'Click for PET'

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Chapter 5
Modified [lys3]-bombesin for [^{18}F]-radiolabelling and multi-modality imaging

*Bombesin analogue **[^{18}F]-BN3** was designed and synthesized for the targeting of gastrin-releasing peptide receptors in prostate cancer cells. The design incorporates a terminal alkyne at the Lys residue of the 14 amino acid peptide, where [^{18}F] can be readily introduced. The tracer proved to have good* in vitro *properties. Furthermore, a molecular scaffold was designed and attached to bombesin to allow for both optical and PET imaging.*

Note: The radiochemistry and *in vitro* studies described in this chapter were performed by Leila Mirfeizi.[1]

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5.1 Introduction

In the last two decades, there has been exponential growth in the development and usage of radiolabeled peptides for the purpose of diagnostic imaging and therapeutics.[1] The automation of peptide synthesis has contributed to this dramatic increase, enabling inexpensive production of peptides in large quantities. However, it is also due to certain key characteristics of peptides, such as fast clearance from non-targeted tissue and blood and rapid tissue penetration that they are being developed as radiopharmaceuticals.[1]

Bombesin (BN 1-14) is a 14 amino acid amphibian neuropeptide (Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2) which binds with high affinity to the gastrin-releasing peptide receptor (GRPR). Amino acids 7-14 are essential for receptor binding.[2] BN(1-14) has received much attention in the field of nuclear imaging as the GRPRs are massively overexpressed in a variety of tumor cells, including prostate tumor cells, which lends bombesin high potential as a radioligand for the diagnosis and imaging of cancer.[3] The specificity of a radioligand for the targeted tissue is crucial to good imaging giving high tumor-to-background ratios. Thus the GRPR-bombesin system is particularly attractive; not only are the GRPRs overexpressed in a variety of primary and metastic malignancies of prostate cancer, but their expression in normal tissue is low.[4] Much effort has been invested in the development of radiolabeled analogues of bombesin as currently available imaging techniques do not yet provide the necessary sensitivity to allow for proper imaging of recurrent or early advanced prostate cancer and metasteses.[2] A wide variety of labeled bombesin analogues have been explored in an effort to develop new probes for high sensitivity imaging. Bombesin is often modified to include tyrosine in the fourth position ([tyr4]-bombesin) or lysine in the third position ([lys3]-bombesin) for facilitation of labeling. [Lys3]-bombesin (Fig. 1) allows for site selective modification at the terminal ε-amino group of lysine. As aforementioned, amino acids 7-14 are those necessary for receptor binding, thus modification in the third position reduces the interference of the affinity of the modified peptide for the targeted receptor. The large majority of existing bombesin analogues are labeled with large metallic radionuclides (^{64}Cu, ^{111}In, ^{68}Ga) through the commonly introduced chelating groups: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA).[5]

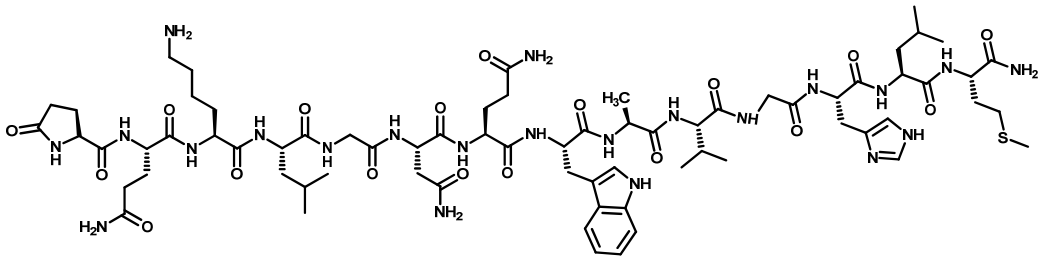


Figure 1 [Lys3]-bombesin

Due predominantly to the synthetic challenges associated with the introduction of [^{18}F] when compared with the ease of chelation techniques used for metallic radionuclides, few instances of [^{18}F]-labeled bombesin can be found in the literature.[6] The synthetic time frame of [^{18}F] ($t_{1/2}$~110 min) is also much reduced as compared to metallic radionuclides such as [^{64}Cu] ($t_{1/2}$~12 h). A further drawback to the use of [^{18}F] is the necessity for multi-step synthetic procedures to synthesize current prosthetic groups such as succinimidyl-[^{18}F]-4-fluorobenzoate ([^{18}F]SFB) or [^{18}F]-4-fluorobenzaldehyde, which are commonly used to label bombesin.[7]

An ideal prosthetic group is one that can be easily synthesized, in which the radionuclide is introduced in the last step of the synthesis, and which requires only mild conditions to attach it to the biomolecule of interest.

Many research groups have begun to exploit the bioorthogonality of the copper-catalyzed azide-alkyne cycloaddition (CuAAC) to allow for straightforward labeling of sugar, protein and peptide targets with [^{18}F] and other radionuclides by introducing a 'click' handle into the target molecules.[8] An advantage of introducing a 'click' handle such as an alkyne into bombesin is the ability to easily tune the properties of the analogue by designing various azides of different sizes, linker lengths or hydrophobicities. Since the 'click' reaction is known to be very robust and to have a very wide substrate scope, changing the azide prosthetic group to achieve new tracers should not require much optimization of the conditions for the labeling protocol.

Positron emission tomography (PET) is one of many available molecular imaging techniques (Chapter 1, Section 1.8). While each technique has its advantages, not one single technique is perfect for every application. For instance, PET has very high sensitivity, but compared to a technique such as magnetic resonance imaging (MRI), it has poor resolution.[9] Efforts have been made to develop multi-modality imaging probes to combine the benefits of two or more techniques to enhance visualization.[9] Various combinations have been explored including MRI/optical imaging, PET/MRI and PET/optical imaging. The merging of PET and optical imaging combines the quantifiable

physiological information of PET with the metabolic distribution information available from fluorescence imaging. In this way, as the short-lived radionuclide decays, information about the probe and its metabolic fate can still be accessed by optical imaging.[9] While it is not a necessity that the two imaging techniques be combined in one single probe, it ensures that there is no difference in the pharmacokinetics or localization of the two different signals, providing more reliable data.[10]

5.2 Goal

As of yet, 'click' chemistry has not been used to label bombesin with [^{18}F]. The aim of this project was to modify [lys3]-bombesin with an alkyne or an azide at the lysine residue allowing for facile and rapid radiolabelling with [^{18}F]. The methodology described in Chapter 3 to accelerate the CuAAC was to be applied. The *in vitro* binding affinity of the resulting bombesin analogue to GRPRs was to be tested, and if necessary, the structure of the alkyne or azide would be changed to optimize its properties. A further aim of this project was to modify bombesin with a scaffold that incorporated both a fluorophore for optical imaging and a handle for radiolabelling with [^{18}F] with the aim to produce a multi-modal imaging agent. The goal was to develop a generic scaffold which could be used for targeted imaging.

5.3 Results and Discussion

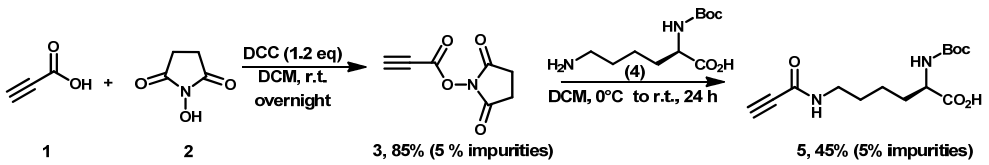
5.3.1 Synthesis of bombesin analogues for CuAAC

There are two possible approaches to the synthesis of an alkyne or azide modified peptide. One approach involves the synthesis of the unnatural alkyne or azide containing amino acid, followed by its introduction into the peptide sequence by solid phase peptide synthesis (SPPS). The alternative is to make use of chemical ligation techniques to modify the existing peptide; this ligation is readily achievable using the terminal ε-amino group of lysine in [lys3]-bombesin.

In this chapter, both methods are used. In this first section, the desired peptide was accessed by the first route: synthesis of the unnatural amino acid followed by SPPS to access the bombesin analogue.

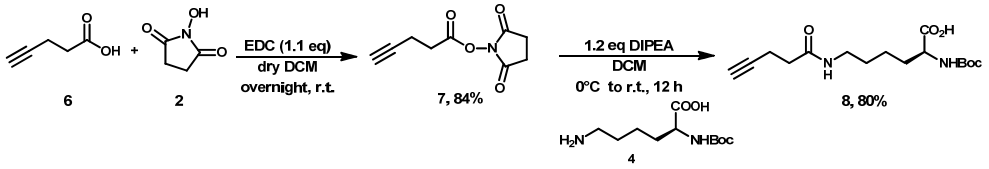
The synthesis of an alkyne containing lysine analogue was initially started with the synthesis of activated ester **3** (Scheme 1). The product was attained by reacting propiolic acid (**1**) with *N*-hydroxysuccinimide (**2**) in the presence of the coupling reagent *N,N'*-dicyclohexylcarbodiimide (DCC). However, as indicated in the literature, purification of the crude reaction mixture by column chromatography resulted in degradation of the target compound **3**.[11] Therefore, crude **3** was reacted with Boc-lys-OH (**4**) to give **5**.

Unfortunately, purification of **5** from the traces of *N,N'*-dicyclohexylurea (DCU) (byproduct of the previous step) proved to be inefficient and resulted in a low yield of product **5** with traces of DCU contamination still present.



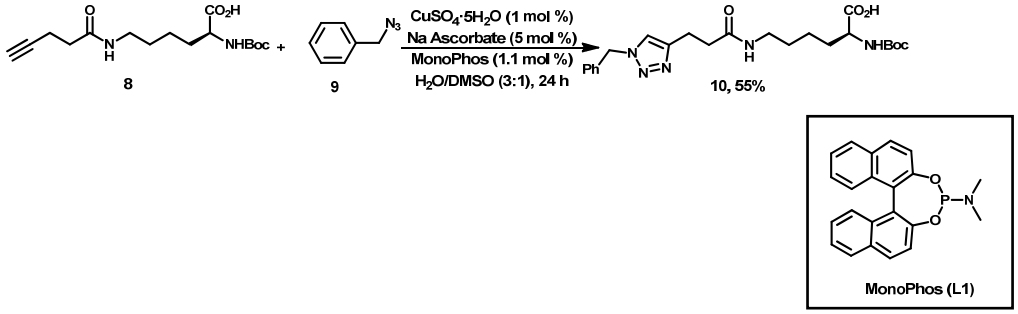
Scheme 1 Attempted synthesis of alkyne modified lysine **5**

Given the instability of alkyne **3** and the resulting purification problems, a slightly longer two carbon linker was chosen, and the synthesis of activated ester **7** was undertaken starting from commercially available 4-pentynoic acid (**6**) (Scheme 2). Treating **6** with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and reacting it with NHS (**2**) in dry DCM overnight yielded activated ester **7** which was coupled to Boc-lys-OH (**4**) in basic conditions giving the acetylene-functionalized amino acid **8**.



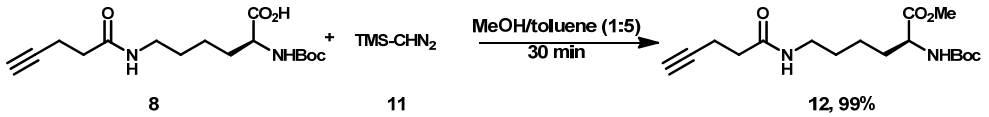
Scheme 2 Synthesis of lysine analogue **8**

The reactivity of **8** was confirmed by reacting it with benzyl azide (**9**) in the presence of 1 mol % of copper catalyst with 1.1 mol % MonoPhos (**L1**) and 5 mol % of sodium ascorbate. Although the reaction produced the expected triazole **10**, it proceeded sluggishly, and required 24 h to reach full conversion (Scheme 3).



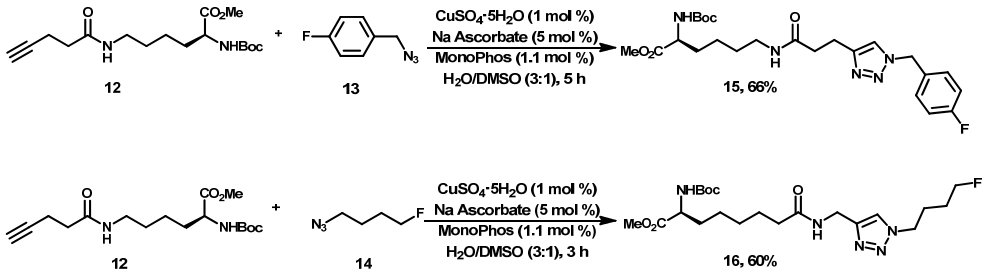
Scheme 3 CuAAC with lysine analogue **8**

We suspected that the carboxylate functionality might be coordinating to the copper center preventing the binding of the alkyne substrate to copper to allow the formation of the necessary copper acetylide (see Chapter 1, section 1.3). To overcome this chelation, methyl ester analogue **12** was prepared by reacting carboxylic acid **8** with trimethylsilyldiazomethane (TMSCHN$_2$, **11**) (Scheme 4).[12]



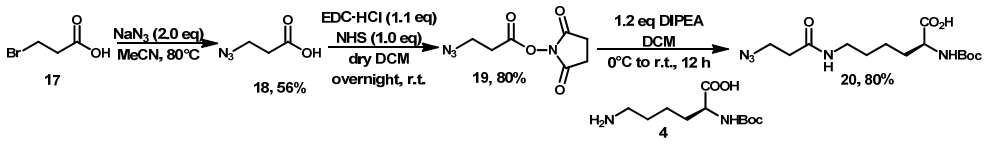
Scheme 4 Synthesis of methyl ester **12**

Compound **12** was reacted with azides **13** and **14** to yield the corresponding triazoles **15** and **16** (Scheme 5). The reactions proceeded relatively quickly to full conversion with 1 mol % of catalyst.



Scheme 5 CuAAC with lysine analogue **12**

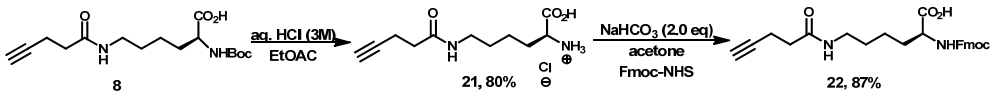
The corresponding azido lysine analogue was also prepared (Scheme 6). Starting from commercially available 3-bromopropanoic acid (**17**), 3-azidopropanoic acid (**18**) was prepared by nucleophilic substitution of bromine with azide anion in MeCN. Activated ester **19** was formed by reacting **18** with EDC·HCl and NHS (**2**) in dry DCM. After purification, it was readily ligated to Boc-lys-OH (**4**) under basic conditions to give the azide-functionalized lysine analogue **20**.



Scheme 6 Synthesis of azido lysine analogue **20**

An important feature of radiochemistry is that the [^{18}F] prosthetic group is generally produced in very small amounts, while the target for labeling is more often present in excess. Based on the results described in Chapter 3, which demonstrated that an excess of alkyne promotes the reaction rate more effectively than does an excess of azide, we opted to first synthesize the alkyne containing analogue of [lys3]-bombesin. In this way, the alkyne will exist in excess as compared to the [^{18}F]-fluoroazide prosthetic group.

To synthesize the desired peptide, we made use of automated solid phase peptide synthesis (SPPS).[13] Although the technique works both with *tert*-butyloxycarbonyl (Boc) and 9-fluorenylmethyloxycarbonyl (Fmoc) protected amino acids, we made use of an automated peptide synthesizer designed for amino acids protected with Fmoc. A change of protecting group for amino acid **8** was thus required before proceeding with the synthesis (Scheme 7). The synthesis was performed up to this stage with the Boc group rather than the Fmoc group, as the Fmoc group can be unstable in basic conditions, such as those used to introduce the azide linker.[14] The Boc group was cleaved with aqueous HCl in ethyl acetate, and Fmoc could be introduced using fluroenylmethoxycarbonyl-*N*-hydroxysuccinimide (Fmoc-NHS) in the presence of sodium bicarbonate.



Scheme 7 Synthesis of amino acid 22

Using unnatural amino acid **22**, we were able to synthesize bombesin analogue **BN1** (Fig. 2). After cleaving **BN1** from the resin and precipitating it from solution, it was purified by preparative RP-HPLC and characterized by mass spectrometry (ESI-MS).

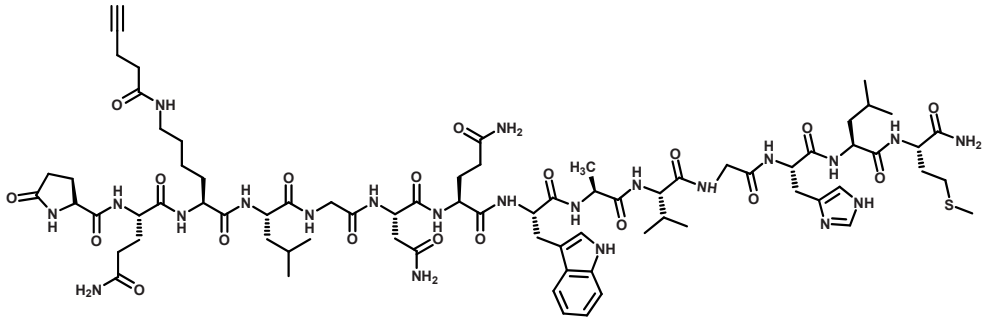
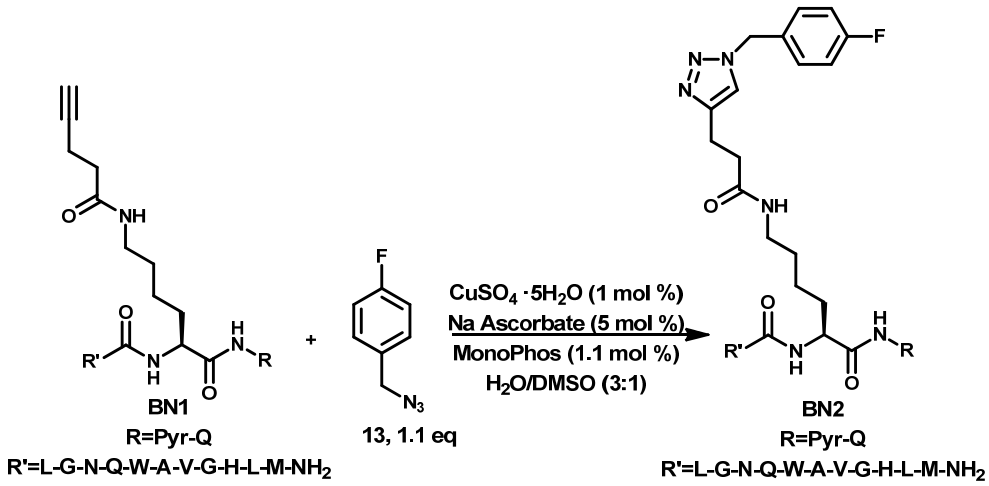


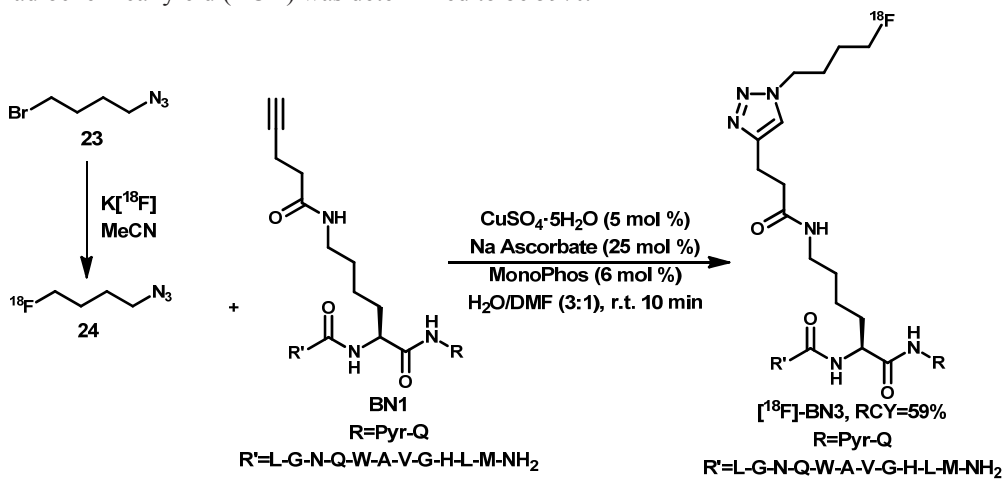
Figure 2 Bombesin analogue **BN1**

A CuAAC of **BN1** with fluorobenzyl azide **13** was performed next. The reaction was monitored by RP-HPLC, and complete conversion to the corresponding triazole **BN2** occurred in 30 min with 1 mol % of copper catalyst (Scheme 8).[15] **BN2** can be purified by RP-HPLC and characterized by mass spectroscopy. When compared to some literature examples of the application of the CuAAC to [^{18}F] labeling of peptides, we found that to achieve reaction times of 20-30 minutes, either a large excess of copper is used, or elevated temperatures are used, or a combination.[16]



Scheme 8 Synthesis of bombesin analogue **BN2**

Having tested the reactivity of **BN1** with a 'cold' [^{19}F] azide, **BN1** was subsequently reacted with [^{18}F]-fluoroazidobutane **24** (prepared from bromoazidobutane **23**) (Scheme 9). Conversion to **BN3** was monitored by radio-thin layer chromatography (radio-TLC). Full conversion was detected after 10 min. The product was purified by RP-HPLC and the radiochemical yield (RCY) was determined to be 59%.



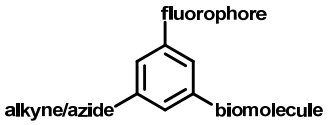
Scheme 9 Synthesis of [^{18}F]-BN3

The binding affinity of [^{18}F]-BN3 for GRP receptors was tested using PC3 cells, a cell line of human prostate cancer. The *in vitro* binding was determined by performing a competitive receptor binding assay using increasing concentrations of unmodified bombesin as a cold blocker. For the purpose of comparison, the assay was also performed using the known GRP receptor specific radioligand [^{125}I]-[tyr4]-BN. The 50% inhibitory concentration (IC_{50}) of [^{18}F]-BN3 was determined to be 44.6 nM. Using the same protocol, the IC_{50} of [^{125}I]-[Tyr4]-BN was determined to be 4.6 nM. Published IC_{50} values of tracers tested on PC3 cells and deemed to have high binding affinities range from <1 nM to 90 nM.[2] We can conclude that [^{18}F]-BN3 retained its ability to target GRPRs even with modification and subsequent labeling.

The tracer stability was tested by incubating [^{18}F]-BN3 in human serum. After 90 min, > 90% of the tracer remained (stability was monitored by radio-TLC). Thus [^{18}F]-BN3 proved to have appropriate *in vitro* binding affinity to GRPRs and demonstrated suitable stability to proceed with *in vivo* studies which will determine whether the binding and stability are retained in a living system. Furthermore, the *in vivo* studies will allow for the pharmacokinetics of the compound to be determined.

5.3.2 Synthesis of a scaffold for multi-modal imaging

The first step towards the synthesis of a scaffold that would provide both a fluorophore for optical imaging and a 'click' handle for radiolabelling was the choice of an appropriate fluorophore. There exists a wide range of available fluorophores with different absorption and emission wavelengths and various functional groups.[17]

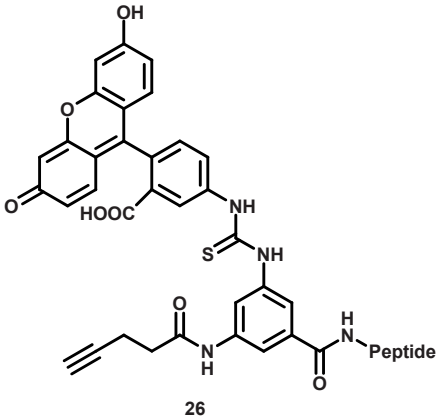


Scheme 10 Schematic design of a scaffold for multimodal imaging

Fluorescein and its functionalized derivatives are a class of dyes extensively used as bio-labeling agents and fluorescent probes due to their strong absorption in the visible range, as well as their high fluorescence quantum yield and stability against photobleaching.[18, 19]

We used fluorescein isothiocyanate (FITC, **27**, Scheme 12) for the synthesis of the scaffold and the optimization of the labeling conditions. FITC is inexpensive compared to many other dyes. Isothiocyanates are convenient functional groups as they react readily with amines without the need for activation.[19]

Our initial attempt to build a multi-functional scaffold was based upon a central aromatic ring with three functional groups for modification with: (1) FITC, (2) an alkyne handle and (3) a biological target (Scheme 11).



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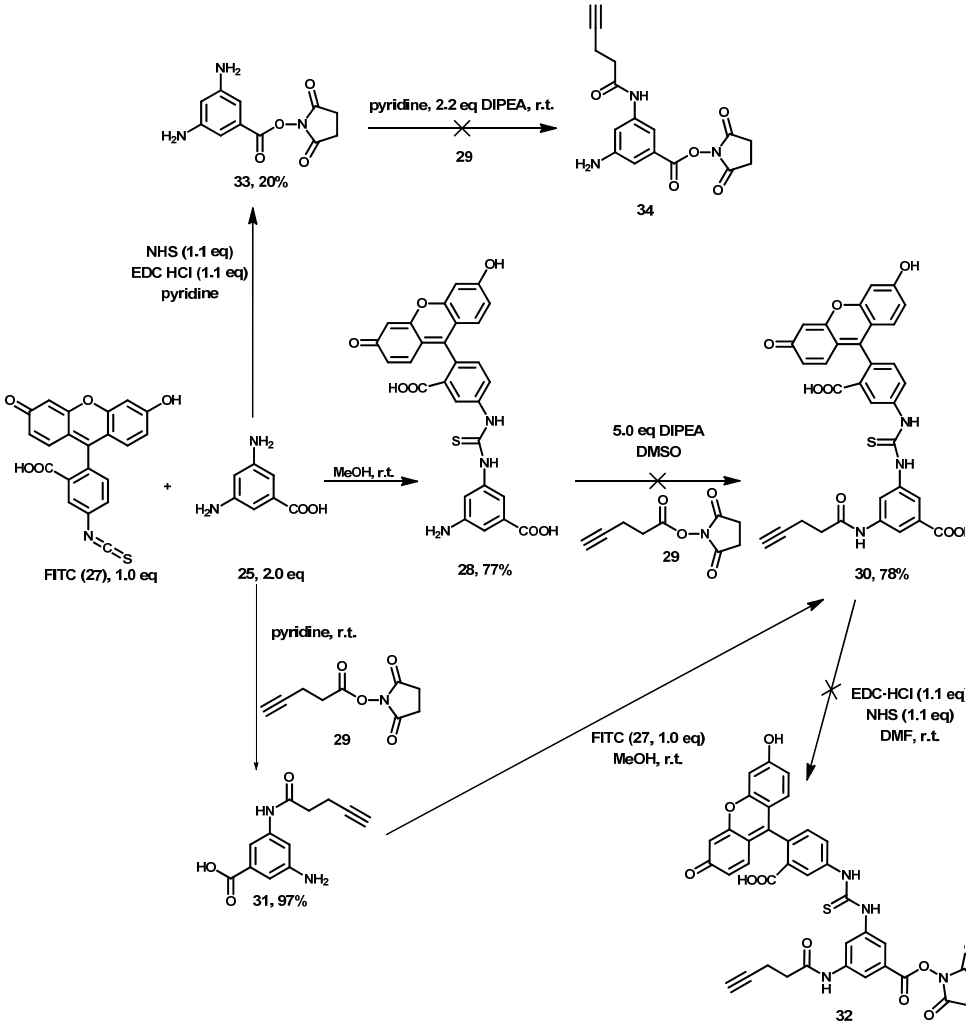
Scheme 11 Design of Scaffold **26**

We started the synthesis from 3,5-diaminobenzoic acid (**25**) (Scheme 12). The molecule contains two amino groups and one carboxylic acid. It was envisioned that one amino group could react with FITC to introduce the fluorophore. The second amino group was to be used to introduce an alkyne, which could serve as a handle for radiolabelling by CuAAC. The carboxylic acid would function as a handle to introduce the target biomolecule. In this case that target is bombesin, but ideally any biomolecule with a free amino group would be amenable to functionalization with this scaffold.

Reacting 2.0 eq of 3,5-diaminobenzoic acid (**25**) with FITC (**27**) in MeOH at room temperature afforded thiourea **28** in 77% yield (Scheme 12). The next step was to introduce an alkyne functionality at the second amino group by reacting **28** with succinimidyl ester **29**. Unfortunately, **30** was not formed, and **28** was fully recovered.

We reasoned that the aniline functional group was perhaps not sufficiently nucleophilic to form amide **30**, given the presence of the electron withdrawing thiourea group in the *meta* position (Hammett constant for $NHC(S)NH_2$ σ_m +0.22).[20] When 3,5-diaminobenzoic acid (**25**) was reacted with activated ester **29**, amide **31** was formed almost quantitatively (Scheme 11). This suggests that indeed, the presence of the electron withdrawing FITC substitution was hindering the introduction of the alkyne. While we suggest that it is an electronic effect, steric effects cannot be excluded from playing a role in this case.

Reacting **31** with FITC (**27**) gave the desired bisfunctionalized scaffold **30**. However, an attempt to form ester **32** failed to give any product and the starting material (**30**) was fully recovered (Scheme 12). Scaffold **30** contains two electron withdrawing substituents, the thiourea (Hammett constant for $NHC(S)NH_2$ σ_m +0.22) and the amide (Hammett constant for $NHC(O)CH_3$ σ_m +0.14), which could explain the failure for **30** to be transformed into **32**. Notably, when 3,5-diaminobenzoic acid **25** was reacted with EDC and NHS, it could be transformed into ester **33** (Scheme 12).

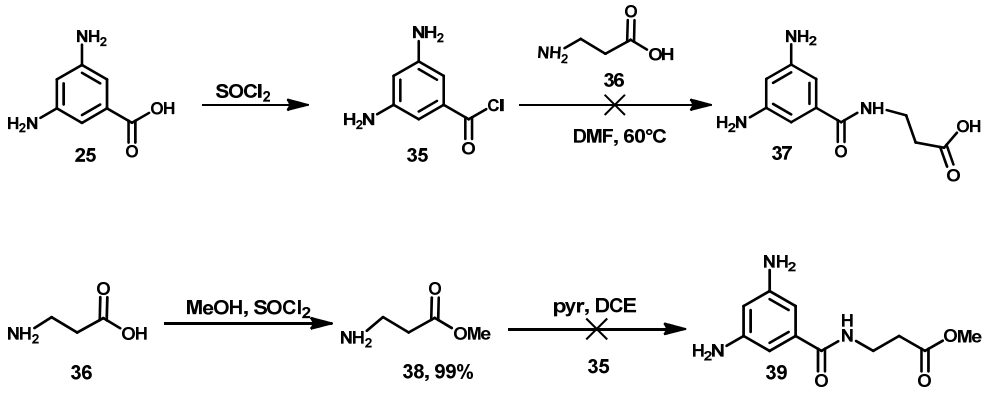


Scheme 12 Attempted synthesis of **32**

However, attempts to transform **33** into the bis-functionalized **34** through reaction with activated ester **29** failed to give any product. The presence of an electron withdrawing ester functionality on the aromatic ring appears to hinder the nuceophilic attack of the aniline functional group.

These findings suggest that introducing three substituents directly on the aromatic ring is challenging due to their individual effects on the electronic properties of the scaffold.

We reasoned that introducing a linker between the carboxylic acid and the aromatic ring might allow for more facile esterification of the carboxylic acid. 3,5-Diaminobenzoic acid **25** was transformed into acid chloride **35** by treatment with thionyl chloride (Scheme 13). 3-Aminopropanoic acid (**36**) was stirred with the acid chloride in DMF at 60°C but this yielded none of the desired product **37**. We noted that 3-aminopropanoic acid (**36**) had limited solubility in DMF. Methyl-3-aminopropanoate **38** was synthesized to improve the solubility of the linker, and added to the reaction mixture containing acid chloride **35** along with pyridine in dichloroethane (Scheme 13). This also failed to produce the desired amide **39**.

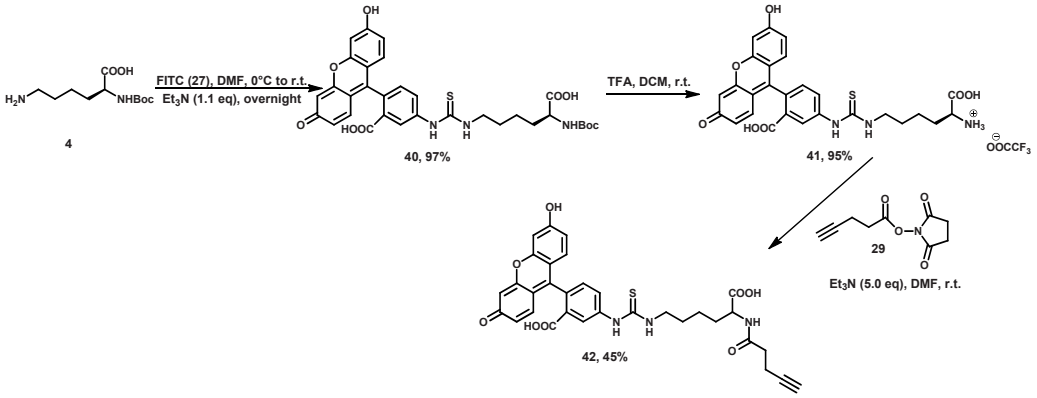


Scheme 13 Attempted syntheses of scaffolds with linker

Given the difficulties that were encountered synthesizing a scaffold based upon a central benzene ring, a new design that excluded any aromatic ring was pursued.

The second design of the scaffold was based on the use of lysine as a trifunctional core (Scheme 14).[21] The concept involves the introduction of FITC at the ε-amino group of lysine, of an alkyne handle at the α-amino group and of the biomolecule at the carboxylic acid.

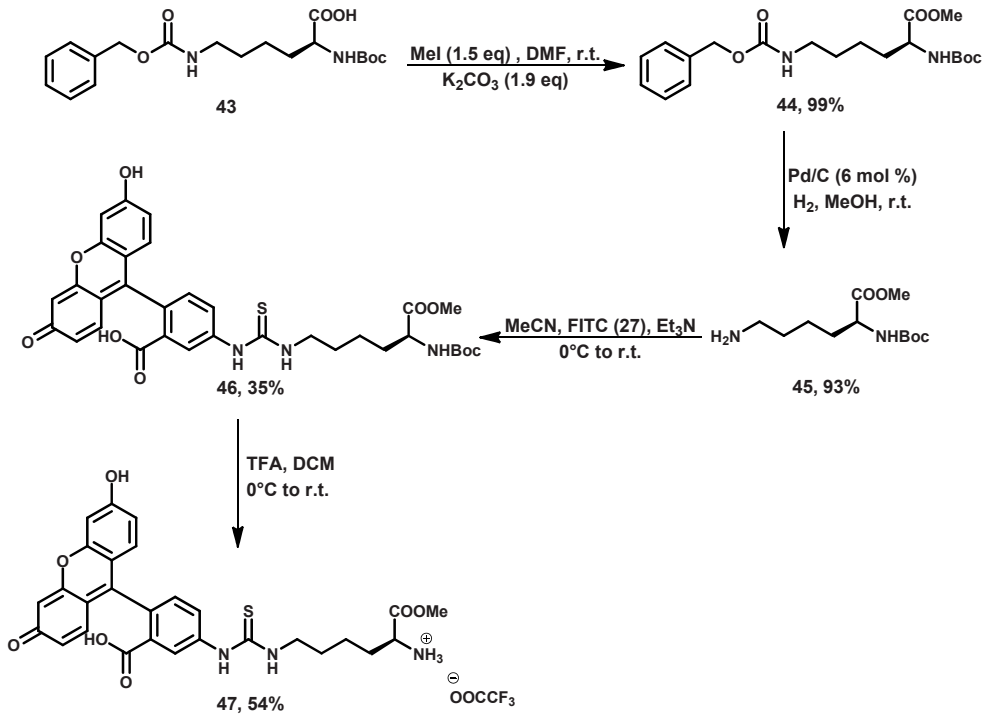
In the presence of Et_3N, the reaction of Boc-lys-OH (**4**) with FITC (**27**) gave **40**. The Boc protecting group was removed by treating **40** with trifluoroacetic acid (TFA) in DCM to afford **41** as a salt. By treating **41** with an excess of Et_3N in the presence of activated ester **29**, **42** could be synthesized.



Scheme 14 Attempted synthesis of lysine based scaffold **43**

In the synthesis depicted in Scheme 14, significant effort was devoted to the optimization of the purification at each step to improve the yields. Purification of such polar molecules can often be challenging. A synthetic route using Boc-lys-OMe (**45**) was designed to improve the solubility and facilitate purification of the molecule at each step (Scheme 15).

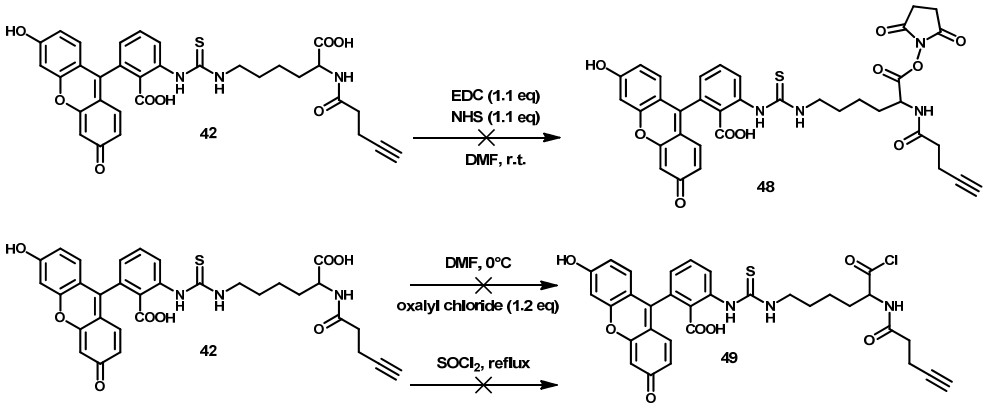
Treatment of Boc-lys-Cbz-OH (**43**) with MeI afforded ester **44**. Hydrogenation over Pd/C removed the carboxybenzyl protecting group to give **45**, which was immediately reacted with FITC (**27**) to give **46**.



Scheme 15 Synthesis of the scaffold from methyl ester protected **44**

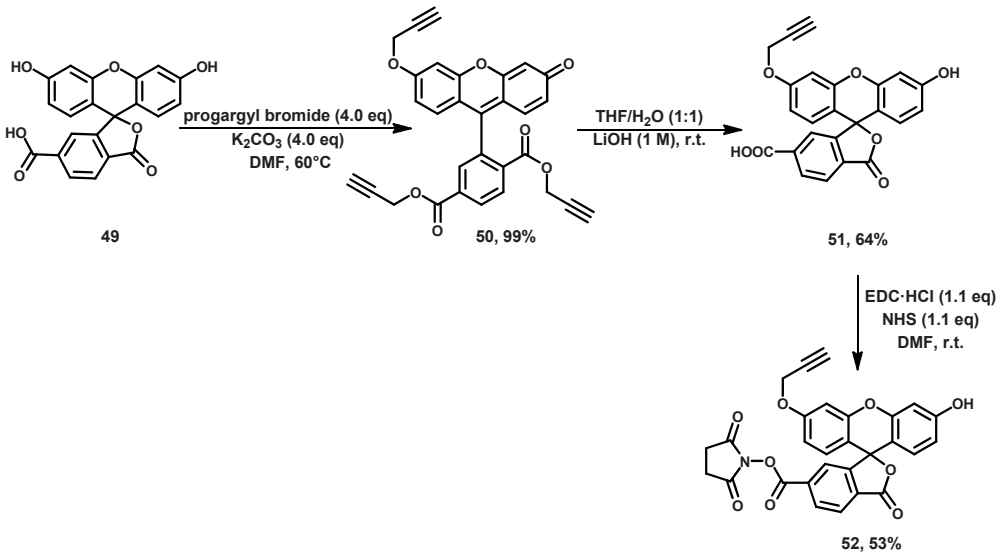
Storing the compound at room temperature led to decomposition of **45** within hours, likely due to side reaction of the free ε-amino group with the methyl ester. The Boc protecting group of **46** was removed by treatment with TFA to afford **47** as a salt. Unfortunately, this route proved to be longer and lower yielding than the original one, and as such, we proceeded with the synthesis as described in Scheme 14 in an attempt to activate the carboxylic acid group of **42** for further functionalization.

Treatment of **42** with EDC·HCl and NHS (**2**) did not give any of product **48** and the starting material was recovered (Scheme 16). We also attempted to form an acid chloride from **42**. Unfortunately neither treatment with oxalyl chloride nor with thionyl chloride afforded the desired product **49** (Scheme 16).



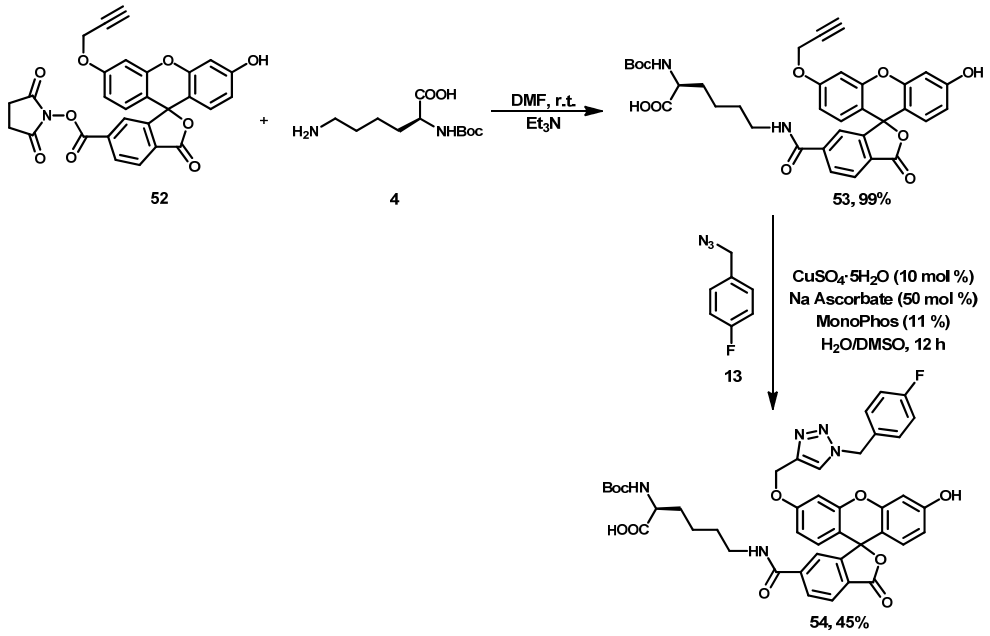
Scheme 16 Attempted synthesis of activatd ester **48** and acid chloride **49**

A third design of the scaffold was synthesized starting from commercially available 6-carboxyfluorescein (**49**) (Scheme 17). The design of this third scaffold differed in that the fluorophore itself serves as the scaffold. By treating **49** with potassium carbonate and reacting it with propargyl bromide trialkyne **50** could be prepared. The ester groups could be cleaved by exposing **50** to LiOH (1 M), giving monopropargyl ether **51**. The succinimidyl ester **52** could be synthesized by reaction with EDC·HCl and NHS (**2**).



Scheme 17 Synthesis of scaffold **52**

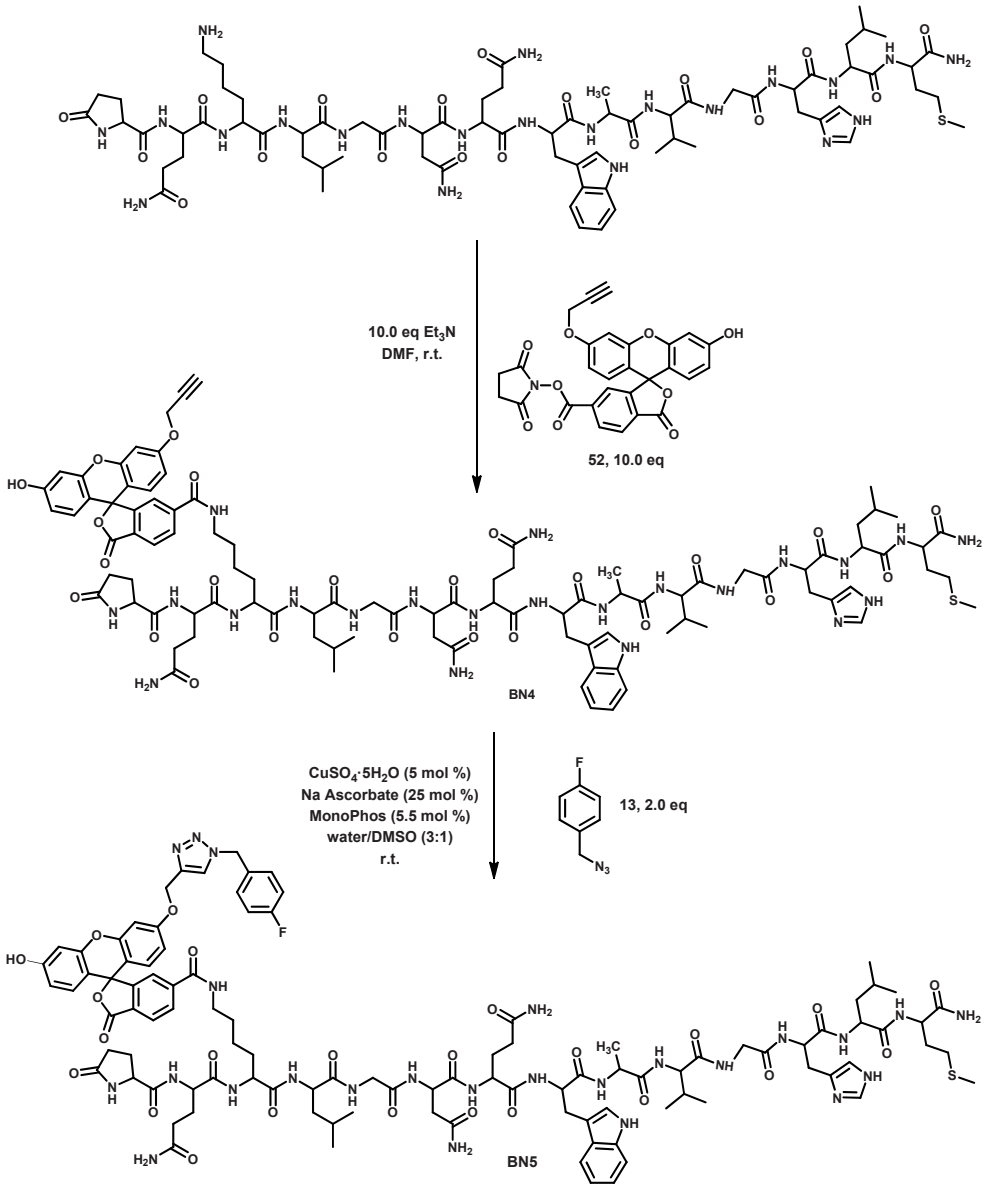
Model compound **53** was synthesized by reaction of **52** with Boc-lys-OH (**4**) giving an excellent yield (Scheme 18). The subsequent CuAAC yielded triazole **54**. This confirmed that both the amide bond formation as well as the subsequent 'click' reaction should proceed smoothly with no intrinsic problems of reactivity in the design.



Scheme 18 Synthesis and CuAAC of model compound **53**

To produce the scaffold functionalized bombesin analogue, chemical ligation techniques were used rather than SPPS. Thus [lys3]-bombesin was reacted with scaffold **52** in the presence of Et_3N to afford the scaffold modified peptide **BN4**, which was purified by RP-HPLC (Scheme 19). **BN4** was reacted with 4-fluorobenzyl azide **13** to afford triazole **BN5**. The reaction was monitored by RP-HPLC. With 5 mol % of the copper catalyst, the reaction had reached 65% conversion after 20 min. Although the reaction had not reached completion, the conversion is sufficient for radiolabelling purposes. **BN4** was purified by RP-HPLC and characterized by mass spectrometry. Comparing the fluorescent properties of **53** and **54** showed that there was no significant decrease of fluorescence.

In conclusion, it was possible to modify bombesin with a scaffold that provides a fluorophore for optical imaging as well as a handle for 'clicking' to a [^{18}F] radionuclide providing a multi-modal imaging tracer.



Scheme 19 Synthesis of **BN4** and **BN5**

5.4 Conclusions and Outlook

In this chapter, it has been demonstrated that it is possible to use the CuAAC to label bombesin with [^{18}F] in a rapid and high yielding fashion using an alkyne modified bombesin and an azido prosthetic group. This approach fulfills many of the requirements that are considered ideal characteristics for a prosthetic group (Chapter 1, Section 1.10). The [^{18}F] is introduced in the last step of the synthesis, the labeling proceeds at room temperature in aqueous solution and can reach full conversion in 15 min. Most importantly, the system is modular in that a wide range of azides can be readily synthesized, altering the properties of the resulting peptide. In this chapter, we demonstrate that labeling with [^{18}F]-fluoroazidobutane results in the formation of a tracer **[^{18}F]-BN3** which retains its high affinity for GRP receptors *in vitro*. The tracer is being pursued for *in vivo* studies.

In the second part of this chapter, three approaches were taken toward building a scaffold incorporating a fluorophore, a 'click' handle for labeling, and a functional group for attachment of bombesin (or an alternative target). While the first two routes proved unsuccessful, the third design of the scaffold, which differs by the use of the fluorophore itself as the core, was synthesized. It can be attached to [lys3]-bombesin at the ε-amino group of lysine and 'clicked' to fluorobenzyl azide **13**.

Further steps in this project would involve exploration of the optimal reaction parameters for the [^{18}F] labeling of **BN4**, as well as determination of the *in vitro* binding affinity to the GRPR after modification and labeling has been performed. Studies on the fluorescence of the resulting peptide would need to be performed, and eventually the system needs to be explored *in vivo* if all of the previous steps yield the desired results. Fluorescent derivative **52** is easily synthesized in high yields over three straightforward steps, and has much potential as a scaffold for copper-catalyzed [^{18}F]-labeling of biomolecules. The activated ester handle allows it to be attached to a wide variety of biologically relevant target molecules, and provides a chromophore for optical imaging as well as a handle for labeling. While the scaffold could be redesigned with relative ease to make use of copper-free 'click' methodology at the ether linkage (see Chapter 6), in some ways it is limited. Ideally, a scaffold is desired that would allow for simple modification to allow access to multi-modal imaging agents not only for the combination of optical imaging/PET, but also for optical imaging/SPECT, PET/CT or PET/MRI. Because the design of this molecule is such that the scaffold is also the fluorophore, there is no possibility to alter the imaging techniques (PET could be substituted for another technology, but optical imaging is fixed by the fluorophore scaffold). It would be interesting not only to have access to a scaffold such as **52**, but to an even more 'generic' scaffold to give access to a wide variety of multi-modal imaging probes.

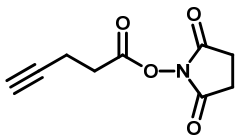
Multi-modality imaging is becoming the focus of much attention in the international research community. While in this chapter we have concentrated on building a tracer for a combination of PET with optical imaging, recently, increasing attention is being lent to the combined technique of PET/MRI. PET and MRI are very much complementary techniques. While MRI provides excellent resolution on the anatomical scale to a sub-millimeter degree of precision, it has poor sensitivity. PET on the other hand is extremely sensitive and lacking resolution.[9] For the combination of PET/MRI, it may be difficult to apply the same 'small molecule' tracer approach that we have taken in this chapter. It is difficult with such an approach to achieve anything significantly greater than a 1:1 ratio of the signal source in one molecule. This limits this approach to the combination of modalities that have similar sensitivities, which is not the case with PET and MRI.[10] Because MRI is much less sensitive than PET is, an individual probe would require a large excess of the MRI source with respect to the PET source in the same molecule, which can be difficult to achieve. Approaches involving macromolecular or nanoparticular conjugates may prove to have more potential in this discipline.[10] PET/MRI has lagged behind similar combinations of technologies such as PET/CT due to the complexity of integrating the two techniques. The magnetic field of MRI can interfere with the sensitive PET detectors, and the PET detector can in turn influence the homogeneity of the magnetic field. Recently these technological hurdles have been overcome, and the first instruments have been tested in research labs around the world.[22] The coming years will be telling in the application of PET/MRI in research and how well it will translate to daily clinical imaging. Regardless, the design and synthesis of multi-modality tracers will be an ongoing endeavor, with a plethora of possibilities for application. It is a field that could benefit enormously from collaborations across disciplines, to avoid an accumulation of designs for different tracers which never get tested for applications.

5.5 Experimental Section

General remarks:

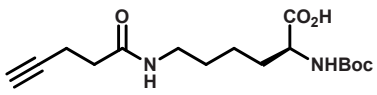
Boc-Lys(Z)-OMe and N-α-Boc-N-ε-propargyloxycarbonyl-(*S*)-lysine methyl ester were prepared according to literature procedure.[23] Reversed phase-high performance liquid chromatography (RP-HPLC) analyses were performed on a Shimadzu LC-10AD VP, Waters Xterra MS C18 column (3.0 x 150 mm, particle size 3.5 µm) using a gradient of H_2O/MeCN (0.1% formic acid); gradient A: from 95:5 at 10 min to 60:40 at 30 min, to 55:45 at 40 min, to 50:50 at 50 min, to 25:75 at 55 min to 44:45 at 60 min, to 95:5 at 65 min until 75 min. Flow 3.0 mL/min. Gradient B: from 95:5 at 0 min to 85:15 at 10 min, to 75:25 at 12 min, to 20:80 at 65 min, to 95:5 at 70 min. Flow 0.5 mL/min. All *N*-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids and reagents used for SPPS were purchased from Novabiochem with the exception of the alkyne modified lysine residue in position 3 (synthesis described below). Solid phase peptide synthesis was performed on an automatic peptide synthesizer (CEM).

_N_-Succinimidyl-4-pentynoate (7)



4-Pentynoic acid (500 mg, 5.10 mmol) was dissolved in dry DCM (20 mL). To this solution was added *N*-hydroxysuccinimide (646 mg, 5.61 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) (1.08 g, 5.61 mmol). The reaction mixture was stirred overnight at room temperature. The reaction mixture was washed with 5% aqueous citric acid (10 mL), $NaHCO_3$ (10 mL) and brine (2 x 10 mL). The organic layer was dried over $MgSO_4$ and the solvent was removed yielding the pure product as a white solid. Yield=84%. Spectroscopic data is in accordance with the literature.[24] ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.89-2.93 (m, 2H), 2.83 (s, 4H), 2.58-2.62 (m, 2H), 2.04 (t, *J*=8.0 Hz, 1H); ^{13}C NMR(100.59 MHz, $CDCl_3$): δ 169.0, 166.9, 80.8, 69.8, 29.9, 25.3, 13.7.

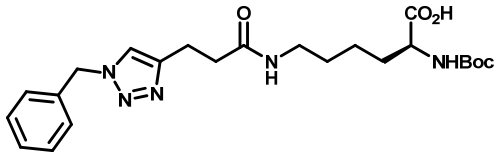
***(S)*-2-(*tert*-Butoxycarbonylamino)-6-pent-4-ynamidohexanoic acid (8)**



Boc-lys-OH (311 mg, 1.27 mmol) was dissolved in 30.0 mL of dry DCM. To this stirred solution was added *N*-succinimidyl-4-pentynoate (**7**) (297 mg, 1.52 mmol). The reaction mixture was cooled in an ice bath and diisopropylethylamine (DIPEA) (193 mg, 0.25 mL) was added in a dropwise fashion. After addition, the reaction mixture was allowed to warm up to room temperature and was left stirring vigorously overnight. The solvent was removed by evaporation, and the crude reaction mixture was purified by column chromatography (20:1 DCM:MeOH) to

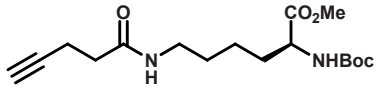
give the product as a white solid. R_f=0.70 (100% MeOH). Yield=80%. Spectroscopic data is in accordance with the literature.[25] ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.71 (br s, 1H), 6.77 (br s, 1H), 4.16-4.19 (m, 1H), 3.20-3.24 (m, 2H), 2.38-2.44-2.47 (m, 4H), 2.00-2.04 (m, 1H), 1.38-1.78 (m, 15H); ^{13}C NMR (100.59 MHz, $CDCl_3$): δ 175.5, 173.2, 156.1, 82.8, 80.3, 69.8, 53.3, 39.5, 35.1, 31.0, 28.6, 25.5, 22.5, 15.1.

_(R)_-6-(3-(1-Benzyl-1H-1,2,3-triazol-4-yl)propanamido)-2-((_tert_-butoxycarbonyl)amino)hexanoic acid (10)



(S)-2-(*Tert*-butoxycarbonylamino)-6-pent-4-ynamidohexanoic acid **8** (100 mg, 0.31 mmol) and benzyl azide (2.06 mg, 0.155 mmol) were dissolved in 5 mL of an H_2O/DMSO (3:1 v/v) mixture. In a separate vial, $CuSO_4 \cdot 5H_2O$ (0.39 mg, 1.56 x 10^{-3} mmol) was dissolved in 0.2 mL of water, and to this vial sodium ascorbate (1.54 mg, 7.75 x 10^{-3} mmol) was added. MonoPhos (0.61 mg, 1.71 x 10^{-3} mmol) was added along with 0.1 mL of DMSO. The reagents were stirred together for 10 min and added to the solution of azide and alkyne. The reaction mixture was allowed to stir at room temperature, and the progress was monitored by thin layer chromatography (100% MeOH). Upon completion of the reaction, the mixture was diluted with water (10 mL) and extracted with EtOAc (3 x 10 mL). The organic layers were combined, dried over $MgSO_4$ and purified by column chromatography (10:1 DCM:MeOH) to give the product as a white solid. R_f=0.10 (100% MeOH). Yield=55%. ^{1}H NMR (400 MHz, CD_3OD): δ 7.68 (s, 1H), 7.28-7.37 (m, 5H), 5.54 (s, 2H), 4.03-4.06 (m, 1H), 3.09 (t, *J*=8.0 Hz, 2H), 3.00 (t, *J*=8.0 Hz, 2H), 2.48-2.52 (m, 2H), 1.28-1.78 (m, 15H); ^{13}C NMR (100.59 MHz, CD_3OD): δ 175.0, 174.6, 158.3, 148.0, 137.1, 130.2, 129.7, 129.3, 123.7, 80.6, 70.6, 55.1, 36.6, 36.3, 32.6, 30.1, 28.9, 26.5, 24.4. HRMS (EI) calcd for $C_{23}H_{34}O_5N_5$ [M+H$^+$] 460.2555, found 460.2550.

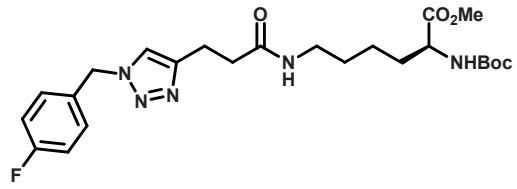
Methyl-(_S_)-2-((_tert_-butoxycarbonyl)amino)-6-(pent-4-ynamido)hexanoate (12)



$TMSCHN_2$ (0.08 mL, 0.153 mmol) was dissolved in 4 mL of a toluene/methanol mixture (5:1 v/v) under an inert atmosphere of N_2. To this solution was added *(S)*-2-(*tert*-butoxycarbonylamino)-6-pent-4-ynamidohexanoic acid **8** (50.0 mg, 0.153 mmol). The reaction mixture was stirred at room temperature for 1.5 h after which it was diluted with diethyl ether (5 mL) and then with 10% AcOH (5 mL). The organic layers were collected, and the aqueous layer was extracted with diethyl ether (2 x 10 mL) and combined with the other organic layers. These were dried over $MgSO_4$ and the solvent was evaporated. The reaction product must be purified immediately, storing the crude reaction mixture leads to degradation of the product. The crude mixture was purified by column

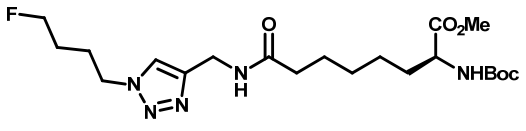
chromatography (50:1 DCM:MeOH) to yield the product as a clear oil. R_f=0.40 (50:1 DCM:MeOH). Yield=99%. After purification, the product appears to be stable and can be stored. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.53 (t, *J*=8.0 Hz, 1H), 5.28 (d, *J*=8.0 Hz, 1H), 4.08-4.09 (m, 1H), 3.59 (s, 3H), 3.11 (q, *J*=8.0 Hz, 2H), 2.35-2.38 (m, 2H), 2.27 (t, *J*=4.0 Hz, 2H), 2.03-2.04 (m, 1H), 1.24-1.67 (m, 15H); ^{13}C NMR (100.59 MHz, $CDCl_3$): δ 173.2, 171.0, 155.5, 83.0, 79.9, 69.3, 53.1, 52.3, 39.0, 35.2, 32.3, 28.8, 28.3, 22.5, 14.9. HRMS (EI) calcd for $C_{17}H_{28}O_5N_2Na$ [M+Na$^+$] 363.1890, found 363.1883.

Methyl-(*S*)-2-((*tert*-butoxycarbonyl)amino)-6-(3-(1-(4-fluorobenzyl)-1H-1,2,3-triazol-4-yl)propanamido)hexanoate (15)



(S)-methyl-2-((*tert*-butoxycarbonyl) amino)-6-(pent-4-ynamido)hexanoate **12** (20.0 mg, 0.054 mmol) and 4-fluorobenzyl azide (8.10 mg, 0.054 mmol) were dissolved in 3 mL of an H_2O/DMSO (3:1 v/v) mixture. In a separate vial, $CuSO_4 \cdot 5H_2O$ (0.13 mg, 5.40 x 10^{-4} mmol) was dissolved in 0.1 mL of water, and to this vial was added sodium ascorbate (0.53 mg, 2.67 x 10^{-3} mmol). MonoPhos (0.61 mg, 5.83 x 10^{-4} mmol) was added along with 0.1 mL of DMSO. The reagents were stirred together for 10 min and added to the solution of azide and alkyne. The reaction mixture was allowed to stir at room temperature, and the progress was monitored by thin layer chromatography (100% DCM). Upon completion of the reaction, the mixture was diluted with water (10 mL) and extracted with DCM. The organic layers were combined, dried over $MgSO_4$ and purified by column chromatography (1:1 DCM:MeOH) to give the product as a white solid. R_f=0.20 (100% MeOH). Yield=66%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.31 (s, 1H), 7.23-7.26 (m, 2H), 7.04 (t, *J*=8.0 Hz, 2H), 5.94 (br s, 1H), 5.45 (s, 2H), 5.22 (br d, *J*=8.0 Hz, 1H), 4.21-4.27 (m, 1H), 3.72 (s, 3H), 3.14-3.17 (m, 2H), 3.01 (t, *J*=8.0 Hz, 2H), 2.50-2.54 (m, 2H), 1.32-1.83 (m, 15H); ^{13}C NMR (100.59 MHz, $CDCl_3$): δ 173.2, 172.0, 164.4, 161.1, 147.1, 130.7, 129.8 (d, *J*=11.3 Hz), 121.4, 116.0 (d, *J*=29.4 Hz), 79.9, 53.2, 53.1, 52.2, 38.8, 35.6, 32.1, 29.7, 28.3, 22.7, 21.4; ^{19}F NMR (200 MHz, $CDCl_3$): -113.4 (m). HRMS (EI) calcd for $C_{24}H_{35}O_5N_4F$ [M+H$^+$] 478.2586, found 478.2554.

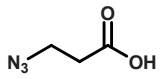
***(S)*-Methyl 2-((*tert*-butoxycarbonyl)amino)-8-(((1-(4-fluorobutyl)-1H-1,2,3-triazol-4-yl)methyl)amino)-8-oxooctanoate (16)**



(S)-methyl-2-((*tert*-butoxycarbonyl)amino)-6-(pent-4-ynamido)hexanoate **12** (67.5 mg, 0.20 mmol) and fluoroazidobutane **14** (28.0 mg, 0.24 mmol) were dissolved in 10

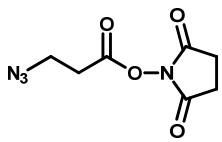
mL of an H_2O/DMSO (3:1) mixture. In a separate vial, $CuSO_4 \cdot 5H_2O$ (0.50 mg, 2.08 x 10^{-3} mmol) was dissolved in 0.1 mL of water, and sodium ascorbate (1.97 mg, 9.95 x 10^{-3} mmol) was added to this solution. MonoPhos (0.79 mg, 2.20 x 10^{-3} mmol) was added along with 0.1 mL of DMSO. The reagents were stirred together for 10 min and added to the solution of azide and alkyne. The reaction mixture was allowed to stir at room temperature, and the progress was monitored by ^{1}H NMR. Upon completion of the reaction, the mixture was diluted with water (10 mL) and extracted with DCM. The organic layers were combined and dried over $MgSO_4$ and purified by column chromatography (1:1 DCM:MeOH) to give the product as a white solid. R_f=0.20 (100% MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39 (br s, 1H), 6.14 (br s, 1H), 5.23 (br d, *J*=8.0 Hz, 1H), 4.50 (t, *J*=5.6 Hz, 1H), 4.34-4.37 (m, 3H), 4.21-4.22 (m, 1H), 3.70 (s, 3H), 3.14-3.18 (m, 2H), 3.01 (t, *J*=8.0 Hz, 2H), 1.97-2.04 (m, 2H), 1.23-1.72 (m, 17H); ^{13}C NMR (100.59 MHz, $CDCl_3$): δ 173.2, 172.1, 157.0, 123.9, 118.4, 84.2, 79.8, 53.2, 52.1, 49.6, 40.9, 38.8, 35.6, 31.9, 29.6, 28.9, 28.2, 26.4, 22.3; ^{19}F NMR (200 MHz, $CDCl_3$): 46.1-46.5 (m). HRMS (EI) calcd for $C_{21}H_{37}O_5N_5F$ [M+H$^+$] 458.2773, found 458.2768.

3-Azidopropanoic acid (18)



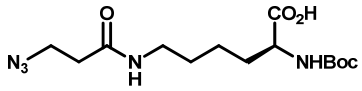
To a stirred solution of 3-bromopropionic acid (1.5 g, 9.8 mmol) in MeCN (4.0 mL), was added NaN_3 (956 mg, 14.7 mmol). The reaction mixture was reluxed for 3 h. After the reaction mixture cooled down, the crude brown mixture was diluted with DCM (5 mL) and washed with 0.1 N aqueous HCl (10 mL). The organic layer was dried over $MgSO_4$ and the solvent was removed to give the pure product. Yield=56%. Spectroscopic data was in accordance with literature values.[26] ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.70 (s, 1H), 3.54 (t, *J*=4.0 Hz, 2H), 2.58 (t, *J*=4.0 Hz, 2H); ^{13}C NMR (100.59 MHz, $CDCl_3$): δ 176.9, 46.2, 35.5.

3-Azidopropionic acid succinimidyl ester (19)



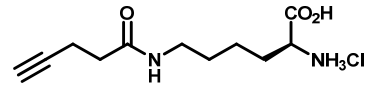
To a stirred solution of 3-azidopropanoic acid **18** (260 mg, 2.25 mmol) and *N*-hydroxysuccinimide (260.0 mg, 2.25 mmol) in 1.0 mL DCM was added EDC·HCl (434 mg, 2.26 mmol). The reaction mixture was stirred at room temperature overnight. The crude reaction mixture was poured into brine and extracted with DCM (3 x 10 mL). The organic layers were combined and dried over $MgSO_4$. The crude mixture was purified by column chromatography (96:4 DCM:EtOAc) to yield the product as a yellow oil. Yield=80%. Spectroscopic data was in accordance with the literature.[3] ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.60 (t, *J*=8.0 Hz, 2H), 2.82 (t, *J*=8.0 Hz, 2H), 2.76 (s, 4H); ^{13}C NMR (100.59 MHz, $CDCl_3$): δ 168.9, 166.3, 45.8, 30.8, 25.3.

(*S*)-6-(3-Azidopropanamido)-2-((*tert*-butoxycarbonyl)amino)hexanoic acid (20)



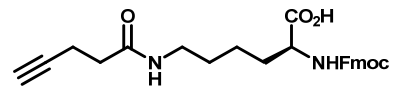
To a solution of Boc-lys-OH protected lysine (259 mg, 1.05 mmol) in DCM (5.0 mL) was added 3-azidopropionic acid succinimidyl ester (245 mg, 1.15 mmol) in DCM (2.0 mL). The solution was cooled down in an ice bath, and diisopropylethylamine (0.21 mL, 1.26 mmol) was added dropwise. After addition, the solution was warmed up to room temperature and stirred overnight. The solution was washed with 0.1 N aqueous HCl (5 mL) and the organic layer was dried over $MgSO_4$ and the solvent evaporated. The crude reaction mixture was purified by column chromatography (gradient 100:1 to 10:1 DCM:MeOH) to yield the product as a white solid. R_f=0.20 (50:1 DCM:MeOH). Yield=87%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.11 (br s, 1H), 6.69 (br s, 1H), 5.47 (br s, 1H), 4.19 (s, 1H), 3.58 (t, *J*=6.8 Hz, 2H), 3.23-3.25 (m, 2H), 2.44 (t, *J*=6.8 Hz, 2H), 1.41-1.80 (m, 15H); ^{13}C NMR (100.59 MHz, $CDCl_3$): δ 176.1, 170.9, 155.9, 70.0, 53.4, 47.4, 39.2, 35.6, 31.9, 28.3, 22.4. HRMS (EI) calcd for $C_{14}H_{26}O_5N_5$ [M+H$^+$] 344.1928, found 344.1914.

***(S)*-1-Carboxy-5-(pent-4-ynamido)pentan-1-amine hydrochloride (21)**



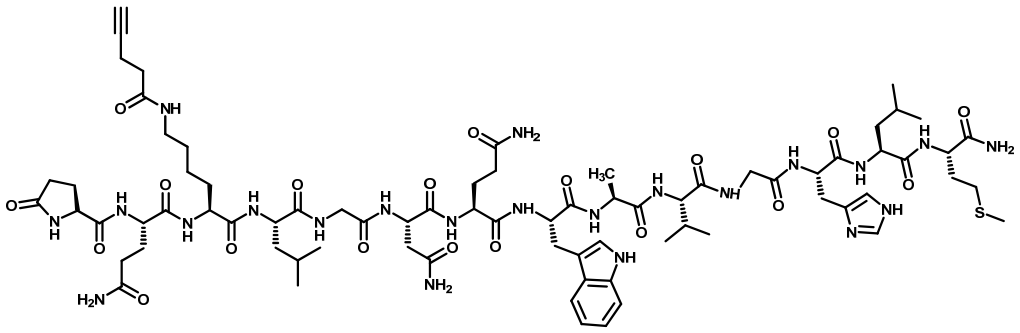
(S)-2-(*Tert*-butoxycarbonylamino)-6-pent-4-ynamidohexanoic acid **8** (50.0 mg, 0.15 mmol) was dissolved in a 3M HCl/EtOAC solution (8.5 mL). The reaction mixture was stirred at room temperature for 30 min, after which the solvent was evaporated and the residue was triturated with hot diethyl ether (3 x 10 mL). The product was recrystallized from methanol to yield a white solid. Yield=80%. Spectroscopic data is in accordance with the literature.[2] ^{1}H NMR (400 MHz, CD_3OD): δ 3.86-3.89 (m, 1H), 3.17-3.22 (m, 2H), 2.42-2.46 (m, 2H), 2.34-2.37 (m, 2H), 2.24-2.26 (m, 1H), 1.84-1.94 (m, 2H), 1.44-1.57 (m, 2H), 1.29 (t, *J*=8.0 Hz, 2H); ^{13}C NMR (100.59 MHz, CD_3OD): δ 172.9, 170.9, 82.4, 69.2, 52.9, 38.7, 34.9, 30.1, 28.8, 22.2, 14.6. HRMS (EI) calcd for $C_{11}H_{19}O_3N_2$ [M+H$^+$] 227.1400, found 227.1388.

***(S)*-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-(pent-4-ynamido)hexanoic acid (22)**

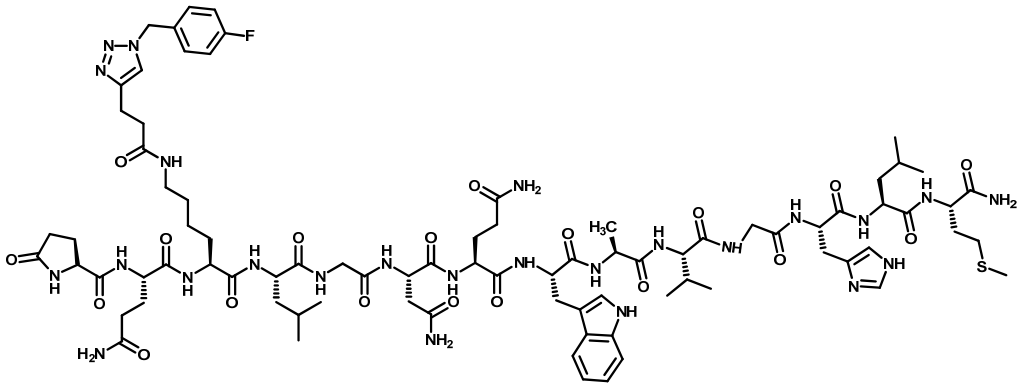


21 (100 mg, 0.38 mmol) was dissolved in a solution of water/acetone (1:1 v/v, 2.0 mL) along with sodium bicarbonate (64.0 mg, 0.76 mmol). To this stirred solution was added fluroenylmethoxycarbonyl-*N*-hydroxy-succinimide (128 mg, 0.38 mmol). The reaction was left stirring at room temperature overnight. Concentrated HCl was added to the solution to adjust the pH to 2. The solvent was then removed and the crude solid was taken up with chloroform. The organic layer was washed with 0.1 N aqueous HCl (2 x 10 mL) and with water (1 x 10 mL) to yield the pure compound as a

white solid. Yield=87%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.00 (br s, 1H), 7.77 (d, *J*=8.0 Hz, 2H), 7.62-7.68 (m, 2H), 7.37 (t, *J*=7.6 Hz, 2H), 7.30 (t, *J*=7.2 Hz, 2H), 4.35-4.38 (m, 2H), 4.18 (t, *J*=6.8 Hz, 1H), 4.12-4.21 (m, 1H), 3.16-3.19 (m, 2H), 2.43-2.47 (m, 2H), 2.33-2.37 (m, 2H), 2.25-2.27 (m, 1H), 1.82-1.86 (m, 1H), 1.68-1.72 (m, 4H), 1.40-1.51 (m, 2H); ^{13}C NMR (100.59 MHz, CD_3OD): δ 172.9, 172.8, 157.5, 144.2, 144.0, 127.6, 127.0, 125.1, 119.8, 82.4, 69.3, 66.8, 54.3, 39.1, 39.0, 34.9, 31.2, 28.7, 23.1, 14.7. HRMS (EI) calcd for $C_{26}H_{29}O_5N_2$ [M+H$^+$] 449.2071, found 449.2059.



BN1. Peptide synthesis was performed on a 0.1 mmol scale. The Fmoc solid phase method was performed on a Rink amide resin (bead size 100-200 mesh, loading 0.7 mmol/g).[27] After completion of the synthesis, the resin was filtered off, and washed first with DCM (1 x 10 mL) and then diethyl ether (3 x 10 mL). The resin was transferred to a sample vial, and to this vial was added a cleaving solution, a mixture of trifluoroacetic acid (TFA)/triisopropyl silane (TIS)/1,3-ethanedithiol(EDT)/thioanisole/H_2O (85/1/4/5/5). The peptide was cleaved from the resin, and all of the remaining side chain protecting groups were cleaved off by gently stirring the resin in this solution at room temperature for 5 h. The solution containing the peptide was filtered and concentrated. The peptide was precipitated using cold diethyl ether and centrifuged. The supernatant was decanted and the solid was washed with cold diethyl ether (20 mL), centrifuged, and the supernatant decanted. This was repeated a further two times. The solid was allowed to dry and then dissolved in water (with 1% formic acid) and lyophilized. Purification of the peptide was achieved by RP-HPLC (Gradient A). Retention time=32 min. HRMS (EI) calcd for $C_{26}H_{29}O_5N_2$ [M+H$^+$]1671.9194, found 1671.9900.

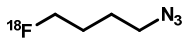


BN2. BN1 (1.00 mg, 5.98 x 10^{-4} mmol) and 4-fluorobenzyl azide (0.14 mg, 9.2 x 10^{-4} mmol) were dissolved in a H_2O/DMSO mixture (3:1, 0.8 mL). In a separate vial, $CuSO_4 \cdot 5H_2O$ (1.5 µg, 5.98 x 10^{-6} mmol) was dissolved in 0.1 mL of water, to this was added sodium ascorbate (5.9 µg, 3.00 x 10^{-5} mmol). MonoPhos (2.51 µg, 6.98 x 10^{-6} mmol) was added along with 0.1 mL of DMSO. The reagents were stirred together for 10 min and added to the solution of azide and peptide. The reaction was monitored by RP-HPLC. Upon completion, the reaction mixture was lyophilized. The crude product was purified by RP-HPLC (Gradient A). HRMS (EI) calcd for $C_{83}H_{121}O_{19}N_{25}FS$ [M+H$^+$] calc 1821.8901, found 1821.8932. Retention time=36.0 min.

Radiochemistry General

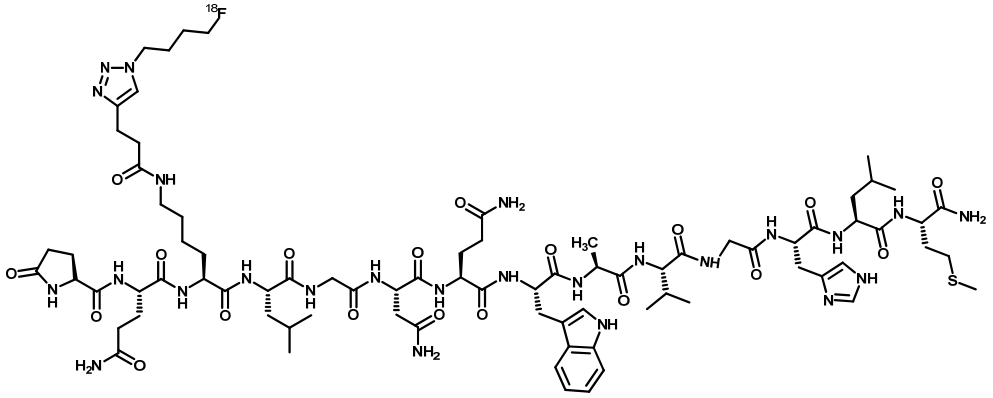
Analytical as well as semipreparative RP-HPLC was performed for monitoring and purification. Isolation of radiolabeled tracers was performed using an RP-C18 column (4.6 mm × 250 mm, 10 μm). The flow was set at 2.5 mL/min using a gradient system starting from 90% solvent A (0.01 M phosphate buffer, pH=6.0) and 10% solvent B (MeCN) (0-2 min) and ramped to 45% solvent A and 55% solvent B at 35 min. The analytic HPLC was performed using the same gradient system but with a reversed-phase Grace Smart RP-C18 column (4.6 mm × 250 mm, 5 μm) and a flow of 1 mL/min.

[^{18}F]-Fluoroazidobutane



Non-carrier added [^{18}F] fluoride was obtained by proton bombardment of an [^{18}O] enriched water target via the ^{18}O (p,n)^{18}F reaction. The radioactivity was trapped by passing the target water through a preactivated Sep-Pak light QMA cartridge (Waters). A 1 mL aqueous solution of K_2CO_3 (4.5 mg) and Kryptofix 222 (20 mg) was used to elute the [^{18}F]fluoride from the cartridge into a conical glass vial. This eluate was evaporated to dryness by three consecutive azeotropic distillations with acetonitrile (3 × 500 μL) under a gentle stream of nitrogen gas (130°C). The dried [^{18}F]-

fluoride was then added to 3.0 mg of bromoazidobutane **(**17 μmol) in 0.5 mL dry DMSO, and the mixture was heated at 140°C for 10 min. The labeled product was absorbed on a C18-light Sep-Pak cartridge followed by washing with 10 mL H_2O and eluted with 5 mL pure methanol. The Sep-Pak eluate containing [^{18}F]-fluoroazidobutane was then purified and separated from its precursor with semi-preparative HPLC on a semi-preparative C18-reversed phase column (mobile phase 60/40 MeCN/H_2O, retention time=10 min). In addition, conversion of the reaction was monitored by radio-TLC (silica gel, hexane/EtOAc (4:1)) (R_f=0.40) and HPLC analysis: retention time= 10 min).



[^{18}F]-BN3. [^{18}F]-fluoroazidobutane and **BN3** were dissolved in a mixture of water and DMF (3:1 v/v). In a separate vial, $CuSO_4 \cdot 5H_2O$ (5 mol %) was dissolved in 0.1 mL of water, and to this was added sodium ascorbate (25 mol %. MonoPhos (6 mol %) was added along with 0.1 mL of DMF. The reagents were stirred together for 10 min and added to the solution of azide and peptide. The reaction was monitored by HPLC and radio-TLC until full conversion was reached. **[^{18}F]-BN3** was purified by RP-HPLC. Radiochemical yield=59%. Retention time=18 min.

Cell culture: The GRPR-positive PC-3 human prostate cancer cell line (ATCC, Manassas, Virginia, USA) was cultured at 37°C in a humidified 5% CO_2 atmosphere. The cells were cultured in RPMI 1640 (Lonza, Verviers, France) supplemented with 10% fetal calf serum (Thermo Fisher Scientific Inc., Logan, Utah, USA) and subcultured twice a week after detaching with trypsin-EDTA.

***In Vitro* Competitive Receptor Binding Assay.** The *in vitro* GRPR binding affinity of **[^{18}F]-BN3** was determined by performing a displacement assay. **[^{125}I]-[Tyr4]-BN** was tested using the same assay. Experiments were performed at 37°C with PC-3 human prostate cancer cells. The 50% inhibitory concentration (IC_{50}) values were calculated by fitting the data with nonlinear regression using GraphPad Prism 5.0 (GraphPad Software, San Diego, California, USA). Results were plotted in sigmoidal curves for the displacement of **[^{18}F]-BN3** and **[^{125}I]-[Tyr4]-BN** as a function of increasing concentration of BN(1-14). The tracers displayed high affinity for binding to GRPRs within PC-3 cell with IC_{50} values of 44.6 and 4.6 nM, respectively.

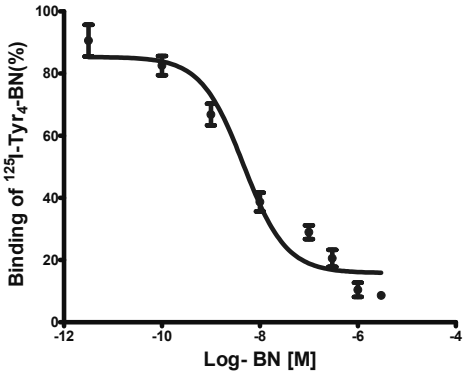


Figure 3 Competitive binding assay on PC-3 cells with **[^{125}I]-[Tyr4]-BN**

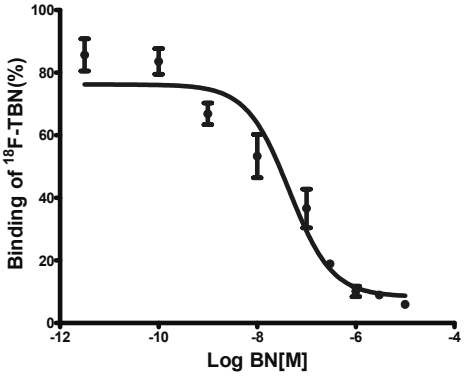


Figure 4 Competitive binding assay on PC-3 cells with **[^{18}F]-BN3**

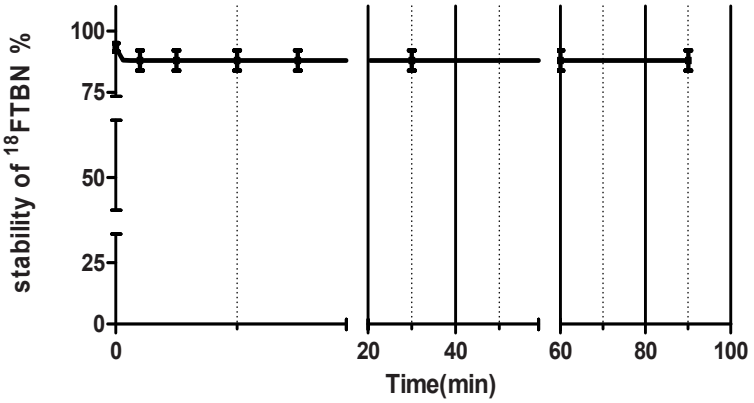
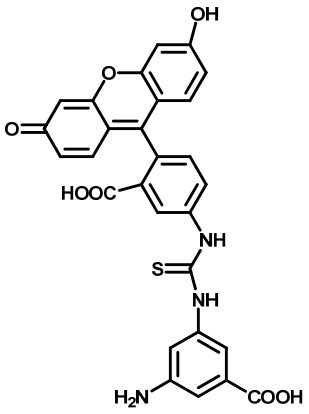


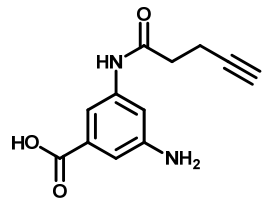
Figure 5 Stability of **[^{18}F]-BN3** in human serum (decay corrected)

5-(3-(3-Amino-5-carboxyphenyl)thioureido)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (28)



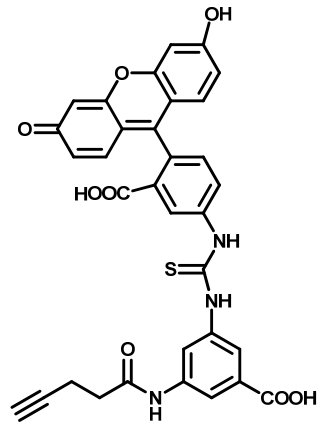
3,5-Diaminobenzoic acid **25** (320 mg, 2.11 mmol) was dissolved in methanol (100.0 mL) by heating. Once dissolved, the solution was allowed to cool, and fluorescein isothiocyanate (FITC) (410 mg, 1.05 mmol) in 5 mL of MeOH was added dropwise. The reaction mixture was left stirring at room temperature overnight. Upon completion of the reaction, the solvent was evaporated and the product was purified by column chromatography (gradient 10:1 to 2:1 DCM:MeOH). Dark red solid. Yield=77%. R_f=0.30 (1:1 DCM:MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ 8.11 (s, 1H), 7.77 (dd, *J*=8.0, 4.0 Hz, 1H), 7.29 (s, 1H), 7.20 (s, 1H), 7.13 (d, *J*=8.0 Hz, 1H), 7.02 (s, 1H), 6.80 (s, 2H), 6.64-6.67 (m, 4H), 6.53-6.56 (m, 2H), 6.35 (s, 2H); ^{13}C NMR (100.59 MHz, CD_3OD): δ 183.0, 180.8, 170.2, 160.6, 153.1, 148.8, 148.3, 147.8, 141.3, 139.3, 133.2, 131.7, 131.3, 129.9, 129.3, 128.7, 127.9, 124.5, 120.2, 118.7, 116.0, 114.8, 113.7, 113.3, 112.8, 110.4, 102.4. HRMS (EI) calcd for $C_{28}H_{20}O_7N_3S$ 542.1016, found 542.1037.

3-Amino-5-(pent-4-ynamido)benzoic acid (31)



3,5-Diaminobenzoic acid **25** (117 mg, 0.77 mmol) was dissolved in pyridine (30 mL). To this stirred solution, *N*-succinimidyl-4-pentynoate (74.8 mg, 0.38 mmol) was added dropwise. The reaction mixture was stirred at room temperature overnight. Pyridine was evaporated and the crude reaction mixture was purified by column chromatography (gradient 30:1 to 10:1 DCM:MeOH) to yield the product as an orange solid. Yield=97%. R_f=0.80 (2:1 DCM:MeOH). Spectroscopic data is in accordance with literature values.[28] ^{1}H NMR (400 MHz, CD_3OD): δ 7.43 (s, 1H), 7.29 (s, 1H), 7.12 (s, 1H), 6.35 (s, 2H), 2.53-2.57 (m, 4H), 2.28 (s, 1H). ^{13}C NMR (100.59 MHz, CD_3OD): δ 173.2, 168.3, 147.9, 132.0, 111.8, 110.7, 107.3, 106.7, 69.0, 60.2, 35.4, 14.1.

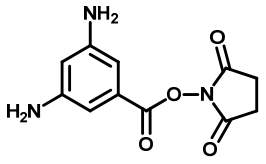
5-(3-(3-Carboxy-5-(pent-4-ynamido)phenyl)thioureido)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (30)



31 (52.0 mg, 0.22 mmol) and FITC (87.2 mg, 0.22 mmol) were dissolved in MeOH (2.0 mL). The reaction mixture was stirred at room temperature overnight. The solvent was evaporated and the crude reaction mixture was purified by column chromatography (3:1 DCM:MeOH, 1% Et_3N) to give the pure product as a dark red solid. R_f=0.20 (3:1 DCM:MeOH, 1% Et_3N). Yield=78%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.13-8.19 (m, 1H), 7.74-7.78 (m, 1H), 7.31 (s, 1H), 7.20 (s, 1H), 7.09-7.14 (m, 1H), 7.01-7.04 (m, 1H), 6.63-6.67 (m, 4H), 6.51-6.55 (m, 2H), 2.53-2.57 (m, 4H), 2.00 (s, 1H). ^{13}C NMR (100.59 MHz, CD_3OD): δ 180.6, 169.8, 169.7, 168.8, 160.2, 152.8, 148.7, 141.1, 140.9, 139.2, 138.9, 132.6, 131.5, 131.1, 129.0, 128.9, 127.6, 124.2, 124.1, 114.1, 114.2, 113.2, 112.4, 111.7, 110.7, 110.1, 102.1, 69.0, 60.2, 35.4, 19.5, 14.1.HRMS (EI) calcd for $C_{33}H_{24}O_8N_3S$ [M+H$^+$] 622.1279, found 622.1297.

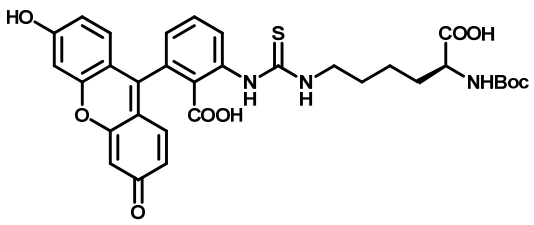
2,5-Dioxopyrrolidin-1-yl 3,5-diaminobenzoate (33)

3,5-Diaminobenzoic acid **25** (250 mg, 1.64 mmol) was dissolved in pyridine (5.0 mL). To this stirred solution was added NHS (208.0 mg, 1.81 mmol) and EDC·HCl (347 mg, 1.81 mmol). The reaction mixture was stirred at room temperature for 4 h after which it was diluted with H_2O (10 mL) and DCM (10 mL). The organic layer was separated and washed with (subsequently) a saturated solution of aqueous $NaHCO_3$ (10 mL), brine (10 mL) and water (10 mL). The organic layer was dried



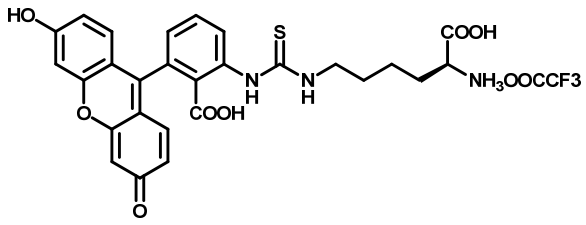
over $MgSO_4$ and the solvent evaporated. This yielded the pure product as a white solid. Spectroscopic data is in accordance with the literature.[29] R_f=0.85 (1:1 DCM:MeOH). Yield=20%. ^{1}H NMR (400 MHz, CD_3OD): δ 6.79 (s, 2H), 6.37 (s, 1H), 2.86 (s, 4H); ^{13}C NMR (100.59 MHz, CD_3OD): δ 169.0, 162.8, 147.7, 133.0, 112.8, 110.6, 106.3, 106.7, 25.3.

***(S)*-2-(3-(5-((*tert*-Butoxycarbonyl)amino)-5-carboxypentyl)thioureido)-6-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (40)**



Boc-lys-OH (70.8 mg, 0.29 mmol) was dissolved in DMF (10 mL). To this stirred solution at 0°C was added FITC (101 mg, 0.26 mmol) and Et_3N (28 mg, 0.04 mL). After 15 min at 0°C, the reaction mixture was allowed to warm to room temperature and left stirring overnight. The reaction mixture was diluted with EtOAc (10 mL) and the organic solution was washed with 0.6 M aqueous citric acid (10 mL), water (10 mL) and brine (10 mL) sequentially to yield the product as a pure white solid. R_f=0.60 (3:1 DCM:MeOH). Yield=97%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.13 (s, 1H), 7.87 (s, 2H), 7.74 (d, *J*=8.0 Hz, 1H), 7.14 (d, *J*=16.0 Hz, 1H), 6.64-6.71 (m, 4H), 6.51-6.57 (m, 2H), 4.05-4.08 (m, 1H), 3.57-3.64 (m, 2H), 1.46-1.89 (m, 6H), 1.42 (s, 9H); ^{13}C NMR (100.59 MHz, CD_3OD): δ 186.8, 183.6, 177.8, 169.7, 163.3, 159.9, 157.1, 152.7, 150.2, 143.8, 143.4, 138.2, 128.9, 124.3, 117.6, 112.2, 104.9, 102.1, 88.5, 53.4, 40.7, 31.2, 30.3, 27.4, 22.9. HRMS (EI) calcd for $C_{32}H_{34}O_9N_3S$ [M+H$^+$] 636.2010, found 636.2026.

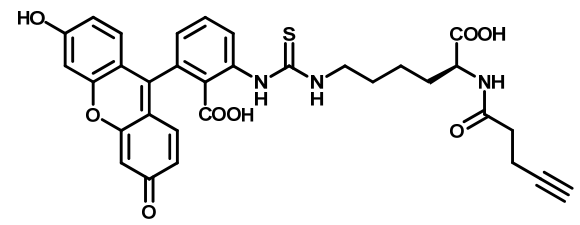
***(S)*-1-Carboxy-5-(3-(2-carboxy-3-(6-hydroxy-3-oxo-3H-xanthen-9-yl)phenyl)thioureido) pentan-1-amine trifluoroacetate (41)**



40 (67.0 mg, 0.11 mmol) was dissolved in a DCM/trifluoroacetic acid (TFA) mixture (9 mL, 5:1 v/v) and the reaction mixture was stirred at room temperature for 1 h. TFA and DCM were evaporated and the reaction flask was left under vacuum overnight. The solid residue was triturated with diethyl ether (3 x 5 mL) to yield the pure salt as a white solid. R_f=0.30 (3:1 DCM:MeOH). Yield=95%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.41 (s, 1H), 8.02 (br d, *J*=8.0 Hz, 1H), 7.89-7.91 (m, 1H), 7.63 (br s, 1H), 7.23-7.31 (m, 3H), 7.03-7.07 (m, 2H), 6.91-6.95 (m, 2H), 3.95-4.02 (m, 1H), 3.67 (br

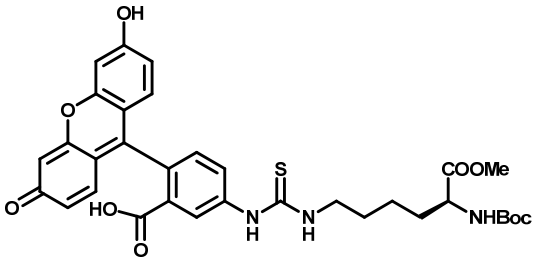
t, *J*=12.0 Hz, 2H), 1.26-2.00 (m, 6H). HRMS (EI) calcd for $C_{27}H_{26}O_7N_3S$ [M+H$^+$] 536.1486, found 536.1499.

(*S*)-2-(3-(5-Carboxy-5-(pent-4-ynamido)pentyl)thioureido)-6-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (42)



41 (130 mg, 0.25 mmol) was dissolved in DMF (3.0 mL) and to this solution Et_3N (0.17 mL) was added. To the stirred solution was added *N*-succinimidyl-4-pentynoate (48.8 mg, 0.25 mmol). The reaction mixture was allowed to stir at room temperature until full conversion was detected by thin layer chromatography. The reaction mixture was diluted with EtOAc (5.0 mL). The resulting organic layer was washed with 0.6 N aqueous citric acid (5.0 mL), followed by brine (5 x 5 mL). The organic layer was dried over $MgSO_4$ and the solvent was removed. The resulting residue was dissolved in a small amount of methanol (0.2 mL) and precipitated with cold diethyl ether, centrifuged to yield the pure product as an orange solid. R_f=0.85 (4:1 DCM:MeOH). Yield=45%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.18 (s, 1H), 7.96 (br s, 1H), 7.79 (d, *J*=8.0 Hz, 1H), 7.18 (d, *J*=8.0 Hz, 1H), 6.76-6.82 (m, 5H), 6.63 (m, 2H), 4.40-4.43 (m, 1H), 3.57-3.63 (m, 2H), 2.44-2.47 (m, 4H), 2.00 (s, 1H), 1.21-1.75 (m, 6H). HRMS (EI) calcd for $C_{32}H_{30}O_8N_3S$ [M+H$^+$] 616.1748, found 616.1771.

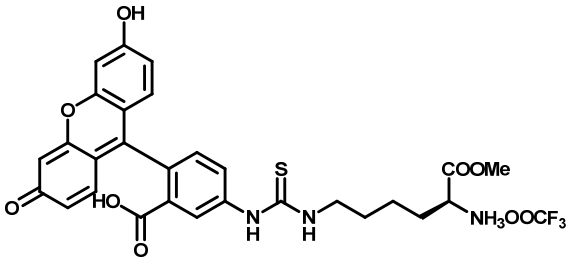
(*S*)-5-(3-(5-((*Tert*-butoxycarbonyl)amino)-6-methoxy-6-oxohexyl)thioureido)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (46)



N-α-Boc-*N*-ε-propargyloxycarbonyl-(*S*)-lysine methyl ester 45 (186 mg, 0.713 mmol) and fluorescein isothiocyanate (304 mg, 0.78 mmol) were dissolved in MeCN (20 mL). To this stirred solution was added Et_3N (0.11 mL, 0.780 mmol). After stirring at room temperature for 12 h, the reaction mixture was diluted with DCM (20 mL) and washed with a saturated aqueous solution of NH_4Cl. The organic layer was dried over $MgSO_4$ and the solvent removed by evaporation. The product was dissolved in a minimal amount of MeOH and precipitated with cold diethyl ether to give a solid orange product. Yield=35%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.06 (s, 1H), 7.89 (d, *J*=8.0 Hz, 1H), 7.73 (d, *J*=8.0 Hz, 1H), 7.16 (d, *J*=8.0 Hz,

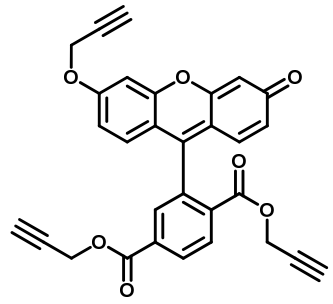
1H), 6.55-6.77 (m, 5H), 4.09-4.13 (m, 1H), 3.71 (s, 3H), 3.51-3.55 (m, 2H), 1.45-1.83 (m, 6H), 1.31 (s, 9H). HRMS (EI) calcd for $C_{33}H_{36}O_9N_3S$ [M+H$^+$] 650.2167, found 650.2177.

(*S*)-6-(3-(3-Carboxy-4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)phenyl)thioureido)-1-methoxy-1-oxohexan-2-amine- trifluoroacetate (47)



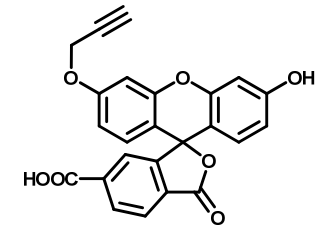
46 (134 mg, 0.21 mmol) was dissolved in DCM (10 mL). To this solution was added trifluoroacetic acid (2.0 mL) at 0°C after which the reaction mixture stirred at room temperature for 3 h. The solvent was removed by evaporation and the salt was used in the next step without any further purification. R_f=0.20 (3:1 DCM:MeOH). Yield=54%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.21 (s, 1H), 7.88 (d, *J*=8.0 Hz, 1H), 7.76 (d, *J*=8.0 Hz, 1H), 7.13 (d, *J*=8.0 Hz, 1H), 6.53-6.68 (m, 5H), 4.08-4.12 (m, 1H), 3.83 (s, 3H), 3.53-3.55 (m, 2H), 1.35-1.73 (m, 6H).

Di(prop-2-yn-1-yl)-2-(3-hydroxy-6-(prop-2-yn-1-yloxy)-9H-xanthen-9-yl)terephthalate (50)



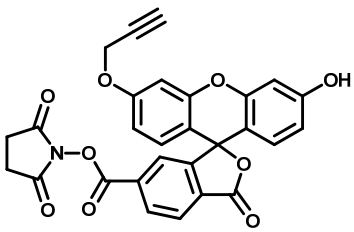
6-carboxyfluorescein **49** (40.0 mg, 0.11 mmol) was dissolved along with K_2CO_3 (66.1 mg, 0.48 mmol) and propargyl bromide (57.0 mg, 0.48 mmol) in DMF (5.0 mL). The reaction mixture was heated to 60°C for 2 h when the reaction reached completion as determined by thin layer chromatography (100 % EtOAc), it was poured into a mixture of EtOAC and 1 N aqueous HCl (1:1 v/v, 10 mL). This solution was extracted with EtOAc. The organic layers were combined, dried over $MgSO_4$ and the solvent was removed to yield the product with sufficient purity to continue the synthesis. R_f=0.70 (100 % EtOAc). Yield=99%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.39-8.44 (m, 2H), 8.03 (s, 1H), 7.31 (s, 1H), 7.19 (s, 1H), 7.01-7.09 (m, 4H), 4.95 (s, 2H), 4.91 (s, 2H), 4.59 (s, 2H), 2.66 (s, 1H), 2.54 (s, 1H), 2.37 (s, 1H); ^{13}C NMR (100.59 MHz, CD_3OD): δ 163.7, 162.4, 159.4, 159.3, 155.9, 134.1, 133.7, 133.6, 133.5, 131.8, 131.5, 131.3, 130.7, 128.7, 127.4, 125.7, 117.9, 116.6, 115.8, 105.1, 101.7, 76.0, 77.6, 57.1, 53.4. HRMS (EI) calcd for $C_{30}H_{19}O_7$ [M+H$^+$] 491.1125, found 491.1126.

3'-Hydroxy-3-oxo-6'-(prop-2-yn-1-yloxy)-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylic acid (51)



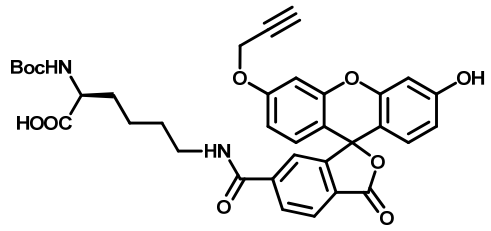
To a solution of 51 (52.0 mg, 0.11 mmol) in THF/H_2O (1:1 v/v, 2 mL) was added LiOH (1 M, 0.32 mmol). The reaction mixture was stirred at room temperature for 1 h, and then diluted with H_2O (5 mL). The aqueous solution was extracted with DCM (2 x 10 mL). The aqueous layer was acidified to pH=4 using 2 N aqueous HCl. The aqueous layer was extracted with DCM followed by EtOAc. The organic layers were collected, dried over $MgSO_4$ and the solvent was evaporated. The crude mixture was purified by column chromatography (3:1 DCM:MeOH) to yield the product as a white solid. R_f=0.55 (3:1 DCM:MeOH). Yield=64%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.23 (d, *J*=8.0 Hz, 1H), 7.99 (d, *J*=8.0 Hz, 1H), 7.70 (s, 1H), 6.94 (s, 1H), 6.70-6.74 (m, 3H), 6.60-6.62 (m, 1H), 6.53-6.55 (m, 1H), 4.79 (s, 2H), 2.98-2.99 (m, 1H); ^{13}C NMR (100.59 MHz, CD_3OD): δ 174.3, 169.8, 160.2, 159.8, 153.2, 152.7, 152.6, 131.0, 129.0, 128.7, 124.8, 124.0, 112.4, 112.0, 111.9, 109.8, 102.5, 102.1, 84.7, 78.0, 76.1, 55.8. HRMS (EI) calcd for $C_{24}H_{15}O_7$ [M+H$^+$] 415.0812, found 415.0816.

2,5-Dioxopyrrolidin-1-yl-3'-hydroxy-3-oxo-6'-(prop-2-yn-1-yloxy)-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylate (52)

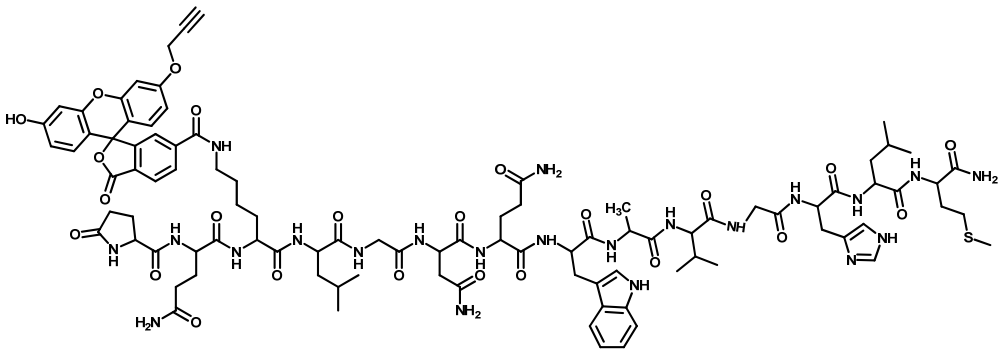


To a solution 51 (28.0 mg, 0.07 mmol) in DMF (2 mL) was added NHS (8.55 mg, 0.07 mmol) and EDC·HCl (14.2 mg, 0.07 mmol). The reaction was stirred overnight at room temperature. The reaction mixture was diluted with EtOAc and washed with brine (1 x 3 mL). The organic layer was dried over $MgSO_4$ and the solvent was removed. The crude reaction mixture was purified by column chromatography (30:1 DCM:MeOH). After purification, the product still contained some impurities which could be precipitated with MeOH to give the pure product as a clear oil. R_f=0.90 (3:1 DCM:MeOH). Yield=53%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.41 (d, *J*=8.0 Hz, 1H), 8.21 (d, *J*=8.0 Hz, 1H), 7.91 (s, 1H), 6.98 (s, 1H), 6.58-6.77 (m, 5H), 4.81 (s, 2H), 3.00 (s, 1H), 2.86 (s, 4H); ^{13}C NMR (400 MHz, CD_3OD): δ 173.6, 168.5, 166.0, 159.6, 152.0, 151.6, 137.2, 130.7, 128.7, 128.6, 124.8, 124.7, 112.5, 112.0, 111.2, 102.3, 101.9, 76.0, 68.2, 67.0, 55.6, 24.9. HRMS (EI) calcd for $C_{28}H_{18}O_9N$ [M+H$^+$] 512.0976, found 512.0988.

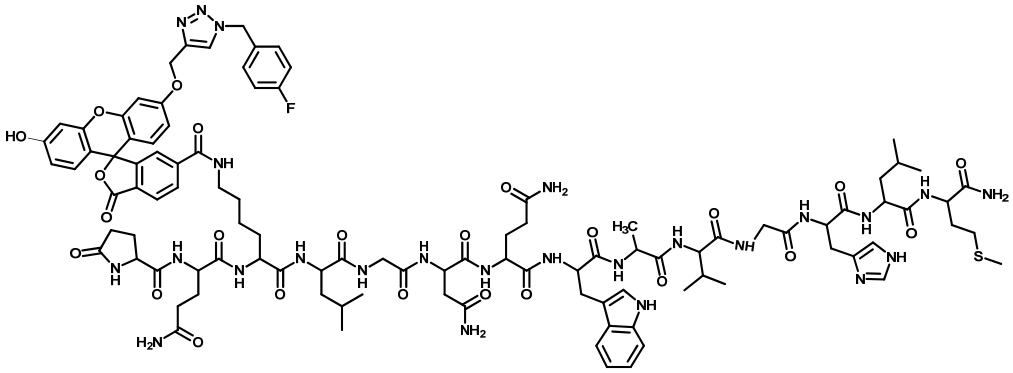
(*S*)-2-((*tert*-butoxycarbonyl)amino)-6-(3'-hydroxy-3-oxo-6'-(prop-2-yn-1-yloxy)-3H-spiro[isobenzofuran-1,9'-xanthen]-6-ylcarboxamido)hexanoic acid (53)



52 (2.5 mg, 4.88 x 10^{-3} mmol) and Boc-lys-OH (1.2 mg, 4.87 x 10^{-3} mmol) were dissolved in DMF (1.0 mL). To this solution was added Et_3N (4 µL) and the reaction mixture was stirred at room temperature overnight. The solution was diluted with EtOAc (5 mL) and washed with a saturated solution of aqueous NH_4Cl (3 mL). The organic layer was dried over $MgSO_4$ and the solvent was removed to yield the pure product as a yellow solid. R_f=0.70 (3:1 DCM:MeOH). Yield=99%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.14 (d, *J*=8.0 Hz, 1H), 8.08 (d, *J*=8.0 Hz, 1H), 7.60 (s, 1H), 6.95 (s, 1H), 6.71-6.73 (m, 3H), 6.57-6.52 (m, 2H), 4.80 (s, 2H), 3.99-4.03 (m, 1H), 3.64-3.67 (m, 2H), 2.67 (s, 1H), 1.50-1.81 (m, 6H), 1.39 (s, 9H). HRMS (EI) calcd for $C_{35}H_{35}O_{10}N_2$ [M+H$^+$] 643.2286, found 643.2284.



BN4. **52** (0.77 mg, 1.51 x 10^{-3} mmol) was dissolved along with [lys3]-bombesin (0.24 mg, 1.51 x 10^{-4} mmol) in dry DMF (0.1 mL). To this stirred solution under inert atmosphere (N_2) was added Et_3N (0.61 mg, 0.83 µL). The reaction mixture was stirred vigorously at room temperature for 24 h after which it was lyophilized. The crude solid was then dissolved in MeCN/H_2O (with 1 % formic acid) and purified by RP-HPLC (Gradient B). Retention time=35 min. HRMS (EI) calcd for $C_{95}H_{123}O_{24}N_{22}S$ 1988.8829, found 1988.8982. HRMS (EI) calcd for $C_{95}H_{122}O_{24}N_{22}SNa$ [M+Na$^+$] 2010.86488, found 2010.85706.



BN5. Fluorobenzyl azide (0.83 μg, 5.49 x 10^{-6} mmol) and **BN3** (0.01 mg, 5.03 x 10^{-6} mmol) were dissolved in a mixture of H_2O/DMSO (3:1 v/v, 0.5 mL). In a separate vial, $CuSO_4 \cdot 5H_2O$ (0.06 μg, 2.52 x 10^{-7} mmol) was dissolved in 0.01 mL of water and to this solution, sodium ascorbate (0.24 μg, 1.21 x 10^{-6} mmol) was added. MonoPhos (0.10 mg, 2.78 x 10^{-7} mmol) was added along with 0.01 mL of DMSO. The reagents were stirred together for 10 min and added to the solution of azide and peptide. The reaction was monitored by RP-HPLC. Upon completion, the reaction mixture was lyophilized. The resulting solid was dissolved in a mixture of MeCN:H_2O (with 1 mol % formic acid) and purified by RP-HPLC (Method B). Retention time=46 min. HRMS (EI) calcd for $C_{102}H_{128}O_{24}N_{25}SNa$ [M+H^+] 2163.9216, found 2163.9267.

5.6 References and Notes

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