Novel N-Derivatized-7-Nitroindolines For The Synthesis Of Photocleavable Crosslinkers, And For Solid Phase Peptide Synthesis

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NOVEL N-DERIVATIZED-7-NITROINDOLINES FOR THE SYNTHESIS OF PHOTOCLEAVABLE CROSSLINKERS, AND FOR SOLID PHASE PEPTIDE SYNTHESIS

HECTOR PATRICIO DEL CASTILLO VAZQUEZ

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Dedication

To my mother, Lucia Vázquez, whose love injected a hunger for knowledge and success in me.

To my father, Héctor Del Castillo, whose love taught me about perseverance and hard work.

To my brother, Adrián Del Castillo, whose love carved discipline and passion from within.

To my husband, Brian Grajeda, whose understanding and unprejudiced heart inspired me to grow.

To my dear sister-in-law, Trinidad Chavarín; and my nephew, Máximo Del Castillo.
NOVEL N-DERIVATIZED-7-NITROINDOLINES FOR THE SYNTHESIS OF PHOTOCLEAVABLE CROSSLINKERS,
AND FOR SOLID PHASE PEPTIDE SYNTHESIS

by

HECTOR PATRICIO DEL CASTILLO VAZQUEZ, B. Sc.

DISSERTATION

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Abstract

7-Nitroindoline-based photochemistry has focused mostly on N-acyl-7-nitroindolines, which have been used as photocleavable protecting groups for carboxylic acids that can be efficiently removed by illumination with UV-light. They offer an alternative to the more commonly used o-nitrobenzyl-based photocleavable protecting groups. N-acyl-7-nitroindolines can also be used as photochemical acylating agents in inert solvents. For example, they have found application in peptide fragment condensation, the convergent synthesis of N-glycopeptides, and the synthesis of peptide thioesters, forming amide and thioester bonds with amine and thiol nucleophiles, respectively, under neutral conditions. The photochemical activation of N-acyl-7-nitroindolines can occur with UV light (\( \lambda = 350 \) nm) by a one-photon absorption process, or with infrared femtosecond laser light (\( \lambda = 710 \) nm) by a two-photon absorption mechanism. Other 7-nitroindoline-based photoreactive moieties have been reported in the literature such as 7-nitroindoline-based ureas and carbamates, which inspired our discovery of 7-nitroindoline-based thiocarbamates, or N-thiocarbamoyl-7-nitroindolines.

In this work, we developed a new N-acyl-7-nitroindoline-based linker for SPPS and devised a strategy for the concomitant cleavage and cyclization of a homodetic RGD-pentapeptide on Sieber Amide resin, which was compared to the photochemical cyclization of the same peptide in solution. We also discovered the novel N-thiocarbamoyl-7-nitroindoline moiety and studied its photolysis kinetics by UV-Vis spectroscopy, and characterized its photolysis product, a 7-nitroso-indoline, to pave the way for the development of a new class of photocleavable crosslinker. Lastly, we incorporated both, N-acyl-7-nitroindoline and N-thiocarbamoyl-7-nitroindoline moieties, into three photocleavable homobifunctional crosslinkers equipped with N-hydroxysuccinimide moieties for crosslinking amine-containing polymers. These photocleavable crosslinkers can generate functional materials with the capability of crosslinking reversal by light illumination. These crosslinkers were used to crosslink gelatin type B in dimethyl sulfoxide. Upon
replacement of the dimethyl sulfoxide with water, hydrogels were obtained, which can be degraded by illumination with UV-light in water. Such photoreactive hydrogels could be useful in biophotolithography applications or as biomaterials whose rigidity or softness can be changed with light.
# Table of Contents

Abstract ............................................................................................................................................................... v

Table of Contents ............................................................................................................................................... vii

List of Figures ...................................................................................................................................................... ix

List of Schemes ................................................................................................................................................... xiii

Chapter 1: Synthesis of a New N-acyl-7-Nitroindoline Crosslinker by a Suzuki Cross-Coupling Reaction and its Application in the Synthesis of a Cyclic RGD-Containing Peptide ................................................................. 1

1.1 Introduction .................................................................................................................................................. 1

1.2 Specific Aims ............................................................................................................................................ 2

1.3. Background and Significance .............................................................................................................. 2

1.3.1 Introduction to N-acyl-7-Nitroindoline Chemistry ........................................................................ 2

1.3.1 Application of N-acyl-7-Nitroindolines in Photochemical Acylations on the Resin .......... 6

1.3.1 Cyclic Peptides, their Cyclization Strategies and the RGD Motif ............................................. 8

1.4 Results and Discussion ........................................................................................................................... 10

1.4.1 Synthesis of a New Linker by a Suzuki Cross-Coupling Reaction ........................................ 10

1.4.2 Synthesis of c(RGDfV) through On-Resin and Solution-Phase Chemistry ........................... 12

1.5 Conclusions ............................................................................................................................................. 15

1.6 Experimental Section ............................................................................................................................. 16

1.6.1 General Information ......................................................................................................................... 16

1.6.2 Experimental Procedures and Spectroscopic Data .................................................................. 17

Chapter 2: Discovery of the Photoreactive N-Thiocarbamoyl-7-Nitroindoline Moiety, Study of its Photolysis by 1 and 2-Photon Absorption ................................................................................................. 26

2.1 Introduction ............................................................................................................................................. 26

2.2 Specific Aims .......................................................................................................................................... 28

2.3. Background and Significance ........................................................................................................... 28

2.3.1 The Discovery of Photolabile 7-Nitroindoline Carbamic Acid Derivatives .......................... 28

2.3.1 A Brief Introduction to Two-Photon Absorption (2PA) ............................................................ 30

2.3.3 Two Photon Absorption (2PA) in the Context of 7-Nitroindoline Photochemistry ........ 33

2.4 Results and Discussion ........................................................................................................................... 35

2.4.1 Synthesis of Three N-Thiocarbamoyl-7-Nitroindoline Model Compounds ..................... 35
2.4.2 Kinetic Study of the 1-Photon Absorption (1PA) Photolyses of N-Thiocarbamoyl-7-Nitroindolines and its Comparison to a Model N-Acyl-7-Nitroindoline ................................................................. 36
2.4.3 Two-Photon Absorption (2PA) Photolyses of N-Thiocarbamoyl-6-Nitroindoline 25 and its Comparison to a Model N-acyl-7-Nitroindoline X ......................................................... 45
2.4.4 Characterization of Photolysis Product “5-Bromo-7-Nitrosoindoline (9-A)’ and its Reversible Conversion to its Azodioxy Dimer (9-B) ................................................................. 48
2.4.5 Structural Computational Studies on Photolysis Product “5-Bromo-7-Nitroindoline” ................................................................................................................................. 53

2.5 Conclusions and Future Work ................................................................................................................................. 57
2.6 Experimental Section ................................................................................................................................................. 58
    2.6.1 General Information .......................................................................................................................................... 58
    2.6.2 Experimental Procedures and Spectroscopic Data ............................................................................................. 59

Chapter 3: Synthesis of N-Derivatized-7-Nitroindoline Homobifunctional Crosslinkers for the Elaboration of Photoerodible Hydrogels ..................................................................................... 64
    3.1 Introduction ............................................................................................................................................................ 64
    3.2 Specific Aims .......................................................................................................................................................... 65
    3.3. Background and Significance ............................................................................................................................. 65
        3.3.1 Biopolymeric Hydrogels as Scaffolds for 3D Tissue Engineering ................................................................. 65
        3.3.2 Photocleavable Crosslinkers and their Application in Material Science ....................................................... 67
    3.4 Results and Discussion ........................................................................................................................................ 71
        3.4.1 Synthesis of HP1, HP2, and HP3 .................................................................................................................... 71
        3.4.2 Crosslinking of HP1, HP2, and HP3 to Gelatin Type-B .................................................................................. 74
    3.5 Conclusions and Future Work ............................................................................................................................ 76
    3.6 Experimental Section ........................................................................................................................................... 77
        3.6.1 General Information ....................................................................................................................................... 77
        3.6.2 Experimental Procedures and Spectroscopic Data ........................................................................................ 78

References ..................................................................................................................................................................... 86
Appendix ...................................................................................................................................................................... 90
Vita 150
List of Figures

**Figure 1**: Kinetic trace graph of the formation for 17 from the resin (Route A). ................................................ 14

**Figure 2**: Time vs absorbance kinetic graph linearized by an exponential curve. The resulting equation was $Y = -0.804e^{-0.010 + 1.01}$, where constant B described the rate of reaction $\beta$. .......................................................... 14

**Figure 3**: (Left) Kinetic trace graph of the photolysis of a model N-acyl-7-nitroindoline in anhydrous conditions (solid) and in excess water conditions (dashed). (Right) Molar % water in acetonitrile vs relative yield of 7-nitroindoline (white squared) and 7-nitrosoindoline (black squares). ............................... 29

**Figure 4**: (Left) Kinetic trace graph of the photolysis of a model 7-nitroindoline carbamate in low concentrations of water. (Right) Kinetic trace graph of the photolysis of a model 7-nitroindoline urea in low concentrations of water. 6 .............................................................................................................. 30

**Figure 5**: Simplistic representation of the Jablonski diagram of an N-acyl-7-nitroindoline in its ground state (GS) being excited towards its excited state (ES) by 1 photon ($\lambda = 350$ nm) in a single-photon absorption process [left], while it is promoted to its ES by 2 photons ($\lambda = 710$ nm) in a two-photon absorption process [right]. ......................................................................................................................... 31

**Figure 6**: Schematic of the two-photon (2PA) laser scanning microscope developed by Dr. Chunqiang Li at the biophotonics laboratory at UTEP. ........................................................................................................... 32

**Figure 7**: (Left) Fluorescent decay graphs of a 34-mer N-peptidyl-7-nitroindoline generated by a 2PA fluorescent microscope at different laser powers. (Right) Double-log $\beta$ plot of reaction rate vs laser intensity with a slope of 2.007 which indicates a 2PA process. 13 .......................................................... 33

**Figure 8**: (A) Initial fluorescent tree pattern generated by two-photon excitation of the 34mer peptide with femtosecond laser at 710 nm light through a tree mask at time 0 min; (B) fluorescent tree pattern after 10 min of irradiation, fluorescence decay is observed; (C) tree pattern after 30 min of irradiation, fluorescence is barely visible due to progressive photolysis to non-fluorescent products; (D) after removal of mask and immediate recording of the fluorescence only the background fluoresces while the tree appears dark; (E) tree under white light. 29 .............................................................................................................. 34

**Figure 9**: Human mesenchymal stem cells, pre-stained with cell membrane stain PKH26, and seeded atop 34mer photoreactive peptide pre-immobilized onto tissue culture grade wells. (Left) Images taken from center of the well. (Right) Cells grew only where the peptide was present. 29 .............................................................................................................. 34

**Figure 10**: Kinetic trace graph of the photolysis of 25 in 20% H2O in acetonitrile at a concentration of $8 \times 10^{-3}$ M ........................................................................................................................................ 36

**Figure 11**: Kinetic trace graph of the photolysis of X in 20% H2O in acetonitrile at a concentration of $8 \times 10^{-3}$ M ........................................................................................................................................ 37

**Figure 12**: Kinetic trace graph of the photolysis of 26 in 20% H2O in acetonitrile at a concentration of $8 \times 10^{-3}$ M ........................................................................................................................................ 38

**Figure 13**: Kinetic trace graph of the photolysis of 27 in 20% H2O in acetonitrile at a concentration of $8 \times 10^{-3}$ M ........................................................................................................................................ 38
**Figure 14:** Time vs absorbance graph describing the kinetic behaviour of 25. (Circles) Describe the formation of photolysis products at 504 nm. [Triangles] Describe the decay of 25 at 352 nm ............... 39

**Figure 15:** Time vs absorbance graph describing the kinetic behaviour of X. (Circles) Describe the formation of photolysis reactions products at 450 nm. .......................................................... 39

**Figure 16** Time vs absorbance graph describing the kinetic behaviour of 26. (Circles) Describe the formation of photolysis products at 462 nm. (Triangles) Describe the decay of 26 at 359 nm ............... 40

**Figure 17:** Time vs absorbance graph describing the kinetic behaviour of 27. (Circles) Describe the formation of photolysis products at 487 nm .......................................................... 35

**Figure 18:** Time vs absorbance graph describing the photochemical decay of 25. The equation that describes this reaction is $Y = 0.75e^{-0.0014} + 0.27$, where the rate of reaction was $1.4 \times 10^{-3}$ s$^{-1}$ ............... 41

**Figure 19:** Time vs absorbance graph describing the linearization of the formation of 29 from 25. The equation that describes this reaction is $Y = -1.10e^{-0.0018} + 1.11$, where the rate of reaction was $1.7 \times 10^{-3}$ s$^{-1}$ .......................................................... 41

**Figure 20:** Time vs absorbance graph describing the linearization of the formation of 5-bromo-7-nitroindoline and 5-bromo-7-nitroso indole from X. The equation that describes this reaction is $Y = -0.95e^{-0.0032} + 1.01$, where the rate of reaction was $3.2 \times 10^{-3}$ s$^{-1}$ .......................................................... 42

**Figure 21:** Time vs absorbance graph describing the photochemical decay of 26. The equation that describes this reaction is $Y = 0.73e^{-0.0061} + 0.30$, where the rate of reaction was $6.1 \times 10^{-3}$ s$^{-1}$ ............... 42

**Figure 22:** Time vs absorbance graph describing the linearization of the formation of 29 from 26. The equation that describes this reaction is $Y = -1.06e^{-0.0087} + 1.04$, where the rate of reaction was $8.7 \times 10^{-3}$ s$^{-1}$ .......................................................... 43

**Figure 23:** Time vs absorbance graph describing the linearization of the formation of 29 from 27. The equation that describes this reaction is $Y = -1.08e^{-0.0021} + 1.07$, where the rate of reaction was $2.1 \times 10^{-3}$ s$^{-1}$ .......................................................... 43

**Figure 24:** Experiments carried out on a film of X. (Top left) Fluorescence decay plots with exponential fitting at varying laser power. (Top right) Double log β plot vs. laser intensity for photoreactive graph. (Bottom left) A fluorescent logo of UTEP was generated by femtosecond laser at 710 nm through a UTEP mask. (Bottom middle) the letters of the logo were the only area of the film that underwent photolysis. After removal of the mask only the background fluoresces. (Bottom right) UTEP logo under white light. .................................................................................. 45

**Figure 25:** Fluorescent microscope image depicting the fluorescence of 25 over the surface of a chip of silica TLC. (Left) Visualization of the fluorescence at t=0. (Right) Visualization of the fluorescence decay after 1 hour of exposure only at a specific region in the center (white circle)................................. 46

**Figure 26:** Decay of 25 as seen through white light through the microscope. Yellow starting material 25 is seen on the left, while photolysis product 29 is seen in on the right ........................................... 46

**Figure 27:** Comparison of the photolysis of 25 and X by femtosecond laser light at a power of 150 mW. Both reactions were completed in around 60 minutes, with the photolysis of X being slightly faster .............................. 47

**Figure 28:** Decay curves of 25 at different laser intensities ........................................................................ 47
**Figure 29:** Double log plot ($\beta$ vs laser intensity) for the photolysis of 25. A slope of 2.1 represented a preliminary result that validates a 2PA absorption process. ................................................................. 48

**Figure 30:** $^1$H-NMR spectrum of commercially available 5-bromo-7-nitroindoline vs (Bottom) $^1$H-NMR spectrum of the unknown pink photolysis product. ................................................................................................................................. 49

**Figure 31:** Variable temperature $^1$H-NMR study of unknown pink spot from -60 to 50°C. Only signals above 8.0 ppm are affected by the change in the temperature. .................................................................................................................. 50

**Figure 32:** Zoomed area from Figure 31 depicting both signals affected by the temperature change .... 51

**Figure 33:** (Top) Mass spectrum of 5-bromo-7-nitrosoindoline 29-A $m/z$ [M+H]$^+$ calcd.: 226.9815; obs.: 226.9813. (Bottom) Zoomed area depicting the presence of the azodioxy dimer 29-B, with its characteristic dibrominated isotopic pattern $m/z$ [M+Na]$^+$ calcd.: 474.9381; obs.: 474.9387. ..................... 52

**Figure 34:** UV-Vis spectrum of pure 5-bromo-7-nitrosoindole, depicting two maxima at 302 and 481 nm. ........................................................................................................................................... 53

**Figure 35:** Dimerization of C-nitroso compounds and dissociation of azodioxy dimers under different conditions................................................................................................................................. 53

**Figure 36:** (Top) Structure of 9-A, all positions of the indoline are highlighted. (Bottom) Theoretical $^1$H-NMR calculation of 9-A. ............................................................................................................................................... 54

**Figure 37:** (Top) Structure of 9-B at an angle where the proton at the position 6 and the azodioxy oxygens are not closest. (Bottom) Theoretical $^1$H-NMR calculation of 9-B. .................................................................................................................. 55

**Figure 38:** (Top) Structure of 9-B at an angle where the proton at the position 6 and the azodioxy oxygens are closest. (Bottom) Theoretical $^1$H-NMR calculation of 9-B. .................................................................................................................. 56

**Figure 39:** Organ-scale FRESH 3D bioprinting of the neonatal-scale human heart. (Left) MRI-derived 3D human heart scaled to neonatal size. (Middle) FRESH-printed collagen heart. (Right) Cross-sectional view of the collagen heart, showing left and right ventricles and interior structures. ................................................. 66

**Figure 40:** (Left) Elastic and (Right) compressive modulus of polyacrylamide hydrogels varying the chain length of the PEG crosslinker. ........................................................................................................ 67

**Figure 41:** (Top) Schematics demonstrating photoinduced patterning of a PEG diacrylate network at the microscale. (Bottom) 3D OsiriX rendering based on a confocal image series of a hydrogel photopatterned with two-photon absorption light with a 3D spiral of fluorescently labelled ACRL-PEG-peptide H) 3D OsiriX confocal image with fluorescently labelled ACRL-PEG-peptide in parallelograms of equal cross-sectional area (100 µm x 100 µm) but of varying heights. ................................................. 68

**Figure 42:** (Top) Structure of the PEG-diacrylate photocleavable crosslinker designed by the Anseth group for radical initiated crosslinking. (Middle) Interconnected 3D channels fabricated within the photodegradable hydrogel covalently labeled with rhodamine B, using a two-photon laser scanning microscope. (Bottom) Migration of a cell along the edge of a photolyzed channel in time-lapsed brightfield images (left) and its corresponding position trace (right). ........................................................................................................ 69

**Figure 43:** Structure of the PEG tetra alkyne photocleavable crosslinker designed by Kanamori et al. for crosslinking by a SPAAC reaction with azide-derivatized gelatin. ........................................................................................................ 70
Figure 44: Top) Structure of the PEG tetra alkyne crosslinker (right) designed by the DeForest group for crosslinking by a SPAAC reaction with an azide-containing peptide (left). (Bottom) A) Microvessels of different sized generated by photoreactive degradation by femtosecond laser light in a 2PA phenomenon. Perfusion of the vessels using fluorescent polymer beads confirms the diffusion inside of the capillary-like structure. B) Photographic image with regular light of the hydrogel showing the vasculature-like architecture on the right. 71

Figure 45: Gelatin-type B gels using the following crosslinkers: 1) HP1; 2) HP2; 3) HP3. 76
List of Schemes

**Scheme 1**: The photochemical condensation of two protected peptide fragments by Pass et al\(^2\) .......... 2

**Scheme 2**: Reaction scheme showing the solvent-dependent photochemical reactions of N-acyl-7-nitroindolines\(^3\) ................................................................................................................................. 3

**Scheme 3**: Mechanism of reaction of Pathway A, proposed by Morrison et al\(^3\) ............................................ 4

**Scheme 4**: Mechanism of reaction of Pathway B, proposed by Morrison et al\(^3\) ............................................ 4

**Scheme 5**: Mechanism of reaction of Pathway A, proposed by Mendez et al\(^4\) ............................................ 5

**Scheme 6**: Mechanism of reaction of Pathway B, proposed by Mendez et al\(^4\) ............................................ 5

**Scheme 7**: On-resin photochemical transamidations via on-resin N-acyl-7-nitroindoline photochemistry\(^14\) ......................................................................................................................... 6

**Scheme 8**: Formation of peptide-\(\alpha\)-thioesters by the photochemical substitution of N-acyl-7-nitroindolines with pyridine (top) and HOBt (bottom), and the subsequent nucleophilic substitution with ethyl mercaptan. Only the experiment using D-alanine is shown, but L-alanine was also utilized\(^11\) .......... 7

**Scheme 9**: On-resin synthesis of cyclic peptides via tandem N-to-S acyl migration and intramolecular NCL by Serra et al\(^20\) .................................................................................................................................................. 9

**Scheme 10**: 7-step synthesis of N-acyl-7-nitroindoline linker for SPPS developed in our laboratory ...... 10

**Scheme 11**: Suzuki cross-coupling reaction of 5-bromo-7-nitroindoline and phenylboronic acid patented by Masaki et al\(^24\) ............................................................................................................................................. 10

**Scheme 12**: Mechanism of reaction of the Suzuki-Miyaura cross-coupling using 5-bromo-7-nitroindoline and 5-(4-carboxyphenyl)boronic acid pinacol ester (11) ................................................................................................................................. 11

**Scheme 13**: 4-step synthesis of a N-acyl-7-nitroindoline linker for SPPS using a Suzuki-Miyaura cross-coupling reaction .................................................................................................................. 12

**Scheme 14**: Reaction pathway depicting Route A, B and C for the synthesis of c(RGDfV). a) \(\lambda = 350\) nm, anhydrous, oxygen-free DCM, conc = \(10^{-4}\). b) TFA/H\(_2\)O/TIPS 95/2.5/2.5. c) DPPA, NaHCO\(_3\) in DMF, conc = \(10^{-3}\) M .................................................................................................................................................. 13

**Scheme 15**: Representation of a N-(Fmoc-glycyl)-5-bromo-7-nitroindoline (X) using 5-bromo-7-nitroindoline and the acyl chloride from Fmoc-glycine-OH\(^29\) .................................................................................................................. 13

**Scheme 16**: Reaction pathway for the synthesis of 7-nitroindoline-based ureas and carbamates established by Hasser et al\(^6\) .............................................................................................................................................. 29

**Scheme 17**: Synthesis of three different N-thiocarbamoyl-7-nitroindolines using different 7-nitroindolines and thiols to validate the efficiency of the strategy ................................................................. 35

**Scheme 18**: Photolysis of N-thiocarbamoyl-7-nitroindoline 25 to form 5-bromo-7-nitrosoindoline (29-A), its corresponding azodioxy dimer (29-B), ethanethiol, and carbon dioxide ................................................................................................................................. 48

**Scheme 19**: Photolysis of the \(\alpha\)-nitrobenzyl functional group ............................................................................. 65

**Scheme 20**: Synthesis of N-acyl-7-nitroindoline homobifunctional crosslinker HP1 ..................................... 72
Scheme 21: Synthesis of N-acyl-7-nitroindoline homobifunctional crosslinker HP2 ........................................ 73
Scheme 22: Synthesis of N-thiocarbamoyl-7-nitroindoline homobifunctional crosslinker HP3 ............... 74
Scheme 23: Elaboration of a photoerodible gelatin-based hydrogels using HP1 as a model homobifunctional crosslinker ................................................................. 75
Chapter 1

Synthesis of a New \textbf{N-Acyl-7-Nitroindoline} Crosslinker by a Suzuki Cross-Coupling Reaction and its Application in the Synthesis of a Cyclic RGD-Containing Peptide

1.1 Introduction

In the 1970s, the Patchornik group studied the photoreactive capabilities of \textbf{N-acyl-7-nitroindolines} when illuminated with UV-light ($\lambda = 350$ nm) and proposed them as efficient photocleavable protecting groups for carboxylic acids, and as versatile acylating agents for different nucleophiles. Since then, they have been used as precursors for the synthesis of esters, carbamic acid derivatives, amino acid derivatives, glycosyl amino acids, glycopeptides, peptide thioesters, peptide hydrazides, and peptide acids; and they have also found various applications in peptide chemistry, neurochemistry, biophotolithography and 3D tissue bioengineering. Ever since \textbf{N-acyl-7-nitroindolines} were first applied to solid phase synthesis in 2001, various research groups including our own have developed different linkers suitable for their coupling to functionalized polymeric resins for Fmoc/t-Bu SPPS protocols. One of the many applications in which \textbf{N-acyl-7-nitroindoline} have not yet been explored is in the production of homodetic backbone cyclized peptides, which have attracted many research groups for their superior pharmacological properties over their linear counterparts. Concomitant peptide cleavage and homodetic backbone cyclization on the resin is a strategy that shortens synthetic and purification steps, and it has been explored using other strategies like Native Chemical Ligation (NCL). We believe that a photochemical approach to a simultaneous cleavage and cyclization of a peptide on the resin can overcome the problem of NCL requiring thiol-containing elements on the peptide sequence and can become a more versatile tool for other types of peptides.

\textit{We hypothesized that the use of a Suzuki cross-coupling reaction will allow for a short synthesis of a \textbf{N-acyl-7-nitroindoline}-based linker for solid phase peptide synthesis. Such linker will be suitable for on-resin homodetic cyclization of peptides and simultaneous cleavage from the solid support.}
1.2 Specific Aims

1.2.1 To synthesize a new photoreactive N-acyl-7-nitroindoline-based linker using a Suzuki cross-coupling reaction.

1.2.2 To synthesize an RGD-containing pentapeptide through three different approaches: photochemical on-resin concomitant cleavage and backbone cyclization, photochemical backbone cyclization in solution and backbone cyclization in solution using a conventional azide approach; and compare them.

1.3 Background and Significance

1.3.1 Introduction to N-Acyl-7-Nitroindoline Chemistry

The Patchornik group studied the photocleavable capabilities of the amide bond of N-substituted α-nitroanilines, 8-nitro-1,2,3,4-tetrahydroquinolines and N-acyl-7-nitroindolines, which produced free carboxylic acids in high yields under mild, neutral conditions\(^1\). N-acyl-7-nitroindolines were initially thought as promising orthogonal protecting groups for carboxylic acids, and their unique acylating capabilities were discovered later in the photocondensation of peptide fragments\(^2\) (Scheme 1).

![Scheme 1](image)

**Scheme 1:** The photochemical condensation of two protected peptide fragments by Pass et al\(^2\).

Morrison et al. later elucidated two different solvent-dependent mechanisms of reaction that validated the findings made by the Patchornik group. Upon illumination with UV-light (\(λ = 350\) nm), N-acyl-7-nitroindolines 1 form the highly reactive nitronic anhydride 2. This intermediate can undergo two
different photolysis reactions: **Pathway A**, where a nucleophile substitutes the photolabile moiety under anhydrous aprotic conditions, forming the 7-nitroindoline 3 and a new acylated compound 4; and **Pathway B**, where an intramolecular reduction furnishes nitrosoindole 5 and a carboxylic acid 6 in media with high excess of water (**Scheme 2**). 

**Scheme 2:** Reaction scheme showing the solvent-dependent photochemical reactions of N-acyl-7-nitroindolines. 

In their mechanistic studies Morrison et al. proposed that, upon illumination with UV-light (λ = 350 nm), N-acyl-7-nitroindolines 1 is promoted to a singlet excited state S1, and then triplet excited state T1 where is turns into a tetrahedral intermediate 7. This behaviour has been observed in other nitroaromatic compounds. Rearrangement to the nitronic anhydride intermediate occurs, and in **Pathway A**, a nucleophile in the media attacks the acyl group to form 7-nitroindoline 3 and acylated compound 4 (**Scheme 3**).
In Pathway B, the excess of water in the media acts as a base that removes a hydrogen from the position 2, releasing a carboxylic acid 6 by an intramolecular redox rearrangement, and forming the nitrosoindole 5 upon a last sigmatropic rearrangement (Scheme 4). Further theoretical studies were performed by Mendez et al. employing semi-empirical calculations (AMPAC 9.2) to study the potential energy surface of an acyl shift to form the nitronic anhydride.
in their studies they validated Morrison et al.’s theory of a triplet state T1 that existed only in a conical intersection between S1/T1/S0 states, a second theory was also supported. It was found that on the S1 excited state N-acyl-7-nitroindoline 1 overcomes a small energy barrier to become nitronic anhydride 2. This acyl shift occurs through a 1,5 sigmatropic rearrangement of the carbonyl from the nitrogen to one of the oxygens in the nitro group. The next step of the reaction is solvent dependent, just as explained by Morrison et al (Shemes 5 and 6)\(^4\).

\[\textbf{Scheme 5:} \text{Mechanism of reaction of Pathway A, proposed by Mendez et al}^4.\]

\[\textbf{Scheme 6:} \text{Mechanism of reaction of Pathway B, proposed by Mendez et al}^4.\]
1.3.2 Application of \textit{N}-Acyl-7-Nitroindolines in Photochemical Acylations on the Resin

The acylating capabilities of \textit{N}-acyl-7-nitroindolines have found many applications in organic synthesis. Methodologies for the synthesis of esters\textsuperscript{5}, carboxylic acid derivatives\textsuperscript{6}, amino acid derivatives\textsuperscript{7}, glycosyl amino acids\textsuperscript{5,8,9}, glycopeptides\textsuperscript{10}, peptide thioesters\textsuperscript{11,12}, peptide hydrazides, and peptide acids\textsuperscript{13} have been established in the literature. One of the most important applications of these for our research group is the formation of linkers for SPPS. While many different photochemical peptide reactions have been explored, we will focus only on the photochemical heterogeneous reactions made by the illumination of the peptide still anchored to polymeric solid supports. Nicolau et al. first explored the utilization of \textit{N}-acyl-7-nitroindoline-based linkers for solid phase photochemistry. They derivatized the surface of a SPPS amino methylated resin to contain a system comprised of a 7-nitroindoline attached to benzoic acid or a series of amino acids through an alkyl spacer. The on-resin \textit{N}-acyl-7-nitroindoline containing the benzoic acid and amino acids were illuminated with UV light ($\lambda = 290$ nm) in the presence of various primary and secondary amines to produce various simultaneous photochemical cleavage and transamidations, in both inter and intramolecular fashion (Scheme 7)\textsuperscript{14}.

\textbf{Scheme 7}: On-resin photochemical transamidations via on-resin \textit{N}-acyl-7-nitroindoline photochemistry\textsuperscript{14}.
Other attempts to photorelease peptides in an on-resin way were made by Hogenauer et al in our laboratory. They synthesized a crosslinker suitable for SPPS in a more efficient way than Nicolaou et al., and they used it to produce three different tripeptides: one that contained non-chiral glycine in its C-terminus, and two that contained chiral L-alanine and D-alanine, respectively. Upon illumination with UV-light and in the presence of pyridine or 1-Hydroxybenzotriazole (HOBt), they were able to produce the corresponding activated amide 8 or ester 9. Followed by the nucleophilic substitution with ethyl mercaptan, they produced three different peptide-α-thioesters. While pyridine was effective for glycine, significant epimerization was observed in the case of L-alanine and D-alanine. HOBt, on the other hand, acts more as an acidic nucleophile, unable to abstract the peptide’s alpha-hydrogen, and maintaining the original configuration of the peptide (Scheme 8).11

![Scheme 8: Formation of peptide-α-thioesters by the photochemical substitution of N-acyl-7-nitroindolines with pyridine (top) and HOBt (bottom), and the subsequent nucleophilic substitution with ethyl mercaptan. Only the experiment using D-alanine is shown, but L-alanine was also utilized.](image)
1.3.3 Cyclic Peptides, their Cyclization Strategies and the RGD Motif

Cyclic peptides have been studied for their excellent potential as therapeutics with respect to their linear counterparts. Synthetic strategies for the cyclization of peptides have been described in the literature since the early 1950’s, and their biological studies demonstrate their potency as antibiotics, hormone analogues, immunosuppressants, and antitumorals. Among their properties we can find low toxicity, good binding affinity, target selectivity, cell permeability, and resistance to hydrolysis by exo and endopeptidases due to their lack of amino and carboxy ends. Their rigid ring structures are related to a decrease in the degrees of freedom and entropy term of the Gibbs free energy, which ultimately translates to a decrease in the number of conformers. C-N cyclizations require the prior activation of the carboxyl function, which could occur by means of the formation of active esters such as nitrophenol, azides, carbodiimides, direct methods and cyclization on polymeric support. Homodetic cyclic peptides are those in which the cyclization’s backbone is formed by amide bonds among the amino acid residues, from a junction between the C-terminus and the N-terminus. These type of cyclizations are usually preferred because of their resemblance and affinity to biological systems, over cyclodepsipeptides which are cyclic peptides connected by unconventional or non-natural covalent bonds such as lactones, ethers, thioethers, disulfides, etc. Solution-phase homodetic cyclizations require the generation of mutually reactive chain ends by means of a free amine and a carboxyl function activated to be susceptible to intramolecular nucleophilic attack at high dilution concentrations (10^{-3}, 10^{-4} M), and in yields that are usually not greater than 50%. A recent approach to concomitant cleavage from the resin and homodetic cyclization of peptides was performed by Serra et al. via tandem N-to-S acyl migration and intramolecular thiol additive-free NCL assisted by N-ethylcysteine and using cysteine-containing peptides (Scheme 9).
While this strategy is promising for the on-resin homodetic cyclization of peptides, and the yields of reaction are similar to those of conventional solution-phase methods (18-50%), this approach is only customed to peptide fragments with thiol-containing sequences derived from cysteines and/or unnatural amino acids.

In the field of drug design, the RGD motif constitutes a major recognition system for cell adhesion. The Kessler group performed spatial screening of many pentapeptides containing the RGD motif and found out that the cyclic sequence c(RGDfV) was one of the few that showed high activity in the treatment of glioblastomas in-vitro. This molecule binds and inhibits the activities of the αvβ3 and αvβ5 integrins, thereby inhibiting endothelial cell-cell interactions, endothelial cell matrix interaction, and angiogenesis\textsuperscript{21,22}. One of their greatest achievement in this field was the production of the antitumoral drug Cilengtidine, a cyclic peptide with the sequence c(RGDF[N-Me]V), containing a N-methylation of one the peptide bonds, and with potent inhibition action towards Glioblastoma and other types of cancers in-vitro and in-vivo\textsuperscript{23}. 

**Scheme 9:** On-resin synthesis of cyclic peptides via tandem N-to-S acyl migration and intramolecular NCL by Serra et al\textsuperscript{30}. 
1.4 Results and Discussion

1.4.1 Synthesis of a New Linker by a Suzuki Cross-Coupling Reaction

Hogenauer et al. developed a strategy for the synthesis of an \textbf{N-acyl-7-nitroindoline} linker for SPPS in 7 steps, which represented a shorter, and less time-consuming option than that developed by Nicolaou et al.\textsuperscript{11} (Scheme 10). This linker has been utilized for the synthesis of peptide-\(\alpha\)-thioesters, peptide-\(\alpha\)-phenylthioesters, for photoreactive amino acids and for collagen-like peptides.

\begin{center}
\textbf{Scheme 10}: 7-step synthesis of a N-acyl-7-nitroindoline linker for SPPS developed in our laboratory\textsuperscript{13}.
\end{center}

Masaki Katai et al. explored the Suzuki-Miyaura cross-coupling of commercially available \textbf{5-bromo-7-nitroindoline} with phenylboronic acid in the presence of the catalyst tetrakis(triphenylphosphine) palladium (0) [\text{Pd(Ph}_3\text{)}_4], which had an overall yield of 68%\textsuperscript{24} (Scheme 11).

\begin{center}
\textbf{Scheme 11}: Suzuki cross-coupling reaction of \textbf{5-bromo-7-nitroindoline} and phenylboronic acid patented by Masaki et al\textsuperscript{24}.
\end{center}
Inspired in their findings we explored the cross-coupling of 5-bromo-7-nitroindoline (10) and 5-(4-carboxyphenyl)boronic acid pinacol ester (11), resulting in a quantitative yield of reaction. A Suzuki-Miyaura cross-coupling reaction requires of a halogenated compound and a boronic acid or ester, where any of them could contain an aryl, alkynyl or alkenyl group. By the action of a metal palladium\(^9\) catalyst such as Pd(PPh\(_3\))\(_4\) a new carbon-carbon bond is formed. The three main steps behind this process are: 1) the oxidative addition of the carbon electrophile to the zero-valent and coordinitionally unsaturated palladium reagent; 2) the transmetalation of a nucleophilic carbon from a boron-containing compound; 3) the rapid reductive elimination of the cross-coupling product with the regeneration of the palladium catalyst\(^{25,26,27}\) (Scheme 12).

**Scheme 12:** Mechanism of reaction of the Suzuki-Miyaura cross-coupling using 5-bromo-7-nitroindoline (10) and 5-(4-carboxyphenyl)boronic acid pinacol ester (11).
The resulting carboxylic acid 12 was protected using equimolar amounts of allyl bromide, furnishing ester 13. We prepared the acyl chloride of Fmoc-protected glycine in-situ through an Appel reaction, and subsequently amidated the position 1 to furnish 14. Finally, we deprotected the C-terminal ester using Pd(PPh\textsubscript{3})\textsubscript{4} and N-methyl aniline (NMA), to furnish photoreactive linker 15. The overall yield of reaction for this strategy was 92%, with only 4 synthetic steps, and with only one step of purification by column chromatography, representing a formidable improvement to previous strategies (Scheme 13).

![Chemical diagram]

Scheme 13: 4-step synthesis of a N-acyl-7-nitroindoline linker for SPPS using a Suzuki-Miyaura cross-coupling reaction.

1.4.2 Synthesis of c(RGDfV) through On-Resin and Solution-Phase Chemistry

We tested our photoreactive linker in the synthesis of an RGD-containing cyclic pentapeptide. The sequence c(RGDfV) is an analogue of Cilengitide with a high pharmacological activity, and we chose it to be our model cyclic peptide for this experiment. We want to explore a new approach to the synthesis of cyclic peptides through concomitant photocleavage and cyclization on the resin using our newly
synthesized linker. We also want to compare this with other novel photoreactive and conventional cyclization solution strategies. For this, we synthesized twice on Sieber Amide Resin the linear sequence \textbf{14} (Route A and B) and designed a control peptide \textbf{19} on SASRIN Wang resin (Route C). Standard Fmoc protocols for the synthesis of these peptides were used, and a final deprotection of the N-terminal aspartic acid was done, furnishing a resin-bound peptide primary amine for all cases\textsuperscript{28} (Scheme 14).

\textbf{Route A}

\begin{align*}
\text{H-D(OrBu)-f-V-R(Pbf)-G} & \quad \xrightarrow{a) \text{ cyclo}[R(Pbf)-G-D(OrBu)-f-V]} \quad \text{cyclo}[RGDfV] \\
\text{90\%} & \quad \text{34\%} & \quad \text{92\%}
\end{align*}

\textbf{Route B}

\begin{align*}
\text{H-D(OrBu)-f-V-R(Pbf)-G} & \quad \xrightarrow{c) \text{ H-D(OrBu)-f-V-R(Pbf)-G}} \quad \text{cyclo}[R(Pbf)-G-D(OrBu)-f-V] \\
\text{88\%} & \quad \text{17} & \quad \text{21} & \quad \text{95\%} & \quad \text{24\%}
\end{align*}

\textbf{Route C}

\begin{align*}
\text{H-D(OrBu)-f-V-R(Pbf)-G} & \quad \xrightarrow{c) \text{ H-D(OrBu)-f-V-R(Pbf)-G}} \quad \text{H-D(OrBu)-f-V-R(Pbf)-G-OH} \\
\text{75\%} & \quad \text{17} & \quad \text{20} & \quad \text{22\% in two steps}
\end{align*}

\begin{align*}
\text{H-D(OrBu)-f-V-R(Pbf)-G-OH} & \quad \xrightarrow{d) \text{ cyclo}[R(Pbf)-G-D(OrBu)-f-V]} \quad \text{cyclo}[RGDfV] \\
\text{17} & \quad \text{21} & \quad \text{94\%}
\end{align*}

\textbf{Scheme 14:} Reaction pathway depicting Route A, B and C for the synthesis of c(RGDfV). a) $\lambda = 350$ nm, anhydrous, oxygen-free DCM, conc. $= 10^{-4}$ M. b) TFA/H$_2$O/TIPS 95/2.5/2.5. c) 1% TFA in DCM. d) DPPA, NaN$_2$CO$_3$ in DMF, conc. $= 10^{-4}$ M.

For route A, we illuminated the resin-bound peptide \textbf{16} with UV-light in anhydrous, oxygen-free DCM, furnishing protected cyclic peptide \textbf{17} in 34\% yield. The photolysis of \textbf{16} was completed in 300 min (5 hours) and it was monitored by UV-Vis spectroscopy. The trace graph of this photolysis showed maxima
of absorption at 229 and 256 nm (Figure 1). Time vs absorbance graphs were also designed to study the kinetic of the photoreaction, and the rate of reaction was obtained fitting the data to the exponential Equation 1: $Y = \alpha e^{-\beta t} + \gamma$, where $\beta$ is equal to the rate constant in s$^{-1}$ (Figure 2). The rate of reaction was $1.0 \times 10^{-2}$ s$^{-1}$.

![Figure 1](image1.png)

**Figure 1:** Kinetic trace graph of the formation of 17 from the resin (Route A).

![Figure 2](image2.png)

**Figure 2:** Time vs absorbance kinetic graph linearized by an exponential curve. The resulting equation was $Y = -0.804e^{0.010t} + 1.01$, where constant $B$ describes the rate of reaction $\beta$. 

14
For route B, photoreactive 16 was cleaved off the resin using 1% TFA in DCM, furnishing 18. Illumination of 18 with UV-light under dilute concentrations (10^{-4} M) using anhydrous, oxygen-free DCM, furnished protected peptide 17 in a yield of 24%. For route C, resin bound peptide 19 was cleaved off the resin using 1% TFA in DCM. The resulting linear peptide crude 20 was cyclized using a conventional approach with diphenyl phosphoryl azide (DPPA) and NaHCO\textsubscript{3} at a concentration of 10^{-4} M, furnishing protected cyclic peptide 17 in a yield of 22% over two steps. A similar deprotection step of peptides 17 from all strategies was done using TFA/H\textsubscript{2}O/TIPS 95:2.5:2.5, to furnish deprotected cyclic peptide 21 in yields over 90%.

1.5 Conclusions

A new N-acyl-7-nitroindoline linker has been developed by a Suzuki reaction, suitable for SPPS and in an excellent yield of reaction of 92%. It has been utilized in the SPPS of an RGD-containing peptide. Two new strategies for photochemical cyclization have been established: 1) concomitant photocleavage and backbone cyclization and 2) solution-phase photochemical cyclization in the synthesis of c(RGDfV), an analogue of antitumoral drug Cilengitide. The on-resin cyclization was the best of the strategies, achieves a 34% yield of reaction, which matches the cyclization yields of peptides by traditional methods. The solution-phase cyclization was achieved in a 24% yield, which is also expected from the cyclization of peptides by traditional methods. A comparison using an azide approach to the cyclization of the peptide was effected, and the yield obtained was 22% over two steps. In our studies, the photochemical backbone cyclization of peptides on the resin stands as a promising strategy over other methods because: 1) it is more efficient, 2) it is carried out under mild, anhydrous conditions, 3) it is versatile enough to work with peptides that do not contain any type of thiol chemistry for NCL. A solution phase photochemical approach also offers a thiol-free approach mild, anhydrous conditions.
1.6 Experimental Section

1.6.1 General Information

All chemicals were purchased as reagent grade from Thermo Fisher Scientific, Sigma-Aldrich, or Acros Organic, and used without further purification. The ACS grade solvents used for reactions were obtained from Thermo Fisher Scientific and they were distilled from the appropriate drying agents. Molecular sieves (3Å and 4Å) were purchased from Alfa Aesar and Thermo Fisher Scientific, respectively, and activated under high vacuum and heat prior to use. Reactions were performed under an argon atmosphere, strictly anhydrous conditions and monitored by TLC on silica gel 60 F254 plates from EMD Millipore or Dynamic Adsorbents, Inc. Spots were detected under UV light (254 nm). The purification of the compounds was performed by flash column chromatography on silica gel (40-60 µm) from Thermo Fisher Scientific, and the ratio between silica and crude product ranged from 50:1 to 120:1 (dry w/w). $^1$H and $^{13}$C NMR spectra were recorded on a Bruker Avance III HD 400 MHz NMR spectrometer at 400 and 101 MHz or on a JEOL 600 MHz NMR spectrometer at 600 and 150 MHz, respectively. Chemical shifts (in ppm) were determined relative to tetramethyl silane ($\delta$ 0.00 ppm) as an internal standard in CDCl$_3$ and DMSO-d$_6$, or relative to the CDCl$_3$ signal ($\delta$ 77.0 ppm) and to DMSO-d$_6$ signal ($\delta$ 39.5 ppm) in $^{13}$C NMR spectra. Coupling constant(s) [Hz] were measured from one-dimensional $^1$H-NMR spectra. Full or partial assignments were made by standard COSY, HSQC, TOCSY and 1D TOCSY experiments. MS analyses were performed on a high resolution JEOL AccuTOF mass spectrometer using an electrospray ionization (ESI) source or with a Direct Analysis in Real Time (DART) ion source. Photochemical reactions were performed in a Rayonet RPR200 reactor (Southern New England Ultraviolet Company, Branford, CT) equipped with 16 UV lamps (350 nm). UV-vis absorption spectra were obtained with a Shimadzu UV-3101PC UV-VIS-NIR scanning spectrophotometer. Peptides were synthesized semi-automatically using a Tribute peptide synthesizer from Protein Technologies, Inc. (USA).
1.6.2 Experimental Procedures and Spectroscopic Data

**Scheme 13:** 4-step synthesis of a N-acyl-7-nitroindoline linker for SPPS using a Suzuki-Miyaura cross-coupling reaction.

4-(7-nitroindolin-5-yl)benzoic acid (12): 5-bromo-7-nitroindoline (117.60 mg, 0.48 mmol, 1.20 equiv), 4-(carboxy)phenylboronic acid pinacol ester (100.00 mg, 0.40 mmol, 1.00 equiv.), sodium carbonate (192.30 mg, 1.81 mmol, 4.5 equiv.), and Pd(PPh₃)₄ (46.60 mg, 0.040 mmol, 0.10 equiv) were suspended in 2.5 mL of a solution toluene/ethanol/water 2:1:1, and set to reflux overnight at 85°C. The mixture was transferred to a separatory funnel and extracted using water and ethyl acetate (200 mL each), keeping the deep red aqueous phase. The aqueous phase was acidified to pH = 2 using HCl 1 M. The product was transferred to a separatory funnel and extracted using ethyl acetate (200 mL) and water (100 mL). The organic layer was washed with brine (200 mL), dried over anhydrous MgSO₄, filtered, and concentrated under reduced pressure, furnishing 111.10 mg of an orange solid (97%). Rᵣ = 0.34 (DCM/MeOH 12:1); ¹H-NMR (400 MHz, 298K, DMSO-d₆) δ 12.92 (s, 1H, -COOH); 8.19(s, 1H, NH); 7.99(s, 1H, ArH); 7.97(s, 1H, ArH); 7.77(s, 1H, ArH); 7.75(s, 1H, ArH); 7.73(s, 1H, ArH); 3.82(t, 2H, ³J = 8.5 Hz, H2); 3.18(t, 2H, ³J = 8.5Hz,
\[^{13}\text{C-NMR (400 MHz, DMSO-d6)} \delta 167.10 (\text{COOH}); 148.64, 142.82, 136.55, 130.02, 128.92, 128.08, 127.52, 126.45, 125.70, 119.61 \text{ [aromatic]; 46.96 [C2]; 27.32 [C3]. HR-ESI-MS DART: m/z [M+H]^+ calcd.: 285.0870; obs.: 285.0872.}

**Allyl 4-(7-nitroindolin-5-yl)benzoate (13):** Indoline 12 (100.00 mg, 0.40 mmol, 1 equiv.) and caesium carbonate (131.30 mg, 0.40 mmol, 1 equiv.), were dissolved in 1.5 mL of anhydrous DMF, and stirred under argon atmosphere at room temperature. AllBr (418.50 µL, 0.48 mmol, 1.2 equiv.) was added dropwise and the solution was stirred for 2 hours. 30 mL of toluene were dissolved in the mixture and coevaporated with DMF under reduced pressure. The crude solid was transferred to a separatory funnel using ethyl acetate and water (200 mL each), and the organic layer was washed with a concentrated solution of \( \text{NaHCO}_3 \) (200 mL x 3 times), brine (100 mL), dried over anhydrous \( \text{MgSO}_4 \), filtered, and concentrated under reduced pressure, furnishing a bright red solid (97%). \( R_f = 0.31 \) (EtOAc/Hex 1:4). \[^1\text{H-NMR (400 MHz, CDCl}_3\] \delta 8.09 (s, 1H, ArH); 8.07 (s, 1H, ArH); 8.05 (s, 1H, ArH); 7.60 (s, 1H, ArH); 7.57 (s, 1H, ArH); 7.49 (s, 1H, ArH); 6.86 (s, 1H, NH); 6.12-6.01 (1H, m, \( \text{CH}_2=\text{CH}^- \)); 5.45-5.41 (dd, 1H, \(^3\text{J} = 17.2 \text{ Hz, } \(^2\text{J} = 1.4 \text{ Hz, Allyl, olef. trans} \)); 5.32-5.29 (dd, 1H, \(^3\text{J} = 17.2 \text{ Hz, } \(^2\text{J} = 1.4 \text{ Hz, Allyl, olef. cis} \)); 4.85-4.84 (dt, 2H, \(^3\text{J} = 5.7 \text{ Hz, } \(^2\text{J} = 1.4 \text{ Hz, allyl CH}_2 \)); 3.93 (2H, t, \(^3\text{J} = 8.6 \text{ Hz, } \text{H32} \)); 3.24 (2H, t, \(^3\text{J} = 8.6 \text{ Hz, } \text{H3} \)). \[^{13}\text{C-NMR (400 MHz, CDCl}_3\] \delta 166.02, 148.78, 143.87, 134.91, 130.28, 128.99, 128.48, 128.46, 126.04, 120.87 \text{ [aromatic]; 132.27 [allyl CH}_2=\text{CH-CH}_2^- \); 118.27 [allyl CH}_2=\text{CH-CH}_2^- \); 65.58 [allyl CH}_2=\text{CH-CH}_2^- \); 47.02 [C2]; 28.19 [C3]. HR-ESI-MS DART: m/z [M+H]^+ calcd.: 325.1183; obs.: 325.1188.

**Allyl 4-(((9H-fluoren-9-yl)methoxy)carbonyl)glycyl)-7-nitroindolin-5-yl)benzoate (14):** Fmoc-glycine-OH (265.90 mg, 0.90 mmol, 1.5 equiv.) was suspended and stirred in 8.5 mL anhydrous DCM under argon atmosphere at room temperature. Trichloroacetonitrile (0.18 mL, 1.79 mmol, 3.00 equiv.) was added
dropwise. Triphenylphosphine (469.20 mg, 1.79 mmol, 3.00 equiv.) was added to the suspension. Formation of the Fmoc-Gly-Cl is confirmed by the medium turning into a clear, pale-yellow solution. Indoline 13 (193.40 mg, 0.60 mmol, 1 equiv.) was added, covered from the light at 30°C and let react for 2 hours. The solution was transferred to a separatory funnel and extracted with ethyl acetate and water (200 mL each). The organic layer was then washed with brine (100 mL), dried over anhydrous MgSO₄, filtered and concentrated under reduced pressure. The crude product was purified by silica chromatography (EtOAc/Hex 1:2), furnishing a bright yellow solid, (95%).

**1H-NMR (400 MHz, 298K, CDCl₃)** δ 8.16 (s, 1H, ArH); 8.14 (s, 1H, ArH); 7.90 (s, 1H, ArH); 7.77 (s, 1H, ArH); 7.75 (s, 1H, ArH); 7.70 (s, 1H, ArH); 7.64 (s, 1H, ArH); 7.62 (s, 1H, ArH); 7.60 (s, 1H, ArH); 7.40 (m, 2H, ArH); 7.31 (m, 2H, ArH); 6.12-6.01 (m, 1H, CH₂=CH₂); 5.79 (t, 1H, J = 4.5 Hz, glycine NH); 5.46-5.42 (dq, 2H, J = 17.2 Hz, Alanyl, olef. trans); 5.33-5.31 (dq, 1H, J = 10.5 Hz, Alanyl, olef. cis); 4.87-4.85 (dt, 2H, J = 5.6 Hz, J = 1.4 Hz, CH₂=CH₂); 4.39 (d, 2H, J = 7.0 Hz, Fmoc CH₂); 4.30 (t, 2H, J = 8.0 Hz, H3); 4.26-4.21 (m, 3H, glycine α CH₂ and Fmoc CH); 3.34 (t, 2H, J = 8.0 Hz, H2).

**13C-NMR (400 MHz, 298K, CDCl₃)** δ 167.14, 165.86, 156.48 [C=O]; 143.86, 142.75, 141.34, 141.03, 137.58, 137.41, 133.79, 130.53, 129.91, 127.81, 127.31, 127.20, 126.94, 125.25, 121.67, 120.06 [aromatic]; 132.22 [allyl CH₂=CH-CH₂-]; 118.54 [allyl CH₂=CH-CH₂-]; 67.40 [Fmoc CH₂]; 65.85 [allyl CH₂=CH-CH₂-]; 49.00 [C2]; 47.13 [Fmoc CH]; 44.35 [glycine α CH₂]; 29.24 [C3]. HR-ESI-MS: m/z [M+Na]⁺ calcd.: 626.1903; obs.: 626.1901.

4-((9H-fluoren-9-ylmethoxy)carbonyl)glycyl-7-nitroindolin-5-yl)benzoic acid (15) – 14 (179.40 mg, 0.37 mmol, 1 equiv.) and Pd(PPh₃)₄ (4.30 mg, 3.7 x 10⁻³ mmol, 1.0 x 10⁻² equiv.) were dissolved in 6.80 mL of anhydrous THF under argon atmosphere at room temperature. NMA (0.40 mL, 3.74 mmol, 10.00 equiv.) was added dropwise and let stir for 1 hour. The THF was removed under reduced pressure and transferred to a separatory funnel using ethyl acetate and water (200 mL each). The organic layer was washed with
brine (100 mL), dried over anhydrous MgSO₄, filtered and concentrated under reduced pressure, yielding 209.1 mg of a yellow solid (99%). \( R_f = 0.34 \) (DCM/MeOH 9:1). \(^1\)H-NMR (400 MHz, 298K, DMSO-d₆) \( \delta \): 13.03 (s, 1H, -COOH); 8.04 (1H, s, aromatic); 8.02 (1H, s, aromatic); 7.98 (s, 1H, aromatic); 7.89 (m, 4H, aromatic); 7.77-7.73 (m, 3H, aromatic and Gly NH); 7.42 (m, 2H, aromatic); 7.34 (m, 2H, aromatic); 4.34-4.25 (m; 5H; H₂, Fmoc CH₂ and CH); 4.10 (d, 2H, \(^3\)J = 17.3 Hz, Gly CH₂); 3.31 (t, 2H, \(^3\)J = 8.0 Hz, H₃). \(^{13}\)C-NMR (400 MHz, 298K, DMSO-d₆) \( \delta \): 168.00, 166.99, 156.49 [C=O]; 143.83, 141.86, 140.72, 140.40, 138.46, 135.58, 133.78, 130.04, 129.48, 127.62, 127.36, 127.08, 126.84, 125.28, 122.54, 120.41 [16 aromatic carbons]; 65.74 [Fmoc CH₂]; 48.64 [C3]; 46.64 [Fmoc CH]; 43.57 [Glycine α CH₂]; 28.70 [C2]. HR-ESI-MS: \( m/z \) [M-H] calcd.: 562.1620; obs.: 562.1612.

**Scheme 14**: Reaction pathway depicting Route A, B and C for the synthesis of c(RGDfV). a) hv = 350 nm, anhydrous, oxygen-free DCM, conc. = \( 10^{-4} \) M. b) TFA/H₂O/TIPS 95/2.5/2.5. c) 1% TFA in DCM. d) DPPA, NaHCO₃ in DMF, conc. = \( 10^{-4} \) M.
Sieber amide resin-bound photoreactive pentapeptide 16 for route A and B: Resin bound photoreactive peptide 16 was synthesized on Sieber amide resin (loading capacity 0.65 mmol/g). The resin (400 mg, 0.260 mmol active site, 1 equiv.) was swollen in dry DCM (10 mL) under shaking for 30 min and washed with NMP (5 x 5 mL). The Fmoc group was removed with 20% piperidine in NMP (3 mL) under shaking for 15 min followed by washing with NMP (5 x 5 mL). 15 (146.53 mg, 0.260 mmol, 1 Eq), HBTU (98.7 mg, 0.260 mmol, 1 Eq), HOBt (35.1 mg, 0.260 mmol, 1 Eq), and DIPEA (0.08 mL, 0.520 mmol, 2 Eq) were dissolved in 0.5 mL NMP, immediately added to the resin, mixed for 1 h, then washed with NMP (5 X 5 mL). The resin turns bright yellow after this first coupling step, and resin loading was qualitatively assessed by Kaiser test. The resin was capped with 10% Ac₂O, 5% DIPEA in NMP (10 mL, 15 min) and then washed with NMP (5 x 5 mL). Fmoc removal was accomplished as described before using 20% piperidine in NMP. The next coupling steps followed the same protocol, but using 5 equiv. HBTU (1.3 mmol, 493.0 mg), HOBt (1.3 mmol, 175.5 mg), 10 equiv. DIPEA (2.6 mmol, 0.4 mL) and the corresponding amino acid [5 equiv. Fmoc-Arg(Pbf)-OH (1.3 mmol, 843.4 mg), Fmoc-Val-OH (1.3 mmol, 441.2 mg), Fmoc-D-Phe-OH (1.3 mmol, 503.7 mg), or Fmoc-Asp(OtBu)-OH] (1.3 mmol, 534.9 mg)] in 0.75 mL NMP. Each loading step was determined by the quantification of dibenzofulvene by UV-VIS spectroscopy (290nm, ε = 5253 M⁻¹cm⁻¹, 1 cm. path length), achieving an overall loading yield of 41% for route A, and 43% for route B.

SASRIN resin-bound reference peptide 19 for route C: Resin bound pentapeptide 19 was synthesized on Super Acid Sensitive Resin (SASRIN, loading capacity 0.9 mmol/g). The resin (250 mg, 0.225 mmol, 1 Eq) was swollen in dry DCM (10 mL) for 30 min and washed with NMP (5 x 5 mL). The first amino acid coupling was prepared by the symmetric anhydride strategy. Fmoc-Gly-OH (669.0 mg, 2.250 mmol, 10 Eq) and EDCI (215.7 mg, 1.125 mmol, 5 Eq) were suspended in 4.0 mL of anhydrous DCM at 0°C. The resulting solution was mixed with the resin and DMAP (27.5 mg, 0.225 mmol, 1 Eq), and stirred for 2 h. The resin was washed
with NMP (5 x 5 mL), and the resin loading was qualitatively assessed by Kaiser test. The Fmoc group was removed with 20% piperidine in NMP (3 mL) under shaking for 15 min followed by washing with NMP (5 x 5 mL). The next coupling steps were followed by the same protocol, but using 5 equiv. HBTU (1.125 mmol, 426.6 mg), HOBt (1.125 mmol, 152.0 mg), 10 equiv. DIPEA (2.5 mmol, 0.44 mL) and 5 equiv. of the corresponding amino acid [Fmoc-Arg(Pbf)-OH (1.125 mmol, 730.0 mg), Fmoc-Val-OH (1.125 mmol, 381.8 mg), Fmoc-D-Phe-OH (1.125 mmol, 435.9 mg), or Fmoc-Asp(OtBu)-OH] (1.125 mmol, 462.9 mg)] in 0.75 mL NMP. Each loading step was determined by the quantification of dibenzofulvene by UV-VIS spectroscopy (290nm, ε = 5253 M⁻¹cm⁻¹, 1 cm. path length), achieving an overall loading yield of 75%.

Cyclo-[R(Pbf)-G-D(OtBu)-f-V] (17) -- **For route A:** 200 mg of resin-bound pentapeptide 16 was dispersed in 37 mL of dry, oxygen-free DCM. The mixture was introduced to a Rayonet chamber and illuminated with UV light (λ = 350 nm), where a water condenser would keep the reaction to room temperature. Monitoring of this reaction was carried out by UV-Vis spectroscopy and TLC. After 5 hours, the dark orange resin is filtered, and a pale-yellow liquid is concentrated by reduced pressure. The crude product was purified by silica chromatography (DCM/MeOH 15:1). 32.1 mg of a white solid were obtained (35%). Rf = 0.550 (DCM/MeOH 9:1). **For route B:** 100 mg of photoreactive peptide 18 were dissolved in 37 mL of anhydrous, oxygen-free DCM (3x10⁻³ M final concentration). The mixture was introduced to a Rayonet chamber and illuminated with UV light (λ = 350 nm) where a water condenser would keep the reaction to room temperature. There was monitoring of this reaction by TLC, until complete consumption of the starting material. After 8 hours, a dark orange solution was concentrated under reduced pressure. The crude product was purified by silica chromatography (DCM/MeOH 15:1). 22.0 mg of a white solid were obtained (22%). **For Route C:** 250 mg of resin-bound peptide 19 were suspended in 10 mL of a solution of 1% TFA in DCM and stirred for 2 minutes. The transparent solution was filtered over 10% pyridine in
MeOH, being this fraction 1. The process was repeated a total of ten times. The last fraction was monitored by TLC to verify no more cleavage product from the resin. The fractions were combined and concentrated to 5% volume in reduced pressure. The product was precipitated by adding 40 mL of cold water and let to further precipitate at 4°C for 2 h. The precipitate was centrifuged and decanted, and 143 mg of a white crude product 20 were obtained. 100 mg of the crude peptide and NaHCO₃ (47.6 mg, 0.57 mmol, 5.0 equiv.) were dispersed in 37 mL anhydrous DMF (3x10⁻³ M final concentration). At 0°C, DPPA (73.1 µL, 0.34 mmol, 3.0 equiv.) were added and the reaction was run at room temperature for 2 days. The solvent was coevaporated with toluene, and the crude product was lyophilized in a mixture water/acetonitrile 1:1. The solid crude was purified by silica chromatography (DCM/MeOH 15:1). 33.0 mg of a white solid were obtained (24%). ¹H-NMR (400 MHz, 298K, DMSO-d₆) δ 8.41-8.34 (m, 1H, Gly NH); 8.08 (d, 1H, ³J = 7.6 Hz, Phe NH); 7.98 (m, 1H, ³J = 8.3 Hz, Asp NH); 7.81-7.73 (m, 2H, Val and Arg NH); 7.27-7.10 (m, 5H, Phe aromatic); 6.68 (bs, 1H, Arg guanidine NH); 6.37 (bs, 1H, Arg guanidine NH); 4.66-4.55 (m, 2H, Phe Hα and Asp Hα); 4.12-4.00 (m, 2H, Arg Hα and Gly Hα); 3.83 (t, 1H, ³J = 7.5 Hz, Val Hα); 3.28-3.21 (m, 1H, Gly Hα); 3.02 (m, 2H, Arg Hδ); 2.98-2.90 (m, 4H, Arg guanidine NH, Phe Hβ, Pbf CH₂); 2.80 (dd, 1H, ³J = 13.7, ²J = 6.2 Hz Phe Hβ); 2.63 (dd, 1H, ³J = 15.3, ²J = 8.8 Hz Asp Hβ); 2.47 (s, 3H, Pbf aromatic CH₃); 2.41 (s, 3H, Pbf aromatic CH₃); 2.36 (dd, 1H, ³J = 15.3, ²J = 8.8 Hz, Asp Hβ); 2.00 (s, 3H, Pbf aromatic CH₃); 1.84 (m, 1H, Val Hβ); 1.72-1.63 (m, 1H, Arg Hβ); 1.52-1.43 (m, 1H, Arg Hδ); 1.41 (s, 6H, Pbf non-aromatic CH₃); 1.34 (s, 9H, Asp(OtBu) CH₃); 1.324 (2H, m, Arg Hγ); 0.69 (dd, 6H, ³J = 26.1, ²J = 6.6 Hz, Val Hγ). ¹³C-NMR (400 MHz, 298K, DMSO-d₆) δ 171.10, 171.02, 170.73, 169.84, 169.43, 169.06 [C=O]; 157.43, 156.01, 137.39, 137.27, 131.40, 129.227, 128.285, 126.423, 124.478, 116.431 [aromatic]; 86.479 [OtBu quaternary carbon]; 80.148 [Pbf non-aromatic quaternary]; 60.316 [Val C₆]; 54.104 [Asp C₆]; 52.397 [Arg C₆]; 49.103 [Phe C₆]; 43.321 [Gly C₆]; 42.654 [Pbf CH₂]; 37.315 [Phe C₇]; 36.370 [Asp C₇]; 29.678 [Val C₇]; 28.415 Pbf non-aromatic CH₃; 28.299 [OtBu CH₃]; 27.610 [Arg C₇]; 21.051 [Arg C₅]; 19.200 [Arg C₅]; 18.96
Val C₉; 18.96, 17.6, 12.29 [Pbf CH₃]. HR-ESI-MS: m/z [M+H]^+ calcd.: 883.4382; obs.: 883.4388; m/z [M+Na]^+ calcd.: 905.4207; obs.: 905.4201.

Cyclo-[RGDFV] (21) – Peptide 17 (24.0 mg, 0.0272 mmol, 1 equiv.) is dissolved in 35 ml of a mixture TFA/H₂O/TIPS and let to react without stirring for 2 hours. The mixture is concentrated under reduced pressure to 5% volume, and the peptide is precipitated by adding 10 mL of cold ether. The precipitate is centrifugated and the ether decanted five times. After liophilizing the product in a mixture water/acetonitrile 1:1, 19.2 mg of a white solid were obtained (92%). ¹H-NMR (400 MHz, 298K, DMSO-d₆) δ 13.039 (1H, s, COOH); 8.043 (1H, s, Aromatic); 8.022 (1H, s, Aromatic); 7.980 (1H, s, Aromatic); 7.888 (4H, m, Aromatic); 7.774 (1H, m, Glycine NH); 7.602 (1H, s, Aromatic); 7.422 (2H, 2, Aromatic); 7.338 (2H, m, Aromatic); 4.338 (1H, s, Aromatic); 4.319 (2H, t, ³J CH/CH₂ = 7.0 Hz, Nitroindoline CH₂ in position 3); 4.275 (2H, d, ³J = 8.0 Hz, Fmoc CH₂); 4.250 (1H, t, ³J = 8.0 Hz, Fmoc CH); 4.096 (d, 2H, ³J = 4.9 Hz, Glycine CH₂); 3.306 (2H, t, ³J = 8.0 Hz, indoline CH₂ in position 2). ¹³C-NMR (400 MHz, 298K, DMSO-d₆) δ 172.09, 171.44, 170.63, 170.08, 156.69, 137.56 [C=O], 128.88, 127.89, 126.10 [arom.], 56.25 [Val Ca], 52.44 [Phe Ca], 50.69 [Arg Ca], 43.29 [Asp Ca], 40.29 [Gly Ca], 40.04 [Arg Ca], 36.70 [Phe Cb], 35.66 [Asp Cb], 28.26 [Val Cb], 24.62 [Arg Cb], 19.17 [Arg Cb], 18.73 [Val Cb]. HR-ESI-MS: m/z [M+H]^+ calcd.: 575.2936; obs.: 575.2930.

H-Linker-G-R(Pbf)-V-f-D(OtBu)-OH 18 – 200 mg of resin-bound peptide 16 were suspended in 10 mL of a solution of 1% TFA in DCM and stirred for 2 minutes. The yellow solution was filtered over 10% pyridine in MeOH, being this fraction 1. The process was repeated a total of ten times. The last transparent fraction was monitored by TLC to verify no more cleavage product from the resin. The fractions were combined and concentrated to 5% volume in reduced pressure. The product was precipitated by adding 40 mL of
cold water and let to further precipitate at 4°C for 2 h. The yellow precipitate was centrifuged and
decanted, and a yellow crude product was obtained. The crude product was purified by silica
chromatography (DCM/MeOH (9:1)). 151.5 mg of a yellow solid (80%) were obtained. Rf = 0.28
(DCM/MeOH 9:1). \(^1\)H-NMR (400 MHz, 298K, DMSO-d6) \(\delta\) 8.29-8.20 (m, 2H, indoline CONH₂); 8.07 (s, 1H, Arg NH); 8.03-7.95 (m, 4H, indoline aromatic);
7.83 (d, 2H, J = 7.8 Hz, indoline aromatic); 7.44 (s, 1H, Val NH); 7.26-7.21 (m, 4H, Phe aromatic); 7.20-7.14
(m 1H, Phe aromatic); 6.76 (bs, 1H, Arg guanidine NH); 6.42 (bs, 1H, Arg guanidine NH); 4.72 (q, 1H, Phe
H\(\alpha\)); 4.39-4.27 (m, 3H, Arg H\(\alpha\) and indoline C\(\text{H}_2\) in position 2); 4.22-4.10 (m, 3H, Val H\(\alpha\) and Gly H\(\alpha\)); 3.53-
3.47 (m, 1H, Asp H\(\alpha\)); 3.29 (t, 2H, J = 16.5 Hz, indoline C\(\text{H}_2\) in position 2); 3.08-2.91 (m; 5H; Phe H\(\beta\), Arg H\(\delta\), and Pbf C\(\text{H}_3\)); 2.85-2.77 (m, 1H, Phe \(^2\)H\(\beta\)); 2.48 (s, 3H, Pbf aromatic CH\(\text{H}_3\)); 2.42 (s, 3H, Pbf aromatic CH\(\text{H}_3\));
2.39-2.37 (m, 1H, Asp \(^1\)H\(\beta\)); 2.19 (dd; 1H; \(^3\)J = 16 Hz, \(^2\)J =  8.3 Hz, Asp \(^2\)H\(\beta\) ); 2.00 (s, 3H, Pbf aromatic CH\(\text{H}_3\));
1.98-1.93 (m, 1H, Val H\(\beta\)); 1.71-1.62 (m, 1H, Arg \(^1\)H\(\beta\)); 1.59-1.43 (m, 3H, Arg H\(\gamma\) and Arg \(^2\)H\(\beta\)); 1.4 (s, 6H, Pbf
non aromatic CH\(\text{H}_3\)); 1.36 (S, 9H, Asp OtBu CH\(\text{H}_3\)); 0.76 (t, 6H, J = 6.5 Hz, Val H\(\beta\)). \(^13\)C-NMR (400 MHz, 298K,
DMSO-d6) \(\delta\) 171.70, 170.89, 170.77, 170.14, 167.49, 167.34 [C=O]; 157.43, 156.08, 140.41, 140.31,
138.47, 137.39, 137.29, 137.28, 135.92, 133.58, 133.50, 131.43, 129.27, 128.29, 128.02, 127.97, 127.36,
126.49, 126.29, 124.32, 120.27, 116.27 [aromatic]; 86.30 [OtBu quaternary]; 80.03 [Pbf non-aromatic quaternary]; 57.47 [Val C\(\alpha\)]; 53.62 [Phe C\(\alpha\)]; 52.06 [Arg C\(\alpha\)]; 51.17 [Asp C\(\alpha\)]; 48.92 [indoline CH\(\text{H}_2\) in position 2]; 42.47 [Gly C\(\alpha\)]; 42.27 [Pbf CH\(\text{H}_3\)]; 38.43 [Asp C\(\beta\)]; 30.71 [Val C\(\beta\)]; 30.53 [Pbf non-aromatic CH\(\text{H}_3\)]; 28.72
[OtBu CH\(\text{H}_3\)]; 28.31 [Arg C\(\delta\)]; 27.73 [Arg C\(\delta\)]; 27.63 [indoline CH\(\text{H}_2\) in position 3]; 19.20 [Arg C\(\beta\)]; 18.98 [Val C\(\gamma\)];
17.86 [Pbf CH\(\text{H}_3\)], 17.63 [Pbf CH\(\text{H}_3\)], 12.29 [Pbf CH\(\text{H}_3\)]. HR-ESI-MS: m/z [M+H]\(^+\) calcd.: 1166.5339; obs.
1166.5350.
Chapter 2

Discovery of the Photoreactive N-Thiocarbamoyl-7-Nitroindoline Moiety, Study of its Photolysis by 1 and 2-Photon Absorption.

2.1 Introduction

When studying the photochemistry of 7-nitroindoline-based compounds, most research groups have focused on the N-acyl-7-nitroindoline moiety. This photoreactive amide was first studied in 1976 by the Patchornik group and, since then, it has found multiple applications in different areas of chemistry, such as in the photorelease of biologically active neuroactive amino acids, as linkers for SPPS, and in the biophotolithography of new generation biomaterials. The precursor for N-acyl-7-nitroindolines are 7-nitroindolines, which are aromatic secondary amines that contain a nitro group in the ortho position. This electron withdrawing nitro group in the 5th position is essential for the photoreactive capabilities of N-acyl-7-nitroindolines, but it also decreases the nucleophilicity of the 7-nitroindoline amine. Therefore, only excellent leaving groups such as acyl chlorides can form the photoreactive amide bonds for N-acyl-7-nitroindolines in anhydrous conditions (Scheme 15)

![Scheme 15](image)

Scheme 15: Representation of a N-(Fmoc-glycyl)-5-bromo-7-nitroindoline (X) using 5-bromo-7-nitroindoline and the acyl chloride from Fmoc-glycine-OH.

While various strategies involving the in-situ formation of acyl chlorides from exposing their corresponding carboxylic acids to SOCl₂, the Appel reaction or to the Ghosez reagent; there are still some drawbacks that challenge both experienced and unexperienced chemists for the formation of these
amides. Preparation of totally anhydrous conditions, overall variable yields of reaction depending on the structural properties of different acyl chlorides, and the presence of acid sensitive protecting groups call for the exploration of other 7-nitroindoline-based photoreactive moieties that can be prepared more efficiently and in more robust ways; especially when envisioning purification of molecules with multiple active sites, or with variable molecular weights such as PEGs. Hassner et al. proposed the formation of 7-nitroindoline-based carbamic acid derivatives, which encompassed a wide variety of ureas and carbamates that were prepared in excellent yields of reaction. The photolytic properties of these molecules using UV-light (λ = 350 nm) by a 1-photon absorption (1PA) phenomenon were also studied, and while they were found to be kinetically slower than N-acyl-7-nitroindolines, they were still considered as promising candidates for future applications in our laboratory. We were able to validate the excellent yields of reaction from some of these ureas and carbamates, but most importantly, we discovered the new 7-nitroindoline thiocarbamate or N-thiocarbamoyl-7-nitroindoline moiety. This new moiety was prepared efficiently using Hassner’s strategy, and its photolytic kinetic properties by 1PA resembled those of N-acyl-7-nitroindolines. The photolysis of the N-thiocarbamoyl-7-nitroindoline moiety by 2-photon absorption (2PA) was also studied in collaboration with Dr. Chunqiang Li’s physics research group at UTEP using a two-photon fluorescence microscope. “7-nitrosoindoline” was identified as the major photolysis product of N-thiocarbamoyl-7-nitroindolines, which did not correspond to the regular photolysis products observed in N-acyl-7-nitroindolines photochemistry.

We hypothesized that, similar to the well-established N-acyl-7-nitroindolines, N-thiocarbamoyl-7-nitroindolines are photoreactive compounds that can undergo photolysis by a 1PA and 2PA mechanism.
2.2 Specific Aims

2.2.1 To synthesize 3 N-thiocarbamoyl-7-nitroindolines varying the 7-nitroindoline and thiol.

2.2.2 To study the kinetics of photolysis reactions of N-thiocarbamoyl-7-nitroindolines by 1PA using UV-Vis spectroscopy and compare it to model N-acyl-7-nitroindoline.

2.2.3 To study the photolytic properties of a model N-thiocarbamoyl-7-nitroindoline by 2PA using a multiphoton fluorescence microscope in collaboration with Dr. Li from the physics department at UTEP.

2.2.4 To characterize the photolysis product “7-nitrosindoline” by NMR spectroscopy, UV-Vis spectroscopy, and mass spectrometry, and validate its reversible conversion to its azodioxy dimer.

2.2.5 To further study the structural properties of 7-nitrosindoline and its azodioxy dimer by computational calculations. This work was done by Dr. Dirk from the Department of Chemistry and Biochemistry, who mentored our undergraduate student Philip Baily.

2.3 Background and Significance

2.3.1 The Discovery of Photolabile 7-Nitroindoline Carbamic Acid Derivatives

In 2007, Hassner et al. synthesized the first 7-nitroindoline-based carbamic acid derivatives. They stated that the use of photocleavable 7-nitroindoline groups focused mostly on protecting and/or activating the carboxylic acid function (N-acyl-7-nitroindolines), and they envisioned new protecting groups for amines and alcohols. They prepared efficiently the 5-bromo-7-nitroindoline and 5,7-dinitroindoline carbamoyl chlorides (22), which would then be used to protect several alcohols and amines in the form of ureas (23) and carbamates (24), respectively (Scheme 16). The yields of reaction for the syntheses of most carbamates and ureas were above 85%, and their photolytic capabilities were studied for the first time.
In 2002, Corrie et al. proposed two solvent-dependent mechanisms of reaction for the photolysis of N-acyl-7-nitroindolines by 1PA, which were explained in depth in Section 1.3.1. In a single experiment, they illuminated model N-acyl-7-nitroindolines using UV light, and took many aliquots at different times of the photolysis experiment. N-acyl-7-nitroindolines show a maximum of absorption at around $\lambda = 310$-350 nm, while photolysis reactions products 7-nitroindolines and 7-nitrosoindoles show a maximum of absorption at around 410-450 nm. They carried out a series of experiments using anhydrous acetonitrile and water at different percentages (10-100%), they observed the difference in photolysis products obtained per experiment, and thus proposed Pathway A and B. Photolysis times ranged between 5 minutes to 1 hour, depending on the water content. 

**Figure 3:** (Left) Kinetic trace graph of the photolysis of a model N-acyl-7-nitroindole in anhydrous conditions (solid) and in excess water conditions (dashed). (Right) Molar % water in acetonitrile vs relative yield of 7-nitroindoline (white squares) and 7-nitrosoindole (black squares).
Hassner et al. built kinetic trace graphs of 7-nitroindoline ureas and carbamates using this same methodology. Their experiments were carried out at a single water content of 8.3% in dioxane-dichloromethane, obtaining 7-nitroindolines as their sole photolysis products in all cases. The maximum of absorption for 7-nitroindoline ureas and carbamates was also around 350 nm, and the yields of photolysis were also mostly above 80% (Figure 4).

Nevertheless, the photolysis times for 7-nitroindoline ureas and carbamates ranged between 2 to 13 hours of illumination, representing a significant difference to those of N-acyl-7-nitroindolines. Prolonged exposure to UV-light in biological systems could result in the genetic damage of cells, which would then represent a drawback for the utilization of these moieties in in-vitro experiments such as tissue engineering and biophotolithography in cellularized scaffolds.

2.3.2 A Brief Introduction to Two-Photon Absorption (2PA)

Photoreactions that are activated by a single high energy photon are said undergo a 1PA processes. This is true for the reactions of N-acyl-7-nitroindolines, which require of a high energy photon whose wavelength is close to 350 nm. In 1931, Dr. Maria Göppert-Mayer first predicted the absorption of two
photons by the same molecule or 2PA in her thesis dissertation\textsuperscript{30}, and her theory was validated experimentally in the 1960s after the discovery of the laser\textsuperscript{31}. Lasers are devices that emit a high density of photons or photon beams that are both spatially and temporally focused. In a 2PA process, two photons of similar energy collide with the reactive “cross-section” area of a molecule, thus activating photoreactions in a similar way that 1PA absorption reactions do. These less energetic pair of photons must have a wavelength that is slightly greater than double the wavelength from a 1PA experiment, since some of the energy is lost in non-radiative relaxation processes. In the case of \textbf{N-acyl-7-nitroindolines}, 1PA experiments are carried out using $\lambda = 350 \text{ nm}$, while experiments by 2PA would require photons of $\lambda = 710 \text{ nm}$. A very simplistic way to represent both processes is described in the following Jablonski diagrams depicted in \textbf{Figure 5}:

\textbf{Figure 5}: Simplistic representation of the Jablonski diagram of an \textbf{N-acyl-7-nitroindoline} in its ground state (GS) being excited towards its excited state (ES) by 1 photon ($\lambda = 350 \text{ nm}$) in a single-photon absorption process [left], while it is promoted to its ES by 2 photons ($\lambda = 710 \text{ nm}$) in a two-photon absorption process [right].

Two-photon fluorescence microscopes are devices that are used to emit and focus femtosecond laser beams into a small area. The beams are emitted in pulses of $10^{-15} \text{ s}$ or 1 femtosecond (fs), to increase the probability of two photons colliding with the same cross-section of a molecule. These instruments are also
used to image the fluorescent decay as femtosecond laser light is illuminated to a certain photoreactive sample. Fluorescent decay intensity curves can be generated to provide of kinetic information of the chemical reaction that is being studied by two-photon absorption\textsuperscript{32,33}. In Figure 6, a schematic of the two-photon laser scanning fluorescence microscope developed in the biophotonics laboratory of the Physics department at UTEP is shown:

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Schematic of the two-photon (2PA) laser scanning microscope developed by Dr. Chunqiang Li at the biophotonics laboratory at UTEP.}
\end{figure}

2.3.3 Two-Photon Absorption (2PA) in the Context of 7-Nitroindoline Photochemistry
Photochemistry of **N-acyl-7-nitroindolines** by 2PA processes has found applications in different areas of neurochemistry, peptide chemistry and biomaterials with photolithography capabilities. Matsuzaki et al. studied the structure-function relationship of dendritic spines, and they developed a method for the photochemical uncaging of glutamate from a nitroindolino-glutamate derivative, to allow mapping of functional glutamate receptors at the level of a single synapse\(^{34}\). Smith et al. proved that the number of postsynaptic AMPA receptors per dendritic spine increases with a higher distance from the soma by experiments involving the uncaging of 4-methoxy-7-nitroindolinyl-caged L-glutamate by 2PA experiments\(^{35}\). Work done in our laboratory in collaboration with the Li group in the Physics Department at UTEP showed that a **34-mer collagen-like N-peptidyl-7-nitroindoline** can be photolyzed by a 2PA process upon illumination with femtosecond laser light. Fluorescence decay graphs were generated by illuminating a thin film of the peptide material with different laser powers, and a double-log plot of reaction rate vs laser intensity showed a slope of 2.007, which is indicative of a 2PA process\(^{36}\) (**Figure 7**).

**Figure 7**: (Left) Fluorescent decay graphs of a **34-mer N-peptidyl-7-nitroindoline** generated by a 2PA fluorescent microscope at different laser powers. (Right) Double-log β plot of reaction rate vs laser intensity with a slope of 2.007 is indicative of a 2PA process\(^{36}\).

Further studies described by Ornelas et al. show the potential applications of this material for biophotolithography. A thin film of the same **34-mer photoreactive peptide** was illuminated with femtosecond laser light through a mask in the shape of a tree. The progression of the reaction through its fluorescent decay was monitored by fluorescence microscope imaging (**Figure 8**)\(^{29}\).
Figure 8: (A) Initial fluorescent tree pattern generated by two-photon excitation of the 34mer peptide with femtosecond laser at 710 nm light through a tree mask at time 0 min; (B) fluorescent tree pattern after 10 min of irradiation, fluorescence decay is observed; (C) tree pattern after 30 min of irradiation, fluorescence is barely visible due to progressive photolysis to non-fluorescent products; (D) after removal of mask and immediate recording of the fluorescence only the background fluoresces while the tree appears dark; (E) tree under white light.

Peptides derivatized to contain a photoreactive moiety and that are susceptible for crosslinking to form hydrogels, could be used for future applications in new generation biomimetic scaffolds were cells can grow and proliferate. Preliminary cytotoxic studies using human mesenchymal stem cells shows that a layer of the 34mer photoreactive peptide might be biomimetic and suitable for cell growth (Figure 9).

Figure 9: Human mesenchymal stem cells, pre-stained with cell membrane stain PKH26, and seeded atop 34mer photoreactive peptide pre-immobilized onto tissue culture grade wells. (Left) Images taken from center of the well. (Right) Cells grew only where the peptide was present.

2.4 Results and Discussion

2.4.1 Synthesis of Three N-Thiocarbamoyl-7-Nitroindoline Model Compounds
During our experiment design phase, we decided to form a new type of functional group that would act as a protecting group of thiols. We decided to shorten Hassner’s approach, and synthesized carbamoyl chloride 22 of commercially available 5-bromo-7-nitroindoline in-situ, and then used ethanethiol to form the first 5-bromo-N-thiocarbamoyl-7-nitroindoline (25). With the success and efficiency of our first thiocarbamate we wanted to prove that the synthesis of other N-thiocarbamoyl-7-nitroindolines could be equally efficient. Therefore, we utilized different 7-nitroindolines that were previously used as precursors for SPPS linkers (28 and 13); and aromatic thiols: thiophenol and p-thiocresol, to synthesize photoreactive thiocarbamates 26 and 27 (Scheme 17).

\[ \text{Scheme 17: Synthesis of three different N-thiocarbamoyl-7-nitroindolines using different 7-nitroindolines and thiols to validate the efficiency of the strategy.} \]

2.4.2 Kinetic Study of the 1-Photon Absorption (1PA) Photolyses of N-Thiocarbamoyl-7-Nitroindolines and its Comparison to a Model N-Acyl-7-Nitroindoline.
The formation of a new photolysis product on the TLC led us to the task of isolating it and characterizing it. Until now, N-acyl-7-nitroindoline chemistry validated the formation of 7-nitroindoline and 7-nitrosoindole as the major photolysis products ruled over two different solvent-dependent mechanisms addressed as Pathway A and B, respectively. An experiment was carried out using N-thiocarbamoyl-7-nitroindoline 25 in a solution of 20% water in anhydrous acetonitrile, at a concentration of $8 \times 10^{-3}$ M. A kinetic trace graph was generated by taking aliquots at different reaction times and analyzing them by UV-Vis spectroscopy (Figure 10).

![Figure 10: Kinetic trace graph of the photolysis of 25 in 20% H$_2$O in acetonitrile at a concentration of $8 \times 10^{-3}$ M.](image)

The reaction was finished in around 22 minutes, and an unknown photolysis dark-pink product was observed as the major photolysis product by TLC. A maximum of absorption was observed at 503 nm, also proving the formation of a different photolysis product. We reproduced the same experiment using N-(Fmoc-glycyl)-5-bromo-7-nitroindoline X (Scheme 15) as our model nitroindoline amide, and the photolysis spectrum is shown in Figure 11. The synthesis for this intermediate is described by Ornelas et al.$^{29}$
Figure 1: Kinetic trace graph of X in 20% H₂O in acetonitrile, at a concentration of 8 x 10⁻³ M.

The reaction was also finished in around 22 minutes, and a maximum of absorption was observed at around 450 nm, which is characteristic of 7-nitroindoline and 7-nitrosoindole. These photolysis products were also observed by TLC. We wanted to prove that the rates of photolysis of other N-thiocarbamoyl-7-nitroindolines were fast too, so we photolyzed 26 and 27 using the same parameters and built kinetic trace graphs of them (Figure 12 and 13).

Figure 12: Kinetic trace graph of 26 in 20% H₂O in acetonitrile, at a concentration of 8 x 10⁻³ M.
Photolysis of 26 occurred in 6 minutes, while the photolysis of 27 occurred in 22 minutes, thus proving that this new moiety could be a kinetic analogue of N-acyl-7-nitroindolines when compared to 7-nitroindoline ureas and carbamates. From the kinetic trace graphs, we decided to construct time vs absorbance graphs that would describe the kinetic behaviour of the photolysis better, and from which we could be able to obtain rate constants for a general comparison of the photolysis reactions (Figure 14-17).

Figure 13: Kinetic trace graph of 27 in 20% H₂O in acetonitrile, at a concentration of 8 x 10⁻³ M.

Figure 14: Time vs absorbance graph describing the kinetic behaviour of 25. (Circles) Describe the formation of photolysis products at 504 nm. (Triangles) Describe the decay of 25 at 352 nm.
**Figure 15:** Time vs Absorbance graphs describing the kinetic behaviour of $X$. (Circles) Describe the formation of photolytic reactions products at 450 nm.

**Figure 16:** Time vs Absorbance graph describing the kinetic behaviour of $26$. (Circles) Describe the formation of photolytic products at 462 nm. (Triangles) Describe the decay of $26$ at 359 nm.
Figure 17: Time vs Absorbance graph describing the kinetic behaviour of 27. (Circles) Describe the formation of photolysis products at 487 nm.

We used Equation 1 to find the rate of reaction of both decay and formation of our products:

\[ Y = 0.75e^{-0.0014} + 0.27, \text{ where the rate of reaction was } 1.4 \times 10^{-3} \text{ s}^{-1}. \]

Figure 18: Time vs absorbance graph describing the photochemical decay of 25. The equation that describes this reaction is
Figure 19: Time vs absorbance graph describing the linearization of the formation of 29 from 25. The equation that describes this reaction is $Y = -1.10e^{-0.0018} + 1.11$, where the rate of reaction was $1.7 \times 10^{-3} \text{s}^{-1}$. 
Figure 20: Time vs absorbance graph describing the linearization of the formation of 5-bromo-7-nitroindoline and 5-bromo-7-nitroso indole from X. The equation that describes this reaction is $Y = -0.95e^{0.0032} + 1.01$, where the rate of reaction was $3.2 \times 10^{-3} \text{s}^{-1}$.

Figure 21: Time vs absorbance graph describing the photochemical decay of 26. The equation that describes this reaction is $Y = 0.73e^{-0.0061} + 0.30$, where the rate of reaction was $6.1 \times 10^{-3} \text{s}^{-1}$. 
**Figure 22:** Time vs absorbance graph describing the linearization of the formation of 29 from 26. The equation that describes this reaction is \( Y = -1.06e^{-0.0087} + 1.04 \), where the rate of reaction was \( 8.7 \times 10^{-3} \) s\(^{-1}\).

![Graph showing absorbance vs time for the formation of 29 from 26.](image)

**Figure 23:** Time vs absorbance graph describing the linearization of the formation of 29 from 27. The equation that describes this reaction is \( Y = -1.08e^{-0.0021} + 1.07 \), where the rate of reaction was \( 2.1 \times 10^{-3} \) s\(^{-1}\).

**Table 1** summarizes the values obtained from the graphs, including the maxima of absorption, the isosbestic point of each graph that separates the absorbances of starting materials vs products (Figures 10-13); and the rate constants, which were obtained by the exponential linearization method using Equation 1 (Figures 18-23):

**Table 1:** Data obtained from the kinetic trace graphs and the kinetic linearization on all photolysis reactions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Maxima of absorption of starting material (nm)</th>
<th>Maxima of absorption of photolysis products (nm)</th>
<th>Isosbestic Point (nm)</th>
<th>Photolysis time (min)</th>
<th>Kinetic rate constant of starting material decay (s(^{-1}))</th>
<th>Kinetic rate constant of photolysis product formation (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>352</td>
<td>504</td>
<td>394</td>
<td>22</td>
<td>1.4x10(^{-3})</td>
<td>1.8x10(^{-3})</td>
</tr>
<tr>
<td>26</td>
<td>359</td>
<td>462</td>
<td>382</td>
<td>6</td>
<td>6.1x10(^{-3})</td>
<td>8.8x10(^{-3})</td>
</tr>
<tr>
<td>27</td>
<td>-</td>
<td>487</td>
<td>400</td>
<td>22</td>
<td>-</td>
<td>2.1x10(^{-3})</td>
</tr>
<tr>
<td>X</td>
<td>-</td>
<td>450</td>
<td>375</td>
<td>22</td>
<td>-</td>
<td>3.2x10(^{-3})</td>
</tr>
</tbody>
</table>
The isosbestic points are specific wavelengths at which the total absorbance of a sample does not change during a chemical reaction. This is an indicative of a clean photolysis reaction. For these experiments, they also serve as visual confirmations of the progression of the reactions since we can easily observe both maxima (starting material and photolysis products) changing over time and affected by each other’s formation/consumption. As observed from the experimental data, 25 and 26 are slightly slower than X, but still under the same order of photolysis time. This is certainly very promising for the future of application of the N-thiocarbamoyl-7-nitroindoline moiety where N-acyl-7-nitroindolines have been utilized. 26 was surprisingly the fastest of the photolyses. We believe that this happens because of two main reasons: 1) The thiophenol substituent is an electron withdrawing moiety that can favour the formation of the nitronic anhydride by the nucleophilic substitution of the nitro group, and 2) The use of indoline 28, which is substituted directly to an allyl ester, when comparing it to the strong electron withdrawing allyl benzoate group (27). A more detailed kinetic study is needed to explain in greater depth the differences between both moieties and their corresponding substituents.

2.4.3 Two-Photon Absorption (2PA) Photolysis Reactions of N-Thiocarbamoyl-7-Nitroindoline 25 and its Comparison to a Model N-Acyl-7-Nitroindoline X

Photolysis of 25 was tested on a 2PA fluorescence microscope. Ornelas et al. had already illuminated femtosecond laser light to a thin film of X, and they proved that the photolysis underwent a 2PA process. In their work, they built a series of decay graphs using laser powers from 100 to 200 mW. With these decay graphs they obtained every rate of reaction, represented by the constant β from the Equation 1, and they built a double log β plots vs laser intensity graph. The slope of this graph was close to 2 (1.996), which is indicative of a 2PA process. They also designed a special mask with the shape of the UTEP logo,
and upon radiation of the laser light in this specific shape, an image was printed on its surface visible by both femtosecond and white light:

![Image](image1.png)

**Figure 24:** Experiments carried out by Ornelas et al. on a film of X. (Top left) Fluorescence decay plots with exponential fitting at varying laser power. (Top right) Double log plot (β vs. laser intensity). (Bottom left) A fluorescent logo of UTEP was generated by femtosecond laser at 710 nm through a UTEP mask. (Bottom middle) the letters of the logo were the only area of the film that underwent photolysis. After removal of the mask only the background fluoresces. (Bottom right) UTEP logo under white light.

We wanted to prove that N-thiocarbamoyl-7-nitroindolines could also undergo 2PA processes. A solution of 25 (1mg in 25 µL acetone) was prepared, and with the help of a capillary tube, a spot was marked on the surface of a silica TLC chip. This spot was then irradiated with femtosecond laser light (720 nm) for 1 hour, and we could observe a fluorescent decay from the sample (Figure 25 and 26).
**Figure 25:** Fluorescent microscope image depicting the fluorescence of 25 over the surface of a chip of silica TLC. (Left) Visualization of the fluorescence at t=0. (Right) Visualization of the fluorescence decay after 1 hour of exposure only at a specific region in the center (white circle).

**Figure 26:** Decay of 25 as seen through white light through the microscope. Yellow starting material 25 is seen on the left, while photolysis product 29 is seen in on the right.

In **Figure 27**, the decay curves from the photolysis of 25 were done at a power of 150 mW. X was also run using the same sample preparation and conditions, and both decays were compared.

**Figure 27:** Comparison of the photolysis of 25 and X by femtosecond laser light at a power of 150 mW. Both reactions were completed in around 60 minutes, with the photolysis of X being slightly faster.
We can observe that both compounds decay completely after 1 hour of exposure. The β for X was 0.17 min$^{-1}$, which was about twice as faster as 25, whose β was 0.08 min$^{-1}$. Still, both decays are in the same order, and we can validate that N-thiocarbamoyl-7-nitroindolines behave very similar to N-acyl-7-nitroindolines kinetically under femtosecond laser light. Using the same method, 25 was illuminated with femtosecond laser light at different laser intensities (Figure 28):

![Image of decay curves of 25 at different laser intensities.](image)

**Figure 28**: Decay curves of 25 at different laser intensities.

A double-log plot (β vs intensity) was generated with the decay curve graph, and a slope of 2.1 was obtained, representing a preliminary result of 2PA for N-thiocarbamoyl-7-nitroindoline 25 (Figure 29).
**Figure 29:** Double log plot (β vs laser intensity) for the photolysis of 25. A slope of 2.1 represented a preliminary result that validates a 2PA absorption process.

### 2.4.4 Characterization of Photolysis Product “5-Bromo-7-Nitrosoindoline (9-A)” and its Reversible Conversion to its Azodioxy Dimer (9-B).

Upon total photolysis of 25 we evaporated the solvent under reduced pressure and purified the unknown pink spot by silica column chromatography (Scheme 18).

![Scheme 18](image)

**Scheme 18:** Photolysis of N-thiocarbamoyl-7-nitroindoline 25 to form 5-bromo-7-nitrosoindoline (29-A), its corresponding azodioxy dimer (29-B), ethanethiol and carbon dioxide.

A very efficient yield of photolysis was obtained from this strategy and 5-bromo-7-nitrosoindoline was identified as the photolysis product by its characterization by NMR-spectroscopy, mass spectrometry and UV-Vis spectroscopy. The $^1$H-NMR spectrum of the unknown pink spot resembled the spectrum of 5-bromo-7-nitroindoline (Figure 30).
Figure 30: (Top) $^1$H-NMR spectrum of commercially available 5-bromo-7-nitroindoline vs (Bottom) $^1$H-NMR spectrum of the unknown pink photolysis product.

Analysis of the unknown pink product by COSY showed correlation among three signals: two triplets at 3.09 and 3.83 ppm corresponding to methylene in the position 3 and 2 respectively, and a singlet at 7.19 ppm corresponding to the amine in the position 1. This fact suggested the formation of an indoline photolysis product through this new pathway. Nevertheless, two broad singlets were observed at 8.72 and 9.10 ppm, which are not conventionally characteristic for aromatic protons. Broad signals are usually a result of rotamers, so a temperature-dependent $^1$H-NMR study was done from temperatures of -60°C to 50°C in chloroform. We observed these two different behaviors: a) The signal at 8.72 ppm, presumably the proton in the position 4 became sharper as the temperature was reduced, and b) the signal at 9.10 ppm, presumably the proton in the position 6, became sharper and it was displaced to lower field at around 9.5 ppm as the temperature was reduced (Figure 31 and 32). This is due to both dynamic effects of NMR spectra, and presumably by the coalescence the signals that are affected structurally (rotamers) by temperature.
**Figure 31:** Variable temperature $^1$H-NMR study of unknown pink spot from -60 to 50°C. Only signals above 8.0 ppm are affected by the change in the temperature.
Figure 32: Zoomed area from Figure 31 depicting both signals affected by the temperature change.

By mass spectrometry we observed the mass corresponding to 5-bromo-7-nitrosoindoline (29-A). Zooming into the spectrum, we were able to observe the azodioxy dimer (29-B) of this molecule, with the characteristic isotopic pattern of dibrominated molecules (Figure 33).
**Figure 33:** (Top) Mass spectrum of 5-bromo-7-nitrosoindoline 29-A m/z [M+H]+ calcd.: 226.9815; obs.: 226.9813. (Bottom) Zoomed area depicting the presence of the azodioxy dimer 29-B, with its characteristic dibrominated isotopic pattern m/z [M+Na]+ calcd.: 474.9381; obs.: 474.9387.

UV-Vis spectroscopy of pure 29-A shows a very broad maximum of absorption at 481 nm, which is not characteristic of 7-nitroindoline or 7-nitrosoindole from Pathway A or B (Figure 34).
2.4.5 Structural Computational Studies on Photolysis Product “5-Bromo-7-Nitrosoindoline”

It is known that aromatic nitroso compounds undergo dimerization to their azodioxy forms in an equilibrium. Biljan et al. discuss the reversibility of this process, and how it is favored by thermal and photochemical conditions (Figure 35)\textsuperscript{37,38}.

Since our preliminary results in \textsuperscript{1}H-NMR and mass spectrometry led us to believe that the unknown pink spot corresponded to 5-bromo-7-nitrosoindoline, an insight into the reversibility of 29-A to 29-B can give us further structural proof for the elucidation of a new mechanism of reaction. Together Dr. Dirk from the department of Chemistry and Biochemistry, and our undergraduate student Philip Baily who was under
his mentorship, planned and performed computational calculations of the theoretical $^1$H-NMR of 29-A and 29-B. The theoretical $^1$H-NMR calculations were carried out using AMPAC/Gaussian Software using B3LYP-6-31G(d,2p)/B3LYP-6-311H+(2d,p) as the theory level. For the calculations of 29-A, the aromatic protons corresponding to the position 4 and 6 were around 6.8 and 7.2 ppm, respectively (Figure 36).

Figure 36: (Top) Structure of 9-A, all positions of the indoline are highlighted. (Bottom) Theoretical $^1$H-NMR calculation of 9-A.
Since the simulated $^1$H-NMR did not correspond to the value obtained by experiments, the azodioxy dimer 29-B was calculated by $^1$H-NMR. The calculations took into consideration the rotation of the C-N bond in the 7th position in different angles. At angles where the proton in the position 6 is farthest from the azodioxy oxygens, we observed a very small displacement from protons in the position 4 and 6 (7.6 and 8.8 ppm respectively) (Figure 37).

Figure 37: (Top) Structure of 9-B at an angle where the proton at the position 6 and the azodioxy oxygens are not closest.

(Bottom) Theoretical $^1$H-NMR calculation of 9-B.
At the angle where the proton in the position 6 is closer to the azodioxy oxygens, we observed a more significant displacement from protons in the position 4 and 6. For the case of the proton in the position 6, the signal matched the experimental value, which was close to 9.1 ppm. The displacement for the proton in the position 4 was slightly higher (7.8 ppm) than in the previous experiment, but still away from the experimental 8.7 ppm that we observed (Figure 3B).

**Figure 3B**: (Top) Structure of 9-B at an angle where the proton at the 6th position and the azodioxy oxygens are closest.

(Bottom) Theoretical ^1H-NMR calculation of 9-B.
Thanks to the computational studies, we could prove that the signal corresponding the aromatic proton in the position 6 is displaced to lower field because of the formation of particular rotamers, where this proton is closer to the azodioxy oxygen. Further calculations are needed to explain the displacement of the proton in the position 4.

2.5 Conclusions and Future Work

The N-thiocarbamoyl-7-nitroindoline moiety was discovered as a photoreactive protecting group for thiols, inspired in Hassner et al’s work. Three N-thiocarbamoyl-7-nitroindolines were synthesized varying the indoline and the thiol, and they all photolyzed into unique 7-nitrosoindoline-based products. Kinetic experiments carried out on each thiocarbamate showed that their kinetic rates are very similar to that of N-acyl-7-nitroindolines, contrary to ureas and carbamates synthesized by Hassner et al. 5-bromo-7-nitrosoindoline (9-A) was identified and isolated from the photolysis of 25, and characterized by NMR spectroscopy, mass spectrometry and UV-Vis spectroscopy. Mass spectrometry experiments validated the presence of the 5-bromo-nitrosoindoline (9-A) and its azodioxy dimer (9-B), while variable temperature $^1$H-NMR experiments showed the presence of rotamers that account for the shift into lower field from the aromatic signals. UV-Vis spectroscopy experiments showed a maximum of absorption at 481 nm, which is not characteristic of regular N-acyl-7-nitroindoline photolysis products. Preliminary 2PA photolysis reactions were carried on 25, where we could observe the fluorescent decay using a 2PA fluorescence microscope. We found out that the rate of photolysis of N-acyl-7-nitroindoline X was twice as fast and 25, but it is still in the same order of reaction, proving that their kinetics of photolysis is very similar. We obtained a slope of 2.1 from a double log plot, proving that 25 undergoes 2PA photolysis upon illumination with femtosecond laser light. Structural computational calculations of the $^1$H-NMR of 9-B explained the displacement to lower field of the proton at the position 6 as a function of the rate of
rotation, which is temperature dependent; more specifically the rotation of the C-N bond in the position 7. Further studies are needed to account for the displacement of the proton at the position 4.

2.6 Experimental Section

2.6.1 General Information

All chemicals were purchased as reagent grade from Thermo Fisher Scientific, Sigma-Aldrich, or Acros Organic, and used without further purification. The ACS grade solvents used for reactions were obtained from Thermo Fisher Scientific and they were distilled from the appropriate drying agents. Molecular sieves (3Å and 4Å) were purchased from Alfa Aesar and Thermo Fisher Scientific, respectively, and activated under high vacuum and heat prior to use. Reactions were performed under an argon atmosphere, strictly anhydrous conditions and monitored by TLC on silica gel 60 F254 plates from EMD Millipore or Dynamic Adsorbents, Inc. Spots were detected under UV light (254 nm). The purification of the compounds was performed by flash column chromatography on silica gel (40-60 µm) from Thermo Fisher Scientific, and the ratio between silica and crude product ranged from 50:1 to 120:1 (dry w/w). \(^1\)H and \(^{13}\)C NMR spectra were recorded on a Bruker Avance III HD 400 MHz NMR spectrometer at 400 and 101 MHz or on a JEOL 600 MHz NMR spectrometer at 600 and 150 MHz, respectively. Chemical shifts (in ppm) were determined relative to tetramethyl silane (δ 0.00 ppm) as an internal standard in CDCl\(_3\) and DMSO-d\(_6\), or relative to the CDCl\(_3\) signal (δ 77.0 ppm) and to DMSO-d\(_6\) signal (δ 39.5 ppm) in \(^{13}\)C NMR spectra. Coupling constant(s) [Hz] were measured from one-dimensional \(^1\)H-NMR spectra. Full or partial assignments were made by standard COSY, HSQC, TOCSY and 1D TOCSY experiments. MS analyses were performed on a high resolution JEOL AccuTOF mass spectrometer using an electrospray ionization (ESI) source or with a Direct Analysis in Real Time (DART) ion source. Photochemical reactions were performed in a Rayonet RPR200 reactor (Southern New England Ultraviolet Company, Branford, CT) equipped with 16 UV lamps (350 nm).
UV-vis absorption spectra were obtained with a Shimadzu UV-3101PC UV-VIS-NIR scanning spectrophotometer. Peptides were synthesized semi-automatically using a Tribute peptide synthesizer from Protein Technologies, Inc. (USA). The details of the in-housed developed two-photon microscope will be summarized. The light source is a mode-locked Ti:Sapphire laser (Maitai HP, 690-1040 nm, 100 fs, 80 MHz, Newport, Santa Clara, CA). The home-built x-y scanner (polygon, galvanometer) can achieve 30 frames/s scanning rate. The laser power at the sample site is varied by rotating a half-wave plate in front of a polarizer. The fluorescence signal from the sample is detected in three spectral channels with photomultiplier tubes (PMTs): red (570-616 nm), green (500-550 nm), and blue (417-477 nm). The outputs of these three PMTs are fed into red/green/blue channels of a frame grabber (Solios eA/XA, Matrox, Quebec, Canada). Two-dimensional images in the x-y plane are acquired through a home-built software program. Each frame has 500x500 pixels. Each final static image is an average of 30 frames.

### 2.6.2 Experimental Procedures and Spectroscopic Data of Compounds

![Scheme 17](image)

**Scheme 17**: Synthesis of three different N-thiocarbamoyl-7-nitroindolines using different 7-nitroindolines and thiols to validate the efficiency of the strategy.
S-ethyl 5-bromo-7-nitroindoline-N-carbothioate (25) – 5-bromo-7-nitroindoline (100 mg, 0.41 mmol, 1 equiv.) and pyridine (100 µL, 1.23 mmol, 3 equiv.) were dissolved in distilled DCM (2 mL) dried over molecular sieves (3Å) at 0°C under argon atmosphere. Triphosgene (81.4 mg, 0.27 mmol, 2/3 equiv.) was added into the dark orange mixture, and the reaction was stirred for 30 minutes until the medium turned bright yellow at room temperature, and total consumption of the starting material was monitored by thin layer chromatography (TLC). Then DMAP (151 mg, 1.23 mmol, 3 equiv.) and ethanethiol (89 µL, 1.23 mmol, 3 equiv.) were added and the mixture was stirred for 30 more minutes. 2 mL of a cold solution of citric acid (237.1 mg, 1.23 mmol, 3 equiv.) in acetone is added to neutralize the bases. The reaction medium is diluted in ethyl acetate (100 mL) and washed 3 times with water (100 mL each), once with a concentrated NaHCO₃ solution (100 mL), and once with brine. The organic layer was separated, dried over magnesium sulfate, filtered, and evaporated under reduced pressure to furnish bright yellow solid 25 (133.5 mg, yield: 98%) r.f. = 0.3 in EtOAc/Hex 1:4. ¹H-NMR (CDCl₃, 400 MHz) δ 7.78 (s, 1H, H₆); 7.53 (s, 1H, H₄); 4.26 (t, 2H, J = 7.0 Hz, H₂); 3.13 (t, 2H, J = 8.2 Hz, H₃); 2.98 (q, 2H, J = 7.3 Hz, ethanethiol CH₂); 1.31 (t, 3H, J = 7.4 Hz, ethanethiol CH₃). ¹³C-NMR (CDCl₃, 400 MHz) δ 167.7 [C=O], 140.3, 138.3, 133.9, 131.8, 125.5, 116.1 [aromatic carbons] 50.0 [C₂], 28.6 [C₃], 25.3 [ethanethiol CH₂], 15.2 [ethanethiol CH₃].

Allyl 7-nitro-N-((phenylthiol)carbonyl)indoline-5-carboxylate (26) – Indoline 28 (100 mg, 0.41 mmol, 1 equiv.) and pyridine (100 µL, 1.23 mmol, 3 equiv.) were dissolved in distilled DCM (2 mL) dried over molecular sieves (3Å) at 0°C under argon atmosphere. Triphosgene (81.4 mg, 0.27 mmol, 2/3 equiv.) was added into the yellow mixture, and the reaction was stirred for 30 minutes at room temperature, until total consumption of the starting material was monitored by thin layer chromatography (TLC). Then DMAP (151 mg, 1.23 mmol, 3 equiv.) and thiophenol (89 µL, 1.23 mmol, 3 equiv.) were added and the mixture was stirred for 30 more minutes. 2 mL of a cold solution of citric acid (237.1 mg, 1.23 mmol, 3 equiv.) in
acetone is added to neutralize the bases. The reaction medium is diluted in ethyl acetate (100 mL) and washed 3 times with water (100 mL each), once with a concentrated NaHCO₃ solution (100 mL), and once with brine. The organic layer was separated, dried over magnesium sulfate, filtered, and evaporated under reduced pressure to furnish orange solid 26. (133.5 mg, yield: 98%) r.f. = 0.4 in EtOAc/Hex 1:6. **¹H-NMR** (400 MHz, 298K, DMSO-d₆) δ 8.33 (s, 1H, H6); 8.07 (s, 1H, H8); 7.54-7.49 (m, 2H, thiophenol arom.); 7.41-7.36 (m, 3H, thiophenol aromatic); 6.07-5.95 (m, 1H, CH₂=CH-); 5.40 (dq, 1H, ³J = 17.0 Hz, ²J = 1.2 Hz, allyl olef. trans); 5.30 (dq, 1H, ³J = 10.4 Hz, ²J = 1.1 Hz, allyl olef. cis); 4.81 (dt, 2H, ³J = 5.8 Hz, ²J = 1.2 Hz, allyl aliph.); 4.43 (t, 2H, J = 8.0 Hz, H2); 3.30 (t, 2H, J = 8.2 Hz, H3). **¹³C-NMR** (400 MHz, 298K, DMSO-d₆) δ 166.52, 164.14 [C=O], 139.57, 138.26, 136.87, 135.41, 129.96, 129.56, 129.37, 126.64, 126.63, 125.25 [arom.], 131.78 [CH2=CH-] 119.10 [CH2=CH-], 66.30 [allyl aliph.], 50.63 [C2], 28.51 [C3]. HR-ESI-MS: m/z [M+H]+ calcd.: 407.0678; obs. 407.0673.

**Allyl 4-(7-nitro-N-(p-tolythio)carbonyl)indolin-5-yl)benzoate (27) – Indoline 13** (100 mg, 0.41 mmol, 1 equiv.) and pyridine (100 µL, 1.23 mmol, 3 equiv.) were dissolved in distilled DCM (2 mL) dried over molecular sieves (3 Å) at 0°C under argon atmosphere. Triphosgene (81.4 mg, 0.27 mmol, 2/3 equiv.) was added into the dark red mixture, and the reaction was stirred for 30 minutes until the medium turned bright orange at room temperature, and total consumption of the starting material was monitored by thin layer chromatography (TLC). Then DMAP (151 mg, 1.23 mmol, 3 equiv.) and p-thiocresol (89 µL, 1.23 mmol, 3 equiv.) were added and the mixture was stirred for 30 more minutes. 2 mL of a cold solution of citric acid (237.1 mg, 1.23 mmol, 3 equiv.) in acetone is added to neutralize the bases. The reaction medium is diluted in ethyl acetate (100 mL) and washed 3 times with water (100 mL each), once with a concentrated NaHCO₃ solution (100 mL), and once with brine. The organic layer was separated, dried over magnesium sulfate, filtered, and evaporated under reduced pressure to furnish orange solid 27. (133.5
mg, yield: 98%) r.f. = 0.3 in EtOAc/Hex 1:6. ¹H-NMR (400 MHz, 298K, DMSO-d6) δ 8.11 (d, 2H, J = 3.4 Hz, arom.); 7.85 (s, 1H, arom.); 7.66 (s, 1H, arom.); 7.59 (d, 2H, J = 8.3 Hz, arom.); 7.40 (d, 2H, J = 8.0 Hz, thiophenol arom.); 7.19 (d, 2H, J = 8.0 Hz, thiophenol arom.); 6.12-6.00 (m, 1H, CH₂=CH-); 5.42 (d, 1H, J = 17.1 Hz, allyl olef. trans); 5.31 (d, 1H, J = 10.4 Hz, allyl olef. cis); 4.84 (d, 2H, J = 5.7 Hz, allyl aliph.); 4.40 (t, 2H, J = 8.2 Hz, H₂); 3.30 (t, 2H, J = 8.0 Hz, H3), 2.34 (s, 3H, thiophenol CH₃).¹³C-NMR (400 MHz, 298K, DMSO-d6) δ 166.53, 165.86 [C=O], 142.88, 140.36, 140.16, 137.46, 136.85, 135.39, 134.57, 130.47, 130.13, 129.72, 127.43, 126.87, 123.40, 121.75 [arom.], 132.21 [CH₂=CH-], 118.49, [CH₂=CH-], 65.79 [allyl aliph.], 50.48 [C₂], 28.96 [C₃], 21.41 [CH₃]. HR-ESI-MS: m/z [M+H]+ calcld.: 497.1147; obs. 497.1142.

Reference N-Fmoc-glycyl-5-bromo-7-nitroindoline – Fmoc-Gly-OH (609.5 mg, 2.02 mmol, 2.5 equiv.) was dispersed in 5 mL anhydrous DCM dried over molecular sieves (3Å) and stirred under argon atmosphere. Ghosez reagent (325 µL, 2.46 mmol, 3.0 equiv.) was added dropwise at room temperature and the reaction was stirred for 30 minutes. Formation of the Fmoc-Gly-Cl is confirmed by the medium turning into a clear, pale-yellow solution. 5-bromo-7-nitroindoline (200 mg, 0.82 mmol, 1 equiv.) was added, covered from the light at 30°C and the reaction was stirred overnight. The reaction medium was diluted with ethyl acetate (100 mL each) and washed once with water (100 mL), once with a saturated solution of NaHCO₃ (100 mL), and once with brine (100 mL). The organic layer was separated, dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by column chromatography (EtOAc/hexanes 1:2) to furnish a yellow solid (406.9 mg, yield: 95%) r.f. = 0.3 in EtOAc/Hexanes 1:2. ¹H-NMR (CDCl₃, 400 MHz) δ 7.77-7.71 (m, 3H, aromatic); 7.60-7.56 (m, 2H, aromatic); 7.52 (s, 1H, aromatic); 7.38 (t, 2H, J = 6.6 Hz, Fmoc aromatic); 7.29 (t, 2H, J = 6.6 Hz, Fmoc aromatic); 5.82 (bs, 1H, Gly NH); 4.35 (d, 2H, J = 6.8 Hz Gly CH₂); 4.23-4.14 (m; 5H; H₂, Fmoc CH₂ and Fmoc CH); 3.21 (t, 2H, J = 8.0 Hz, H3).¹³C-NMR (CDCl₃, 400 MHz) δ 167.03, 156.39 [C=O], 143.73, 141.24, 140.79,
138.39, 133.24, 131.82, 127.71, 127.10, 125.45, 125.14, 119.96, 117.01 [aromatic], 67.31 [Fmoc CH₂], 48.77 [C₂], 47.02 [Fmoc CH], 44.19 [Gly CH₂], 28.91 [C₃]. HR-ESI-MS: m/z [M+Na]⁺ calcd.: 544.0484; obs.: 544.0482.

5-bromo-7-nitrosoindoline (29) – 25 (50 mg, 0.15 mmol, 1 equiv.) was dissolved in 188 mL of a solution of 20% HPLC-grade water in distilled, oxygen-free ACN dried over molecular sieves (3Å) (final concentration 8.0 x 10⁻⁴ M). The mixture was illuminated with UV-light (λ = 350 nm) with a Rayonet UV reactor for 2 hours. The reaction medium was evaporated under reduced pressure and the crude product was purified by column silica chromatography (EtOAc/hexanes 1:4) to furnish dark pink solid 29. (34.7 mg, yield: 94%) r.f. = 0.3 in EtOAc/hexanes 1:4. ¹H-NMR (CDCl₃, 400 MHz) δ 9.10 (bs, 1H, H₆); 8.72 (bs, 1H, H₄); 7.19 (s, 1H, NH), 3.85 (t, 2H, ³J = 8.2 Hz, H₂); 3.09 (t, 2H, H₃, ³J = 8.2 Hz, H₃). ¹³C-NMR (CDCl₃, 400 MHz) δ 167.7 (C=O), 140.3, 138.3, 133.9, 131.8, 125.5, 116.1 [aromatic] 50.0 [C₂], 28.6 [C₃], 25.3 [ethanethiol CH₂], 15.2 [ethanethiol CH₃]. HR-ESI-MS: m/z [M+H]⁺ calcd.: 226.9815; obs.: 226.9705.
Chapter 3

Synthesis of N-Derivatized-7-Nitroindoline Homobifunctional Crosslinkers for the Elaboration of Photoerodible Hydrogels

3.1 Introduction

In 3D tissue engineering, biomimetic polymers such as gelatin, collagen, chitosan, and different types of PEGs have been proposed as suitable matrices for the fabrication of hydrogel-based scaffolds where cells can grow and proliferate. Crosslinkers are mostly used for the elaboration of these soft materials, and endothelial cells have the capacity to grow in monolayers on their surfaces. The main challenge that these scaffolds face towards 3D thick tissue engineering is the pre-formation of a complex pseudovascular network, which is a fundamental structural and functional requirement for the self-sustaining of complex thick tissue in-vivo. By means of subtractive manufacturing, select research groups have proposed the utilization of crosslinkers that contain a photocleavable α-nitrobenzyl moiety, which has been proven to be photolyzed by non-cutting femtosecond laser light. The high spacio-temporal resolution of multiphoton excitation technologies has facilitated the formation of hollow 3D vascular networks inside the biopolymeric matrix, spanning nearly all size scales of native human vasculature. The successful proliferation of many different types of cells on these biopolymer-based new generation materials stands as a promising option for future organ cloning technologies, along with other cut-edge 3D tissue engineering techniques such as 3D bioprinting. Based on our group experience with N-acyl-7-nitroindolines, and our recent discovery of N-thiocarbamoyl-7-nitroindolines, we decided to develop our own photocleavable crosslinkers, and produce our own novel gelatin-based soft materials. The main advantage of using 7-nitroindoline crosslinkers over α-nitrobenzyl-based ones is the absence of aldehyde by-products. These functional groups are not bioinert, since they can form undesired bonds with primary amine-containing macromolecules.
We hypothesized that N-acyl or N-thiocarbamoyl-7-nitroindolines are suitable core moieties for the development of novel photocleavable crosslinkers.

3.2 Specific Aims

3.2.1 To synthesize three 7-nitroindoline homobifunctional crosslinkers that two contain N-hydroxysuccinimide ends, which are reactive with primary amines.

3.2.2 To test their crosslinking capabilities in the elaboration of gelatin type B gels.

3.2.3 To test the photocleavable capabilities of the gels by illumination with UV-light (λ = 350 nm)

3.3 Background and Significance

3.3.1 Biopolymeric Hydrogels as Scaffolds for 3D Tissue Engineering

A hydrogel can be defined as a hydrophilic material made of a three-dimensional cross-linked polymer network with the ability to swell and retain large amounts of water inside its matrix\(^9\). These soft materials have attracted the attention of material scientists because of their tunable properties, their versatile fabrication methods; and have found a wide range of applications in tissue engineering\(^{40}\), regenerative medicine\(^{41}\), wastewater treatment\(^{42}\), and soft robotics\(^{43}\). Over the years, new generation hydrogels have been designed with properties such as environment-response, self-healing, shape memory, which stem from the need of mimicking the structural and functional properties of biological systems such as tissue, extracellular matrix and organs. Collagens are major components in many extracellular matrices, and they
play central roles in all phases of wound healing, including cell proliferation, remodeling, homeostasis, and inflammation\textsuperscript{44}. These natural scaffolds support most soft tissues and organs inside the body, and a vascular system ensures the correct transport of oxygen and nutrients. Biomimetic scaffolds for thick 3D tissue engineering would represent a major impactful solution to current healthcare problems such as organ shortage, and the use of more accurate drug test models that could reduce the necessity of animal testing. To date, a series of biopolymer and PEG-based artificial biomimetic scaffolds with integrated vasculature-like microarchitectures have been generated spanning all sizes of native human vasculature, and research groups have managed to grow cells on their surfaces. Lee et al. used the “Freeform Reversible Embedding of Suspended Hydrogels (FRESH)” bioprinting method and produced components of the human neonatal heart at various scales. The heart tissue printed by this technique accurately reproduces patient-specific anatomical structure, and specific cardiac ventricles grown with human cardiomyocytes showed synchronized contractions, directional action potential propagation, and wall thickening up to 14% during peak systole (Figure 39)\textsuperscript{45}.

![Figure 39](image.png)

\textbf{Figure 39:} Organ-scale FRESH 3D bioprinting of the neonatal-scale human heart. (Left) MRI-derived 3D human heart scaled to neonatal size. (Middle) FRESH-printed collagen heart. (Right) Cross-sectional view of the collagen heart, showing left and right ventricles and interior structures\textsuperscript{45}.

In recent literature, the role of the mechanical properties of the extracellular matrix have been discussed, as they have been proven to play an important part in regulating the cellular responses that occur during tissue regeneration, wound healing, and disease progression. Fabricating successful artificial scaffolds is
yet a very challenging topic, as many variables like the degree of substitution, type of matrix, type of covalent crosslinking bond, and length of the crosslinker can influence the stiffness or softness of the biomaterial. In their work, Zaragoza et al. correlated the length of the crosslinker and the elastic and compressive modulus from different polyacrylamide hydrogels. PEG crosslinkers of higher length increased the mechanical properties of the material, presumably by an increase of the order structure within the hydrogel network (Figure 40). 

![Graph](image)

**Figure 40:** (Left) Elastic and (Right) compressive modulus of polyacrylamide hydrogels varying the chain length of the PEG crosslinker.

### 3.3.2 Photocleavable Crosslinkers and their Application in Material Science

Manipulation of 3D materials at the microscale has been considered important for the engineering of complex tissue, biophotolithography, development of biosensors, elucidation of cell-material interactions and guidance of cellular differentiation. In their work, Hahn et al. explored the photochemical crosslinking of a PEG hydrogel containing acrylate-terminal ends to fluorescently labeled acryloyl (ACRL)-PEG-peptide by formation of radicals. Micropatterns were formed by two-photon absorption laser across user-defined regions of interest. The patterns were generated by the laser at 720 nm and observed by confocal fluorescence microscopy (Figure 41).
This research inspired the Anseth group, who thought of an approach to micropatterning through subtractive manufacturing. They designed a homobifunctional crosslinker containing a base PEG core connected through a diamide bond to two o-nitrobenzyl moieties with an acrylate moiety in each end. Through redox-initiated free radical co-polymerization of the crosslinker and PEG monoacrylate, a hydrogel with photocleavable properties was fabricated. Channels from 5 to 50 µm of diameter were generated with a two-photon confocal laser scanning microscope, on which fibrosarcoma cells were able to migrate and proliferate (Figure 42).
Figure 42: (Top) Structure of the PEG-diacrylate photocleavable crosslinker designed by the Anseth group for radical initiated crosslinking. (Middle) Interconnected 3D channels fabricated within the photodegradable hydrogel covalently labeled with rhodamine B, using a two-photon laser scanning microscope. (Bottom) Migration of a cell along the edge of a photolyzed channel in time-lapsed brightfield images (left) and its corresponding position trace (right)\textsuperscript{49}.

In 2015, Kanamori et al. synthesized a photodegradable hydrogel using a 4-arm PEG o-nitrobenzyl-based tetraalkyne crosslinker and connected it to azide-functionalized gelatin via strain-promoted-azide-alkyne-cycloaddition (SPAAC) to form their photoreactive hydrogel. They encapsulated murine breast cancer cells inside their photoreactive polymer and used optical techniques for cell sorting (Figure 43)\textsuperscript{50}. 
Figure 43: Structure of the PEG tetra alkyne photocleavable crosslinker designed by Kanamori et al. for crosslinking by a SPAAC reaction with azide-derivatized gelatin\textsuperscript{50}.

The DeForest group designed a photodegradable hydrogel using a four-armed PEG-tetraalkyne joined to an o-nitrobenzyl-oligopeptide-based diazide by SPAAC. The resulting biomaterial was photoablated using femtosecond laser light to build microchannel networks spanning nearly all size scales of native human vasculature for directed cell function and cut-edging microfluidics\textsuperscript{51} (Figure 44).
3.4 Results and Discussion

3.4.1 Synthesis of HP1, HP2, and HP3

We decided to build three homobifunctional crosslinkers that had two different photoreactive moieties: the classical N-acyl-7-nitroindoline moiety that has been studied thoroughly in the literature, and our newly discovered N-thiocarbamoyl-7-nitroindoline moiety. For our first crosslinker, we utilized 7-nitroindoline (13), described in the Chapter 1, and coupled it to mono-t-butyl succinate by a Ghosez reaction. The resulting diester (30) was deprotected using ZnCl₂ and DCM to remove the t-butyl group (31), and then using Pd(PPh₃)₄ and NMA to remove the allyl ester. The resulting diacid (32) was treated with EDCI and NHS to furnish homobifunctional crosslinker HP₁, which is suitable for the crosslinking of amine-containing polymers. The overall yield of this synthesis was 50% (Scheme 20).
Most photocleavable crosslinkers used in the literature use PEG-based compounds because they have been proven to be biomimetic, they are long molecules that have more probability to crosslink macromolecules such as biopolymers, they have a higher probability to be water soluble, and their longer structures can result in biomaterials with different mechanical properties. Regular N-acyl-7-nitroindoline coupling involves the formation of in-situ acyl chlorides, whose yields of reaction are variable as discussed in Chapter 2. To avoid struggling with the possibility of a low yield synthesis and difficult purification procedures of mono and disubstituted molecules, we took N-acyl-7-nitroindoline (14) and deprotected the Fmoc group using DBU and EtSH. The resulting primary amine (33) was coupled to a PEG diacid using a mild carbodiimide approach with EDCI to furnish a crude containing diester (34). A total deprotection of the crude allyl diester using Pd(PPh)$_3$$_4$ ensured the quantitative formation of diacid (35), which was further treated with EDCI and NHS to furnish homobifunctional crosslinker HP2, in an overall yield of 77% (Scheme 21).
We also decided to include a crosslinker that contained our newly discovered N-thiocarbamoyl-7-nitroindoline moiety. Starting from 7-nitroindoline 13, we synthesized and isolated its corresponding carbamoyl chloride (36). We utilized a PEG-dithiol in the presence of pyridine to furnish dialkene 37. This diester was deprotected using Pd(PPh₃)₄, and the resulting diacid 38 was treated with EDCI and NHS to furnish homobifunctional crosslinker HP3 (Scheme 22). The overall yield of reaction for this crosslinker was 85%, representing the most efficient synthetic strategy of them all.
3.4.2 Crosslinking of HP1, HP2, and HP3 to Gelatin Type-B

The name collagen is given to a group of related fibrous proteins, found in the mesodermal tissues of animals. It constitutes the main organic component of skin, bone, tendon, and loose connective tissue. Gelatin is a derived protein that can be prepared as a breakdown product of collagen; therefore, it has been utilized in many biomaterials' application as a biomimetic analogue of collagen. Our homobifunctional crosslinkers are designed to react with amines, so we found in the literature that there is an average of 4.0 - 4.7 g of lysine in 100 g of different types of gelatins. Therefore, we designed a series of experiments in which we calculated the total molar amount of lysines in 10 g of gelatin and utilized half of this amount of crosslinker to account for its two active sites. Some of our crosslinkers are inherently...
water insoluble, so we found out that gelatin is soluble in DMSO at 40°C. Preliminary experiments in our laboratory proved that after total solubilization, gelatin type B remains in solution at room temperature for over a period of months. Experiments were carried out using HP1, HP2 and HP3, and rubber-like biomaterials were produced (Scheme 23). HP3 was soluble in water because of the longer PEG chain, and we believe that optimizing a strategy for the crosslinking of gelatin type B in an aqueous medium could be a major advantage over HP1 and HP2 for biological applications.

![Scheme 23](image)

Scheme 23: Elaboration of a photoerodible gelatin-based hydrogel using HP1 as a model homobifunctional crosslinker.

Table 2 summarizes the experiments carried out for the production of the biomaterials:

<table>
<thead>
<tr>
<th>Crosslinker</th>
<th>Amount of gelatin (mg)</th>
<th>mmol of Gelatin Type B (Equiv.)</th>
<th>Amount of crosslinker (mg)</th>
<th>mmol of crosslinker (Equiv.)</th>
<th>Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP1</td>
<td>30</td>
<td>$8.1 \times 10^{-4}$ (1.0)</td>
<td>0.24</td>
<td>$4.0 \times 10^{-4}$ (0.5)</td>
<td>Formed</td>
</tr>
<tr>
<td>HP1</td>
<td>30</td>
<td>$8.1 \times 10^{-4}$ (1.0)</td>
<td>0.48</td>
<td>$8.1 \times 10^{-4}$ (1.0)</td>
<td>Not Formed</td>
</tr>
<tr>
<td>HP1</td>
<td>30</td>
<td>$8.1 \times 10^{-4}$ (1.0)</td>
<td>0.73</td>
<td>$12.2 \times 10^{-4}$ (1.5)</td>
<td>Not Formed</td>
</tr>
<tr>
<td>HP2</td>
<td>30</td>
<td>$8.1 \times 10^{-4}$ (1.0)</td>
<td>0.42</td>
<td>$4.0 \times 10^{-4}$ (0.5)</td>
<td>Formed</td>
</tr>
<tr>
<td>HP2</td>
<td>30</td>
<td>$8.1 \times 10^{-4}$ (1.0)</td>
<td>0.84</td>
<td>$8.1 \times 10^{-4}$ (1.0)</td>
<td>Debris formed</td>
</tr>
<tr>
<td>HP3</td>
<td>30</td>
<td>$8.1 \times 10^{-4}$ (1.0)</td>
<td>1.26</td>
<td>$12.2 \times 10^{-4}$ (1.5)</td>
<td>Not Formed</td>
</tr>
<tr>
<td>HP3</td>
<td>30</td>
<td>$8.1 \times 10^{-4}$ (1.0)</td>
<td>1.0</td>
<td>$4.0 \times 10^{-4}$ (0.5)</td>
<td>Formed</td>
</tr>
<tr>
<td>HP3</td>
<td>30</td>
<td>$8.1 \times 10^{-4}$ (1.0)</td>
<td>2.0</td>
<td>$8.1 \times 10^{-4}$ (1.0)</td>
<td>Not Formed</td>
</tr>
<tr>
<td>HP3</td>
<td>30</td>
<td>$8.1 \times 10^{-4}$ (1.0)</td>
<td>3.0</td>
<td>$12.2 \times 10^{-4}$ (1.5)</td>
<td>Not Formed</td>
</tr>
</tbody>
</table>
These materials were washed with water over 10 times to diffuse the DMSO out of their matrices, and they were stored inside the fridge at 4°C. Their structure was kept after weeks of being dispersed in water and refrigerated. We made preliminary photodegradation experiments using tiny pieces of the biomaterials, and upon illumination with UV-light 350 nm over a period of six hours, the hydrogels were completely degraded and solubilized in water, thus proving their photoerodible properties. In Figure 45 we present an image of the biomaterials that were obtained for these experiments:

![Figure 45: Gelatin-type B gels using the following crosslinkers: 1) HP1; 2) HP2; 3) HP3.](image)

### 3.5 Conclusions and Future Work

Three 7-nitroindoline-based homobifunctional crosslinkers were developed using both traditional N-acyl-7-nitroindoline and newly discovered N-thiocarbamoyl-7-nitroindoline photoreactive moieties. The three alkyl and PEG-based crosslinkers had NHS termini that were suitable for the crosslinking of amine-containing polymers. Gelatin type-B was utilized as the biomimetic matrix for the design of these novel soft materials. Illumination with UV-light over a period of 6 hours resulted in the complete decomposition and solubilization of the soft material in water. For future work, we believe that these biomaterials will be suitable for biophotolithography using femtosecond laser light from a 2PA fluorescence microscope. We propose for future work optimizing a strategy for the crosslinking of Gelatin type-B in an aqueous...
medium using water soluble HP3. This could have advantages when envisioning biological application for this soft material.

3.6 Experimental Section

3.6.1 General Information

All chemicals were purchased as reagent grade from Thermo Fisher Scientific, Sigma-Aldrich, or Acros Organic, and used without further purification. The ACS grade solvents used for reactions were obtained from Thermo Fisher Scientific and they were distilled from the appropriate drying agents. Molecular sieves (3Å and 4Å) were purchased from Alfa Aesar and Thermo Fisher Scientific, respectively, and activated under high vacuum and heat prior to use. Reactions were performed under an argon atmosphere, strictly anhydrous conditions and monitored by TLC on silica gel 60 F254 plates from EMD Millipore or Dynamic Adsorbents, Inc. Spots were detected under UV light (254 nm). The purification of the compounds was performed by flash column chromatography on silica gel (40-60 µm) from Thermo Fisher Scientific, and the ratio between silica and crude product ranged from 50:1 to 120:1 (dry w/w). ¹H and ¹³C NMR spectra were recorded on a Bruker Avance III HD 400 MHz NMR spectrometer at 400 and 101 MHz or on a JEOL 600 MHz NMR spectrometer at 600 and 150 MHz, respectively. Chemical shifts (in ppm) were determined relative to tetramethyl silane (δ 0.00 ppm) as an internal standard in CDCl₃ and DMSO-d₆, or relative to the CDCl₃ signal (δ 77.0 ppm) and to DMSO-d₆ signal (δ 39.5 ppm) in ¹³C NMR spectra. Coupling constant(s) [Hz] were measured from one-dimensional ¹H-NMR spectra. Full or partial assignments were made by standard COSY, HSQC, TOCSY and 1D TOCSY experiments. MS analyses were performed on a high resolution JEOL AccuTOF mass spectrometer using an electrospray ionization (ESI) source or with a Direct Analysis in Real Time (DART) ion source. Photochemical reactions were performed in a Rayonet RPR200 reactor (Southern New England Ultraviolet Company, Branford, CT) equipped with 16 UV lamps (350 nm).
UV-vis absorption spectra were obtained with a Shimadzu UV-3101PC UV-VIS-NIR scanning spectrophotometer. Peptides were synthesized semi-automatically using a Tribute peptide synthesizer from Protein Technologies, Inc. (USA). The details of the in-housed developed two-photon microscope will be summarized. The light source is a mode-locked Ti:Sapphire laser (Maitai HP, 690-1040 nm, 100 fs, 80 MHz, Newport, Santa Clara, CA). The home-built x-y scanner (polygon, galvanometer) can achieve 30 frames/s scanning rate. The laser power at the sample site is varied by rotating a half-wave plate in front of a polarizer. The fluorescence signal from the sample is detected in three spectral channels with photomultiplier tubes (PMTs): red (570-616 nm), green (500-550 nm), and blue (417-477 nm). The outputs of these three PMTs are fed into red/green/blue channels of a frame grabber (Solios eA/XA, Matrox, Quebec, Canada). Two-dimensional images in the x-y plane are acquired through a home-built software program. Each frame has 500 x 500 pixels. Each final static image is an average of 30 frames.

3.6.2 Experimental Procedures and Spectroscopic Data of Compounds

**Allyl 4-(N-(4-(t-butoxy)-4-oxobutanoyl)-7-nitroindolin-5-yl)benzoate (30)** – Indoline 13 (300.0 mg, 0.93 mmol, 1.0 equiv.) and mono-t-buty succinate (483.7 mg, 2.78 mmol, 3.0 equiv.) are dissolved in 4.0 mL of anhydrous pyridine at 0°C. Ghosez reagent (429 µL, 3.24 mmol, 3.5 equiv.) is added dropwise, and the solution is stirred at 90°C for 1 day. The reaction is quenched adding drop by drop a solution of NaHCO₃ in the medium at 0°C, until the pH is neutral, and then diluted in EtOAc (200 mL). The organic phase is washed twice with water (100 mL), and once with brine (100 mL). The organic layer was separated and dried over anhydrous MgSO₄, filtered and concentrated under reduced. The dark-orange crude was purified by silica column chromatography (EtOAc/hex 1:1), furnishing 284.6 mg of a bright yellow solid (yield = 64%). Rf = 0.3 (EtOAc/Hex 1:1) 3H-NMR (400 MHz, 298K, DMSO-d6) δ 8.13 (d, 2H, J = 8.0 Hz, arom.); 7.88 (s, 1H, arom.); 7.67 (s, 1H, arom.); 7.62 (d, 2H, J = 8.0 Hz, arom.); 6.12-6.00 (m, 1H, CH₂=CH-
4-(5-((allyloxy)carbonyl)phenyl)-7-nitroindolin-N-yl)-4-oxobutanoic acid (31) – 30 (200.0 mg, 0.42 mmol, 1.0 equiv.) and ZnCl₂ (567.0 mg, 4.16 mmol, 10.0 equiv.) are dispersed and stirred in 5 mL of anhydrous DCM at room temperature for 5 hours. The reaction is diluted in EtOAc (200 mL). The organic phase is washed twice with water (100 mL), and once with brine (100 mL). The organic layer was separated, dried over anhydrous MgSO₄, filtered and concentrated under reduced pressure. The yellow crude was purified by silica column chromatography (DCM/MeOH 12:1), furnishing 167.8 mg of a bright yellow solid (yield = 95%), Rᶠ = 0.35 (DCM/MeOH 12:1) ¹H-NMR (400 MHz, 298K, DMSO-d₆) δ 12.25 (s, 1H, -COOH); 8.06 (d, 2H, J = 8.4 Hz, arom.); 7.98 (d, 2H, J = 13.5 Hz, arom.); 7.90 (d, 2H, J = 8.4 Hz, arom.); 6.14-6.02 (m, 1H, CH₂=CH-); 5.43 (dd, 1H, ᵃHelvetica J = 17.3 Hz, ᵃHelvetica J = 1.6 Hz, allyl olef. trans); 5.30 (dd, 1H, ᵃHelvetica J = 10.5 Hz, ᵃHelvetica J = 1.3 Hz, allyl olef. cis); 4.84 (d, 2H, J = 5.3 Hz, allyl aliph.); 4.35 (t, 2H, J = 8.0 Hz, H₂); 3.30 (t, 2H, J = 8.0 Hz, H₃); 2.78 (t, 2H, J = 6.0 Hz, succinate CH₂); 2.52 (succinate CH₂). ¹³C-NMR (400 MHz, 298K, DMSO-d₆) δ 173.56, 170.30, 165.07 [C=O], 142.39, 140.38, 138.62, 135.07, 133.94, 129.94, 128.81, 127.33, 126.98, 120.44 [arom.], 132.63 [CH₂=CH-], 118.00 [CH₂=CH-], 65.19 [allyl aliph.], 49.40 [C₂], 28.79 [succinate CH₂], 28.50 [C₃]. HR-ESI-MS: m/z [M+Na]+ calcd.: 447.1168; obs. 447.1169.
4-\((\text{N-(3-carboxypropanoyl)-7-nitroindolin-5-yl)benzoic acid (32)} – 31\) (150.0 mg, 0.35 mmol, 1.0 equiv.) and Pd(PPh\(_3\))\(_4\) (81.6 mg, 0.071 mmol, 0.2 equiv.) are dissolved in 3 mL anhydrous DMF. 382.5 µL NMA are added dropwise, and the reaction is stirred at room temperature for 30 minutes. The reaction medium is quenched with a saturated solution of citric acid in acetone, dropwise until the pH is neutral. The medium is coevaporated with toluene and diluted in EtOAc (100 mL). The organic phase is washed twice with water (100 mL), once with a solution of 1M HCl (100 mL), and once with brine (100 mL). The organic layer was separated, dried over MgSO\(_4\), filtered, and concentrated under reduced pressure, furnishing 135.5 mg of a bright yellow solid. (yield = Quant.). 

\(^1\)H-NMR (400 MHz, 298K, DMSO-d\(_6\)) \(\delta\) 12.63 (s, 2H, -COOH); 8.03 (d, 2H, J = 8.4 Hz, arom.); 8.00 (s, 1H, arom.); 7.96 (s, 1H, arom.); 7.86 (d, 2H, J = 8.4 Hz, arom); 4.35 (t, 2H, J = 8.2 Hz, H\(_2\)); 3.30 (t, 2H, J = 8.0 Hz, H\(_3\)); 2.78 (t, 2H, J = 7.0 Hz, succinate CH\(_2\)); 2.51 (succinate CH\(_3\)). \(^{13}\)C-NMR (400 MHz, 298K, DMSO-d\(_6\)) \(\delta\) 173.60, 170.33, 167.12 [C=O], 141.92, 140.44, 138.66, 135.40, 133.87, 130.19, 130.10, 127.38, 126.85, 120.40 [arom.], 49.44 [C2], 29.86 [succinate CH2], 28.80 [succinate CH2], 28.56 [C3].

**HP1 NHS crosslinker – 31** (100.0 mg, 0.26 mmol, 1.0 equiv.), EDCI (161.5 mg, 1.04 mmol, 4 equiv.), and NHS (120.0 mg, 1.04 mmol, 4 equiv.) are dissolved in 2 mL of anhydrous DMF and stirred at room temperature for 6 hours. The reaction medium is coevaporated with toluene and diluted in EtOAc (100 mL). The organic phase is washed with once water (100 mL), once with a concentrated solution of NaHCO\(_3\) (100 mL), once with brine (100 mL), dried over MgSO\(_4\), filtered, and concentrated under reduced pressure. The yellow crude was purified by silica column chromatography (pure EtOAc), furnishing 284.6 mg of a bright yellow solid (yield = 85%). R\(_f\) = 0.3 (pure EtOAc). \(^1\)H-NMR (400 MHz, 298K, DMSO-d\(_6\)) \(\delta\) 8.18 (s, 1H, arom.); 8.17 (s, 1H, arom.); 8.08 (s, 1H, arom.); 8.06 (s,1H, arom.); 8.04 (m, 1H, arom); 8.03 (m, 1H, arom.); 4.36 (t, 2H, J = 8.2 Hz, H\(_2\)); 3.31 (t, 2H, J = 8.0 Hz, H\(_3\)); 2.97 (s, 4H, CH\(_2\) NHS); 2.91 (s, 4H, CH\(_2\) NHS); 2.80 (s,
1H, succinate CH2). 13C-NMR (400 MHz, 298K, DMSO-d6) δ 170.80, 170.36, 170.12, 169.17, 168.42 [C=O], 167.05, 161.54, 151.82, 144.33, 140.41, 138.81, 134.71, 134.18, 130.76, 127.68, 123.62, 120.86 [aromatic], 49.52 [C2], 28.49 [CH2 NHS], 25.72 [CH2 NHS], 25.58 [CH2 succinate], 25.46 [CH2 succinate], 25.29 [C3]. HR-ESI-MS: m/z [M+Na]+ calcd.: 601.1183; obs.

Allyl 4-(N-glycyl-7-nitroindolin-5-yl)benzoate (33) – 14 (300 mg, 0.5 mmol, 1.0 equiv.) is dissolved in 6 mL of anhydrous DCM. EtSH (107.7 µL, 1.5 mmol, 3 equiv.) is added, followed by DBU (148.4 µL, 1.0 mmol, 2 equiv.) dropwise. The reaction is stirred for 10 minutes, and then diluted in EtOAc (200 mL). The organic phase is washed once with concentrated NaHCO3 solution (100 mL), once with water (100 mL), and once with brine (100 mL). The organic phase is separated, dried over MgSO4, filtered, and concentrated under reduced pressure. The yellow crude was purified by alumina column chromatography (DCM/MeOH 9:1), furnishing 284.6 mg of a bright yellow solid (yield = quant.). Rf = 0.3 (DCM/MeOH 9:1). 1H-NMR (400 MHz, 298K, DMSO-d6) δ 8.18 (s, 1H, NH2 and arom.); 8.01 (s, 1H, arom.); 7.98 (s, 1H, arom.); 7.92 (s, 1H, arom.); 7.89 (s, 1H, arom.); 6.13-6.01 (m, 1H, CH2=CH-); 5.43 (dq, 1H, 3J = 17.4 Hz, 2J = 1.7 Hz, allyl olef. trans); 5.30 (dd, 1H, 3J = 10.5 Hz, 2J = 1.4 Hz, allyl olef. cis); 4.83 (dt, 2H, 3J = 5.4 Hz, 2J = 1.4 Hz allyl aliph.), 4.24 (t, 2H, J = 8.0 Hz, H2); 3.28 (t, 2H, J = 8.0 Hz, H3). 13C-NMR (400 MHz, 298K, DMSO-d6) δ 172.21, 165.09 [C=O], 142.39, 140.30, 138.47, 135.04, 134.11, 129.93, 128.79, 127.34, 126.99, 120.45 [arom.], 132.62 [CH2=CH-], 118.00 [CH2=CH-], 65.18 [allyl aliph.], 48.40 [C2], 44.81 [Gly CH2], 28.60 [C3].

Crude HP2 allyl diester (34) – 33 (150 mg, 0.40 mmol, 1.0 equiv.), and EDCI (119.7 mg, 0.79 mmol, 2.0 equiv.) are dissolved in 3 mL of anhydrous THF. PEG-diacid (65.5 mg, 0.30 mmol, 0.75 equiv.) is added, and the reaction mixture is stirred at room temperature for 2 hours. The THF is evaporated under reduced pressure, and the crude is diluted in EtOAc (200 mL). The organic phase is washed once with a
concentrated NaHCO₃ solution (100 mL), once with water (100 mL), and once with brine (100 mL). The organic phase is separated, dried over MgSO₄, filtered, and concentrated under reduced pressure. The yellow crude was purified by silica column chromatography (DCM/MeOH 15:1), furnishing 301.2 mg of a bright yellow solid crude (yield = quant. by TLC). Rf = 0.3 (DCM/MeOH 9:1). The product comigrated with a carbodiimide-based impurity, so we decided to continue with the crude for the deprotection.

**HP2 diacid (35) – 34** (301.2 mg crude containing 0.29 mmol, 1 equiv.) and Pd(PPh₃)₄ (68.1 mg, 0.042 mmol, 0.6 equiv.) are dissolved in 4 mL of anhydrous DMF. NMA (319 µL, 2.94 mmol, 10 equiv.) is added dropwise, and the solution is stirred at room temperature for 30 minutes. The reaction medium is quenched with a cold saturated solution of citric acid in acetone, dropwise until the pH is neutral. The medium is coevaporated with toluene and diluted in EtOAc (100 mL). The organic phase is washed twice with water (100 mL), once with a solution of 1M HCl (100 mL), and once with brine (100 mL). The organic layer was separated, dried over MgSO₄, filtered, and concentrated under reduced pressure, furnishing 256.0 mg of a yellow solid (yield = quant.). Rf = 0.35 (DCM/MeOH 5:1).

**1H-NMR (400 MHz, 298K, DMSO-d6)** δ 13.10 (bs, 2H, -COOH); 8.04-7.94 (m, 5H, NH and arom.); 7.86 (s, 1H, arom.); 4.83 (dt, 2H, J = 5.4 Hz, 3J = 1.4 Hz allyl aliph.); 4.32 (t, 2H, J = 8.0 Hz, H2); 4.21 (d, 2H, J = 5.4 Hz, PEG CH₂); 3.99 (s, 2H, gly CH₂); 3.69-3.57 (m, 4H, PEG CH₂); 3.29 (t, 2H, J = 8.0 Hz, H3). **13C-NMR (400 MHz, 298K, DMSO-d6)** δ 169.60, 167.45, 167.10 [C=O]; 141.66, 140.37, 138.47, 135.70, 133.61, 130.53, 130.03, 127.39, 126.79, 120.42 [arom.], 70.33 [PEG CH₂], 69.83 [PEG CH₂], 69.56 [PEG CH₂], 48.77 [C2], 41.66 [glycine CH₂], 28.66 [C3].

HR-ESI-MS: m/z [M+Na]^+ calcd.: 891.2449; obs.: 891.2440.
**HP2 NHS crosslinker – 34** (150 mg, 0.17 mmol, 1 equiv.), **NHS** (59.6 mg, 0.52 mmol, 3 equiv.), and EDCI (80.4 mg, 0.52 mmol, 3 equiv.) are dissolved in 3 mL of anhydrous DMF and stirred at room temperature for 6 hours. The reaction medium is coevaporated with toluene and diluted in EtOAc (100 mL). The organic phase is washed with once water (100 mL), once with a concentrated solution of NaHCO$_3$, once with brine (100 mL), dried over MgSO$_4$, filtered, and concentrated under reduced pressure. The yellow crude was purified by silica column chromatography (DCM/MeOH 20:1), furnishing 155.2 mg of a bright yellow solid (yield = 85%). $R_f$ = 0.35 (DCM/MeOH 20:1). $^1$H-NMR (400 MHz, 298K, DMSO-d$_6$) δ 8.17 (s, 1H, arom.); 8.15 (s, 1H, arom.); 8.07-8.00 (m, 4H, arom.); 7.97 (t, 1H, J = 5.6 Hz, NH); 4.34 (t, 2H, J = 8.0 Hz, H$_2$) 4.21 (d, 2H, J = 5.7 Hz, Glycine CH$_2$), 3.99 (s, 2H, PEG CH$_2$); 3.68-3.61 (m, 4H, PEG CH$_2$); 3.31 (t, 2H, J = 8.0 Hz, H$_3$); 2.92 (s, 4H, NHS CH$_2$). $^{13}$C-NMR (400 MHz, 298K, DMSO-d$_6$) δ 170.79, 170.35, 170.12, 167.05 [C=O], 151.81, 144.36, 144.26, 140.36, 138.64, 134.80, 130.74, 127.67, 127.58, 123.62, 120.87 [arom.], 70.32 [PEG CH$_2$], 69.83 [PEG CH$_2$], 69.55 [PEG CH$_2$], 36.41 [C$_2$], 30.11 [Glycine CH$_2$], 25.46 [NHS CH$_2$], 25.28 [C$_3$]. HR-ESI-MS: m/z [M+Na]$^+$ calcd.: 1085.2777; obs.: 1085.2770.

**Allyl 4-(N-(chlorocarbonyl)-7-nitroindolin-5-yl)benzoate (36) – Indoline 13** (300 mg, 0.93 mmol, 1 equiv.) were dissolved in 6 mL of anhydrous DCM. Pyridine (186.9 uL, 1.85 mmol, 2 equiv.) was added dropwise at 0°C. Triphosgene (183.1 mg, 0.62 mmol, 2/3 equiv.) was added at 0°C, and the solution was stirred at room temperature for 1 hour. The reaction medium is quenched with a saturated solution of citric acid in acetone, dropwise until the pH is neutral. The medium is evaporated under reduced pressure and diluted in EtOAc (100 mL). The organic phase is washed twice with water (100 mL), once with a concentrated solution of NaHCO$_3$ (100 mL), and once with brine (100 mL). The organic layer was separated, dried over MgSO$_4$, filtered, and concentrated under reduced pressure. $^1$H-NMR (400 MHz, 298K, DMSO-d$_6$) δ 8.16 (s, 1H, arom.); 8.13 (s, 1H, arom.); 7.90 (s, 1H, arom.); 7.72 (s, 1H, arom.); 7.63 (s, 1H, arom.); 7.61 (s, 1H,
arom.); 6.12-6.00 (m, 1H, CH2=CH-); 5.43 (dq, 1H, 3J = 17.4 Hz, 2J = 1.7 Hz, allyl olef. trans); 5.32 (dd, 1H, 3J = 10.5 Hz, 2J = 1.4 Hz, allyl olef. cis); 4.86 (dt, 2H, 3J = 5.4 Hz, 2J = 1.4 Hz allyl aliph.), 4.52 (t, 2H, J = 8.0 Hz, H2); 3.34 (t, 2H, J = 8.0 Hz, H3). \[^{13}\text{C-NMR (400 MHz, 298K, DMSO-d6)}\] \(\delta\) 165.82, 146.99 [C=O], 142.48, 140.69, 138.63, 133.20, 130.61, 130.18, 127.90, 127.05, 122.13 [arom.]; 132.20 [CH2=CH-]; 118.61 [CH2=CH-]; 65.92 [allyl aliph.]; 53.60 [C2], 28.37 [C3].

**HP3 allyl diester (37) – 36** (100 mg, 0.26 mmol, 2.5 equiv.) and **PEG-dithiol 1.5K** (173.8 mg, 0.10 mmol, 1 equiv.) were dissolved in 3 mL of anhydrous DCM. At 0°C, pyridine (17 \(\mu\)L, 0.21 mmol, 2 equiv.) is added dropwise, and the reaction is stirred at room temperature for 8 hours. The reaction medium is quenched with a cold saturated solution of citric acid in acetone, dropwise until the pH is neutral. The medium is evaporated under reduced pressure. The yellow crude was purified by silica column chromatography (DCM/MeOH 15:1), furnishing 221.0 mg of a bright yellow solid (yield = 90%). \(R_f\) = 0.3 (DCM/MeOH 15:1). HR-ESI-MS when \(n = 35\) PEG units: \(m/z\) [M+2+Na]\(^{13}\) calcd.: 801.8672; obs.: 801.6879.

**HP3 diacid (38) – 37** (100 mg, 0.042 mmol, 1 equiv.) and **Pd(PPh\(_3\))\(_4\)** (9.7 mg, 0.2 mmol, 0.2 equiv.) are dissolved in 2 mL of anhydrous DMF. NMA (46.0 \(\mu\)L, 0.42 mmol, 10.0 equiv.) is added dropwise and the reaction medium is stirred for 30 minutes. The reaction medium is quenched with a saturated solution of citric acid in acetone, dropwise until the pH is neutral. The medium is coevaporated with toluene, and the product is precipitated by adding 20 mL of THF. The solid is centrifuged and decanted 5 times, removing the palladium and NMA-salts. The product is lyophilized using a mixture water/acetonitrile 1:1; furnishing 97.0 mg of a yellow solid (yield = quant.). \(R_f\) = 0.4 (DCM/MeOH 4:1). HR-ESI-MS when \(n = 35\) PEG units: \(m/z\) [M+2+Na]\(^{13}\) calcd.: 789.6751; obs.: 789.6750. Distributions for +2 and +4 charged species are also visible in the spectrum.
**HP3 NHS crosslinker – 38** (50 mg, 0.021 mmol, 1 equiv.), **NHS** (7.5 mg, 0.065 mmol, 3 equiv.) and **EDCI** (10.1 mg, 0.065 mmol, 3 equiv.) were dissolved in 1 mL of anhydrous DMF and stirred at room temperature for 6 hours. The reaction medium is coevaporated with toluene and the yellow crude was purified by silica column chromatography (DCM/MeOH 9:1), furnishing 48.5 mg of a yellow solid (yield = 90%). \( R_f = 0.35 \) (DCM/MeOH 9:1). HR-ESI-MS when \( n = 35 \) PEG units: \( m/z [M+2+Na]^{+3} \) calcd.: 854.3526; obs.: 854.3524.

General procedure for the formation of gelatin hydrogels – 30 mg of **gelatin type B** and different amounts of crosslinker (amounts described in table x) are suspended in 300 µL of DMSO inside 20 mL glass vials. The mixture is set overnight to 40°C. The DMSO is decanted, the resulting hydrogels are scraped carefully from the vial’s floor and suspended in 5 mL of HPLC-grade water. The water is decanted and suspended in 5 mL of water 10 times to diffuse the DMSO out of the biopolymer matrix.
References


Appendix

\[ ^1H\text{-NMR}, \text{400 MHz, DMSO-}d_6, \text{compound 12.} \]
$^{13}$C-NMR, 400 MHz, DMSO-d6, compound 12.
ESI-DART HR mass spectrum of compound 12. \( m/z \) [M+H]\(^+\) calcd.: 285.0870; obs.: 285.0872.
$^{1}$H-NMR, 400 MHz, CDCl$_3$, compound 13.
$^{13}$C-NMR, 400 MHz, CDCl$_3$, compound 13.
ESI-DART HR mass spectrum of compound 13. m/z [M+H]^+ calcd.: 325.1183; obs.: 325.1188.
\(^1\)H-NMR, 400 MHz, CDCl\(_3\), compound 14.
$^{13}$C-NMR, 400 MHz, CDCl$_3$, compound 14.
ESI-TOF HR mass spectrum of compound 14. m/z [M+Na]^+ calcld.: 626.1903; obs.: 626.1901.
$^1$H-NMR, 400 MHz, DMSO-d6, compound 15.
$^{13}$C-NMR, 400 MHz, DMSO-d$_6$, compound 15.
ESI-TOF HR mass spectrum of compound 15. m/z [M-H]− calcd.: 526.1620; obs.: 562.1612.
1H-NMR, 400 MHz, DMSO-d6, compound 17.
$^{13}$C-NMR, 400 MHz, DMSO-d6, compound 17.
ESI-TOF HR mass spectrum of compound 17. $m/z [M+H]^+$ calcd.: 883.4382; obs.: 883.4388; $[M+Na]^+$ calcd.: 905.4207; obs.: 905.4201.
H-NMR, 400 MHz, DMSO-d6, compound 18.
$^{13}$C-NMR, 400 MHz, DMSO-d$_6$, compound 18.
ESI-TOF HR mass spectrum of compound 18. $m/z$ [M+H]$^+$ calcd.: 1166.5339; obs.: 1166.5350.
$^{1}$H-NMR, 400 MHz, DMSO-d$_6$, compound 21.
$^{13}$C-NMR, 400 MHz, DMSO-d$_6$, compound 21.
$^{13}$C-DEPT 135 NMR, 400 MHZ, DMSO-d6, compound 21.
ESI-TOF HR mass spectrum of compound 21. \( m/z \) [M+H]\(^+\) calcd.: 575.2936; obs.: 575.2930.
\textsuperscript{1}H-NMR, 400 MHz, CDCl\textsubscript{3}, compound 25.
$^{13}$C-NMR, 400 MHz, CDCl$_3$, compound 25.
$^1$H-NMR, 400 MHz, CDCl$_3$, compound 26.
$^{13}$C-NMR, 400 MHz, CDCl$_3$, compound 26.
ESI-TOF HR mass spectrum of compound 27. $m/z$ [M+Na]$^+$ calcd.: 407.0678; obs.: 407.0673.
$^1$H-NMR, 400 MHz, CDCl$_3$, compound 27.
$^{13}$C-NMR, 400 MHz, CDCl$_3$, compound 27.
ESI-TOF HR mass spectrum of compound 27. \(m/z\) [M+Na]\(^+\) calcd.: 497.1147; obs.: 497.1142.
$^1$H-NMR, 400 MHz, CDCl$_3$, compound 29.
$^{13}$C-NMR, 400 MHz, CDCl$_3$, compound 29.
Variable Temperature $^1$H-NMR, 400 MHz, CDCl$_3$, compound 29.
ESI-TOF HR mass spectrum of compound 29-A. \( m/z [M+H]^+ \) calcd.: 226.9815; obs.: 226.9813.
ESI-TOF HR mass spectrum of compound 29-B. \( m/z [\text{M+Na}]^+ \) calcd.: 474.9381; obs.: 474.9387.
$\text{H-NMR, 400 MHz, CDCl}_3$, compound 30.
$\text{C-NMR, 400 MHz, CDCl}_3$, compound 30.
ESI-TOF HR mass spectrum of compound 30. $m/z$ [M+H]$^+$ calcd.: 503.1794; obs.: 503.1793.
$^3$H-NMR, 400 MHz, DMSO-d$_6$, compound 31.
$^{13}$C-NMR, 400 MHz, DMSO-$d_6$, compound 31.
ESI-TOF HR mass spectrum of compound 31. $m/z \ [M+Na]^+$ calcd.: 447.1168; obs.: 447.1169.
$^1$H-NMR, 400 MHz, DMSO-d$_6$, compound 32.
$^{13}$C-NMR, 400 MHz, DMSO-d$_6$, compound 32.
$^1$H-NMR, 400 MHz, DMSO-d$_6$, HP1.
$^{13}$C-NMR, 400 MHz, DMSO-d$_6$, HP1.
ESI-TOF HR mass spectrum of HP1. $m/z$ [M+H]$^+$ calcd.: 601.1183; obs.: 601.1186.
$^{1}$H-NMR, 400 MHz, DMSO-d$_6$, compound 33.
$^{13}$C-NMR, 400 MHz, DMSO-d$_6$, compound 33.
ESI-TOF HR mass spectrum of crude containing compound 34. $m/z$ [M+Na]$^+$ calcld.: 971.3075; obs.: 971.3070.
$^1$H-NMR, 400 MHz, DMSO-d$_6$, compound 35.
$^{13}$C-NMR, 400 MHz, DMSO-d6, compound 35.
ESI-TOF HR mass spectrum of compound 35. \( m/z \) [M+Na]+ calcd.: 891.2449; obs.: 891.2440.
$^1$H-NMR, 400 MHz, DMSO-d6, HP2.
$^{13}$C-NMR, 400 MHz, DMSO-d$_6$, HP2.
ESI-TOF HR mass spectrum of HP2. $m/z$ [M+Na]$^+$ calcd.: 1085.2777; obs.: 1085.2770.
$^1$H-NMR, 400 MHz, DMSO-d$_6$, compound 36.
$^{13}$C-NMR, 400 MHz, DMSO-d$_6$, compound 34.
ESI-TOF HR mass spectrum of compound 37. When n = 35 PEG units, $m/z$ [M+2+Na]$^{3+}$ calcd.: 801.8672; obs.: 801.6879. Distributions for +2 and +4 charged species are also visible in the spectrum.
ESI-TOF HR mass spectrum of compound 38. When n = 35 PEG units, $m/z \ [M+2+Na]^3$ calcd.: 789.6751; obs.: 789.6750. Distributions for +2 and +4 charged species are also visible in the spectrum.
ESI-TOF HR mass spectrum of HP3. When $n = 35$ PEG units, $m/z [M+2+Na]^3$ calcd.: 854.3526; obs.: 854.3524. Distributions for +2 and +4 charged species are also visible in the spectrum.
Vita

Patricio Del Castillo was born in the city of Naucalpan de Juarez, Mexico State, Mexico. He graduated high school from Monterrey Institute of Technology and Higher Education (ITESM), campus Cuernavaca City, Mexico, with honors and with the International Baccalaureate Diploma (IBO). He pursued his degree in Chemistry at ITESM, campus Monterrey City, Mexico, being one of the top grades on three occasions. During his junior year, he joined a research group with venue at Monterrey Tech and financed by the company Placosa-Eftec whose research targeted the design of ceramic-plastic nanocomposites for the car paint industry. In his senior year, he was one of the six students who earned the prestigious Mexican CENEVAL-chemistry prize nationwide. Also, during these years, he contacted Dr. Katja Michael and was invited to her research group to work in the development of his thesis project at the University of Texas at El Paso, with the co-advising of ITESM. This research focused on the production and application of a new N-acyl-7-nitroindoline-based photoreactive amino acid linker for Solid Phase Peptide Chemistry (SPPS). Just after graduation, Patricio kept working as an affiliate of the Michael group for the Summer of 2016.

He then joined UTEP’s doctoral program in chemistry in Dr. Michael’s group. Since the beginning of the doctoral work, Patricio was supported as a teaching assistant, and then he earned the CONACyT scholarship from the Mexican government along with the CONTex fellowship from the UT system, which financed his tuition. Patricio was also recipient of the Dodson Grant at UTEP, which financed work for his research. Patricio presented his research at the Society for Biomaterial conference, at international ACS conferences, and at the NSF PREM: Pathway to the Workforce workshop. Patricio’s dissertation entitled “Novel N-derivatized-7-nitroindolines for the chemical crosslinking of photocleavable biomaterials, and for solid phase peptide synthesis” was supervised by Dr. Katja Michael, and it has work in collaboration with Dr. Chunqiang Li from the Physics Department at UTEP, and with Dr. Carl Dirk from the Department of Chemistry and Biochemistry at UTEP.

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