Design and Development of a Near-Infrared (NIR) Fluorogenic Probe for the Detection of Leucine Aminopeptidase

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Abstract

Tracking biologically relevant molecules, such as proteins and enzymes, in cells is an ongoing issue in biomedical research. A small molecule organic probe would be useful in targeting these molecules. For this study, we targeted leucine aminopeptidase (LAP), a protease that hydrolyzes N-terminal leucine residues of proteins and peptides. LAP is overexpressed in liver cancer cells, making it an ideal biomarker to track. We designed a near-infrared naphthalimide-based fluorogenic probe for detection of LAP. Unfortunately, synthesis of the probe could not be completed due to an overall 4% yield from two linear steps. In future work, we will try new methods for synthetic optimization and complete the final two three steps.

Introduction

Fluorescence is a molecular process involving excitation of a molecule with UV light at a particular wavelength, allowing the promotion of electrons from the ground state to the excited, higher energy state. As the molecule relaxes, it undergoes a series of vibrational energy losses. The result is the emission of a photon or light. The specific wavelength emitted corresponds to a particular region of the electromagnetic spectrum, such as infrared, ultraviolet, or visible light (Smith, 2023).

Fluorescence is a valuable tool in various scientific disciplines, particularly in the detection and identification of specific sites or molecules of interest. One prominent application is the use of fluorescent labels, often referred to as "tags," which can be attached to a target molecule or binding site. Once the fluorescent tag is introduced, it can be excited with light of a specific wavelength, and its emission can be monitored, providing insights into the behavior and characteristics of the tagged site. This is particularly useful in biochemical and medical research, where the ability to observe and track specific biomolecules.

In the context of biomedical research, fluorescence is widely utilized to identify and monitor biomarkers—molecules that serve as indicators of various physiological or pathological conditions. Biomarkers can be proteins, nucleic acids, or other molecules whose presence or concentration is directly associated with a specific health condition or disease state. Using fluorescent tags or probes that bind to these biomarkers, researchers can more effectively track their behavior, quantify their levels, and gain insights into the underlying biological processes. Such applications are critical for early detection, diagnosis, and the monitoring of diseases, as well as for the development of novel therapeutic strategies. Thus, fluorescence serves as a powerful tool for advancing both basic scientific research and clinical applications.

An example of one of these research applications is cancer. It is one of the leading causes of death in the US (Johns Hopkins University, 2024). Identifying and locating cancerous cells poses difficulty in surgery (Nadler, 2013), as some cancerous cells can vary in size from a baseball to grains of rice. To detect these tumors fluorescence-guided surgeries have been developed as a method during removal (Zhang et al., 2019). This process works by targeting a biomarker that is unique to cancer and developing a probe to selectively target it (Matsukawa et al., 2023). The biomarker will bind/react with the fluorogenic probe, which will cause an increase of fluorescence at the site of the tumors. This allows a more complete resection of small tumor cells (Choi, 2023).

For this study, we chose to develop a fluorogenic probe to aid in the resection of liver cancer cells. To accomplish this, we designed a probe which will react with leucine aminopeptidase (LAP). LAP hydrolyzes N-terminal leucine residues from proteins and peptides. Previous studies have shown that LAP is upregulated predominantly in the cytoplasm of hepatocellular carcinoma (HCC) cells. LAP overexpression has been linked with metathesis of HCC cells.

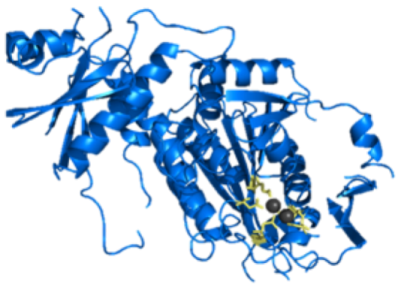
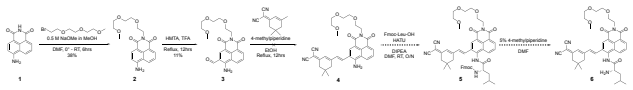
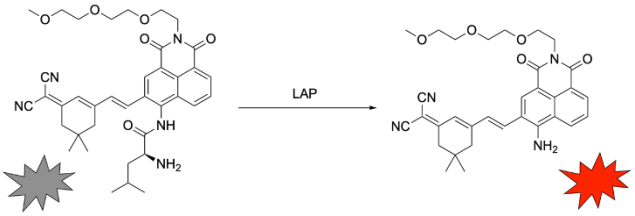


Figure 1. Structure of Leucine Aminopeptidase (Kim et al, 1993).



Scheme 1. Synthesis of NIR probe 6.

Our probe will consist of a naphthalimide dye as the fluorescent core of the dye (see image 1). A polyethylene glycol (PEG) chain will be added to increase solubility. The π conjugation of the dye extended to the near-infrared (NIR) region by adding a dinitrile moiety. The probe needs to emit light in the near-infrared region to avoid autofluorescence of proteins and tissues in the body. Leucine will be added to the molecule to facilitate the reaction between LAP and the probe. The probe will be weakly fluorescent with leucine bound to the naphthalimide structure. Once leucine is cleaved by LAP, the fluorescence intensity will increase.



Scheme 2. Naphthalimide-based near-IR fluorogenic probe is activated in the presence of LAP inducing fluorescence through Internal Charge Transfer (ICT).

Materials and Methods

General Procedures

Solvents were purchased from either Sigma Aldrich, Oakwood Chemicals, Thermo Scientific and Fisher Scientific and used as is. Reagents were purchased from either Ambeed, Oakwood Chemicals, or Thermo Fisher. Reactions were monitored by TLC to determine reaction progress and completion. Products were purified via flash column chromatography. Purity was determined by ^{1}H NMR spectroscopy.

Flash Chromatography Procedures

A Buchi C850 PURE FlashPrep chromatography instrument equipped with disposable Ecoflex silica flash cartridges were used for all flash chromatography purifications. Purification of compound 2 was performed starting at 45% hexanes:55% ethyl acetate to 100% ethyl acetate over 25 minutes. Purification of compound 3 was performed starting at 70% H_2O:30% acetonitrile to 100% acetonitrile over 20 min. Full absorbance spectrum and evaporative light scattering detection (ELSD) was used to identify fractions for isolation.

Instrument(s)

^{1}H-NMR spectra were collected on a JEOL 400YH NMR Spectrometer.

Synthesis of 6-amino-2-(2-(2-(2-methoxyethoxy)ethoxy)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (2).

Compound 1 (212 mg, 1 mmol) was added to a round bottom flask fitted with stir bar and dissolved in DMF (8.4 mL). The flask was placed in an ice bath and 0.5M sodium methoxide in methanol (2.2 mL, 1.1 mmol) was added dropwise. The orange mixture was left to stir for 10 minutes at 0ºC before methyl-PEG_3-bromide (248 mg, 1.1 mmol) was added dropwise to the solution. The reaction was allowed to proceed for 6 hours at room temperature. The solvent was removed under reduced pressure and the crude product was dissolved in dichloromethane and purified through flash chromatography (Hexanes/Ethyl Acetate). Fractions were collected based on absorbance and condensed to afford a yellow/orange oil (38%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.32 (s, 3H), 3.44 (m, 2H), 3.58 (m, 2H), 3.63 (m, 2H), 3.70 (m, 2H), 3.83 (t, 2H), 4.44 (t, 2H) 5.00 (s, 2H), 6.83 (d, 1H), 7.63 (t, 1H), 8.08 (d, 1H), 8.36 (d, 1H), 8.57 (d, 1H).

Synthesis of 6-amino-2-(2-(2-(2-methoxyethoxy)ethoxy)ethyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinoline-5-carbaldehyde (3).

Compound 2 (116.3 mg, 0.325 mmol) and hexamethylenetetramine (90.9 mg, 0.648 mmol) was added to a round bottom flask fitted with stir bar and dissolved in trifluoroacetic acid (10 mL). The flask was placed in an oil bath and refluxed for 12 hours at 60ºC. After 12 hours, the solvent was removed under reduced pressure and the crude product was dissolved in 70% H2O: 30% Acetonitrile and purified through reverse phase flash chromatography (H_2O/Acetonitrile). Fractions were collected based on absorbance and condensed to afford a yellow oil (11%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.32 (s, 3H), 3.44 (m, 2H), 3.58 (m, 2H), 3.63 (m, 2H), 3.70 (m, 2H), 3.83 (t, 2H), 4.44 (t, 2H) 5.00 (s, 2H), 7.86 (t, 1H), 8.10 (s, 1H), 8.58 (d, 1H), 8.68 (d, 1H).

Synthesis of (E)-2-(3-(2-(6-amino-2-(2-(2-(2-methoxyethoxy)ethoxy)ethyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-5-yl)vinyl)-5,5-dimethylcyclohex-2-en-1-ylidene)malononitrile (4)

Compound 3 (5.9 mg, 0.0153 mmol) and 2-(3,5,5-Trimethylcyclohex-2-en-1-ylidene)propanedinitrile (2.9 mg, 0.0156 mmol) was

added to a round bottom flask fitted with stir bar under N_2 and dissolved in absolute ethanol (3 mL). The flask was placed in an oil bath and 4-methylpiperidine (3.62 µL, 0.0306 mmol) was added dropwise. The reaction mixture refluxed for 12 hours at 80ºC. After 12 hours, the solvent was removed under reduced pressure and the crude product was subjected to thin layer chromatography. TLC showed only starting material components present.

Results and Disucssion

Purification and Characterization of Compound 2

Thin layer chromatography (TLC) was run to determine the solvent system and gradient used in flash chromatography. The calculated retention factor (R_f) values were used to determine an optimal solvent system. Our TLC results comparing compound 2 to compound 1 showed the best solvent mixture would be a gradient of hexanes and ethyl acetate. The flash chromatography method used in the purification of compound 2 showed optimal separation at retention time of 13-17 min (Figure 2). The fractions collected from 13-16 resulted in a 38% yield of compound 2.
The proton NMR spectrum of compound 2 shows the addition of the PEG chain (Figure 3). The PEG peaks can be seen in the 3-4ppm region. The integration of these peaks suggests they are properly integrated and there are no significant impurities.

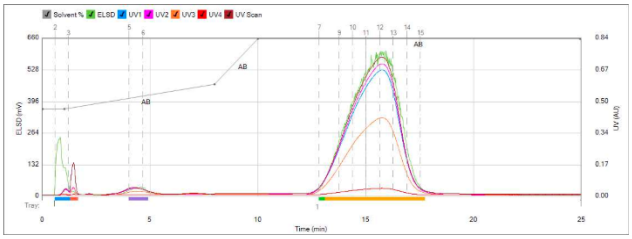


Figure 2. Flash Chromatography Absorbance vs ELSD spectrum for Compound 2.

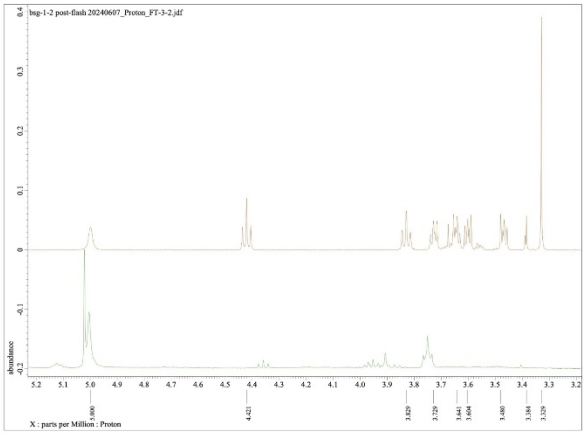


Figure 3. Overlayed Proton NMR comparing compound 1 and 2.

Purification and Characterization of Compound 3

Thin layer chromatography (TLC) was run to determine the solvent system and gradient used in flash chromatography. The calculated retention factor (R_f) values were used to determine an optimal solvent system. Our TLC results comparing compound 3 to compound 2 showed the best solvent mixture would be a gradient of H_2O and acetonitrile. The flash chromatography method used in the purification of compound 2 showed optimal separation at retention time of 10 min (Figure 4). Concentration of samples at 10 min resulted in an 11% yield of compound 3.
The proton NMR spectrum of compound 3 shows the disappearance of a doublet at 6.83ppm (Figure 5). In addition, the doublet at 8.08ppm has been converted to a singlet at 8.10ppm. The loss of a doublet peak and the conversion of a doublet to a singlet both indicate formylation occurred ortho to the amine group of 4-amino-naphthalimide derivative.

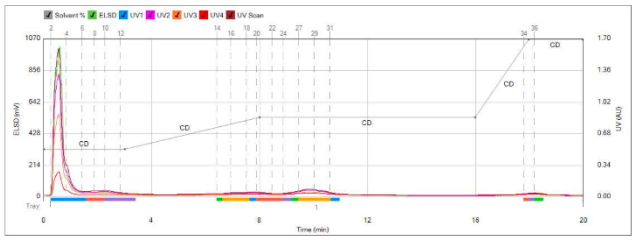


Figure 4. Flash Chromatography Absorbance vs ELSD spectrum for Compound 3.

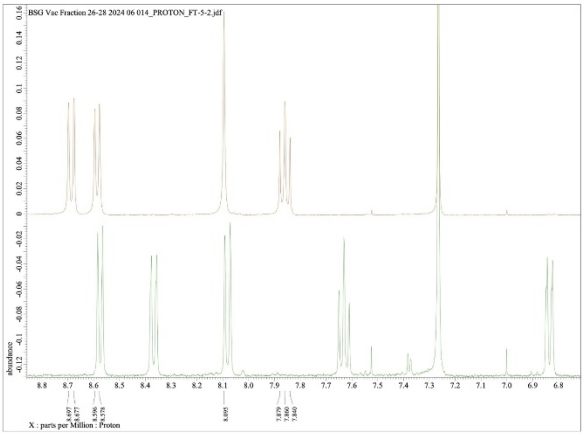


Figure 5. Overlayed Proton NMR comparing compound 2 and 3.

Synthesis of Compound 4

The previous reactions were found to be very low yielding. Due to the very minimal amount of compound 3 present for the synthesis of compound 4, failure at this step requires repeating the two prior synthetic steps. It's possible the reaction didn't

proceed with a good to decent yield due to the limitations of the Duff reaction. It might be more advantageous to add the leucine residue prior to forming the aldehyde using the Vilsmeier-Haack reaction instead. In the future, we will need to work towards re-synthesizing and optimizing the next steps in our synthetic plan.

Conclusion

Compound 2 was successfully synthesized in fair yield. ^{1}H NMR of compound 2 shows addition of PEG_3 chain and optimal purity post-flash chromatography. ^{1}H NMR of compound 3 shows the disappearance of one of the aromatic protons of the naphthalimide core, which indicates the addition of the formyl (aldehyde) group. In addition, the ^{1}H NMR of compound 3 showed the change of another aromatic proton from a doublet to a singlet, indicating removal of a neighboring proton. While the naphthalimide core was formylated, the yield was subpar. In the future, we will need to explore options other than the Duff reaction, like the Vilsmeier-Haack reaction.

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Acknowledgements

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