted, L. H., Tijburg, L. (2001) Investigation of plant extracts for the protection of processed foods against lipid oxidation. Comparison of antioxidant assays based on radical scavenging, lipid oxidation and analysis of the principal antioxidant compounds. Eur. Food Res. Technol., 212, 319–328.

Shan, X., Aw, T. Y., Jones, D. P. (1990) Glutathione-dependent protection against oxidative injury. Pharmacol. Ther., 47(1), 61–71.

Sheenan, J. C., Hess, G. P. (1955) A new method of forming peptide bonds. J. Am. Chem. Soc., 77, 1067.

Sheehan, J. C., Hasspacher, K. J., Yeh, Y. L. (1959) Activated cyclic derivatives of amino acids. J. Am. Chem. Soc., 81, 6086.

Sheh, L., Chen, B. L., Chen, C. F. (1990) Synthesis of cyclic peptide homologs of glutathione as potential antitumor agents. Int. J. Pept. Protein Res., 35, 55–62.

Sheppard, R. C. (1977) Solid phase peptide synthesis – A reassessment, in Molecular Endocrinology, Ed. MacIntyre, I., Szelke, M., Elsevier/North-Holland Biomedical Press, New York, 43.

Sieber, P., Riniker, B. (1987) Protection of histidine in peptide synthesis: a reassessment of the trityl group. Tetrahedron Lett., 28, 6031.

Singh, S. P., Wishnok, J. S., Keshive, M., Deen, W. M., Tannenbaum, S. R. (1996) The chemistry of the S-nitrosoglutathione/glutathione system. Proc. Natl. Acad. Sci. USA, 14428–14433.

Soomets, U., Zilmer, M., Langel, Ü. (2005) Manual Solid-Phase Synthesis of Glutathione analogues: A Laboratory-Based short course. In: Howl, J., editors. Peptide Synthesis and Applications. Totowa, New Jersey: Humana Press Inc.; p 241–257.

Steinauer, R., Chen, F. M., Benoiton, N. L. (1989) Studies on racemization associated with the use of benzotriazol-1-yl-tris(dimethylamino)phosphonium hexafluorophosphate (BOP)., Int. J. Pept. Protein Res., 34(4), 295–298.

Stewart, J. M. (1981) The Peptides, Vol. 3, Protection of Functional Groups in Peptide Synthesis. ed. Gross, E., Meienhofer, J., Academic Press, New York, 169–201.

Stewart, J. M., Klis, W. A. (1990) Polystyrene-based solid phase peptide synthesis: the state of the art, in Innovation and Perspectives in Solid Phase Synthesis, Ed. Epton, R., SPCC (UK) Ldt, Birmingham, UK, 1.

Strickland, S., Palmer, G., Massey, V. (1975) Determination of dissociation constants and specific rate constants of enzyme-substrate (or protein-ligand) interactions from rapid reaction kinetic data., J. Biol. Chem., 250, 4048–4052.

Zeevalk, G. D., Manzino, L., Sonsalla, P. K., Bernard, L. P. (2007) Characterization of intracellular elevation of glutathione (GSH) with glutathione monoethyl ester and GSH in brain and neuronal cultures: relevence to Parkinson's disease. Exp. Neurol., 203, 512–520.

Zhang, S., Chai, F. Y., Yan, H., Guo, Y., Harding, J. J. (2008) Effects of N-acetylcysteine and glutathione ethyl ester drops on streptozotocin-induced diabetic cataract in rats. Mol. Vis., 14, 862–870.

Zilmer, M., Soomets, U., Rehema, A., Langel, Ü. (2005) The Glutathione System as an Attractive Therapeutic Target. Drug Design Reviews – Online, 2, 121–127.

Tam, J. P., Wong, T.-W., Rieman, M. W., Tjoeng, F.-S., Merrifield, R. B. (1979) Cyclo-hexyl ester as a new protecting group for aspartyl peptides to minimize aspartimide formation in acidic and basic treatments. Tetrahedron Lett., 42, 4033–4036.

Tam, J. P. (1985) Enhancement of coupling efficiency in solid phase peptide synthesis by elevated temperature, in Peptides: structure and Function, Proc. 9th Am. Pept. Symp., Ed. Deber, C. M., Hruby, V. J., Kopple, K. D., Pierce Chemical Co., Rockford, IL, 423–425.

Tam, J. P., Merrifield, R. B. (1987) In The Peptides, Vol. 9, eds. Underfreund, S., Meienhofer, J., Academic Press, New York, 185–248.

Tam, J. P. (1988) Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. Proc. Natl. Acad. Sci. USA, 85, 5409.

Thaler, A., Seebach, D. & Cardinaux, F. (1991) Improvement of degree of resin swelling and of efficiency of coupling in solid-phase synthesis. Helv. Chim. Acta, 74, 628–643.

Thieriet, N., Guibe, F., Albericio, F. (2000) Solid-phase peptide synthesis in reverse (N $\rightarrow$ C) direction., Org. Lett., 2(13), 1815–1817.

Townsend, D. M., Tew, K. D., Tapiero, H. (2003) The importance of glutathione in human disease. Biomed. Pharmacother. 57, 145–155.

Valencia, E., Hardy, G. (2001) Glutathione – nutritional and pharmacologic viewpoints: Part V. Nutrition., 17(11–12), 978.

Veber, D. F., Milkowski, J. D., Denkewalter, R. G., Hirschmann, R. (1968) The synthesis of peptides in aqueous medium. IV. A novel protecting group for cysteine. Tetrahedron Lett., 3057.

Wang, S.-S. (1973) P-Alkoxybenzyl alcohol resin and p-alkoxybenzyloxycarbonyl-hydrazide resin for solid phase synthesis of protected peptide fragments. J. Amer. Chem. Soc., 95, 1328.

White, P.D. (1994) Protected derivatives of tryptophan, processes for their preparation and their use for the preparation of dipeptide, polypeptide or protein structures. US Pat. 5 300 651.

Winterbourn, C. C., Metodiewa, D. (1994) The reactions of superoxide with reduced glutathione. Arch. Biochem. Biophys., 314, 284–290.

Wu, Z., Minhas, G. S., Wen, D., Jiang, H., Chen, K., Zimniak, P., Zheng, J. (2004) Design, synthesis, and structure-activity relationships of haloenol lactones: site-directed and isozyme-selective glutathione S-transferase inhibitors. J. Med. Chem., 47, 3282–3294.

Yajima, H., Fujii, N., Ogawa, H., Kawatani, H. (1974) Trifluoromethanesulphonic acid, as a deprotecting reagent in peptide chemistry. J. Chem. Soc., Chem. Commun., 107.

Yajiama, H., Takeyama, M., Kanaki, J., Nishimura, O., Fujino, M. (1978) Studies on peptides. LXXX. N^G-mesitylene–2-sulfonylarginine. Chem. Pharm. Bull., 26, 3752.

Yajima, H., Funakoshi, S., Akaji, K. (1985) Current contributions of peptide synthesis to studies on brain-gut-skin triangle peptides. Int. J. Pept. Protein Res., 26, 337.

Yamamoto, M., Sakamoto, N., Iwai, A., Yatsugi, S., Hidaka, K., Noguchi, K., Yuasa, T. (1993) Protective actions of YM737, a new glutathione analog, against cerebral ishemia in rats. Res. Commun. Chem. Pathol. Pharmacol., 81, 221–232.

Yamashiro, D., Li, C. H. (1973) Protection of tyrosine in solid-phase peptide synthesis. J. Org. Chem., 38, 591–592.

Yamoto, M. S. N., Iwai, A. (1993) Protective actions of YM737, A new glutathione analog, against cerebral ischemia in rats. Research Communication in Chemical Pathology and Pharmacology., 81, 221–232.

Yang, Y., Sweeney, W. V., Schneider, K., Thörnqvist, S., Chait, B. T., Tam, J. P. (1994) Aspartimide formation in the base-driven 9-fluorenylmethoxycarbonyl chemistry. Tetrahedron Lett., 35, 9689–9692.

Yoshizawa-Kumagaye, K., Nishiuchi, Y., Nishio, H., Kimura, T. (2005) Amino acid deletion products resulting from incomplete deprotection of the Boc group from Npi-benzyloxymethylhistidine residues during solid-phase peptide synthesis. J. Pept. Sci., 11(8), 512–515.

Yue, T. L., McKenna, P. J., Lysko, P. G., Gu, J. L., Lysko, K. A., Ruffolo, R. R., Jr., Feuerstein, G. Z. (1994) SB 211475, a metabolite of carvedilol, a novel antihypertensive agent, is a potent antioxidant. Eur. J. Pharmacol., 251, 237–243.

SUMMARY IN ESTONIAN

Uued glutatiooni analoogid ja nende antioksüdatiivne toime

Glutatioon (GSH) on madalmolekulaarne tripeptiid, mis omab antioksüdantses kaitses olulist rolli. Käesoleva töö raames disainiti ja sünteesiti rida GSH analooge ning mõõdeti ja võrreldi nende antioksüdantseid omadusi nii GSH-ga kui omavahel. Omaduste ja struktuuri omavaheliste seoste leidmiseks modifitseeriti glutatiooni struktuuri. Glutatiooni analoogide disainimisel kasutati järgmisi erinevaid võimalusi: neljanda aminohappe lisamine GSH molekulile, GSH ahelas γ-glutamüüljäägi asendamine α-glutamüüljäägiga, L-aminohapete asemel D-aminohapete kasutamine ja C-terminaalse karboksüülrühma amiidimine. Neljanda aminohappena kasutati kõige enam O-metüül-L-türosiini, et suurendada ühendite antioksüdatiivseid omadusi. Lisaks tetrapeptiididele sünteesiti ja iseloomustati ka kahte tripeptiidi: α-GSH, mis sisaldab α-glutamüüljääki, ja α-GOH, milles tsüsteiinijääk on asendatud seriinijäägiga.

Antud töö põhjal võib järeldada, et uute GSH analoogide disainimise tulemusena saadud peptiidid suurendavad hüdroksüülradikaali püüdmisvõimet võrreldes GSH-ga. γ-glutamüülrühma asendamine α-glutamüülrühmaga parandas oluliselt UPF peptiidide hüdroksüülradikaali püüdmisomadusi, kusjuures teised struktuurimodifikatsioonid ei andnud nii häid efekte. DPPH radikaali elimineerimisel on glutatioonil veidi madalam EC_{50} väärtus kui kõikidel UPF peptiididel. Võrreldes aga antiradikaalseid efektiivsusi, mille arvutamisel on arvesse võetud ka radikaali elimineerimise kiirus, ületavad UPF peptiidid GSH, kusjuures UPF17 on uuritud peptiididest kõige efektiivsem. Kineetiliselt iseloomustati GSH ja selle α-glutamüülderivaadi poolt DPPH radikaali püüdmise mehhanismi ja leiti, et selles protsessis ilmneb mittekovalentse kompleksi tekke staadium. Kompleksi teke on erinev GSH ja tema α-glutamüülderivaadi korral, mis selgitab nende ainete antioksüdantsete omaduste erinevusi mudelkatses.

Antud töös on ka näidatud, et UPF peptiidid on suhteliselt stabiilsed. Nende dimeeride usaldusväärseid koguseid oli võimalik detekteerida peale 48-tunnilist seismist füsioloogilises lahuses ja enam kui pool peptiidi hulgast oli monomeerses vormis pärast ühte nädalat. Saadud tulemused koos UPF peptiidide andmetega nende mittetoksilisusest lubavad pidada käesolevas dissertatsioonis uuritud ühendeid võimalikeks ravimeellasteks.

ACKNOWLEDGEMENTS

First I would like to express my deepest gratitude to my supervisors, Professor Ülo Langel, Professor Ursel Soomets and Professor Jaak Järv for their guidance, continuous support and optimism supporting me to work in science of biochemistry and finish my thesis.

I am grateful to my dear colleagues at the Chair of Organic Chemistry for their good company, and all the staff members of the Biochemistry Institute for valuable help during my experimental work.

I wish to thank all my co-authors, especially Riina Mahlapuu for fruitfull discussions and useful advises in the lab and for correcting this thesis.

Finally, my warmest thank to my sons Igor, Alar, Rait and their families for their love and bringing sunshine to my life.

The financal support from the Estonian Science Foundation (Grants No. 6503, 7856, SF0180064s08) and from the graduate school "Functional Materials and Processes" receiving funding from the European Social Fund under project 1.2.0401.09-0079 in Estonia.

PUBLICATIONS

CURRICULUM VITAE

Säde Viirlaid

Born:	07.06.1951, Orava, Estonia
Citizenship:	Estonian
Address:	Institute of Chemistry, University of Tartu, Ravila 14a, 50411, Tartu, Estonia
Phone:	+372 5800 2777
E-mail:	sade.viirlaid@ut.ee

Education

2007	PhD student, Institute of Chemistry, University of Tartu
1995	Master of Sciences (MSc, organic chemistry) University of Tartu
1974	BSc chemistry (organic chemistry)
1969–1974	Student, Department of Chemistry, Faculty of Physics and Chemistry, University of Tartu

Professional employment

2008–	Institute of Organic Chemistry, lecturer; program manager of chemistry;
1999–2007	Institute of Organic and Bioorganic Chemistry, lecturer;
1993–1999	Institute of Organic Chemistry, assistant;
1987–1993	Laboratory of Bioorganic Chemistry, senior engineer;
1978–1987	Laboratory of Organic Synthesis, senior engineer;
1974–1978	Department of Organic Chemistry, senior engineer

Main scientific publications

1. **S. Viirlaid**, A. Tuulmets. Effect of Solvents on Grignard Reaction. XI Anisole, Reakts. Sposobn. Org. Soedin. 1974, 11 (1), 65.
2. **S. Viirlaid,** S. Kurrikoff, A. Tuulmets. Effect of Solvents on Grignard Reaction. XII The Reaction of Diphenylmagnesium with Bensophenone, Reakts. Sposobn. Org. Soedin. 1974, 11 (1), 73.
3. V. Buleza, I. Bokotei, U. Mäeorg, M. Babidorich, A. Sorochinskaya, S. Kolonistova (**S. Viirlaid**). Sex Pheromone *Synanthedon myopaeformis* (Lepitoptera: *Aegiriidae*): Biological Activity, *Abstracts* 1990, 314(4), 1002–6.
4. V. Buda, U. Mäeorg, V. Karalius, G.H.L. Rothschild, S. Kolonistova (**S. Viirlaid**), P. Ivinskis, R. Mozuraitis. C_{18}-Dienes as attractants for Eighteen

Clearwing (*Sessidae*), Tineid *(Tineidae*) and Choreutid (*Choreutidae*) Moth Species. *J. Chem. Ecol.,* 1993, 19(4), 799–813.

5. U. Mäeorg, K. Kallas, **S. Viirlaid**, T. Pehk, A. Meriste. Behavior of Some Enynols and One Diynol in Super Basic Systems $MNH(CH_2)_2NH_2$/ $H_2N(CH_2)_2NH_2$, *Proc. Estonian Acad.Sci. Chem.,* 1996, 45, 3/4, 134–139.
6. U. Mäeorg, E. Loodmaa, **S. Viirlaid**, New Activated Zn-Cu Catalyst-Superior Tool for the Partial Hydrogenation of Triple Bond. *Electronic Conference on Trend in Organic Chemistry*, ECTOC. Jun–July, 1995. http://www.ch.ic.ac.uk:807.ectoc_syn.html. CD-ROM, Published by Royal Society of Chemistry, 1996.
7. U. Mäeorg, **S. Viirlaid**, Ü. Kelder, K. Kallas, S. Mäeorg Convenient Method for the Trimethylsilylation of Acetylenic compounds. *Electronic Conference of Metallorganic Chemistry,* ECTOC-3, Jun-July, 1997.
8. U. Mäeorg, **S. Viirlaid**, H. Hagu, H. D. Verkruijsse, L. Brandsma. On the in situ trimethylsilylation of zinc acetylides. J. Organomet. Chem., 2000, 601, 341–342.
9. L. Adlerz, U. Soomets, L. Holmlund, **S. Viirlaid**, Ü. Langel, K. Iverfeldt. Down-regulation of amyloid precursor protein by peptide nucleic acid oligomer in cultured rat neurons and astrocytes, Neuroscience Letters 336, 2003, 55–59.
10. P. Põder, M. Zilmer, J. Starkopf, J. Kals, A. Talonpoika, A. Pulges, Ü. Langel, T. Kullisaar, **S. Viirlaid**, R. Mahlapuu, A. Zarkovski, A. Arend, U. Soomets. An antioxidant tetrapeptide UPF1 has a neuroprotective effect in transient global brain ischemia. Neuroscience Letters, 2004, 370 (1), 45-50.
11. Merike Vaher, **Säde Viirlaid**, Kersti Ehrlich, Riina Mahlapuu, Jüri Jarvet, Ursel Soomets, Mihkel Kaljurand, Characterization of the antidoxidative activity of novel neuropeptides by using capillary electrophoresis. Electrophoresis 13 (2006);
12. K. Ehrlich, **S. Viirlaid**, R. Mahlapuu, K. Saar, T. Kullisaar, M. Zilmer, Ü. Langel, U. Soomets. Design, synthesis and properties of novel powerful antioxidants, glutathione analogues. Free Radical Research, 2007; 41(7): 779–787.
13. **Säde Viirlaid**, Riina Mahlapuu, Kalle Kilk, Aleksei Kuznetsov, Ursel Soomets, Jaak Järv. Mechanism and stoichiometry of 2,2-diphenyl-1-picrylhydrazyl radical scavening by glutathione and its novel a-glutamyl-derivative. Bioorg. Chem. 37, 126–132 (2009).
14. Kilk, K., Mahlapuu, R., **Viirlaid, S.,** Järv, J., Soomets, U. (2009) Pharmacophores in antioxidative UPF peptides. European Journal of Pharmaceutical Sciences, 38(1), 157.

ELULOOKIRJELDUS

Säde Viirlaid

Sünniaeg ja koht:	07.06.1951, Orava, Eesti
Kodakondsus:	Eesti
Aadress:	Tartu Ülikool, Keemia Instituut Ravila 14a, 50411, Tartu, Eesti
Telefon:	+372 5800 2777
E-mail:	sade.viirlaid@ut.ee

Haridus

2007	Tartu Ülikooli Keemia Instituudi doktorant
1995	MSc (orgaaniline keemia
1974	BSc keemia (orgaaniline keemia)
1969–1974	Tartu Ülikooli keemiaosakonna üliõpilane

Teenistuskäik

2008–	TÜ Keemia Instituudi lektor (orgaaniline keemia); Keemia bakalaureuse- ja magistriõppekava programmijuht;
1999–2007	orgaanilise ja bioorgaanilise keemia instituut, lektor;
1993–1999	orgaanilise keemia instituut, assistent;
1987–1993	TO Bioorgaanilise sünteesi labor, lep.tööde van.-insener;
1978–1987	TUS orgaanilise sünteesi labor, lep.tööde van.-insener;
1974–1978	orgaanilise keemia kateeder, lep.tööde van.-insener;

Tähtsamad teaduspublikatsioonid

1. **S. Viirlaid**, A. Tuulmets. Effect of Solvents on Grignard Reaction. XI Anisole, Reakts. Sposobn. Org. Soedin. 1974, 11 (1), 65.
2. **S. Viirlaid,** S. Kurrikoff, A. Tuulmets. Effect of Solvents on Grignard Reaction. XII The Reaction of Diphenylmagnesium with Bensophenone, Reakts. Sposobn. Org. Soedin. 1974, 11 (1), 73.
3. V. Buleza, I. Bokotei, U. Mäeorg, M. Babidorich, A. Sorochinskaya, S. Kolonistova (**S. Viirlaid**). Sex Pheromone *Synanthedon myopaeformis* (Lepitoptera: *Aegiriidae*): Biological Activity, *Abstracts* 1990, 314(4), 1002–6.
4. V. Buda, U. Mäeorg, V. Karalius, G.H.L. Rothschild, S. Kolonistova (**S. Viirlaid**), P. Ivinskis, R. Mozuraitis. C_{18}-Dienes as attractants for Eighteen Clearwing (*Sessidae*), Tineid *(Tineidae*) and Choreutid (*Choreutidae*) Moth Species. *J. Chem. Ecol.,* 1993, 19(4), 799–813.

5. U. Mäeorg, K. Kallas, **S. Viirlaid**, T. Pehk, A. Meriste. Behavior of Some Enynols and One Diynol in Super Basic Systems $MNH(CH_2)_2NH_2$/ $H_2N(CH_2)_2NH_2$, *Proc. Estonian Acad.Sci. Chem.,* 1996, 45, 3/4, 134–139.
6. U. Mäeorg, E. Loodmaa, **S. Viirlaid**, New Activated Zn-Cu Catalyst-Superior Tool for the Partial Hydrogenation of Triple Bond. *Electronic Conference on Trend in Organic Chemistry*, ECTOC. Jun–July, 1995. http://www.ch.ic.ac.uk:807.ectoc_syn.html. CD-ROM, Published by Royal Society of Chemistry, 1996.
7. U. Mäeorg, **S. Viirlaid**, Ü. Kelder, K. Kallas, S. Mäeorg Convenient Method for the Trimethylsilylation of Acetylenic compounds. *Electronic Conference of Metallorganic Chemistry,* ECTOC-3, Jun–July, 1997.
8. U. Mäeorg, **S. Viirlaid**, H. Hagu, H. D. Verkruijsse, L. Brandsma. On the in situ trimethylsilylation of zinc acetylides. J. Organomet. Chem., 2000, 601, 341–342.
9. L. Adlerz, U. Soomets, L. Holmlund, **S. Viirlaid**, Ü. Langel, K. Iverfeldt. Down-regulation of amyloid precursor protein by peptide nucleic acid oligomer in cultured rat neurons and astrocytes, Neuroscience Letters 336, 2003, 55–59.
10. P. Põder, M. Zilmer, J. Starkopf, J. Kals, A. Talonpoika, A. Pulges, Ü. Langel, T. Kullisaar, **S. Viirlaid**, R. Mahlapuu, A. Zarkovski, A. Arend, U. Soomets. An antioxidant tetrapeptide UPF1 has a neuroprotective effect in transient global brain ischemia. Neuroscience Letters, 2004, 370 (1), 45–50.
11. Merike Vaher, **Säde Viirlaid**, Kersti Ehrlich, Riina Mahlapuu, Jüri Jarvet, Ursel Soomets, Mihkel Kaljurand, Characterization of the antidoxidative activity of novel neuropeptides by using capillary electrophoresis. Electrophoresis 13 (2006);
12. K. Ehrlich, **S. Viirlaid**, R. Mahlapuu, K. Saar, T. Kullisaar, M. Zilmer, Ü. Langel, U. Soomets. Design, synthesis and properties of novel powerful antioxidants, glutathione analogues. Free Radical Research, 2007; 41(7): 779–787.
13. **Säde Viirlaid**, Riina Mahlapuu, Kalle Kilk, Aleksei Kuznetsov, Ursel Soomets, Jaak Järv. Mechanism and stoichiometry of 2,2-diphenyl-1-picrylhydrazyl radical scavening by glutathione and its novel a-glutamylderivative. Bioorg. Chem. 37, 126–132 (2009).
14. Kilk, K., Mahlapuu, R., **Viirlaid, S.,** Järv, J., Soomets, U. (2009) Pharmacophores in antioxidative UPF peptides. European Journal of Pharmaceutical Sciences, 38(1), 157.

DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS

1. **Toomas Tamm.** Quantum-chemical simulation of solvent effects. Tartu, 1993, 110 p.
2. **Peeter Burk.** Theoretical study of gas-phase acid-base equilibria. Tartu, 1994, 96 p.
3. **Victor Lobanov.** Quantitative structure-property relationships in large descriptor spaces. Tartu, 1995, 135 p.
4. **Vahur Mäemets.** The ^{17}O and ^{1}H nuclear magnetic resonance study of H_2O in individual solvents and its charged clusters in aqueous solutions of electrolytes. Tartu, 1997, 140 p.
5. **Andrus Metsala.** Microcanonical rate constant in nonequilibrium distribution of vibrational energy and in restricted intramolecular vibrational energy redistribution on the basis of slater's theory of unimolecular reactions. Tartu, 1997, 150 p.
6. **Uko Maran.** Quantum-mechanical study of potential energy surfaces in different environments. Tartu, 1997, 137 p.
7. **Alar Jänes.** Adsorption of organic compounds on antimony, bismuth and cadmium electrodes. Tartu, 1998, 219 p.
8. **Kaido Tammeveski.** Oxygen electroreduction on thin platinum films and the electrochemical detection of superoxide anion. Tartu, 1998, 139 p.
9. **Ivo Leito.** Studies of Brønsted acid-base equilibria in water and non-aqueous media. Tartu, 1998, 101 p.
10. **Jaan Leis.** Conformational dynamics and equilibria in amides. Tartu, 1998, 131 p.
11. **Toonika Rinken.** The modelling of amperometric biosensors based on oxidoreductases. Tartu, 2000, 108 p.
12. **Dmitri Panov.** Partially solvated Grignard reagents. Tartu, 2000, 64 p.
13. **Kaja Orupõld.** Treatment and analysis of phenolic wastewater with microorganisms. Tartu, 2000, 123 p.
14. **Jüri Ivask.** Ion Chromatographic determination of major anions and cations in polar ice core. Tartu, 2000, 85 p.
15. **Lauri Vares.** Stereoselective Synthesis of Tetrahydrofuran and Tetrahydropyran Derivatives by Use of Asymmetric Horner-Wadsworth-Emmons and Ring Closure Reactions. Tartu, 2000, 184 p.
16. **Martin Lepiku.** Kinetic aspects of dopamine D_2 receptor interactions with specific ligands. Tartu, 2000, 81 p.
17. **Katrin Sak.** Some aspects of ligand specificity of P2Y receptors. Tartu, 2000, 106 p.
18. **Vello Pällin.** The role of solvation in the formation of iotsitch complexes. Tartu, 2001, 95 p.

19. **Katrin Kollist.** Interactions between polycyclic aromatic compounds and humic substances. Tartu, 2001, 93 p.
20. **Ivar Koppel.** Quantum chemical study of acidity of strong and superstrong Brønsted acids. Tartu, 2001, 104 p.
21. **Viljar Pihl.** The study of the substituent and solvent effects on the acidity of OH and CH acids. Tartu, 2001, 132 p.
22. **Natalia Palm.** Specification of the minimum, sufficient and significant set of descriptors for general description of solvent effects. Tartu, 2001, 134 p.
23. **Sulev Sild.** QSPR/QSAR approaches for complex molecular systems. Tartu, 2001, 134 p.
24. **Ruslan Petrukhin.** Industrial applications of the quantitative structure-property relationships. Tartu, 2001, 162 p.
25. **Boris V. Rogovoy.** Synthesis of (benzotriazolyl)carboximidamides and their application in relations with *N*- and *S*-nucleophyles. Tartu, 2002, 84 p.
26. **Koit Herodes.** Solvent effects on UV-vis absorption spectra of some solvatochromic substances in binary solvent mixtures: the preferential solvation model. Tartu, 2002, 102 p.
27. **Anti Perkson.** Synthesis and characterisation of nanostructured carbon. Tartu, 2002, 152 p.
28. **Ivari Kaljurand.** Self-consistent acidity scales of neutral and cationic Brønsted acids in acetonitrile and tetrahydrofuran. Tartu, 2003, 108 p.
29. **Karmen Lust.** Adsorption of anions on bismuth single crystal electrodes. Tartu, 2003, 128 p.
30. **Mare Piirsalu.** Substituent, temperature and solvent effects on the alkaline hydrolysis of substituted phenyl and alkyl esters of benzoic acid. Tartu, 2003, 156 p.
31. **Meeri Sassian.** Reactions of partially solvated Grignard reagents. Tartu, 2003, 78 p.
32. **Tarmo Tamm.** Quantum chemical modelling of polypyrrole. Tartu, 2003. 100 p.
33. **Erik Teinemaa.** The environmental fate of the particulate matter and organic pollutants from an oil shale power plant. Tartu, 2003. 102 p.
34. **Jaana Tammiku-Taul.** Quantum chemical study of the properties of Grignard reagents. Tartu, 2003. 120 p.
35. **Andre Lomaka.** Biomedical applications of predictive computational chemistry. Tartu, 2003. 132 p.
36. **Kostyantyn Kirichenko.** Benzotriazole — Mediated Carbon–Carbon Bond Formation. Tartu, 2003. 132 p.
37. **Gunnar Nurk.** Adsorption kinetics of some organic compounds on bismuth single crystal electrodes. Tartu, 2003, 170 p.
38. **Mati Arulepp.** Electrochemical characteristics of porous carbon materials and electrical double layer capacitors. Tartu, 2003, 196 p.

39. **Dan Cornel Fara.** QSPR modeling of complexation and distribution of organic compounds. Tartu, 2004, 126 p.
40. **Riina Mahlapuu.** Signalling of galanin and amyloid precursor protein through adenylate cyclase. Tartu, 2004, 124 p.
41. **Mihkel Kerikmäe.** Some luminescent materials for dosimetric applications and physical research. Tartu, 2004, 143 p.
42. **Jaanus Kruusma.** Determination of some important trace metal ions in human blood. Tartu, 2004, 115 p.
43. **Urmas Johanson.** Investigations of the electrochemical properties of polypyrrole modified electrodes. Tartu, 2004, 91 p.
44. **Kaido Sillar.** Computational study of the acid sites in zeolite ZSM-5. Tartu, 2004, 80 p.
45. **Aldo Oras.** Kinetic aspects of dATPαS interaction with $P2Y_1$ receptor. Tartu, 2004, 75 p.
46. **Erik Mölder.** Measurement of the oxygen mass transfer through the air-water interface. Tartu, 2005, 73 p.
47. **Thomas Thomberg.** The kinetics of electroreduction of peroxodisulfate anion on cadmium (0001) single crystal electrode. Tartu, 2005, 95 p.
48. **Olavi Loog.** Aspects of condensations of carbonyl compounds and their imine analogues. Tartu, 2005, 83 p.
49. **Siim Salmar.** Effect of ultrasound on ester hydrolysis in aqueous ethanol. Tartu, 2006, 73 p.
50. **Ain Uustare.** Modulation of signal transduction of heptahelical receptors by other receptors and G proteins. Tartu, 2006, 121 p.
51. **Sergei Yurchenko.** Determination of some carcinogenic contaminants in food. Tartu, 2006, 143 p.
52. **Kaido Tämm.** QSPR modeling of some properties of organic compounds. Tartu, 2006, 67 p.
53. **Olga Tšubrik.** New methods in the synthesis of multisubstituted hydrazines. Tartu. 2006, 183 p.
54. **Lilli Sooväli.** Spectrophotometric measurements and their uncertainty in chemical analysis and dissociation constant measurements. Tartu, 2006, 125 p.
55. **Eve Koort.** Uncertainty estimation of potentiometrically measured ph and pK_a values. Tartu, 2006, 139 p.
56. **Sergei Kopanchuk.** Regulation of ligand binding to melanocortin receptor subtypes. Tartu, 2006, 119 p.
57. **Silvar Kallip.** Surface structure of some bismuth and antimony single crystal electrodes. Tartu, 2006, 107 p.
58. **Kristjan Saal.** Surface silanization and its application in biomolecule coupling. Tartu, 2006, 77 p.
59. **Tanel Tätte.** High viscosity $Sn(OBu)_4$ oligomeric concentrates and their applications in technology. Tartu, 2006, 91 p.

60. **Dimitar Atanasov Dobchev**. Robust QSAR methods for the prediction of properties from molecular structure. Tartu, 2006, 118 p.
61. **Hannes Hagu**. Impact of ultrasound on hydrophobic interactions in solutions. Tartu, 2007, 81 p.
62. **Rutha Jäger.** Electroreduction of peroxodisulfate anion on bismuth electrodes. Tartu, 2007, 142 p.
63. **Kaido Viht.** Immobilizable bisubstrate-analogue inhibitors of basophilic protein kinases: development and application in biosensors. Tartu, 2007, 88 p.
64. **Eva-Ingrid Rõõm.** Acid-base equilibria in nonpolar media. Tartu, 2007, 156 p.
65. **Sven Tamp.** DFT study of the cesium cation containing complexes relevant to the cesium cation binding by the humic acids. Tartu, 2007, 102 p.
66. **Jaak Nerut.** Electroreduction of hexacyanoferrate(III) anion on Cadmium (0001) single crystal electrode. Tartu, 2007, 180 p.
67. **Lauri Jalukse.** Measurement uncertainty estimation in amperometric dissolved oxygen concentration measurement. Tartu, 2007, 112 p.
68. **Aime Lust.** Charge state of dopants and ordered clusters formation in CaF_2:Mn and CaF_2:Eu luminophors. Tartu, 2007, 100 p.
69. **Iiris Kahn**. Quantitative Structure-Activity Relationships of environmentally relevant properties. Tartu, 2007, 98 p.
70. **Mari Reinik.** Nitrates, nitrites, N-nitrosamines and polycyclic aromatic hydrocarbons in food: analytical methods, occurrence and dietary intake. Tartu, 2007, 172 p.
71. **Heili Kasuk.** Thermodynamic parameters and adsorption kinetics of organic compounds forming the compact adsorption layer at Bi single crystal electrodes. Tartu, 2007, 212 p.
72. **Erki Enkvist.** Synthesis of adenosine-peptide conjugates for biological applications. Tartu, 2007, 114 p.
73. **Svetoslav Hristov Slavov**. Biomedical applications of the QSAR approach. Tartu, 2007, 146 p.
74. **Eneli Härk.** Electroreduction of complex cations on electrochemically polished Bi(*hkl*) single crystal electrodes. Tartu, 2008, 158 p.
75. **Priit Möller.** Electrochemical characteristics of some cathodes for medium temperature solid oxide fuel cells, synthesized by solid state reaction technique. Tartu, 2008, 90 p.
76. **Signe Viggor.** Impact of biochemical parameters of genetically different pseudomonads at the degradation of phenolic compounds. Tartu, 2008, 122 p.
77. **Ave Sarapuu.** Electrochemical reduction of oxygen on quinone-modified carbon electrodes and on thin films of platinum and gold. Tartu, 2008, 134 p.
78. **Agnes Kütt.** Studies of acid-base equilibria in non-aqueous media. Tartu, 2008, 198 p.

79. **Rouvim Kadis.** Evaluation of measurement uncertainty in analytical chemistry: related concepts and some points of misinterpretation. Tartu, 2008, 118 p.
80. **Valter Reedo.** Elaboration of IVB group metal oxide structures and their possible applications. Tartu, 2008, 98 p.
81. **Aleksei Kuznetsov.** Allosteric effects in reactions catalyzed by the cAMP-dependent protein kinase catalytic subunit. Tartu, 2009, 133 p.
82. **Aleksei Bredihhin.** Use of mono- and polyanions in the synthesis of multisubstituted hydrazine derivatives. Tartu, 2009, 105 p.
83. **Anu Ploom.** Quantitative structure-reactivity analysis in organosilicon chemistry. Tartu, 2009, 99 p.
84. **Argo Vonk.** Determination of adenosine A_{2A}- and dopamine D_1 receptor-specific modulation of adenylate cyclase activity in rat striatum. Tartu, 2009, 129 p.
85. **Indrek Kivi.** Synthesis and electrochemical characterization of porous cathode materials for intermediate temperature solid oxide fuel cells. Tartu, 2009, 177 p.
86. **Jaanus Eskusson.** Synthesis and characterisation of diamond-like carbon thin films prepared by pulsed laser deposition method. Tartu, 2009, 117 p.
87. **Margo Lätt.** Carbide derived microporous carbon and electrical double layer capacitors. Tartu, 2009, 107 p.
88. **Vladimir Stepanov.** Slow conformational changes in dopamine transporter interaction with its ligands. Tartu, 2009, 103 p.
89. **Aleksander Trummal.** Computational Study of Structural and Solvent Effects on Acidities of Some Brønsted Acids. Tartu, 2009, 103 p.
90. **Eerold Vellemäe.** Applications of mischmetal in organic synthesis. Tartu, 2009, 93 p.
91. **Sven Parkel.** Ligand binding to 5-HT_{1A} receptors and its regulation by Mg^{2+} and Mn^{2+}. Tartu, 2010, 99 p.
92. **Signe Vahur.** Expanding the possibilities of ATR-FT-IR spectroscopy in determination of inorganic pigments. Tartu, 2010, 184 p.
93. **Tavo Romann**. Preparation and surface modification of bismuth thin film, porous, and microelectrodes. Tartu, 2010, 155 p.
94. **Nadežda Aleksejeva.** Electrocatalytic reduction of oxygen on carbon nanotube-based nanocomposite materials. Tartu, 2010, 147 p.
95. **Marko Kullapere.** Electrochemical properties of glassy carbon, nickel and gold electrodes modified with aryl groups. Tartu, 2010, 233 p.
96. **Liis Siinor.** Adsorption kinetics of ions at Bi single crystal planes from aqueous electrolyte solutions and room-temperature ionic liquids. Tartu, 2010, 101 p.
97. **Angela Vaasa.** Development of fluorescence-based kinetic and binding assays for characterization of protein kinases and their inhibitors. Tartu 2010, 101 p.

98. **Indrek Tulp.** Multivariate analysis of chemical and biological properties. Tartu 2010, 105 p.
99. **Aare Selberg.** Evaluation of environmental quality in Northern Estonia by the analysis of leachate. Tartu 2010, 117 p.
100. **Darja Lavõgina.** Development of protein kinase inhibitors based on adenosine analogue-oligoarginine conjugates. Tartu 2010, 248 p.
101. **Laura Herm.** Biochemistry of dopamine D_2 receptors and its association with motivated behaviour. Tartu 2010, 156 p.
102. **Terje Raudsepp.** Influence of dopant anions on the electrochemical properties of polypyrrole films. Tartu 2010, 112 p.
103. **Margus Marandi.** Electroformation of Polypyrrole Films: *In-situ* AFM and STM Study. Tartu 2011, 116 p.
104. **Kairi Kivirand.** Diamine oxidase-based biosensors: construction and working principles. Tartu, 2011, 140 p.
105. **Anneli Kruve.** Matrix effects in liquid-chromatography electrospray mass-spectrometry. Tartu, 2011, 156 p.
106. **Gary Urb.** Assessment of environmental impact of oil shale fly ash from PF and CFB combustion. Tartu, 2011, 108 p.
107. **Nikita Oskolkov.** A novel strategy for peptide-mediated cellular delivery and induction of endosomal escape. Tartu, 2011, 106 p.
108. **Dana Martin.** The QSPR/QSAR approach for the prediction of properties of fullerene derivatives. Tartu, 2011, 98 p.