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DEPARTMENT OF ORGANIC CHEMISTRY

Synthesis of unnatural cysteine and selenocysteine amino acids bearing photolabile α-methylnitropiperonyl protecting group, applicable in protein engineering

Bachelor thesis

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ABBREVIATIONS

BNI – 5-bromo-7-nitroindolinyl

BNZ – benzoin

Boc – *tert*-butyloxycarbonyl

Cbz – benzyloxycarbonyl

Cys – cysteine

DEACM – (7-diethylaminocoumarin-4-yl)methyl

DNI – 5,7-dinitroindolinyl

DMF – *N*,*N*-dimethylformamide

DMSO – dimethylsulfoxide

Fmoc – 9-fruorenylmethyloxycarbonyl

HRMS – high resolution mass spectrometry

NDBF – nitrodibenzofuran

NMR – nuclear magnetic resonance

NPE – 1-(*o*-nitropiperonyl)-ethyl

NPhE – 1-(*o*-nitrophenyl)-ethyl

NPPOC – 2-(2-nitrophenyl)propoxycarbonyl

NVOC – nitroveratryloxycarbonyl

MS – mass spectrometry

pHP – *p*-hydroxyphenacyl

Sec - selenocysteine

TES – triethylsilyl

TFA – trifluoroacetic acid

TLC – thin layer chromatography

TsOH – *p*-toluenesulfonic acid

INTRODUCTION

Selenium plays an important biological role in living organisms and is known primary for its antioxidant activity and, in therapeutic aspects, for its chemopreventive, anti-inflammatory, and antiviral properties [**1**]. The main biological form for selenium is selenocysteine (Sec), a cysteine (Cys) analogue bearing a selenium atom instead of sulfur. Sec exhibits higher nucleophilicity, lower pK_a and a lower redox potential compared to Cys, therefore, making it quite attractive for bioengineering. For example, targeted placement of selenocysteine creates sites for selective protein conjugation, labeling, dimerization, or altered protein folding, it may also increase the catalytic properties of enzymes [**2**]. However, incorporating Sec into a polypeptide chain can prove to be difficult due to the high reactivity of the selenol group. Therefore, Sec is introduced as a less reactive derivative in most cases in the form of a selenoether or bearing a protecting group.

Protecting groups are structural modifications of functional groups rendering those groups inactive to certain (reaction) conditions. After the protecting group served its purpose, a chemical reaction is performed for the removal of the protecting group. In the case of photolabile protecting groups no chemical reagent other than light is necessary for deprotection. Light provides a noninvasive external trigger for the group's removal and because light-induced deprotection is almost instantaneous, the control of timing and localization is possible. This category of protecting groups opens a possibility of working with extremely sensitive molecules, which otherwise would be incompatible with acids, bases or other deprotection conditions. This is important for studies in a delicate environment such as a cell and thus is particularly suitable for applications in the fields of biochemistry and microbiology. The term "caged" is used for compounds with specific properties (usually biological), when the relevant functional group is rendered inactive by a protecting group. The original activity of such compound is fully restored upon deprotection.

The aim of this thesis is to improve the synthesis path for the preparation of NPE derivative protected cysteine and selenocysteine for use in protein engineering.

LITERATURE REVIEW

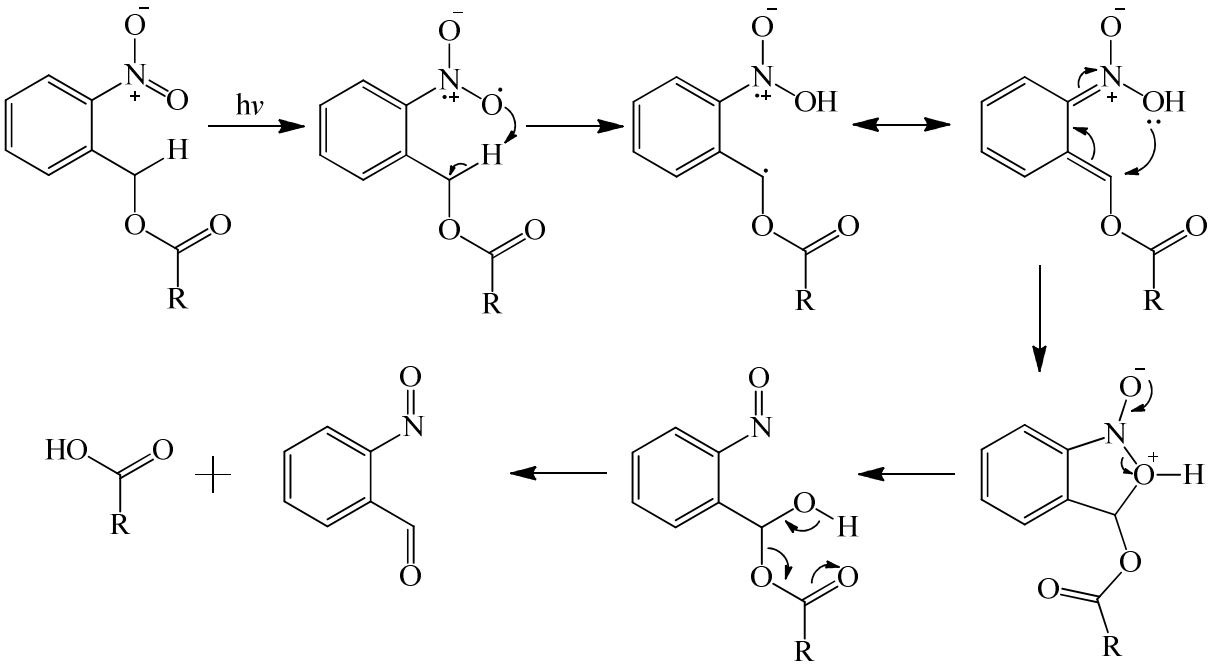
The following literature review contains useful information about some of the most frequently used photolabile protecting groups, their attributes and possible applications focusing on *o*-nitrobenzyl derivatives.

Requirements for photolabile protecting groups

There are complex requirements that must be achieved for a caging group to be useful. First, the protecting group should be easy to introduce to a desired compound with satisfactory yields. Also the protecting group and/or the caged compound should be highly soluble in the given environment and stable under the given conditions. For example, the photolabile protecting group should not decompose due to the system's pH or temperature. It has to have a large extinction coefficient for an effective photolysis. Light-induced deprotection should have a high quantum yield, meaning that the protecting group's removal should occur for as many excited molecules as possible with minimum amounts of light. Deprotection should occur at wavelengths at which the light does not damage the system or other structural fragments of the photocaged molecule. For example, the light should not be phototoxic to a living cell in which the reaction takes place, as well as the protecting group should be compatible with other leaving groups and should not participate in other reactions that do not include the light-induced removal of the protecting group at hand. Furthermore, the deprotection byproducts should not be toxic and should not react with the environment. Lastly, the protecting group should not interfere with the detection or monitoring of the relevant compound or process [**3**].

o-Nitrobenzyl alcohol derivatives

o-Nitrobenzyl alcohol derivatives are most frequently used in bio-research and engineering for photolabile protection of relevant compounds. The removal of this protecting group is based on photochemically induced photoisomerisation of the*o*-nitrobenzyl alcohol derivative into an*o*-nitrosobenzaldehyde or another carbonyl derivative. The mechanism of this process was identified by Norrish and for carbonyl compounds is described as Norrish-type II reaction [**4**].



Scheme 1. *o*-Nitrobenzyl protecting group's deprotection mechanism.

The protecting group is used on amines, alcohols or acids by forming carbamates, carbonates or esters. Deprotection yields a carbonyl derivative forming from the protecting group and the liberated fragment. In the case of carbamates, spontaneous decarboxylation leads to the free amine [**4**]. Before *o*-nitrobenzyl {**fig. 1**} protecting group species could be integrated into living organisms a few modifications have to be made. Because shorter wavelength (260-275 nm for **1**) [**5**] light can potentially cause damage to living organisms, methoxy {**fig. 1**} and methylenedioxy moieties were introduced thus red-shifting the absorbance maximum (355 nm for **3**) so that even the most light-sensitive amino acid, tryptophan, was not affected [**4, 6**]. Another possible problem is a side reaction – the formation of an imine from the photodeprotection produced aldehyde in the presence of a

primary amine. This can be avoided by adding a carbonyl scavenger or using a secondary alcohol derivative {**fig. 1**} instead of primary thus forming a less reactive ketone instead of an aldehyde.

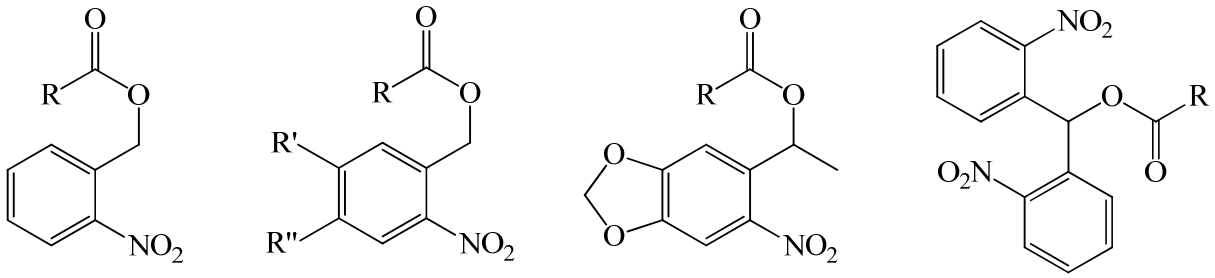
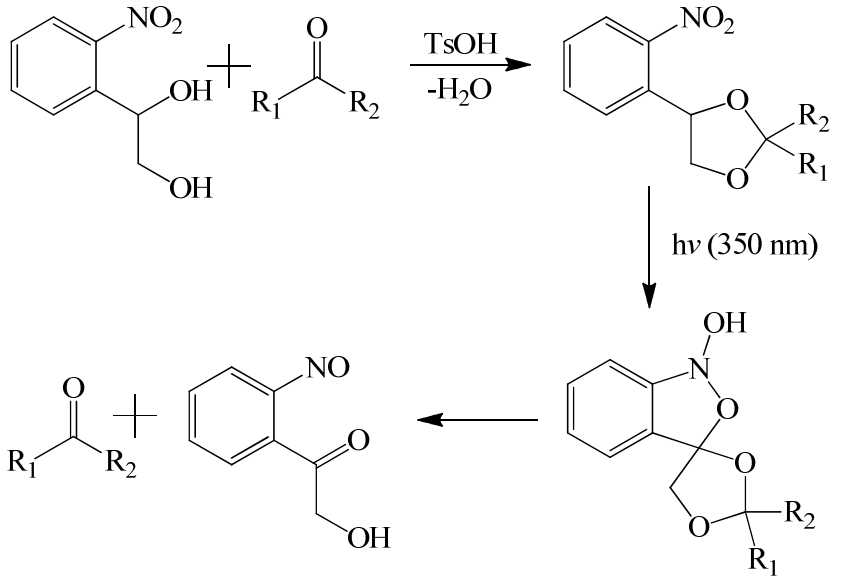


Figure 1.*o*-Nitrobenzyl type protecting groups.

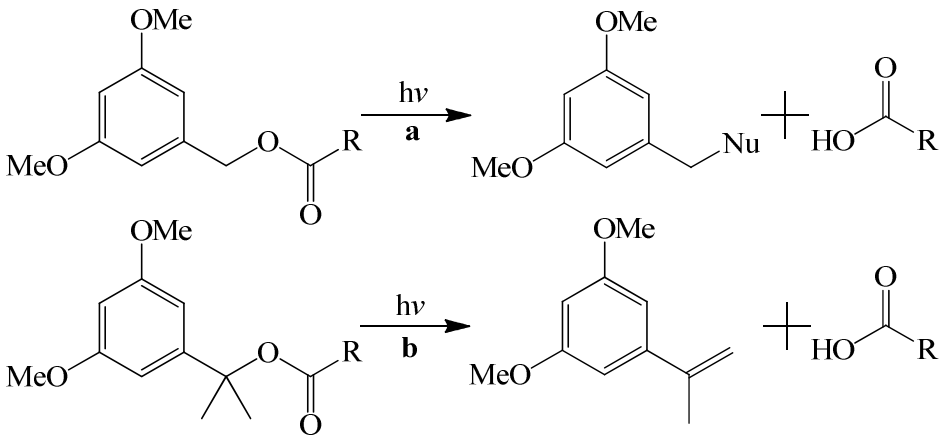
The example described in the reference [**4**] contained the di(nitrobenzyl)oxycarbonyl group {**fig. 1**}. In the latter case the molecule's symmetry prevents the formation of diastereoisomers, also the quantum yield is significantly increased due to an additional hydrogen abstraction-capable scaffold [**4**]. Based on the information gathered in the relevant review [**4**], the exact influence of the substituents at the benzylic centres on the quantum yield has been described as "a complex combination of both steric and electronic effects, as well as the statistics of the hydrogen atom abstraction". Furthermore, an interesting modification of *o*-nitrobenzyl type protecting group was proposed for the protection of carbonyl compounds (**scheme2**)[**4**].



Scheme 2. *o*-Nitrobenzyl photolabile protecting group integration for the carbonyl group's protection.

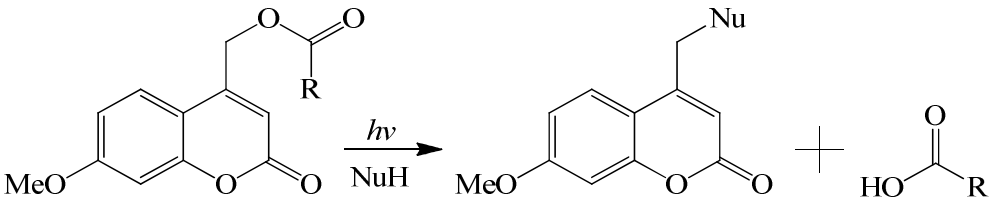
Other common photolabile protecting groups

Benzyl alcohol derivatives have relatively high quantum and chemical yields for the deprotected product. Photolysis causes a heterolythic C-R bond cleavage resulting in a benzylic carbocation, which reacts with a present nucleophile or base, usually the solvent, and a conjugate base. Depending on the substituents in the benzylic position, deprotection can also occur under non-nucleophilic conditions forming a styrene derivative (**scheme 3, b**). Minimal amounts of water in the presence of a non-nucleophilic solvent increase the overall reaction rate. The main drawback is a high lability of the mentioned protecting group in acidic media [**7**].



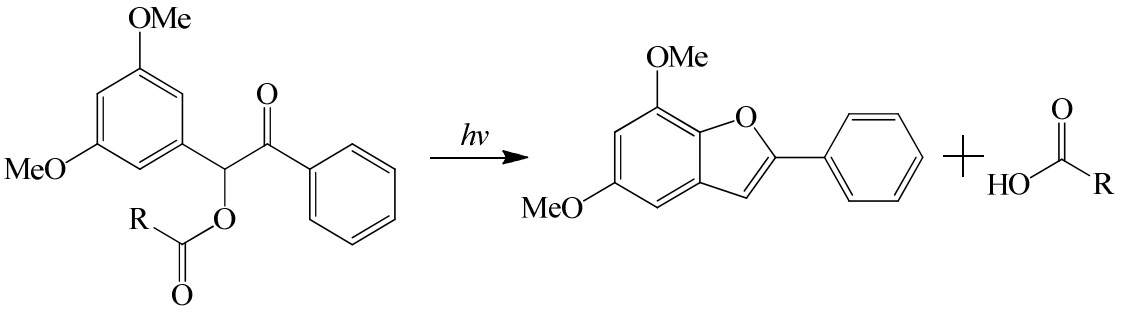
Scheme 3. Benzyl alcohol derivative photolysis pathways under (a) nucleophilic and (b) non-nucleophilic conditions.

Coumarin derivatives upon photodeprotection undergo a heterolytic C-R bond cleavage resulting in a coumarinylmethyl cation and a conjugate base. The carbocation reacts with a present nucleophile forming a coumarinylmethyl derivative. In aqueous media hydoxymethyl coumarin derivative is formed [**7**].



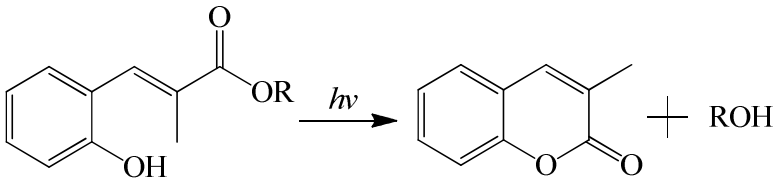
Scheme 4. Coumarin derivative photolysis.

Benzoin derivatives have relatively high quantum and chemical yields making them quite attractive caging groups. The main drawback is that for caged amines the decarboxylation of carbamic acids is much slower than the photolysis thus making benzoin derivatives incompatible with kinetic studies [**7**].



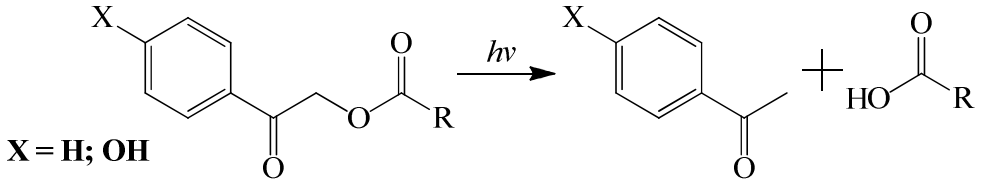
Scheme 5. Benzoin derivative photolysis.

Cinnamyl ester photolysis is based on a cis-trans isomerization followed by an intramolecular transesterification which results in the release of the caged compound. In certain cases (eg. 7-hydroxy or 7-methoxy coumarin derivatives [**8**]), the resulting coumarin derivative byproduct exhibits fluorescent properties which have been applied in tracking the uncaged compound's concentration [**7**].



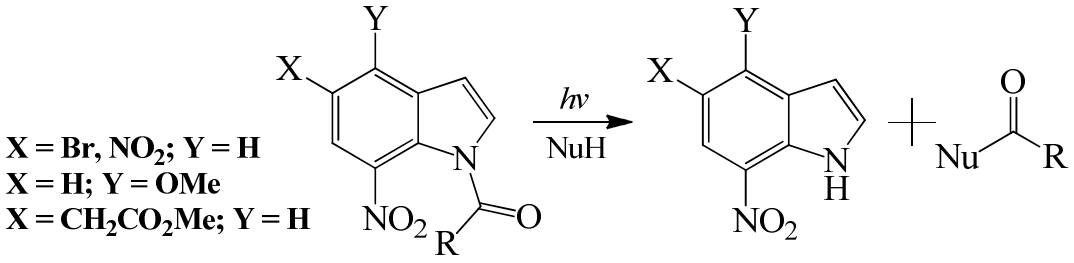
Scheme 6. Cinnamyl ester photolysis.

Phenacyl derivatives are rarely used due to the limitations of their application in account for the high reactivity of their carbonyl groups. Nonetheless, deprotection byproducts are biologically inert and have little absorbance at longer wavelengths thus being an attractive caging group for biological research. Phenacyl derivatives can undergo a number of different reaction pathways depending on the substituents on the benzene ring and the solvent in which the photolysis is carried out. Even small amounts of water can greatly increase deprotection rates [**7**].



Scheme 7. Phenacyl derivative photolysis.

The 7-nitroindoline photolysis mechanism involves light-induced migration of the *N*-acyl group onto the nitro group's oxygen generating an extremely electrophilic *O*-acyl intermediate. Further reaction depends on the present nucleophile. For example, if deprotection occurs in the presence of an amine or in methanol, the liberated fragment would be an amide or a methyl ester. Due to this fact a variety of applications of 7-nitroindoline derivatives were reported. Coupling two peptides using the 7-nitroindoline protection technique has been attempted, but unfortunately, the preparation of 7-nitroindoline protected peptides is difficult, therefore this application (peptide bond formation) had not proven to be useful [**7**].

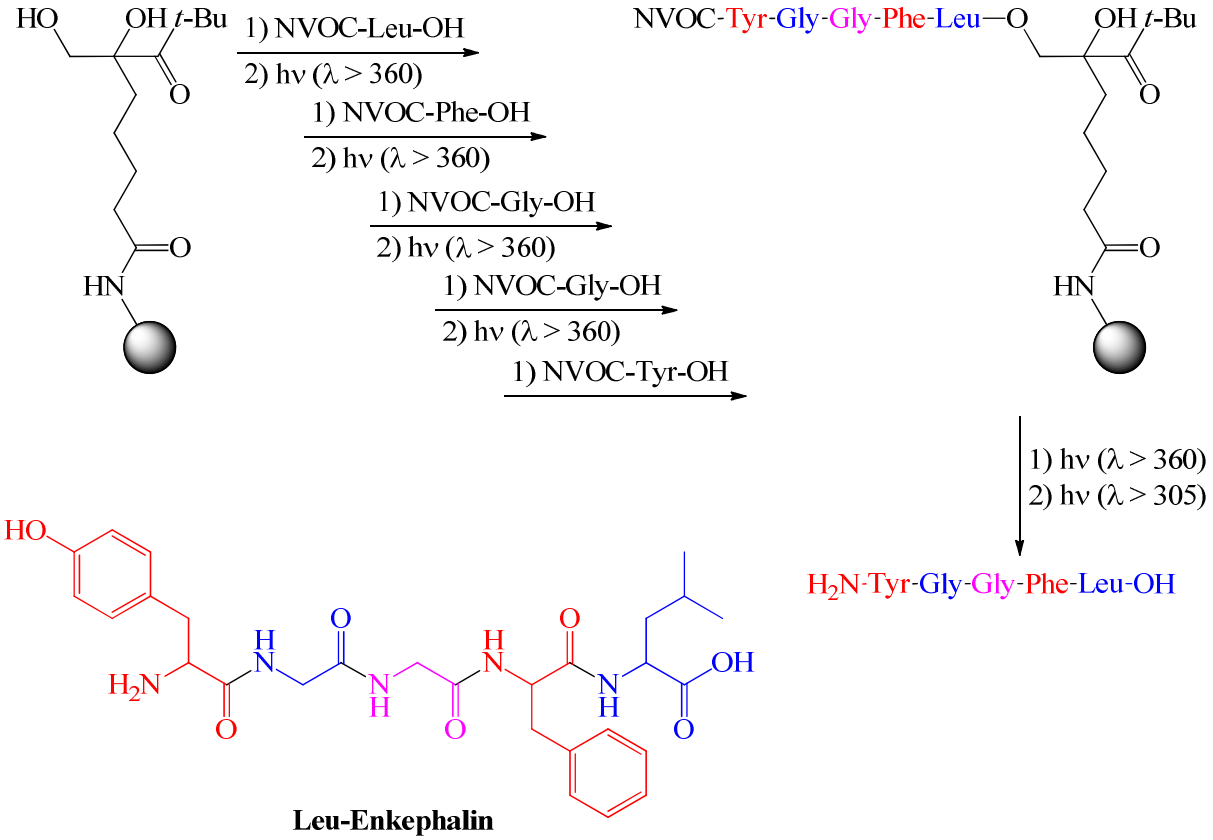


Scheme 8. 7-nitroindoline derivative photolysis.

Wavelength selective photolabile protecting groups

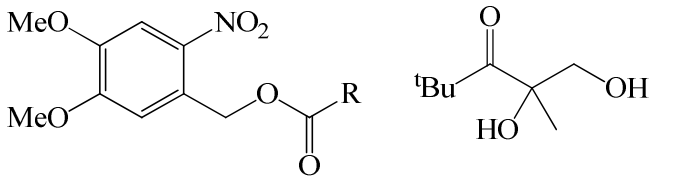
In analogy to chemical orthogonality, for widely used protecting groups such as *tert*-butyloxycarbonyl (Boc), 9-fruorenylmethyloxycarbonyl (Fmoc), triethylsilyl (TES), etc., for photolabile caging groups which could be individually removed with light of different wavelength the term "chromatic orthogonality" was proposed. To design a system containing different orthogonal caging groups, additional requirements have to be met. Stability of each protecting group should be very different at specific wavelengths and the energy gained from the photon absorption should not be transferred between different caging groups. To this day many different successful applications of wavelength selective deprotection were achieved.

M. Kessler et al have successfully applied nitroveratryloxycarbonyl (**NVOC**) {**fig. 2**} protecting group and a *tert*-butyl ketone derivative linker {**fig. 2**}in a solid phase organic synthesis. The cleavage of **NVOC** was obtained at 350 nm and linker at 300 nm gave quantitative yields of the synthesized peptide (**Leu-Enkephalin**) [**9**].



Leu-Enkephalin

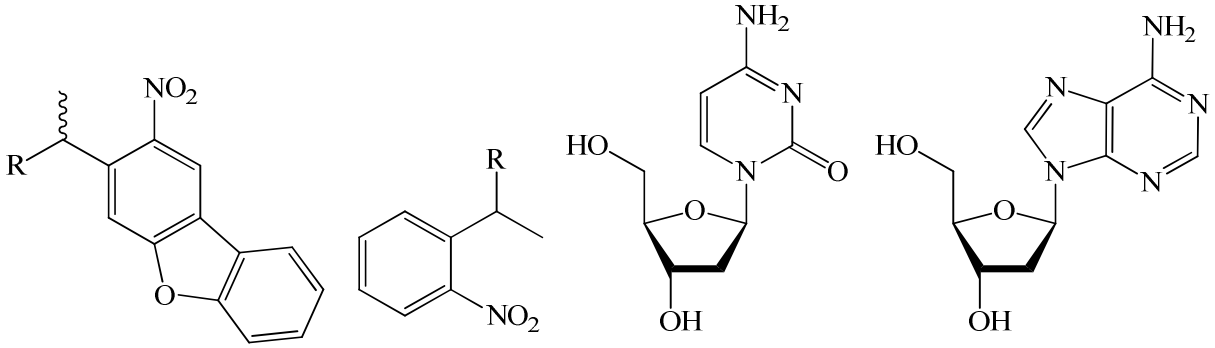
Scheme 9. Synthesis of Leu-Enkephalin.



NVOC **1,2-dihydroxy-2,4,4-trimethylpentan-3-one**

Figure 2. Photolabile groups used in Leu-Enkephalin synthesis.

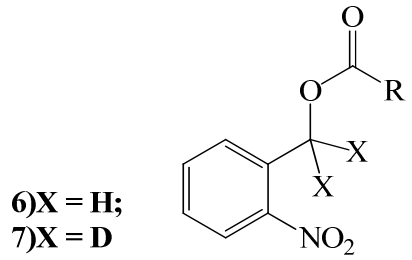
A Heckel et al used a nitrodibenzofuran (**NDBF**) and 1-(*o*-nitrophenyl)-ethyl (**NPhE**) {**fig. 3**} pair to temporary mask the Watson-Crick interaction in deoxycytidine and deoxyadenosine by attaching the mentioned protecting groups to N^4 and N^6 respectively. After the nucleosides were incorporated into an oligonucleotide, the protecting groups were selectively removed. **NDBF** having a considerable absorption above 400 nm was removed with 440 nm wavelength light while **NPhE** was almost unaffected and was removed at typical 365 nm wavelength for its class. The selectivity achieved was in the range of 1 order of magnitude [**10**].



DNBF **NPhE** **Deoxycytidine** **Deoxyadenosine**

Figure 3. Materials used in nucleoside protection.

It is possible to invoke the kinetic isotope effect. The *o*-nitrobenzyl group's photolysis occurs through the abstraction of the benzylic hydrogen atom thus a selectivity of up to 8:1 was acquired between *o*-nitrobenzyl-H and *o*-nitrobenzyl-D {**fig. 4**} derivatives. This effect could be further improved by desymmetrization with different substituents on the two *o*-nitrobenzyl cages [**3**].



6)X = H;
7)X = D

Figure 4. *o*-Nitrobenzyl protecting groups used in kinetic isotope effect studies.

V. S. Miguel et al conducted a study on wavelength selectivity. They prepared seven different caging groups attached to quartz surfaces for this study. Five families of chromophores were addressed: *o*-nitrobenzyl type (**NVOC**, **NPPOC**), benzoin type (**BNZ**), (coumarin-4-yl)methyl type (**DEACM**), 7-nitroindoline type (**DNI**, **BNI**) and *p*-hidroxyphenacyl type (**pHP**){**fig. 5**}. Complete orthogonality was achieved between the pair **pHP** at 300 nm and **DEACM** at 420 nm. Other mentioned pairs required wavelengths below 300 nm for cleavage of the second protecting group. Up to four photoactivatable functional levels were achieved using these protecting groups. However, this includes deprotection using wavelengths below 300 nm [**11**]. Also the selectivity is not complete and a careful variation of light intensities is required. Therefore, an application of more than two functional levels in the field of bioengineering is questionable at this point. Nevertheless, the use of all four levels may be possible in surface modification and in solid phase organic synthesis.

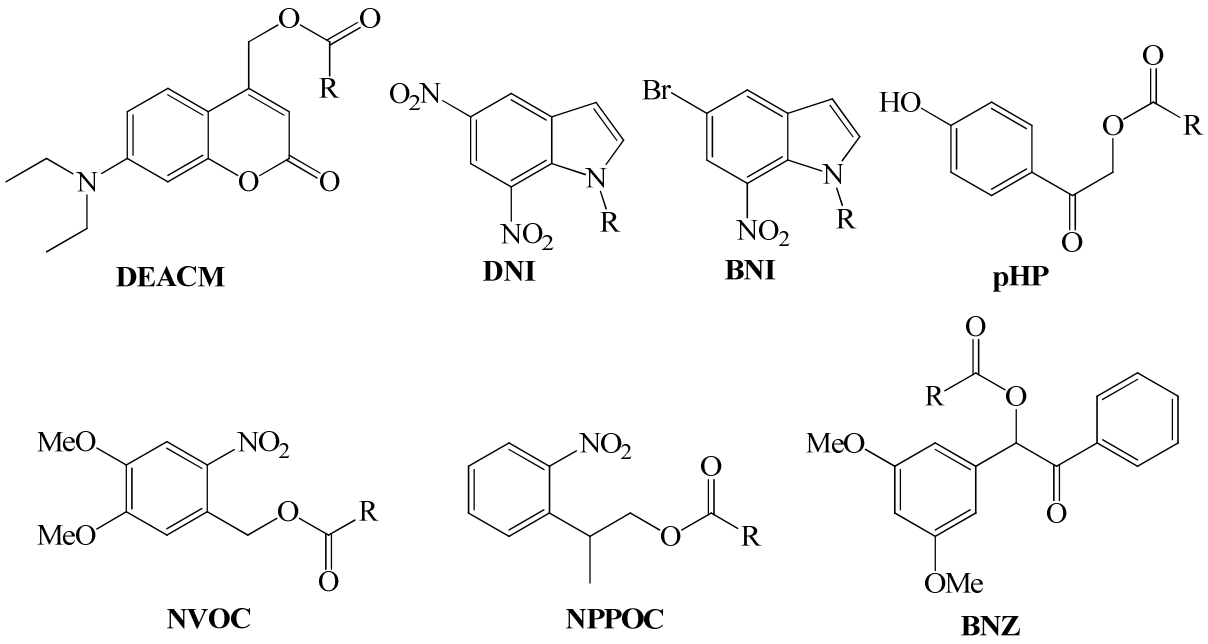
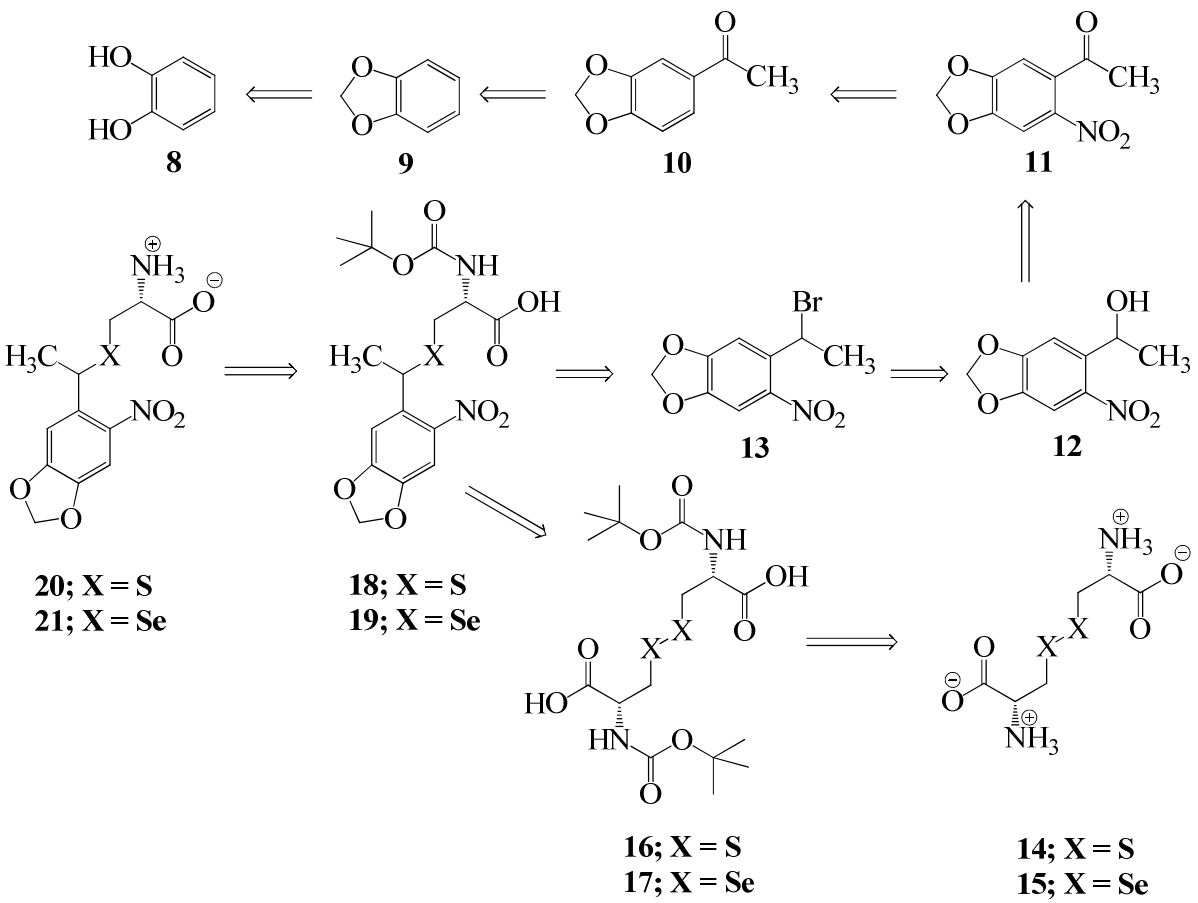


Figure 5. Photolabile protecting groups used in the study on wavelength selectivity.

The literature review shows a large variety of existing photolabile protecting groups, their importance, the requirements and possibilities of application in biochemistry, microbiology and other fields of research and engineering. The advance in various research fields demands a lot of new and improved tools to further the studies. The aim of this review is to summarize various contributions to the photolabile protecting group's research field. The main focus was dedicated to *o*-nitrobenzyl derivatives because it is one of the smallest photolabile protecting groups which can exhibit satisfactory properties with appropriate structural modifications.

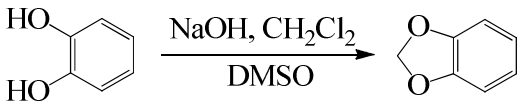
RESULTS AND DISCUSSION

Some applications of photolabile protecting groups have certain limitations. Large caging groups are incompatible with certain biological applications. For example, due to steric hindrance incorporating a caged amino acid into proteins would be impossible using a large caging group. Therefore, we chose an NPE derivative as a suitable caging group for cysteine and selenocysteine, which, in turn, would be used in protein engineering. The synthesis of NPE-Cys had previously been attempted in our laboratory with NPE-Cl. Unfortunately, the nucleophilic substitution with the chalcogene derivative gave poor yields which led to the conclusion that a leaving group at least as good as bromine was necessary in the NPE fragment for an efficient synthesis. Because NPE-Br is not a commercially available substance, we decided to synthesize it by the following scheme with pyrocatechol as the starting material (**scheme 10**).



Scheme 10. Retrosynthesis of photocaged cysteine and selenocysteine.

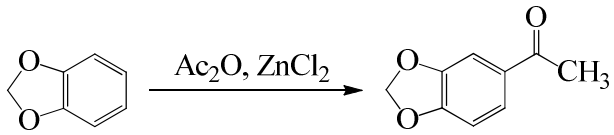
Benzo[1,3]dioxolane (**9**) was synthesized by adding a heated suspension of pyrocatechol (**8**) and 4 equivalents of sodium hydroxide in dimethylsulfoxide dropwise to a boiling mixture of dichloromethane and dimethylsulfoxide as described by Frank R. et al in their method [**12**]. But unfortunately, this step was impossible to carry out following the given method due to the solidification of the first mixture after the addition of sodium hydroxide. It was clear that sodium pyrocatecholate formed from pyrocatechol and two equivalents of sodium hydroxide is vaguely soluble in dimethylsulfoxide. To overcome this problem, sodium hydroxide was added in portions to the first mixture until any signs of solidification. The experiment led to a conclusion that no more than 2.2 equivalents of sodium hydroxide may be added for the reaction to be carried out smoothly. With these changes the yield was boosted up to 64% (54% lit. [**12**]). This stage can be omitted as benzo[1,3]dioxolane (**9**) is a commercially available substance and was synthesized because we had the required material for this reaction.



8 **9**

Scheme 11. Benzo[1,2]dioxolane synthesis.

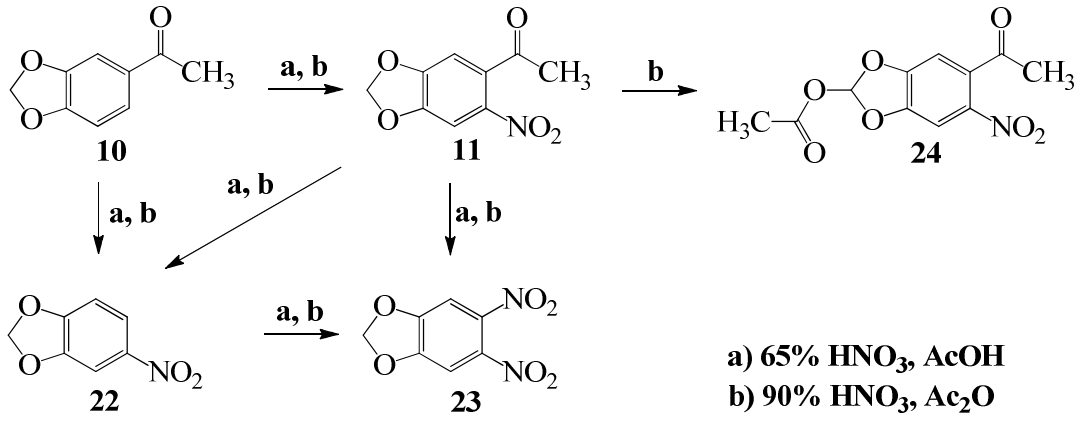
The pure benzo[1,3]dioxolane (**9**) was then acetylated in a stirring suspension of acetic anhydride and zinc chloride as Friedel-Crafts catalyst. While performing the reaction at room temperature only a small part of the starting material reacted. It was thought that the low conversion was due to the lack of $ZnCl_2$ for its capability to complex with not only the acetic anhydride, but also the acetic acid formed as a side product in the reaction. Increasing the amount of the catalyst to 3 equivalents showed promising results. Also the yield when performing the reaction at room temperature was low and erratic (0 – 37%), while the best results were achieved when the reaction temperature was lowered to 0˚C. This boosted the yield two fold (79%) and even gave the product in better purity. The crude product **10** was recrystallized from hexane.



9 **10**

Scheme 12. 3,4-(Methylenedioxy)acetophenone synthesis.

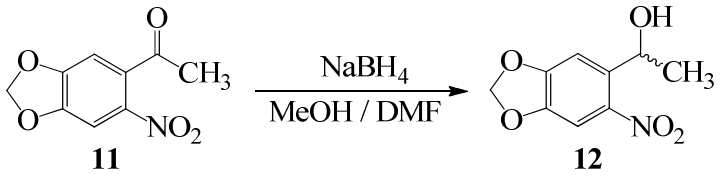
The pure 3,4-(methylenedioxy)acetophenone (**10**) was dissolved in glacial acetic acid and added dropwise to a stirring mixture of nitric and glacial acetic acids. Performing the reaction at 0 - 20˚C resulted in extremely poor yields (11%). It seemed that the starting material was not reactive enough under these reaction conditions, so it was decided to use nitroacetate formed from fuming nitric acid and acetic anhydride instead. Even though this way the yield was drastically increased, unfortunately, it was inconsistent. The yield varied between 5% and 47% and three side products were observed, separated by column chromatography and identified as 1,2-(methylenedioxy)-4-nitrobenzene (**22**), 1,2-dinitro-4,5-(methylenedioxy)benzene (**23**) and 5-acetyl-6-nitrobenzo[*d*][1,3]dioxol-2-ylacetate (**24**). Due to the lack of reliability and the difficulty of controlling the reaction with such an aggressive reagent, we chose to work with the previous method trying to find more favorable conditions for the formation of the target product. After conducting the reaction under a number of different conditions it was found that at low temperatures (up to 30˚C) the reaction does not occur or proceeds very slowly and at temperatures above 50˚C the formation of the aforementioned side products dominates. Hence, we came to a conclusion that the reaction should be carried out at around 50˚C, has to be performed fast, to minimize the possibility for the target product to participate in any possible side reaction and with a large excess of nitric acid that the drop in nitric acid's concentration would not have a big influence on the overall speed of the reaction. Following this method, the target product **11** in a yield of 62% has been formed.



Scheme 13. 4,5-(Methylenedioxy)-2-nitroacetophenone synthesis.

4,5-(Methylenedioxy)-2-nitroacetophenone's (**11**) carbonyl group was then reduced using sodium borohydride in a mixture of methanol and dimethylformamide. It is worth mentioning, that

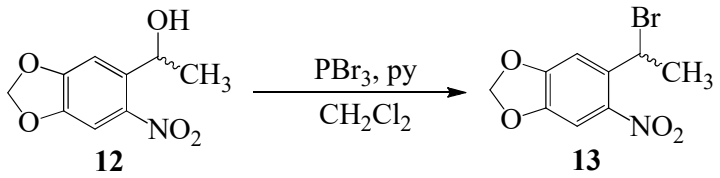
dimethylformamide was added for a better solubility of the starting material, a larger amount of sodium borohydride was used comparing to the one used in the reference [**13**] and the obtained product was not as pure as stated in the reference and required additional purification. All procedures were carried out in the dark to avoid light-induced decomposition of the target product **12** and the pure sample was stored in a dry dark place.



11 **12**

Scheme 14. (*R*/*S*)-1-(4',5'-(methylenedioxy)-2-nitrophenyl)ethanol synthesis.

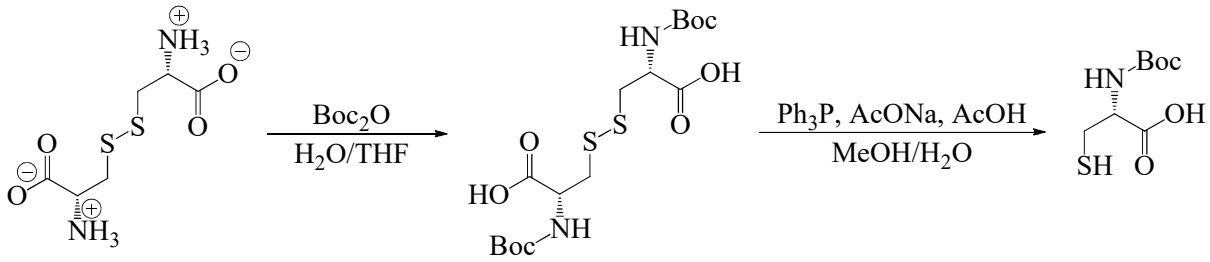
NPE-Br **13** was prepared from (*R*/*S*)-1-(4',5'-(methylenedioxy)-2-nitrophenyl)ethanol (**12**) using PBr_3 as the bromine donor. Additional efforts were made in drying the solvent and the reagents used to avoid the decomposition of PBr_3 in the presence of water. No modifications of the described procedure [**14**] were needed as the reaction proceeded smoothly in moderate yields. All procedures were carried out in the dark to avoid light-induced decomposition of the *o*-nitrobenzyl alcohol derivative and the target product. The pure product **13** was stored in a dry dark place at -20˚C.



12 **13**

Scheme 15. (*R*/*S*)-1-bromo-1-[4',5'-(methylenedioxy)-2-nitrophenyl]ethane synthesis.

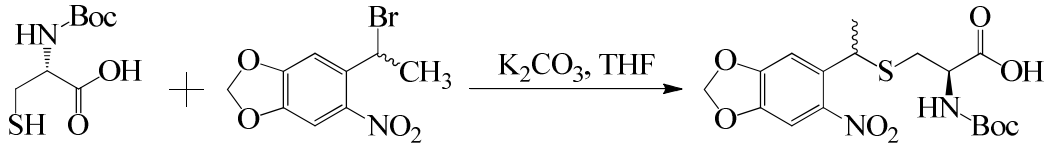
The preparation of *L*-Cys **25** for introduction of the NPE protecting group consisted of the protection of cystine's amino groups with Boc and the reduction of the disulfide to thiol. These procedures were simple and did not require any modification.



14 **16** **25**

Scheme 16.*N*-Boc-*L*-Cysteine synthesis.

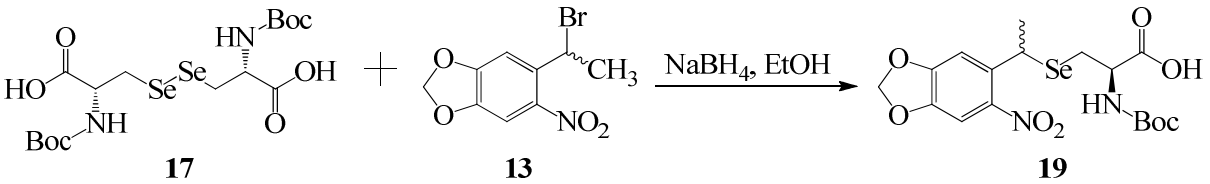
A substitution reaction was attempted by stirring *N*-Boc-*L*-Cys **25**, an excess of NPE-Br **13** (1.5 equivalents) and potassium carbonate as a base in THF at reflux in an inert atmosphere. Using 1 equivalent of potassium carbonate resulted in poor and inconsistent yields (0 – 20%). This was partly overcome by increasing the amount of base present to 2 equivalents. Thus the yield was boosted to 42%. Excess of NPE-Br **13** can be recovered during the purification procedure *via* column chromatography. Also it is worth mentioning that the desired product seems to destruct if exposed to temperatures above 40˚C and must not be left in the presence of any acids for extended periods of time (such as the acetic acid used during purification). Also, the synthesis and purification procedures should be carried out in the dark to avoid light induced deprotection of the caged compound.



25 **13** **18**

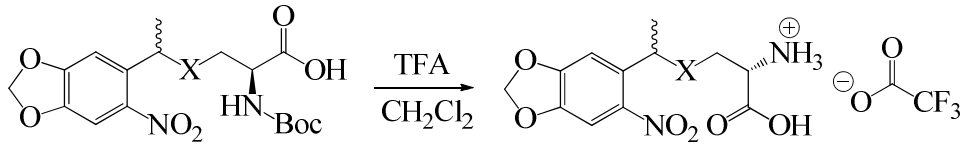
Scheme 17.*N*-Boc-*S*-(1-[4',5'-(methylenedioxy)-2-nitrophenyl]ethyl)-*L*-cysteine synthesis.

Caging of *N*-Boc-*L*-Sec with NPE-Br **13** proceeded better compared to *N*-Boc-*L*-Cys **25**. The reaction resulted in a higher yield (64%) and a shorter reaction time owing to the higher reactivity of the selenolate. Moreover, less side products were observed. This can be explained by the shorter reaction time, which plays a critical role in the reaction. It seems that the longer the time, the lesser the yield and more byproducts are observed.



Scheme 18. *N*-Boc-*Se*-(1-[4',5'-(methylenedioxy)-2-nitrophenyl]ethyl)-*L*-selenocysteine synthesis.

Both Boc protected photocaged amino acids were treated with TFA to afford NPE-Cys **20** and NPE-Sec **21**. Unfortunately, due to the caged amino acid's liability in acidic media only moderate yields (55%) were achieved for deprotection. The yields could be increased, but further experimentation is needed to adjust the reaction conditions for the desired results to be achieved.



Scheme 19. Removal of Boc protecting group.

The synthesis of NPE-Br **13** proved to be not as trivial as expected. One of the hardest tasks was nitro groups introduction to 3,4-(methylenedioxy)acetophenone (**10**) due to its surprisingly low reactivity. Therefore an unusual method had to be used for this reaction. Furthermore, the stability of the caged amino acids also proved to be an issue. Due to the compounds destruction at elevated temperatures the reaction time became a vital aspect. Moreover, the caged amino acid's lability in acidic media made purification and Boc protecting group's removal into another predicament. Regarding these facts the caged compounds should be handled appropriately.

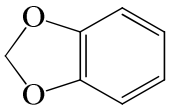
NPE-Br **13** prepared this way was a racemic mixture, but it did not cause any difficulties in synthesizing the caged compounds or in their further use in protein engineering. Nevertheless, a clear difference was observed between the two diastereoisomers in both *N*-protected and deprotected caged amino acid's NMR spectra.

In comparison to DMNB, another protecting group from *o*-nitrobenzyl family used in biotechnology, NPE protected compounds are less accessible due to that NPE derivatives are commercially unavailable. Although, some properties of NPE caged compounds are more attractive than DMNB compounds. Cleavage time for NPE caged molecules is clearly shorter and quantum yield of deprotection is a lot better. Therefore NPE protecting group should be more suitable for certain cases of biotechnological application.

EXPERIMENTAL

Analytical TLC was conducted on silica gel plates Silufol 60 F_{254} (Merck) with detection by UV light or a potassium permanganate solution. Column chromatography was performed on silica gel (Merck 60Å 230-240 mesh). ^{1}H and ^{13}C NMR spectra were recorded on Bruker Ascend 400 (400 MHz and 100 MHz respectively), chemical shifts are given in ppm. Mass spectra were recorded on Bruker SCION SQ 436-GC. High resolution mass spectra were recorded on Agilent 6230 TOF LC/MS. IR spectra were run on a Perkin-Elmer FT-IR spectrophotometer Spectrum BX II in KBr. Melting points were determined in open capillaries using digital melting point IA9100 series apparatus (electrothermal). Solvent evaporation and solution concentration were performed under reduced pressure using a rotary evaporator unless otherwise noted.

Benzo[1,3]dioxolane (9)



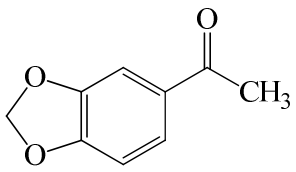
To a solution of pyrocatechol (**8**) (50 g; 0.45 mol) in DMSO (150 ml) sodium hydroxide was added in portions of ~5 g every 15-20 minutes up to 37.8 g (0.945 mol). A mixture of dichloromethane (75 ml) and DMSO (100 ml) was heated to reflux and the previous content was added dropwise. The process was monitored by TLC (chloroform, $R_f = 0.75$; the target product tends to evaporate from the silica gel plate over time). Overall reaction time was 5 hours. On completion the reaction mixture was steam distilled, the organic phase was separated and dried overanhydrous sodium sulphate and distilled to yield the product as a slightly yellow oil.

Yield: 35.2 g (64%); b. p. 172-173˚C; $n_D^{20} = 1.538$.

Lit. data: b. p. 173-175˚C; $n_D^{20} = 1,5377$ [**15**].

^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.90-6.80 (m, 4H, ArH); 5.97 (s, 2H, OCH_2O).

3,4-(Methylenedioxy)acetophenone (10)



A mixture of $ZnCl_2$ (15.5 g; 0.123 mol) and Ac_2O (41 ml; 0.435 mol) was cooled to 0°C and benzo[1,3]dioxolane (**9**) (5 g; 0.041 mol) was added. The reaction mixture was stirred at 0°C for 10 hours. The process was monitored by TLC (chloroform, R_f = 0.30). On completion the reaction mixture was poured over ice and the resulting brown solid was washed with water until neutral pH. A part of the product was obtained by extraction with chloroform (3 ×15 ml) from the filtrate. The combined extracts were washed with saturated sodium carbonate, dried over anhydrous sodium sulphate and the solvent was evaporated. The resulting residue and the previous brown solid were recrystallized from *n*-hexane to yield the product as yellow crystals.

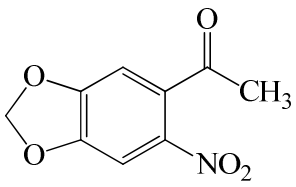
Yield: 5.30 g (79%); m. p. 83-84°C.

Lit. data: m. p. 85–87°C [**16**].

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.56 (dd, ^3J = 8.1 Hz ^4J = 1.7 Hz, 1H, Ar^6-H); 7.45 (d, ^4J = 1.7 Hz, 1H, Ar^2-H); 6.86 (d, ^3J = 8.1 Hz, 1H, Ar^5-H); 6.06 (s, 2H, OCH_2O); 2.55 (s, 3H, CH_3).

MS (EI): m/z calcd [$C_9H_8O_3$]: 164.0; found: 164.0.

4,5-(Methylenedioxy)-2-nitroacetophenone (11)



Method A:

A mixture of HNO_3 (65%; 32 ml; 0.489 mol) and glacial acetic acid (48 ml) was heated to 50°C and 3,4-(methylenedioxy)acetophenone's (**10**) (8 g; 0.0487 mol) solution in glacial acetic acid (24 ml) was added dropwise over 10 minutes maintaining a temperature of 50°C. Afterwards, the reaction mixture was quickly cooled and poured over ice. The crude product was collected by filtration and was washed with water until neutral pH. An additional portion of the crude product was obtained by extraction with chloroform (3 × 15 ml) from the filtrate. The combined extracts were washed with saturated $NaHCO_3$, dried over anhydrous sodium sulphate and the solvent was evaporated. Both portions of the crude product were recrystallized from *n*-octane to obtain the product as yellow crystals.

Yield: 7.19 g (71%); m. p. 110-111°C

Method B:

Fuming nitric acid (~90%; 0.234 ml; 4.6 mmol) was added dropwise to acetic anhydride (3 ml) maintaining a temperature below -20°C. 3,4-(Methylenedioxy)acetophenone (**10**) (500 mg; 3.05 mmol) was dissolved in acetic anhydride (2.25 ml) and added dropwise to the previous mixture at -30°C. The reaction mixture was left stirring for 30 minutes and poured over ice. The crude product was collected by filtration and washed with water until neutral pH. The filtrate was neutralized with saturated $NaHCO_3$ and an additional portion of the crude product was obtained by extraction with chloroform from the filtrate. Both portions of the crude product were recrystallized from *n*-butanol to obtain the product as yellow crystals.

Yield: 295 mg (46%); m. p. 110-111°C.

Lit. data: m. p. 112°C [**13**].

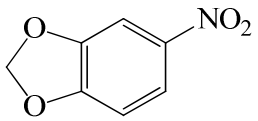
^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.55 (s, 1H Ar^3-H); 6.76 (s, 1H, Ar^6-H); 6.20 (s, 2H, OCH_2O);

2.50 (s, 3H, CH_3).

MS (EI): m/z calcd [$C_9H_7NO_5$]: 209.0; found: 208.9.

Identified side products:

1,2-(Methylenedioxy)-4-nitrobenzene (**22**)



M. p. 145-146°C.

Lit. data: 144-146°C [**17**].

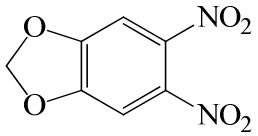
^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.92 (dd, ^3J = 8.6 Hz, ^4J = 2.3 Hz, 1H, Ar^5-H); 7.69 (d, ^4J = 2.3 Hz, 1H, Ar^3-H); 6.89 (d, ^3J = 8.6 Hz, 1H, Ar^6-H); 6.17 (s, 2H, OCH_2O).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 153.1; 148.2; 142.9; 119.9; 107.6; 104.5; 103.0.

IR ν (cm^{-1}): 1503, 1338 (NO_2); 1267 (C-O).

MS (EI): m/z calcd [$C_7H_5NO_4$]: 167.0; found: 167.0.

1,2-Dinitro-4,5-(methylenedioxy)benzene (**23**)



M. p. 95-96°C.

Lit. data: 98-100°C [**18**].

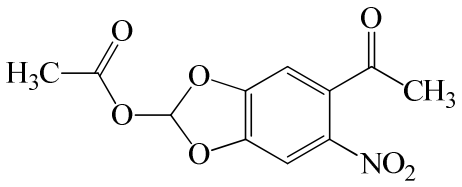
^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.32 (s, 2H, ArH); 6.29 (s, 2H, OCH_2O).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 150.9; 138.8; 105.0; 104.6.

IR ν (cm^{-1}): 1537, 1501, 1368, 1341 (NO_2); 1273 (C-O).

MS (EI): m/z calcd [$C_7H_4N_2O_6$]: 212.0; found: 211.9.

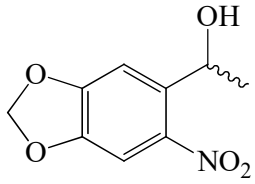
5-Acetyl-6-nitrobenzo[*d*][1,3]dioxol-2-ylacetate (**24**)



^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.85 (s, 1H, OCH(O)O); 7.74 (s, 1H, Ar^1-H); 6.96 (s, 1H, Ar^4-H); 2.52 (s, 3H, $COCH_3$); 2.17 (s, 3H, $OC(O)CH_3$).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 198.6; 168.0; 149.6; 146.0; 141.0; 135.5; 114.4; 107.3; 106.0; 30.2; 20.8.

(*R/S*)-1-(4',5'-(methylenedioxy)-2-nitrophenyl)ethanol (12)



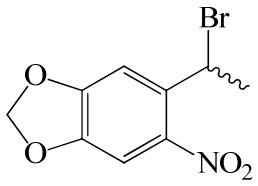
To a solution of 4,5-(methylenedioxy)-2-nitroacetophenone (**11**) (500mg; 2.39 mmol), methanol (2 ml) and DMF (4 ml) cooled to 0°C sodium borohydride was added in portions of 0.045g (1.19 mmol) every 30 minutes up to 0.225 g (5.95 mmol). The process was monitored by TLC (chloroform, R_f = 0.10; R_f may be higher if the sample taken from the reaction mixture contains any DMF). On completion, the reaction mixture was quenched with saturated ammonium chloride until the emission of ammonia ceased. The crude product was extracted with diethyl ether (4 × 2 ml), the combined extracts were washed with brine, dried over anhydrous sodium sulphate and the solvent was evaporated. The resulting oil was purified by column chromatography (petrol ether / chloroform - 2:1) to yield the product as a yellow solid.

Yield: 455 mg (90%); m. p. 84-85°C.

Lit. data: m. p. 86°C [**19**].

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.46 (s, 1H, Ar^3-H); 7.28 (s, 1H, Ar^6-H); 6.13 (s, 2H, OCH_2O); 5.46 (q, ^3J = 6.3 Hz, 1H, CH); 2.46 (s, 1H, OH); 1.54 (d, ^3J = 6.3 Hz, 3H, CH_3).

(*R*/*S*)-1-bromo-1-[4',5'-(methylenedioxy)-2-nitrophenyl]ethane (13)



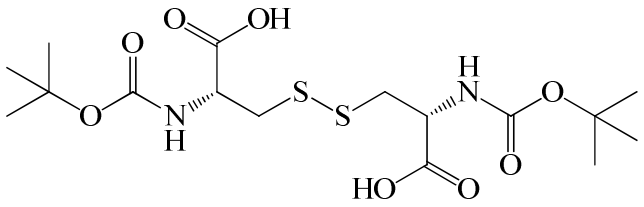
(*R*/*S*)-1-bromo-1-[4',5'-(methylenedioxy)-2-nitrophenyl]ethane (**12**) (2 g; 9.47 mmol) was loaded into an oven-dried flask, then dried under vacuum and purged with dry argon gas (this process was repeated 3 times). The compound was dissolved in CH_2Cl_2 (32.5 ml) and cooled to 0°C under an argon atmosphere. Then PBr_3 (0.36 ml; 1.03 mmol) solution in CH_2Cl_2 (15 ml) was added dropwise over 10 minutes, the mixture was cooled to 0°C and pyridine (0.064 ml; 0.79 mmol) was added. The reaction was left to stir for 15 minutes in the cold, then warmed to room temperature and stirred for additional 1.5 hours under an argon atmosphere (the reaction was monitored by TLC - chloroform, R_f= 0.85). Afterwards, the reaction mixture was cooled to 0°C and quenched with dry methanol (0.5 ml; 12.3 mmol), warmed to room temperature and left stirring for additional 30 minutes under an argon atmosphere. Afterwards, volatiles were removed with rotary evaporator, the resulting residue was dissolved in CH_2Cl_2 (38 ml) and saturated $NaHCO_3$ (38 ml) mixture. The organic layer was separated and washed with saturated $NaHCO_3$ (1 × 38 ml) and brine (3 × 38 ml), then dried over anhydrous sodium sulphate. After solvent evaporation, the resulting crude product was purified by column chromatography (chloroform / petrol ether - 1:2) to yield the product as yellow crystals.

Yield: 1.72 g (66%); m. p. 77-78°C.

Lit. data: 76.1-77.8°C [**14**].

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.37 (s, 1H, Ar^3-H); 7.29 (s, 1H, Ar^6-H); 6.15 (s, 2H, OCH_2O); 5.91 (q, ^3J = 6.8 Hz, 1H, CH); 2.05 (d, ^3J = 6.8 Hz, 3H, CH_3).

N,*N'*-bisboc-*L*-cystine (16)



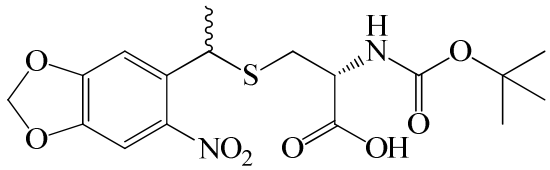
To a stirring suspension of *L*-cystine (**14**) (1.2 g; 5 mmol) in NaOH (1 M; 7 ml) Boc_2O (4.4 g; 20 mmol) solution in dioxane (1.5 ml) was added. The mixture became homogenous in 3 hours and was left to stir for an additional 2 days. The reaction was monitored by TLC (ethyl acetate / dichloromethane / glacial acetic acid - 1:1:0.01, $R_f = 0.55$). On completion, dioxane was evaporated, the residue was diluted with brine, washed with AcOEt (3 × 20 ml) and acidified to pH = 2 with $NaHSO_4$ (1 M). The product was extracted with AcOEt (3 × 20 ml), the combined extracts were dried over anhydrous sodium sulphate and the solvent was evaporated to yield the product as colorless crystals.

Yield: 878 mg (40%); m. p. 136-138°C.

Lit. data: 142-145°C [**20**].

^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.22 (d, ^3J = 8.4 Hz, 2H, NH); 4.17 (ddd, ^3J = 4.2, 8.4, 10.2 Hz, 2H, CH); 3.10 (dd, ^3J = 4.2 Hz, ^2J = 13.5 Hz, 2H, C**H**$_a$H$_b$); 2.89 (dd, ^3J = 10.2 Hz, ^2J = 13.5 Hz, 2H, CH$_a$**H**$_b$); 1.38 (s, 18H, CH_3).

N-Boc-*S*-(1-[4',5'-(methylenedioxy)-2-nitrophenyl]ethyl)-*L*-cysteine (18)



N-boc-*L*-cysteine (**25**) (139 mg; 0.628 mmol), K_2CO_3 (197 mg; 1.32 mmol) and (*R*/*S*)-1-bromo-1-[4',5'-(methylenedioxy)-2-nitrophenyl]ethane (**13**) (259 mg; 0.943 mmol) were dissolved in THF

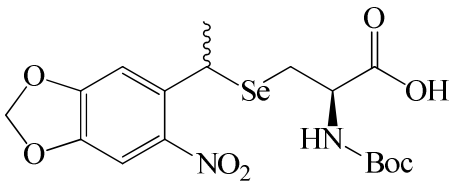
(4.5 ml) and refluxed under an argon atmosphere for 7.5 hours. The reaction was monitored by TLC (toluene / AcOH - 9:1, R_f = 0.35). On completion, the solvent was evaporated, brine (10 ml) was added and the mixture was acidified to pH = 2 with $NaHSO_4$ (1 M). The product was extracted with AcOEt (4 × 5 ml), the combined extracts were dried over anhydrous sodium sulphate and the solvent was evaporated. The thick oily residue was purified by column chromatography (toluene / AcOH - 25:2, R_f = 0.18) to yield the product as a yellow oil.

Yield: 108 mg (42%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.35 - 7.16 (m, 2H, Ar-H); 6.13& 6.12 (s, 2H, OCH_2O); 5.33 - 5.24 (m, 1H, NH); 4.85 & 4.82 (q, ^3J = 6.9 Hz, 1H, ArCH); 4.56 - 4.42 (m, 1H, NCH); 2.97 - 2.77 (m, 2H, SCH_2); 1.58 & 1.56 (d, ^3J = 6.9 Hz, 3H, CH_3); 1.47 (s, 9H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 155.8; 152.3; 147.1; 143.4; 138.2; 136.0; 108.2; 105.2; 103.2; 80.8; 53.3; 39.9; 34.0; 28.6; 23.4.

_N_-Boc-_Se_-(1-[4',5'-(methylenedioxy)-2-nitrophenyl]ethyl)-_L_-selenocysteine (19)



To an ice cold solution of *N*,*N'*-bisboc-*L*-selenocystine (**17**) (150 mg; 0.28 mmol) and (*R*/*S*)-1-bromo-1-[4',5'-(methylenedioxy)-2-nitrophenyl]ethane (**13**) (231 mg; 0.84 mmol) dissolved in ethanol (7.5 ml) $NaBH_4$ (42.4 mg; 1.12 mmol) was added under an argon atmosphere. The reaction mixture was warmed to room temperature and stirred for additional 2.5 hours. On completion the mixture was acidified to pH = 2 with $NaHSO_4$ (1 M) and the product was extracted with AcOEt (3 × 10 ml). The combined extracts were dried over anhydrous sodium sulphate and the solvent was evaporated. The resulting residue was purified by column chromatography (toluene / AcOH - 25:2, R_f = 0.18) to yield the product as a yellow oil.

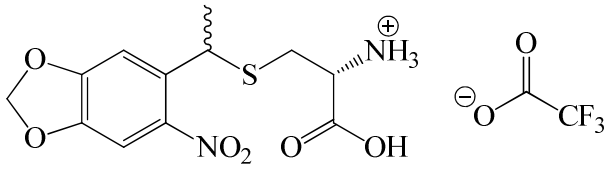
Yield: 166 mg (64%).

^{1}H NMR (400 MHz, $CDCl_3$,): δ = 7.34 & 7.33 (s, 1H, Ar-H); 7.18 & 7.15 (s, 1H, Ar-H); 6.13 & 6.12 (s, 2H, OCH_2O); 5.28 & 5.27 (d, ^3J = 7.8 Hz, 1H, NH); 5.03 (q, ^3J = 7.0 Hz, 1H, ArCH); 4.64 - 4.53 (m, 1H, NCH); 3.04 (dd, ^3J = 4.8 Hz, ^2J = 12.9, 1H, SeC**H**$_a$CH$_b$); 2.92 (dd, ^3J = 5.5 Hz, ^2J =

12.9 Hz, 1H, $SeCH_aC\mathbf{H_b}$); 1.72 & 1.70 (d, ^3J = 7.0 Hz, 3H, CH_3); 1.47 (s, 9H, 3 x CH_3).

HRMS: m/z $[M + Na]^+$ calcd $[C_{17}H_{22}N_2O_8Se + Na]$: 485.0435; found: 485.0456.

S-(1-[4',5'-(methylenedioxy)-2-nitrophenyl]ethyl)-*L*-cysteine trifluoroacetate (20)



N-Boc-*S*-(1-[4',5'-(methylenedioxy)-2-nitrophenyl]ethyl)-*L*-cysteine (**18**) (108 mg; 0.261 mmol) was cooled to 0°C and TFA (0.5 ml; 6.5 mmol) was added. The mixture was warmed to room temperature and stirred for 25 minutes under an argon atmosphere. On completion, dichlorometane (5 ml) was added to the reaction mixtureand volatiles were removed with a stream of argon (this step was repeated until no TFA was present in the outgoing stream). The residue was then dissolved in chloroform, diethyl ether was added to the solution and the target product was collected by filtration. The resulting solid was washed with diethyl ether and dried under vacuum to yield *S*-(1-[4',5'-(methylenedioxy)-2-nitrophenyl]ethyl)-*L*-cysteine trifluoroacetate (**20**) as yellow crystals.

Yield: 45 mg (55%); 90°C (dec.).

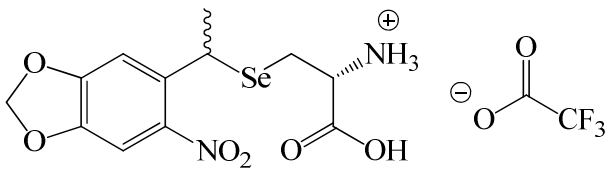
^{1}H NMR (400 MHz, DMSO-d_6,): δ =7.53 & 7.52 (s, 1H, Ar^3-H); 7.36 & 7.35 (s, 1H Ar^6-H); 6.24 & 6.23 (s, 2H, O-CH_2-O); 4.67 & 4.64 (q, ^3J = 6.8 Hz, 1H, Ar-CH); 3.72 (m, 1H, NCH); 2.87 (dd, ^3J = 4.6 Hz, ^2J = 14.0 Hz, 1H, $C\mathbf{H_a}H_b$); 2.77 - 2.66 (m, 1H, $CH_a\mathbf{H_b}$); 1.53 & 1.52 (d, ^3J = 6.8 Hz, 3H, CH_3).

^{13}C NMR (100 MHz, DMSO-d_6): δ = 169.2 & 168.9; 152.1; 147.1; 143.3 & 143.1; 135.3; 108.1; 105.0 & 104.9; 103.8; 53.2; 37.9 & 37.8; 32.9 & 32.6; 22.8.

IR ν (cm^{-1}): 1675 (C=O); 1506 (NO_2); 1337 (NO_2); 1258 (C-O).

HRMS: m/z $[M + H]^+$ calcd $[C_{12}H_{14}N_2O_6S + H]$: 315.0645; found 315.0654.

***Se*-(1-[4',5'-(methylenedioxy)-2-nitrophenyl]ethyl)-*L*-selenocysteine trifluoroacetate (21)**



N-Boc-*S*-(1-[4',5'-(methylenedioxy)-2-nitrophenyl]ethyl)-*L*-cysteine (**19**) (166 mg; 0.359 mmol) was cooled to 0°C and TFA (0.75 ml; 9.8 mmol) was added. The mixture was warmed to room temperature and stirred for 15 minutes under an argon atmosphere. On completion, dichlorometane (5 ml) was added to the reaction mixtureand volatiles were removed with a stream of argon (this step was repeated until no TFA was present in the outgoing stream). The residue was then dissolved in chloroform, diethyl ether was added to the solution and the target product was collected by filtration. The crystals were washed with diethyl ether and dried under vacuum to yield *Se*-(1-[4',5'-(methylenedioxy)-2-nitrophenyl]ethyl)-*L*-selenocysteine trifluoroacetate (**21**) as yellow crystals.

Yield: 84 mg (49%); 50°C (dec.).

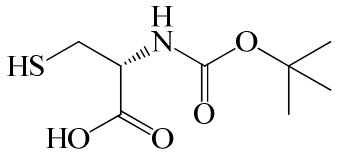
^{1}H NMR (400 MHz, DMSO-d_6,): δ = 8.40 (br s, 3H, NH_3); 7.51 & 7.50 (s, 1H, Ar-H); 7.30 (s, 1H, Ar-H); 6.23 & 6.22 (s, 2H, OCH_2O); 4.85 & 4.82 (q, ^{3}J = 7.0 Hz, 1H, ArCH); 4.12 & 4.04 (m, 1H, NCH), 2.99 & 2.98 (dd, ^{3}J = 5.7 Hz, ^{2}J = 15.9 Hz, 1H, SeC**H**$_a$CH$_b$); 2.86 & 2.82 (dd,^{3}J = 6.0 Hz,^{2}J = 15.9 Hz, 1H, SeCH$_a$C**H**$_b$), 1.68 (d, ^{3}J = 7.0 Hz, 3H, CH_3).

^{13}C NMR (100 MHz, DMSO-d_6): δ = 170.0; 152.1; 147.0; 142.4 & 142.3; 135.6 & 135.4; 108.5 & 108.4; 105.2 & 105.1; 103.8; 52.9 & 52.8; 33.2 & 32.7; 23.4 & 23.2; 22.9 & 22.8.

IR ν (cm^{-1}): 1677 (C=O); 1519 (NO_2); 1333 (NO_2); 1259 (C-O).

HRMS: m/z $[M + H]^+$ calcd [$C_{12}H_{14}N_2O_6Se$ + H]: 363.0090; found: 363.0108.

_N_-Boc-_L_-cysteine (25)



_N_,_N'_-bisboc-_L_-cystine (**16**) (200 mg; 0.454 mmol), triphenylphosphine (143 mg; 0.545 mmol), sodium acetate (24.1 mg; 0.294 mmol) and glacial acetic acid (24 µl) were dissolved in a mixture of MeOH (3 ml) and H_2O (1 ml), heated to reflux and stirred for 14 hours under an argon atmosphere. The reaction was monitored by TLC (ethyl acetate / dichloromethane / AcOH - 1:1:0.01, $R_f = 0.45$). On completion the reaction mixture was concentrated to 1/3 of its volume, diluted with NaOH (1 M; 1.5 ml), washed with AcOEt (3 × 3 ml) and acidified to pH = 2 with $NaHSO_4$ (1 M). The product was extracted with AcOEt (3 × 3 ml), the combined extracts were dried over anhydrous sodium sulphate and the solvent was evaporated to yield the product as a slightly yellow oil.

Yield: 200 mg (99.5%).

^{1}H NMR (400 MHz, D_2O): δ = 4.28 (br s, 1H CH); 2.87 (m, 2H, CH_2); 1.35 (s, 9H, CH_3).

RESULTS AND CONCLUSIONS

- The method for the preperation of 1-bromo-1-[4',5'-(methylenedioxy)-2-nitrophenyl]ethane was improved. The procedures were simplified and the overall yield was increased to 21% (10% lit.).

- Cysteine and selenocysteine derivatives bearing the NPE photolabile protecting group applicable for protein engineering were synthesised.

- It was found that NPE protected amino acids are not stable at elevated temperatures and acidic media and so these conditions should either be avoided, or should be mild as possible when handling these compounds.

Proteinų inžinerijoje pritaikomų negamtinių, fotolabilią α-metil-6-nitropiperonil apsaugą turinčių, cisteino ir selenocisteino aminorūgščių sintezė

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Darbo vadovas: prof. dr. Viktoras Masevičius.

Apibendrinimas

Selenas yra labai svarbus normaliai gyvų organizmų veiklai. Jis dažniausiai yra aptinkamas organizmuose kaip aminorūgšties cisteino analogas turintis seleno atomą vietoje sieros. Selenocisteinas, lyginant su cisteinu, pasižymi stipresniu nukleofiliškumu, žemesne pK_a verte, mažesniu redokso potencialu ir todėl yra labai patrauklus tyrimų objektas bioinžinerijoje. Deja, selenocisteinas yra per daug reaktyvus, kad būtų tiesiogiai naudojamas, todėl pirma suformuojamas mažiau aktyvus jo darinys - selenoeteris - dažnai tai atliekama įvedant apsauginę grupę. Fotolabilios apsauginės grupės suteikia galimybę jas lengvai pašalinti nenaudojant agresyvių medžiagų. Pašalinimas atliekamas apšviečiant apsaugotą darinį artimąja ultravioletine spinduliuote.

Šio bakalaurinio darbo tikslas buvo susintetinti NPE fotolabilią apsauginę grupę turinčius cisteiną ir selenocisteiną. NPE fragmento pasirinkimas buvo sąlygotas nedidelio šios grupės sterinio faktoriaus ir didesnio fotolabilumo. Pastarasis leidžia šią apsauginę grupę taikyti jautriose biologinėse sistemose, kuriose trumpesnio nei 300 nm bangos ilgio šviesa gali pakenkti tos sisemos elementams. NPE fragmentodydis leidžia šią grupę taikyti su fermentais, kurių aktyviuose centruose laisvas tūris yra labai apribotas.

Bakalaurinio darbo metu buvo susintetintas fotolabilios apsauginės grupės įvedimui taikytas pirmtakas, 1-brom-1-[4',5'-(metilendioksi)-2-nitrofenil]etanas, jo sintezės metodika buvo patobulinta - procedūros supaprastintos, pagerinta bendra sintezės išeiga (lyginant su literatūroje aprašyta). Ši apsauginė grupė buvo prijungta prie cisteino ir selenocisteino tiolio ir selenolio grupių patenkinamomis išeigomis. Suformuoti junginiai sėkmingai pritaikyti baltymų inžinerijoje. Taip pat įvertintas anksčiau minėtų apsaugotų aminorūgščių stabilumas aukštesnėse temperatūrose ir rūgštinėse sąlygose.

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