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A ^{18}F-labeled dibenzocyclooctyne (DBCO) derivative for copper-free click labeling of biomolecules†‡

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The new prosthetic group ^{18}F-TEG-DBCO (dibenzocyclooctyne) can be prepared within a total reaction time of 60 min including purification with an overall yield (n.d.c.) of 34 ± 5%. Copper-free click cycloadditions with an azido-cRGD, a folate-azide and two α-MSH analogue azido-peptides resulted in very high RCYs and fast reaction kinetics.

For non-invasive *in vivo* imaging of processes and pharmacokinetics of radiolabeled biomolecules, the positron emission tomography (PET) is one of the most powerful methods.[1] For PET applications, fluorine-18 has ideal nuclear characteristics and is the most commonly applied radionuclide in PET. The relatively long half-life of 110 minutes enables multi-step radiosyntheses, and the rather low β^+-energy ensures a very high spatial resolution in tomography.[2] The challenge for nuclear chemists consists in finding appropriate ^{18}F-labeling strategies, especially for sensitive biomolecules. Most of them are sensitive to the commonly used harsh conditions in direct ^{18}F-labeling reactions such as high temperatures and strong basic conditions.[3,4] As a result, the development of indirect labeling strategies *via* ^{18}F-prosthetic groups, which can subsequently be attached to biomolecules under mild reaction conditions, is needed.[5–7] Besides, the radiolabeling reaction should allow bioorthogonal ^{18}F-labeling to treat the multitude of functional groups in bioactive compounds with respect. The most prominent example of such reactions, which fulfills all the mentioned criteria, is the copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) first published by Sharpless *et al.* in 2001.[8] This variant of the Huisgen 1,3-dipolar cycloaddition of terminal alkynes and azides enables ^{18}F-labeling with high specificity and excellent yields under mild conditions.[9,10] In the past decade, a widespread spectrum of PET tracers has been synthesized using the CuAAC method for ^{18}F-labeling of bioactive compounds.[11] One of the latest developments is based on an amino acid, which is thought to minimize the influence on the pharmacokinetic properties of the intended radiotracer. As an amino acid derived ^{18}F-prosthetic group, it is particularly suitable for peptides and proteins.[12] However, with all the advantages of the copper(I)-catalyzed cycloaddition, there is one major disadvantage. The need for cytotoxic copper species as a catalyst in the click reaction causes an extensive work-up guaranteeing the complete removal of copper for *in vivo* applications. This fact led to the necessity of alternative fast and copper-free click reaction strategies. By using strained alkynes instead of terminal alkynes, copper is no longer needed to catalyze the click reaction. These so-called strain-promoted click reactions were first reported by Baskin *et al.*[13] and can be carried out between cyclooctyne derivatives and azides or tetrazines as 3 + 2 cycloaddition.[11] The use of azadibenzocyclooctynes for copper-free click reactions was first reported by Kuzmin *et al.* in 2010.[14] Recently, Arumugam *et al.* have published the development of a ^{18}F-labeled azadibenzocyclooctyne for ^{18}F-labeling of peptides *via* a strain-promoted click reaction without the use of a copper species, showing the high potential of this concept for ^{18}F-labeling of biomolecules.[15] Our aim was to develop a new ^{18}F-prosthetic group based on (aza)dibenzocyclooctyne (DBCO) for radiolabeling of biomolecules such as peptides and microproteins. For reduced lipophilicity, we introduced a triethylene glycol spacer to the (aza)dibenzocyclooctyne. Two different leaving groups, different bases, base concentrations and precursor amounts during radiolabeling were evaluated for optimized ^{18}F-labeling. Consequently, two DBCO-based precursors and the non-radioactive reference compound were synthesized, and the ^{18}F-labeling reaction was optimized. Finally, we performed a proof-of-principle click reaction with the new ^{18}F-labeled prosthetic group and an azido-functionalized cyclic Arg-Gly-Asp (cRGD) peptide as a model system. This peptide is used as the gold-standard vector in targeting the $\alpha_v\beta_3$ integrin.[16,17] Furthermore, we carried out further copper-free click reactions using a folate-azide for targeting the folate receptor and two α-MSH analogue azido-

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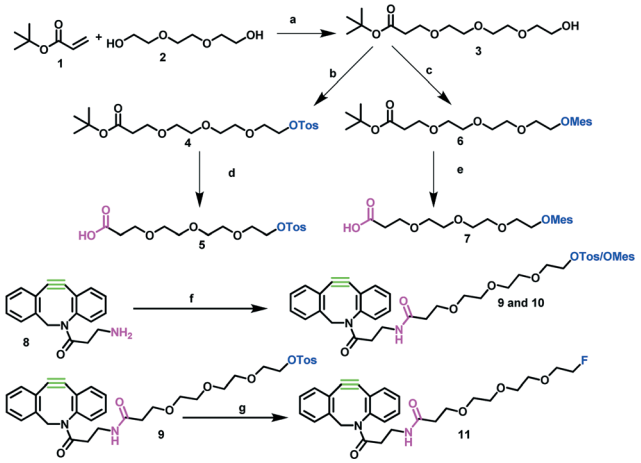
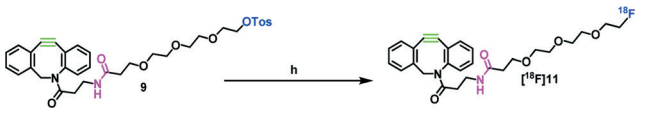


Scheme 1 Synthesis of alkyne-functionalized reference compound **11** and labeling precursors **9** and **10**. Regents and conditions: a) sodium, THF, 24 h, rt; b) TEA, *p*-toluenesulfonyl chloride, DCM, 1 h, 0 °C, rt; c) TEA, methanesulfonyl chloride, 1 h, 0 °C, rt; d) TFA, DCM, 4 h, rt; e) TFA, DCM, 4 h, rt; f) TEG-carboxylic acid, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uranium-hexafluorophosphate (HBTU), *N*,*N*-diisopropylethylamine (DIPEA), DMF, 24 h, rt; g) tetrabutylammonium fluoride, THF, 2 h, 80 °C.

functionalized peptides with high specificities to the melanocortin receptor 1 (MC1R).

The syntheses of reference compound **11** and the ^{18}F-labeling precursors **9** and **10** are depicted in Scheme 1. The synthesis started from commercially available triethylene glycol **2**. In the first step, **2** was reacted with *tert*-butylacrylate **1** to create a carboxylic acid function enabling the desired amide coupling.[18] Compound **3** was then reacted with either *p*-toluenesulfonyl chloride[19] or methanesulfonyl chloride[20] to transfer the hydroxyl function into suitable leaving groups for the nucleophilic radiofluorination reaction. Subsequently, protected intermediates **4** and **6** were deprotected by trifluoroacetic acid in dichloromethane at room temperature to yield **5** and **7**.[21] Both linker groups were coupled *via* an amide bond to the dibenzocyclooctyne (DBCO)-amine **8**, using HBTU *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)-uranium-hexafluorophosphate (HBTU) as coupling reagent and *N*,*N*-diisopropylethylamine (DIPEA) as base. The coupling was performed at room temperature for 12 h to yield the desired precursors for the ^{18}F-fluorination reaction in overall yields of 28% (for precursor **9**) and 56% (for precursor **10**) over four steps. Due to the quite high costs for DBCO-amine **8**, we aimed to insert this component in the last synthesis step. In relation to the amounts of **8**, the yields were good to very high, leading to 56% and 87%, respectively. The reference compound was synthesized through ^{19}F-fluorination of **9** using tetrabutylammonium fluoride (TBAF) at 120 °C for 2 h to yield **11** in excellent yields of 82%.

The radiofluorination of precursor **9** is depicted in Scheme 2. The radiolabeling of precursors **9** and **10** was optimized using different parameters such as various bases, base concentrations, reaction times and different amounts of precursors. Initially, the use of two different bases,

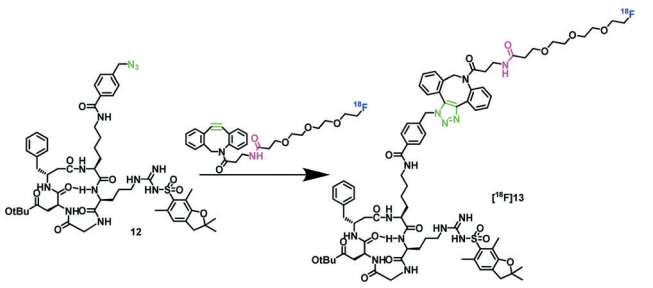


Scheme 2 Synthesis of ^{18}F-prosthetic group [^{18}F]**11**. Reagents and conditions: h) n.c.a. $^{18}F^-$, Et_4NHCO_3, MeCN, 100 °C, 10 min, RCY 91%.

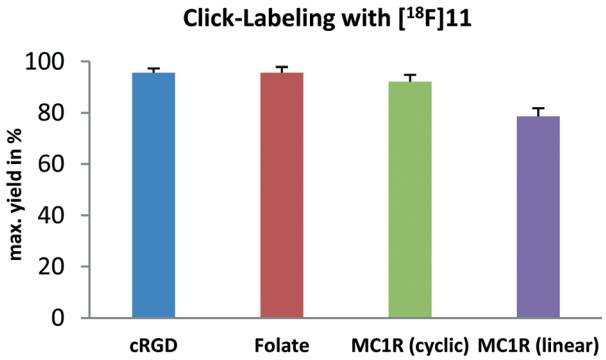
tetrabutylammonium hydroxide (TBA-OH) and tetraethylammonium bicarbonate (Et_4NHCO_3), in acetonitrile was screened. The use of TBA-OH caused decomposition of the precursors, and a RCY of only 30% was achievable. The use of precursor **9** (7.5 mg, 12 μmol) in acetonitrile and tetraethylammonium bicarbonate gave the highest RCY of ≥90% within 10 minutes. For further evaluation of precursors **9** and **10**, tetraethylammonium bicarbonate was used as base. With a base amount below 17 μmol, no ^{18}F-labeling was observed, while increasing the base amount to higher than 17 μmol resulted in reduced yields. Besides, the amount of precursor played an important role. Reaction kinetics were monitored for 2.5, 5.0 and 7.5 mg (4, 8 and 12 μmol) of precursor **9**. By increasing the amount of precursor (12 μmol), RCYs of ≥90% after 10 min were observed. Between the two leaving groups we did not observe significant differences in RCYs.

Isolation of the final ^{18}F-labeled prosthetic group was performed by fixation of the product fraction obtained from semi-preparative HPLC on a C18 reversed phase cartridge, followed by elution of the ^{18}F-prosthetic group from the resin with acetonitrile (1 mL). An exemplary radio-HPLC chromatogram of the crude mixture after radiolabeling of [^{18}F]**11** is shown in the ESI.‡ The solvent was removed under reduced pressure, and the ^{18}F-prosthetic group was resolved in the desired solvent to perform the subsequent click reaction. The new ^{18}F-prosthetic group was synthesized and isolated within only 60 min in an excellent overall yield (n.d.c.) of 34 ± 5%, ready for copper-free click reactions with azido-functionalized biomolecules. For the lipophilicity of the ^{18}F-prosthetic group, a log*D* value of 1.20 ± 0.07 was calculated using the octanol–water distribution coefficient.

To test the viability of [^{18}F]**11**, it was used in the copper-free cycloaddition with azido-functionalized cRGD peptide **12** (1 mg, 1.1 μmol), as shown in Scheme 3, as a model system.



Scheme 3 SPAAC of protected azido-functionalized cRGD **18** and the new prosthetic group [^{18}F]**11**. Click reaction conditions: PBS buffer/acetonitrile (1 : 1), 25 °C or 40 °C, 5 min, RCY 93%.



Scheme 4 Radiolabeling of various azides with [^{18}F]**11**. RCYs after 20 min are displayed in a bar chart. Errors are given as standard deviation representing n = 3.

The copper free ^{18}F-click reaction gave the desired peptide [^{18}F]**13** in excellent RCY of 93% within 5 min, which shows the particularly high potential of the new prosthetic group for ^{18}F-labeling of sensitive biomolecules under very mild conditions (25 °C, phosphate-buffered saline (PBS, pH 7.4), 5 min). An exemplary radio-HPLC chromatogram of [^{18}F]**13** in comparison to the ^{18}F-prosthetic group [^{18}F]**11** is shown in the ESI.‡

Furthermore, an azido-functionalized folate derivative as a well-known tumor targeting vector was ^{18}F-labeled in a copper free click reaction using the new ^{18}F-prostehtic group. Remarkably, quantitative ^{18}F-click labeling was observed after a few minutes at room temperature and a good (low) log$D$ value of 0.6 ± 0.07 was determined for the final ^{18}F-folate. The ^{18}F-labeled folate can be separated from unreacted folate-azide by HPLC and C18 SPE. The stability of the ^{18}F-folate was analyzed in human serum at 37 °C. After 1.0 h and 1.5 h, ≥95% intact ^{18}F-folate was observed. To our best knowledge, this is the first report on a new ^{18}F-folate labeled *via* a copper-free click approach.

Two different azido-functionalized α-MSH analogue peptides, N_3-TEG-Gly-Gly-Nleu-Gly-His-DPhe-Arg-Trp-NH_2 and N_3-TEG-Gly-Gly-Nleu-[Cys-His-DPhe-Arg-Trp-Gly-Cys]-NH_2, with high specificities to the MC1R (melanocortin receptor 1) were prepared by using solid phase peptide synthesis (SPPS).[22] Both peptides were radiolabeled with the ^{18}F-prostehtic group in a copper-free click cycloaddition. For the linear peptide, RCY of up to 79% after 20 min was observed for 0.4 μmol peptide at 40 °C. For the cyclic α-MSH analogue, the click reaction proceeded with excellent RCY of 92% even with a lower amount of only 0.2 μmol peptide at 40 °C.

Summarized conditions, RCY and kinetics for the four biomolecule-azides, which were tested in copper-free click reactions with the new ^{18}F-prosthetic group, are shown in the ESI.‡ The RCYs are also displayed as a bar chart in Scheme 4.

Especially for the radiolabeling of sensitive biomolecules, the use of ^{18}F-prosthetic groups is of particular interest, where the use of harsh conditions for direct ^{18}F-labeling reactions is excluded. Due to the toxicity of copper, the attachment of the ^{18}F-prosthetic groups *via* copper(I)-catalyzed cycloaddition is no longer the first choice. The use of strained alkynes for copper-free cycloaddition enables selective radiolabeling of azido-functionalized biomolecules under very mild conditions.

The herein reported synthesis strategy of a ^{18}F-labeled DBCO-based prosthetic group enables copper-free ^{18}F-click labeling of various biomolecule-azides under very mild conditions and with outstanding efficacy. The organic syntheses provided the two precursors in good to high yields over four steps. The organic syntheses are robust and very reliable, and in reference to DBCO-amine, the strategy and yields were optimized for economic reasons. High to excellent results were obtained for the ^{18}F-labeling of the two different precursors, which are available and ready to use for subsequent ^{18}F-click reactions within only 60 min and in high yields of 34% (n.d.c.).

The new ^{18}F-prosthetic group performs outstandingly in copper-free click reactions with different biomolecule-azides, which are known as excellent tumor targeting vectors of common interest.[16,17,23–25] All click reactions proceeded with excellent to even quantitative yields under very mild conditions (water or PBS, RT or 40 °C) with very fast reaction kinetics. The tested biomolecule-azides (RGD, linear MSH-peptides and folic acid) were not achievable in such good yields with conventional copper(I)-catalyzed click cycloaddition.[11]

In cases of non-quantitative ^{18}F-click labeling, the ^{18}F-labeled products were easily separated by radio-HPLC from the unreacted ^{18}F-prostehtic group. For the ^{18}F-labeled folate derivative, a low log$D$ value of 0.6 ± 0.07 was determined. High stability was observed in human serum at 37 °C over a period of 1.5 h. Further *in vitro* and *in vivo* evaluation of the new ^{18}F-folate using human KB cells and PET imaging is ongoing. Similarly, investigations and evaluation using the other new ^{18}F-tracers derived from copper-free ^{18}F-click labeling in *in vivo* PET imaging are planned.

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