Isotopic Labeling

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Strain-Promoted Copper-Free "Click" Chemistry for ^{18}F Radiolabeling of Bombesin

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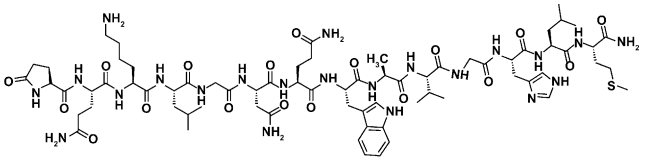
Bombesin is a 14 amino acid (Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2) neuropeptide, which binds with high affinity to the gastrin-releasing peptide receptor (GRPR). Bombesin has received much attention in the field of nuclear imaging because the GRPR is massively overexpressed on a variety of tumor cells, including breast and prostate tumor cells, thus making bombesin a promising radioligand for the diagnosis and imaging of cancer.[1] Much effort has been invested in the development of labeled bombesin analogues.[2] Bombesin is often modified in the form of Lys[3]-bombesin, which allows for site-selective introduction of the radionuclide at the terminal amino



Lys[3]-Bombesin

group of lysine. Amino acids 7–14 are known to be essential for receptor binding, thus modification in the third amino acid reduces potential for interference.[3] A variety of bombesin analogues for nuclear imaging have been synthesized and are predominantly labeled with large metal-based 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).[4]

Positron emission tomography (PET) is a nuclear imaging technique used extensively in diagnostic medicine and drug development. In the last decade, ^{18}F ($t_{1/2} \approx 110$ min) has been popularized as a nonmetallic PET radioisotope. With a longer half-life than other nonmetallic radioisotopes for PET, such as ^{11}C ($t_{1/2} \approx 20$ min) and ^{13}N ($t_{1/2} \approx 10$ min), ^{18}F has the distinct advantage of allowing for off-site production and transportation of the radionuclide as well as allowing for scans to be carried out over several hours. Furthermore, the use of ^{18}F results in images with higher resolution than other radionuclides because of its low positron energy.[5] Very few instances of ^{18}F-labeled bombesin have been reported to date, which is predominantly due to the synthetic challenges associated with the introduction of ^{18}F when compared with simple chelation techniques used for metallic radionuclides.[6] Notably, the synthetic time frame is also much reduced when compared with metallic radionuclides such as ^{64}Cu ($t_{1/2} \approx 12$ h). A major disadvantage is the need for the multistep synthetic procedures required to synthesize current prosthetic groups such as [^{18}F]succinimidyl 4-fluorobenzoate ([^{18}F]SFB) or [^{18}F]4-fluorobenzaldehyde, which are commonly used to introduce ^{18}F in the presence of a free amine.[7] Ideally, a prosthetic group should be easily synthesized, introduce the radionuclide in the last step of the synthesis, and should require only the mildest of conditions to attach it to the biomolecule of interest.

The azide–alkyne cycloaddition has been popularized under the general term "click" chemistry since the discovery that it proceeds regioselectively at room temperature in the presence of catalytic amounts of Cu^I ions.[8] The bioorthogonality of the azide and the alkyne has proven unparalleled. The robustness and versatility of this reaction along with its mild conditions makes it attractive for labeling target molecules with radionuclide-containing prosthetic groups. Many groups have exploited the bioorthogonality of this reaction to allow for fast and straightforward labeling of sugar and peptide targets with ^{18}F and other radionuclides.[9] The obvious limitation of this methodology for biological systems is the cytotoxicity of copper. Potential contamination of labeled compounds with traces of copper is a major drawback and this reaction is thus not suitable for development of in vivo pretargeting methodologies. In recent years, great progress has been made in developing copper-free methodologies through, for example, the use of strained cyclooctynes;[10] in one instance this reaction has been used for the introduction of ^{111}In to a target peptide for single photon emission computed tomography (SPECT) imaging.[11] We envisioned the use of Lys-[3]bombesin modified with a strained alkyne to allow for rapid and facile labeling with ^{18}F in the absence of possible copper contamination. A further advantage of this methodology would be the possibility to fine-tune the properties of the resulting labeled peptide. The azide group can be designed to tune the hydrophilicity, bulk, or charge of the peptide in question. Furthermore, though we

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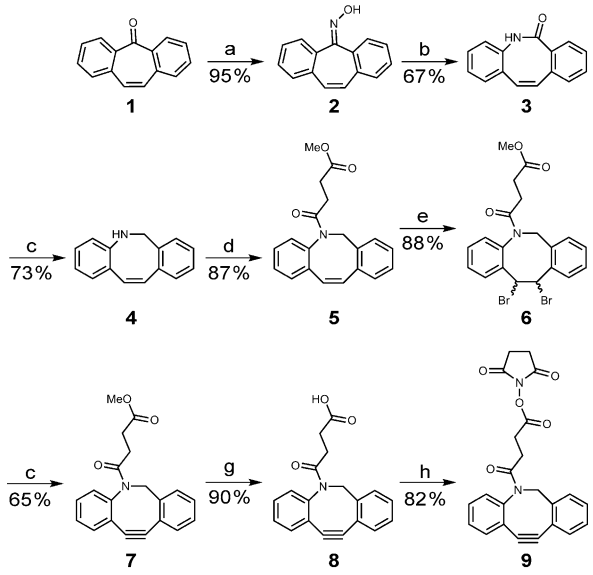
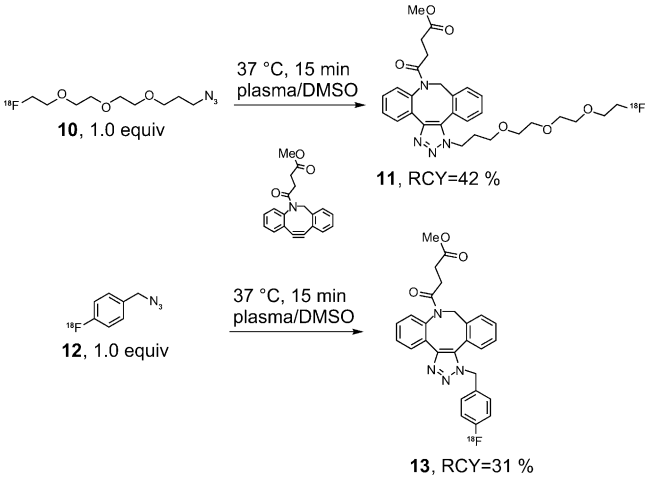
focus in this work on the use of bombesin, we expect that the technique would be amenable to the use of other biomolecules as well. We present herein the first instance of ^{18}F radiolabeling using copper-free "click" chemistry, and its application to the synthesis of a ^{18}F-labeled analogue of bombesin, a potent ligand for tumor imaging. We further demonstrate that the "click" radiolabeling does not compromise the GRP receptor in vitro binding affinity in human prostate cancer cells.

Our starting point was to find a suitable strained alkyne with the optimal balance of reactivity and stability. Van Hest, van Delft, and co-workers reported an aza-dibenzocyclooctyne (**7**), which proved to be simultaneously reactive and stable.[12] For our purposes, which involve rapid "clicking" of a short-lived radioisotope and eventual in vitro and in vivo studies, **7** appeared an ideal choice. However, we opted for an alternate and shorter synthetic route to our target molecule **9** (Scheme 1). Our synthesis started from commercially available dibenzosuberenone (**1**), which was heated to reflux in the presence of hydroxylamine hydrochloride to form oxime **2** in 95% yield. The crucial step of this synthesis is the subsequent Beckmann rearrangement, which gave amide **3** in 67% yield.[13] The amide was reduced with Dibal-H to amine **4**, which was thus reached in three steps, rather than five. The synthesis starts with the very inexpensive precursor **1** (1 g ≈ 1 €) rather than the reported 2-ethynylaniline (1 g ≈ 55 €). A short linker can be introduced by reaction of **4** with methyl succinyl chloride in the presence of triethylamine. The bromination proceeded smoothly in 88% yield and was followed by subsequent debromination with a solution of potassium *tert*-butoxide in THF to form **7**. Basic hydrolysis afforded carboxylic acid **8**, and the *N*-hydroxysuccinimide ester **9** was formed to allow coupling of the strained alkyne to the peptide.



Scheme 1. Reagents and conditions: a) $NH_2OH{\cdot}HCl$ (3.0 equiv), pyridine/ethanol (1:1), reflux; b) TCT (1.0 equiv), DMF, RT; c) Dibal-H (5.0 equiv), CH_2Cl_2, RT; d) Et_3N (2.0 equiv), methyl succinyl chloride (1.5 equiv), CH_2Cl_2, RT; e) Br_2 (1.0 equiv), CH_2Cl_2, 0°C; f) 1.0 M *t*BuOK in THF, THF, −40°C, Ar atmosphere; g) LiOH (2.0 equiv), H_2O, RT; h) EDC (1.1 equiv), NHS (1.1 equiv), CH_2Cl_2, RT. TCT = trichlorotriazine, DMF = dimethylformamide, Dibal-H = diisobutyl aluminum hydride, THF = tetrahydrofuran, EDC = *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide, NHS = *N*-hydroxysuccinimide.

Of key importance to the field of radiochemistry is high reactivity, which allows for the rapid introduction of the short-lived radiolabel under biocompatible conditions. Before we proceeded with the modification of bombesin, we tested the reactivity of the aza-benzocyclooctyne with several fluorine-containing azides in pseudo in vivo conditions (Scheme 2).

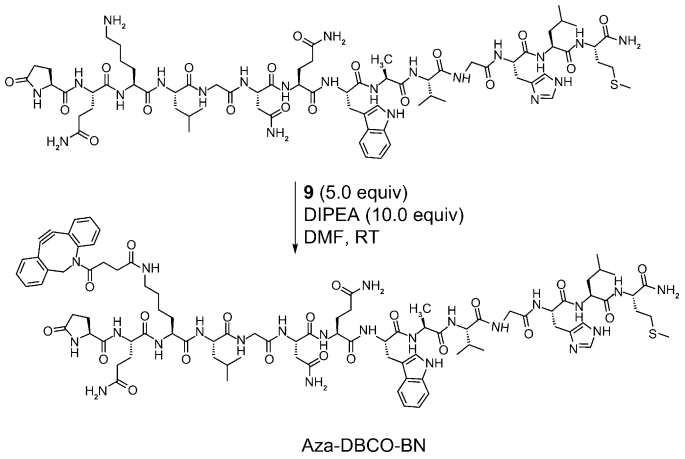


Scheme 2. Strain-promoted "click" chemistry for ^{18}F labeling.

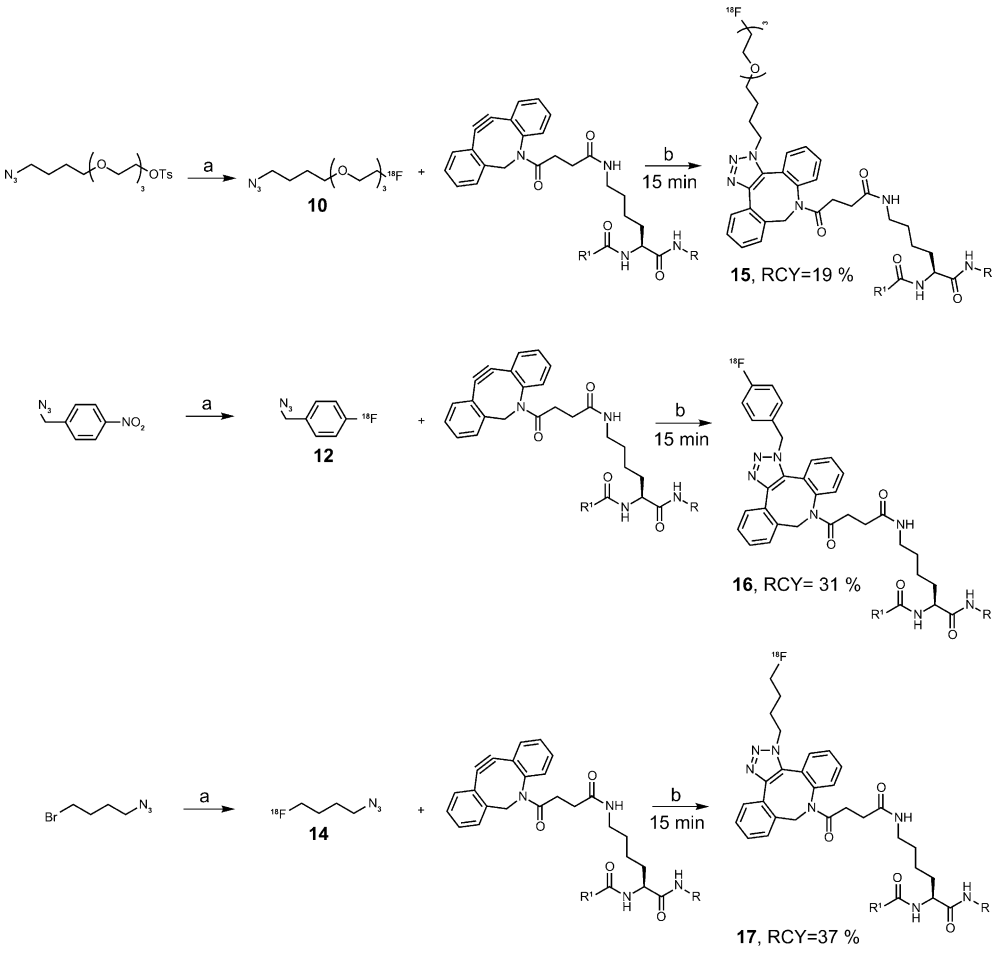
Strained alkyne **7** could be reacted with a ^{18}F-labeled azide by simply stirring the two reactants together in a mixture of human plasma and DMSO. We found that alkyne **7** could be fully converted to the corresponding triazoles within 15 min.[14] With the hydrophilic [^{18}F]PEGylated azide **10**, triazole **11** was isolated with a radiochemical yield (RCY) of 42%. With the more lipophilic [^{18}F]fluorobenzyl azide **12**, the product was isolated with an RCY of 31%. Furthermore, it should be noted that the reaction is performed in human plasma. Considering the eventual application of radiolabeling in vivo, it was important to confirm that the selected strained alkyne was not too fragile to withstand any exposure to a biological environment. Given that alkyne **7** could be fully converted to triazoles in less than 15 min, we concluded that the reaction was fast enough for the desired time scale for labeling with ^{18}F.

Succinimidyl ester **9** was therefore conjugated to Lys[3]-bombesin under basic conditions (Scheme 3). Full conversion to the product Aza-DBCO-BN was achieved after 24 h as determined by reverse-phase (RP) HPLC, and subsequently Aza-DBCO-BN was purified by RP-HPLC and characterized by mass spectrometry (ESI-MS). Now that the target bombesin analogue was modified with a strained alkyne, we tested the efficiency of the copper-free [3+2] cycloaddition with various ^{18}F-containing azides.

Three ^{18}F-containing azides were selected to react with Aza-DBCO-BN (Scheme 4). One advantage of this methodology is the ease with which the properties of the resulting peptidic tracer can be modified by simply changing the azides.



Scheme 3. Modification of bombesin with **9**. DIPEA = *N*,*N*-diisopropylethylamine.



Scheme 4. Reagents and conditions: a) K[^{18}F], MeCN, 110 °C; b) DMF, RT. R = Pyr-Gln, R^1 = Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$, Pyr = pyroglutamic acid.

tively. The logarithmic partition coefficients were determined for all three tracers and were found to be −0.43, 1.27, and 0.26, respectively. This methodology provides us with tracers that range from the quite hydrophilic [^{18}F]PEGTOxBN to the more hydrophobic [^{18}F]BnTOxBN.

The binding affinity of all three tracers for gastrin-releasing peptide receptors was tested using PC3 human prostate cancer cells. The in vitro binding was determined by performing a competitive receptor binding assay with the receptor specific radioligand [^{125}I]Tyr[4]-BN (a displacement assay). The 50% inhibitory concentrations (IC_{50}) were determined to be 40 nM, 29 nM, and 30 nM for **15**, **16**, and **17**, respectively (see Figures 1–3 in the Supporting Information). All three tracers maintain high affinity for the GRPRs even after modification.

We have achieved rapid radiolabeling of bombesin with [^{18}F] by using a very straightforward protocol. Simple stirring of the radionuclide-containing azide with the modified bombesin analogue for 10–15 min at room temperature suffices to reach the target peptides in modest to good yields. Furthermore, the azide can be readily varied from a more lipophilic aromatic azide to a hydrophilic PEGylated azide. As a result, peptides with different properties are readily accessible from the same modified peptide, thus allowing for rapid modification and fine-tuning. In this way, the optimal lipophilicity for cellular uptake and metabolic clearance can be achieved. To the best of our knowledge, this is the first example of ^{18}F radiolabeling using copper-free click chemistry. We have also developed a simplified and relatively inexpensive route to the target aza-dibenzocyclooctyne, and have hopefully rendered it more accessible for future use in a clinical setting. Although we describe herein the modification and labeling of bombesin and demonstrate that it maintains high affinity for the targeted receptors, ideally, this methodology would be applied to imaging by pretargeting. For molecules such as antibodies that have slower pharmacokinetics and are thus not amenable to the use of the short-lived ^{18}F, it would be highly advantageous to be able to administer the ^{18}F radio-

[^{18}F]PEGylated azide **10**, [^{18}F]fluorobenzyl azide **12**, and [^{18}F]fluoroazidobutane **14** were reacted with Aza-DBCO-BN at room temperature in DMF for 15 min, during which time complete conversion of the starting material could be detected by radio-TLC to give triazoles [^{18}F]PEGTOxBN (**15**), [^{18}F]BnTOxBN (**16**) and [^{18}F]BuTOxBN (**17**) respec-

nuclide to the target in vivo. In this way, the use of radionuclides for imaging such targets will not be limited to the longer-lived metallic radioisotopes, and higher-resolution images can be achieved using ^{18}F. Studies in this area will be reported in due course.

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