# Synthesis of alkyne-modified bleomycin disaccharide precursor, its conversion to a $^{18}$F-labeled radiotracer and preliminary in vivo-PET imaging studies

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**Abstract:** The bleomycins (BLMs) are known antitumor antibiotics composed of the tumoricidal and tumor seeking domains. The peptide structure of BLMs is responsible for the cytotoxicity by selective oxidative cleavage of DNA (and RNA), while the tumor cell selectivity and internalization resides in the disaccharide moiety (*i.e.* BLM disaccharide). This has prompted researchers to utilize BLM disaccharide and its derivatives as constituents for the selective recognition of tumor cells, which may find further applications as new tumor imaging tools or drug delivery vehicles. In the present study a high yielding synthesis of an alkyne modified BLM disaccharide precursor that may be used as a useful agent for the click conjugation, its conversion to a $^{18}$F-labeled radiotracer, and preliminary in vivo PET imaging studies of the tracer with breast cancer (MCF-7) xenograft mouse models are described.

## Introduction

2-O-(3-carbamoyl-α-D-mannopyranosyl)-L-gulose (i.e. BLM disaccharide) and its importance for the tumor cell selectivity and internalization of bleomycins (BLMs, antitumor antibiotics used clinically against squamous cell carcinomas and malignant lymphomas) have extensively been studied by Hecht.[1-6] Accordingly, BLM and BLM disaccharide, but not deglycosylated BLM, show binding/uptake to several cancer cells in vitro (breast MCF-7, prostate DU-145, pancreatic BxPC3, C6 rat glioma, SW1088 human brain cancer and SW480 human colon adenocarcinoma cells), whereas no binding has been observed with the corresponding ‘’normal’’ cell lines.1-3 The detailed mechanism of the selective uptake is still unknown, but it has been suggested that the binding is receptor-mediated and the metabolic shift to glycolysis in cancer cells provides vehicle for the improved internalization.[3] An increased binding with clustered BLM disaccharides and the carbamoyl mannose has demonstrated that multivalency takes a place on this receptor-mediated binding.[5,6] It has also been observed that the site of the carbamoyl group on the disaccharide core is crucial for the recognition and the carbamoyl mannose constituent and its presentation to the cells may be sufficient for the selective and efficient cellular uptake.[4-6] Even improved binding/uptake has been observed with modified BLM disaccharide analogs, in which the site and structure ($NH_2$ vs. NHMe) of the carbamoyl group on the sugar core has been varied (Figure 1).[5]

In the present study a straightforward and high yielding synthesis of an alkyne modified BLM disaccharide precursor is described, which may be used as a useful agent for the preparation of BLM disaccharide conjugates with azide-modified cargos via click reaction.[7] In order to evaluate the in vivo delivery potency of the BLM disaccharide alone, the precursor was converted (via a global deprotection and subsequent Cu(I)-catalyzed click reaction with 1-azido-2-(2-(2-(2-[18F]fluoroethoxy)ethoxy)ethoxy)ethane8-10) to a $^{18}$F-labelled BLM disaccharide, and its applicability for in vivo PET-imaging studies in a breast cancer (MCF-7) xenograft mouse model was evaluated.

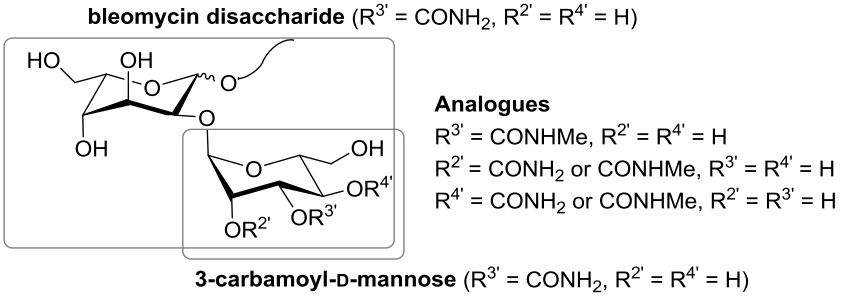


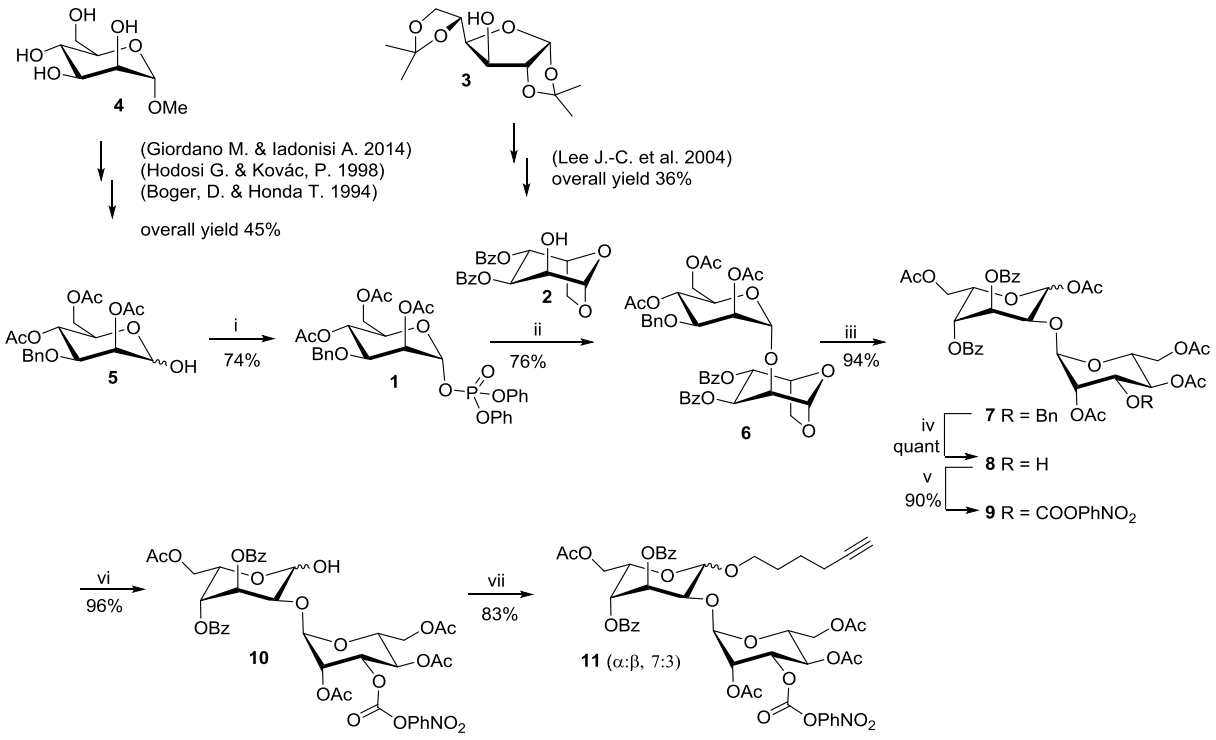

Figure 1. Bleomycin disaccharide, its constituent: 3-carbamoyl-D-mannose, and 2'-, 4'-carbamoyl analogues, studied by Hecht.[1-6]

Results and Discussion

Synthesis of the alkyne modified bleomycin disaccharide. The total synthesis of 2-O-(α-D-mannopyranosyl)-L-gulose has been described for the first time almost four decades ago by Tsuchia et al.[11] This procedure was based on preparation of 6-azido-5-(α-D-mannopyranosyl)-glucofuranoside precursor that via reduction by a $NaBH_4$ treatment and a photolytic azide/carbonyl-conversion gave the desired reconfigured 2-O-mannopyranosyl substituted L-gulo sugar (*i.e.* BLM disaccharide without the carbamoyl group). Since then improvements to produce BLM disaccharide have been reported by several research groups. Boger and Honda prepared 1,3,4,6-tetra-O-benzyl L-gulose glycosyl acceptor from benzyl-α-D-mannopyranoside, via inversion of C5 stereochemistry, and condensed it with 2,4,6-tri-O-acetyl-3-O-carbamoyl-α-D-mannopyranosyl diphenyl phosphate to give the fully protected BLM disaccharide in 93% yield.12 Hecht et al. glycosylated 1,3,4,6-tetra-O-acetyl L-gulose with 2,4,6-tri-O-acetyl-3-O-benzyl-α-D-mannopyranosyl diphenyl phosphate (**1**) in 62% yield.[5] The 3-O-benzyl group of the mannose unit was removed and the carbamoyl group was introduced to the exposed hydroxyl group to give the desired protected BLM disaccharide. An alternative glycosyl acceptor strategy was reported by Lee J.-C. et al.[13]: 1,6-Anhydro-3,4-di-O-benzoyl-β-L-gulopyranoside (**2**) was prepared from diacetone-α-D-glucose (**3**) and condensed with 6-O-acetyl-2,4-di-O-benzoyl-3-O-[(p-nitrophenyl)formyl]-α-D-mannopyranosyl trichloroacetimidate in 82% yield. The α-linked disaccharide obtained was exposed to a $Cu(OTf)_2$-catalyzed acetolysis and gentle treatment with ammonia gave the desired protected BLM disaccharide as a hemiacetal. The advantage of this procedure was the readily available L-gulo glycosyl acceptor (**2**) that could be synthesized from 3 in a relatively high 36% overall yield (Scheme 1).

The preparation of an alkyne modified and fully protected BLM disaccharide precursor (**11**) in the present study is a combination of the previously reported procedures (Scheme 1). 1,6-Anhydro-3,4-di-O-benzoyl-β-L-gulopyranoside (**2**) and 2,4,6-tri-O-acetyl-3-O-benzyl-α-D-mannopyranosyl diphenyl phosphate (1) were first prepared following the published procedures.12-15 Glycosylation of 2 with 1 in the presence of TMSOTf catalyst gave the desired 2-O-(α-D-mannopyranosyl)-L-gulose (**6**) in 76% yield. The 1,6-anydro linkage was cleaved/acetylated using acetic anhydride in the presence $Cu(OTf)_2$-catalyst (**7**, 94%) and the benzyl protection of the mannose unit was selectively and quantitatively removed by hydrogenolysis using $Pd(OH)_2$ as a catalyst (**8**). The exposed OH group of 8 was converted to the *p*-nitrophenyl carbonyl group (**9**, 90%) and the anomeric acetyl group was selectively removed by subsequent treatments with HBr in AcOH (a glycosyl bromide formation) and AgOTf in wet acetone (**10**, 96%). Condensation between 5-hexyn-1-ol and 10 using an in situ formation of a sulfoxide donor in the presence of trifluoromethanesulfonic anhydride, diphenyl

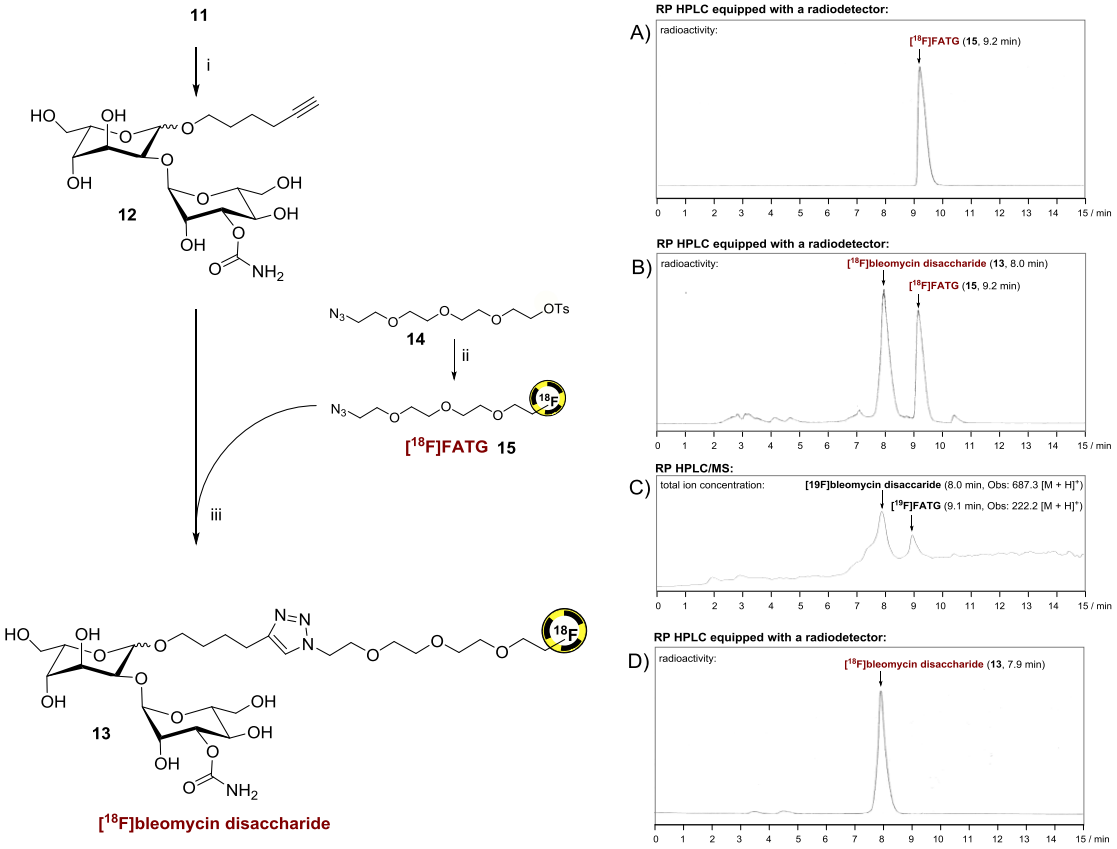
sulfoxide and 2,4,6-tri-tert-butyl pyridine gave **11** in 83% yield with an α/β-ratio of 7:3. Thus the overall yield of **11** from a cheap starting material: diacetone-α-D-glucose (**3**) was as high as 18%. The protecting group scheme of the precursor **11** (bearing the base labile acetyl and benzoyl protections and the p-nitrophenyl carbonyl group) was designed in a manner that the final bleomycin disaccharide structure (**12**) may be readily obtained in a single step using concentrated ammonia.




Scheme 1. Synthesis of alkyne modified BLM disaccharide. Reagents and conditions: i) nBuLi, $(PhO)_2POCl$, THF, -78˚C, 20 min.; ii) TMSOTf, DCM, -20˚C, 20 min.; iii) $Cu(OTf)_2$, $Ac_2O$, rt, 48h; iv) $H_2$, $Pd(OH)_2$, rt, 12h; v) ***p***-nitrophenyl chloroformate, DMAP, pyridine, 40˚C, 10h; vi) 1: 30% HBr-AcOH, $Ac_2O$, DCM; 2: AgOTf, 2,4,6-tri-tert-butyl pyridine, aq. acetone; vii) 5-hexyn-1-ol, $Tf_2O$, $Ph_2SO$, 2,4,6-tri-tert-butyl pyridine, DCM, -60˚C to rt, 4h.

**Synthesis of $^{18}$F-labeled BLM disaccharide**. Synthesis of 18F-labelled BLM disaccharide **13** was accomplished using the procedure outlined in Scheme 2. Prior to the 18F-labeling, precursor **11** (as an anomeric mixture) was quantitatively converted to the alkyne modified BLM disaccharide **12** using 6N ammonia in dry methanol, followed by filtration through a C-18 RP column. The prosthetic agent **15** ([18F]FATG) was prepared by reacting 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl *p*-toluenesulfonate with [18F]fluoride-Kryptofix complex in anhydrous DMSO.[8-10] The obtained 1-azido-2-(2-(2-(2-[18F]fluoroethoxy)ethoxy)ethoxy)ethane ([18F]FATG 15, about 2 GBq), with a radiochemical purity of at least 90 %, was allowed to react with **12** in the presence of pre-activated copper sulfate/sodium ascorbate. The reaction was followed by RP HPLC (C-18, 250 × 10 mm, 10 μm, 90 Å) equipped with a radio detector (Scheme 2B). The retention times of the product (**13**) and starting material (**15**) were additionally compared to a LC-MS data of the corresponding ‘’cold

reaction'' (***i.e***. 1-azido-2-(2-(2-(2-[19F]fluoroethoxy)ethoxy)ethoxy)ethane used for the same reaction, Scheme 2C). The conversion was observed to be optimal at 60 ˚C, with 45 % turnover at 15 min, and it did not increase at longer incubation intervals. Typical yield was about 360 MBq at end of synthesis, with radiochemical purity of 97 %. 13 was isolated by RP HPLC (C-18, 250 × 10 mm, 10 µm, 90 Å), the product fractions were collected, diluted with water, loaded to a RP C18 cartridge, and the product was eluted with ethanol. Before use in biological studies, the homogeneity of 13 was further confirmed by RP HPLC (Scheme 2D). Finally, the homogenized **13** was formulated in phosphate-buffered saline to a maximal ethanol concentration of 10% (v/v). The stability of the tracer (**13**) was additionally evaluated by incubating it in rat serum at 37˚C. According to HPLC analysis (using the same conditions as above, Scheme 2) half-life of ca 0.5 h for the decay was observed.




Scheme 2. Preparation of $^{18}$F-labelled BLM disaccharide. Reagents and conditions: i) 6N $NH^3$ in methanol, 12h at rt.; ii) [18F]fluoride–Kryptofix complex, DMSO, 5 min at 80˚C; iii) CuSO4, sodium ascorbate, water, 15 min. at 60˚C. RP HPLC profiles of A) 15, B) the reaction mixture, C) the corresponding ''cold'' reaction mixture, and D) homogenized 13. RP HPLC conditions: An analytical (C-18, 250 × 5 mm, 10 µm, 90 Å) column, a gradient elution from 5 to 80% acetonitrile in water over 15 min., flow rate 1.0 mL min-1).

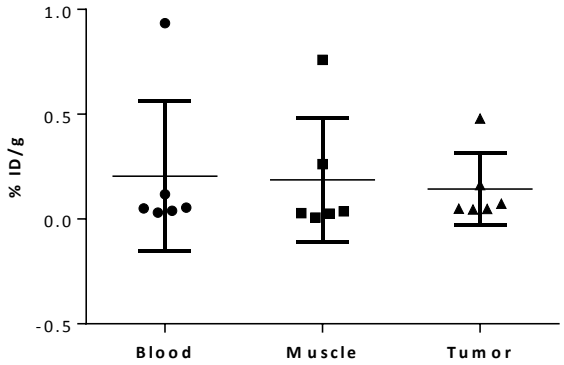



Figure 2. $^{18}$F-labelled BLM disaccharide (**13**) uptake in MCF-7 xenograft bearing mice. The uptake of the tracer into blood, muscle and tumor was determined ex vivo at 120 min. post injection. A relatively low tracer uptake was seen into the tumors. The tumor-to-blood (T/B) and tumor-to-muscle (T/M) ratios were 0.70 and 0.76, respectively. One outlier showed higher, but still low, uptakes in all measured tissues. Exclusion of these measurements resulted in slightly higher T/B and T/M ratios (1.30 and 1.0, respectively). Results are expressed as mean ± SD (n = 6). % ID/g = percentage of injected dose per gram tissue.

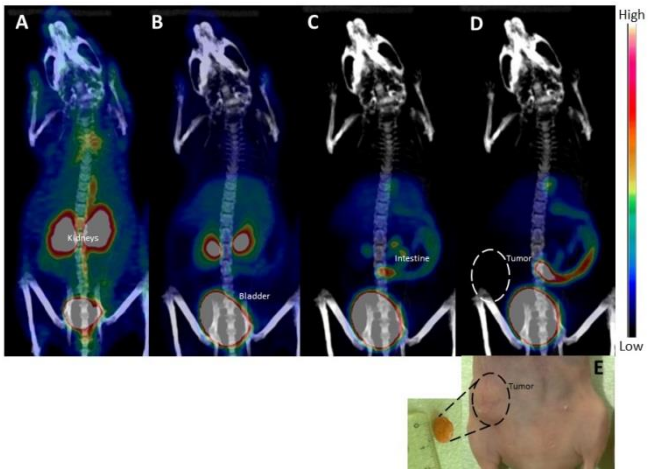



Figure 3. Whole body PET/CT images illustrating the in vivo biodistribution pattern of $^{18}$F-labelled BLM disaccharide (13) in a MCF-7 xenograft bearing mouse as a function of time. Images are summed for time periods of 0 - 5 min (A), 5 - 30 min. (B), 30 - 60 min. (C) and 60 - 120 min. (D). The tracer is rapidly excreted via the renal system (A and B) but also via the hepatobiliary system at later time points (C and D). The uptake of the tracer into the MCF-7 tumor (E) is negligible at all time points (A-D).

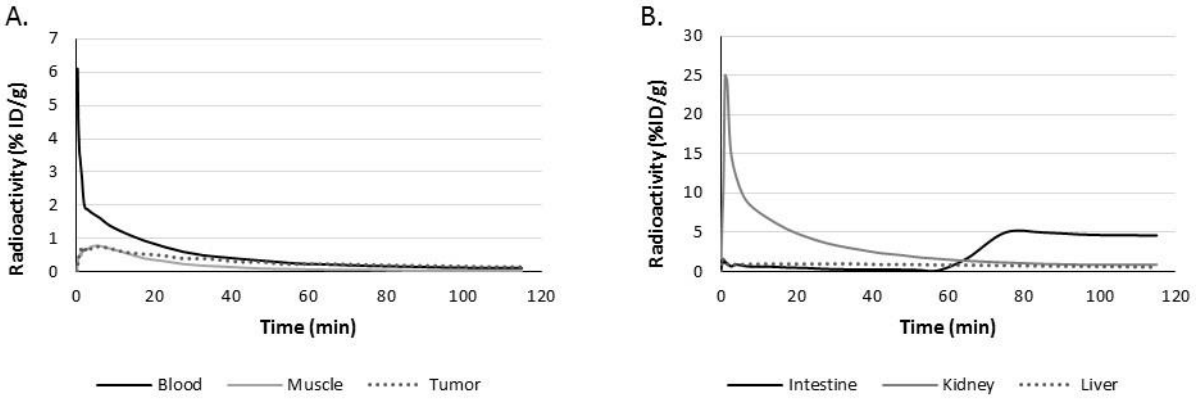



Figure 4. Time-radioactivity curves (TACs) obtained from blood, muscle, tumor (A), intestine, kidney and liver (B) show rapid accumulation of $^{18}$F-labelled BLM disaccharide (**13**) into the kidneys and at later time point also to the intestine. The $^{18}$F-radioactivity uptake into the tumor reached that seen in background tissues, such as blood and muscle. TACs were obtained from a 120-min long dynamic PET scan (Figure 2). The uptake is expressed as percent of the injected dose per gram of tissue (% ID/g).

**Uptake of $^{18}$F-labeled BLM disaccharide into MCF-7 xenograft bearing mice.** In order to evaluate the uptake of 18F-labelled BLM disaccharide (**13**) we choose to use the human breast adenocarcinoma derived MCF-7 cell line.16 Previously, Yu et al.6 showed that BLM selectively targeted MCF-7 cells but not the non-tumorigenic breast epithelium cell line MCF-10A.17 The ex vivo uptake of 18F-labelled BLM disaccharide (13) into MCF-7 xenografts was rather low. The mean ± SD uptake into blood, muscle and tumor was 0.20 ± 0.36, 0.19 ± 0.30 and 0.14 ± 0.17 % ID/g, respectively, at 120 min. after the tracer injection (Figure 1). The tumor-to-blood (T/B) and tumor-to-muscle (T/M) ratios were 0.70 and 0.76, respectively. As visualized in Figure 2 one outlier was detected. The T/B and T/M ratios did increase to 1.30 and 1.05, respectively, when the outlier was eliminated. The corresponding uptake values (mean ± SD) for blood, muscle and tumor was then 0.058 ± 0.034, 0.072 ± 0.107 and 0.075 ± 0.049 % ID/g, respectively. The in vivo dynamic PET/CT scan of a MCF-7 xenograft bearing mouse confirm a negligible uptake of the tracer into the tumor (Figure 3). The highest 18F-radioactivity uptake was detected in the kidneys and the urine bladder immediately after the injection, indicating rapid excretion of $^{18}$F-labelled BLM disaccharide (**13**) via the renal system. After 60 min an increased $^{18}$F-radioactivity uptake was also detected in the intestine. Hence, the tracer is most probably also excreted through the hepatobiliary system. Time-radioactivity curves (TACs) achieved from the PET scan are shown in Figure 4. The tumor uptake in relation to blood and muscle is visualized in panel A, confirming a low background level tumor uptake. Panel B show a high initial uptake into kidney, a steady state uptake into the liver and an increased uptake into the intestine at 60 min post injection.

**Conclusions**

An alkyne modified BLM disaccharide precursor (11) has been synthesized from a cheap starting material, i.e. diacetone-α-D-glucose, in a relatively high 18% overall yield. The precursor may be converted to final bleomycin disaccharide structure in a single step using ammonia in methanol and conjugated with azide-modified cargos using Cu(I)-catalyzed click reaction, being hence a valuable agent for the preparation of new tumor imaging tools or drug delivery vehicles. In order to evaluate the tumor targeting potency of the BLM disaccharide alone in vivo, the precursor was globally deprotected/carbamoylated (**12**), labeled with 1-azido-2-(2-(2-(2-[18F]fluoroethoxy)ethoxy)ethoxy)ethane and the applicability of the BLM disaccharide [18F]radiotracer (**13**) for in vivo PET-imaging studies with breast cancer (MCF-7) xenograft mouse models was evaluated. The $^{18}$F-labelling was optimized in different temperatures and the conversion (from **12** to **13**) was found to be optimal at 60°C, with 45% turnover at 15 min. Radiochemical yield was ca. 360 MBq at the end of the synthesis, with radiochemical purity of 97%. The in vivo dynamic PET/CT scans showed a negligible uptake of the tracer into the tumor and high uptakes in the kidneys and in the urine bladder, which indicated rapid excretion of the tracer via the renal system. Uptake in the intestine demonstrated excretion also through the hepatobiliary system. While notable uptakes with bleomycin disaccharide to breast cancer (MCF-7) cells has previously been observed in vitro, these experiments in vivo demonstrated that BLM disaccharide behaves like most of the small molecular compounds in general, i.e. excreting rapidly via renal clearance. This may partly be related to the premature degradation of the tracer, but suggests that the structural elements of the cargo (like the peptide domain of BLM, cf. previous PET imaging studies[19-24]) expectedly play an important role to gain the desired pharmacokinetic properties and the plausible tumor targeted delivery. Further studies applying BLM disaccaride and its multiplied analogs, paying attention also to the structural requirements of the cargo, are needed to conclude the drug delivery potential of this interesting disaccharide.

## Experimental Section

**General Considerations**. All reaction solvents were dried over molecular sieves. Solid reagents were dried over P2O5 in a vacuum desiccator. The NMR spectra were recorded at 400 or 500 MHz. Chemical shifts are given in ppm using internal TMS or solvent residual signals as reference. Appropriate 1D and 2D NMR methods (e.g., TOCSY, COSY, DEPT and HSQC) were used for peak assignment. The HRMS spectra were recorded using a MS (ESI-TOF) spectrometer. A single quadrupole LC/MS instrument (with the same RP HPLC conditions) was used for the monitoring of the corresponding ‘’cold reaction’’.

**3-O-Benzyl-2,4,6-tri-O-acetyl-α-D-mannopyranosyl diphenyl phosphate (1)**. A solution of n-BuLi (4.4 mL, 11 mmol, 2.5 M in hexane) was added to a stirred solution of 3-O-benzyl-2,4,6-tri-O-acetyl-D-mannopyranose[12,14,15] (3.6 g, 9.1 mmol) in anhydrous THF (27 mL) at -78 °C. The resulting solution was stirred for 10 min at -78 °C before the addition of diphenyl chlorophosphate (2.3 mL, 11 mmol). After stirring for 10 min at -78 °C, the reaction mixture was poured into a two-phase solution of EtOAc (100 mL) and saturated aqueous $NaHCO_3$ (300 mL) with vigorous stirring. The organic layer was collected and washed with brine solution and dried over anhydrous $Na_2SO_4$. The Solution was concentrated in vacuo, and the residue was purified by flash column chromatography (30%

EtOAc-hexane in the presence of 5% $Et_3N$) to afford 1 (4.2 g, 74%) as glassy syrup. The NMR data are in accordance with the reported data.

**2-O-(2,4,6-tri-O-Acetyl-3-O-benzyl-α-D-mannopyranosyl)-3,4-di-O-benzoyl-1,6-anhydro-β-L-gulopyranose (6).** A solution of 1 (2.2 g, 3.5 mmol) and 3,4-di-O-benzoyl-1,6-anhydro-β-L-gulopyranose[13] (2, 1.1 g, 2.9 mmol) in dry $CH_2Cl_2$ (35 mL) was treated with cold solution of TMSOTf (0.83 mL, 3.8 mmol) in dry $CH_2Cl_2$ (5 mL) at -20 °C under argon atmosphere. The reaction mixture was stirred at -20 °C for 20 min and then poured into a two-phase solution of EtOAc (50 mL) and saturated aqueous $NaHCO_3$ (100 mL) with vigorous stirring. The organic layer was collected, and washed with saturated aqueous NaCl (100 mL). Solvents were dried over anhydrous $Na_2SO_4$, and concentrated in vacuo. Flash chromatography (25% EtOAc-hexane) of crude residue gave 6 (1.5 g, 76%) as a colorless syrup. $^1$H NMR (500 MHz, $CDCl_3$) δ 2.00 (s, 3 H), 2.04 (s, 3 H), 2.08 (s, 3 H), 3.78 (dd, 1H, J = 5.0, 8.0 Hz), 3.96 (dd, 1H, J = 3.0, 9.5 Hz), 4.13-4.16 (m, 2 H), 4.21 (dd, 1H, J = 7.0, 12.5 Hz), 4.25 (d, 1 H, J = 8.5 Hz), 4.28 (dd, 1H, J = 2.0, 4.0 Hz), 4.41 (d, 1H, J = 11.5 Hz), 4.60 (d, 1H, J = 12.0 Hz), 4.84 (dd, 1H, J = 4.0, 3.5 Hz), 4.89 (d, 1H, J = 1.5 Hz), 5.19 (dd, 1H, J = 9.5, 10.0 Hz), 5.35 (dd, 1H, J = 2.0, 3.0 Hz), 5.60-5.63 (m, 2H), 5.65 (d, 1H, J = 2.0 Hz), 7.26-7.44 (m, 9 H), 7.53-7.57 (m, 2 H), 8.0-8.02 (m, 4 H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ 20.7, 20.82, 20.84, 62.9, 64.5, 67.3, 67.9, 69.0, 69.6, 70.1, 71.7, 72.3, 74.6, 76.7, 99.7 (C-1', $J_{C-H}$ = 175.0 Hz), 100.5 (C-1, $J_{C-H}$ = 179.2 Hz), 127.9, 128.0, 128.3, 128.4, 128.6, 128.7, 128.97, 129.0, 129.3, 129.6, 129.83, 129.84, 130.5, 133.1, 133.6, 133.7, 134.3, 137.6, 165.5, 165.6, 169.7, 169.8, 170.6; HRMS calcd for $C_{39}H_{40}O_{15}Na$ [M + Na]+: 771.2265, found: 771.2266.

**2-O-(2,4,6-tri-O-Acetyl-3-O-benzyl-α-D-mannopyranosyl)-3,4-di-O-benzoyl-6-O-acetyl-α/β-L-gulopyranosyl acetate (7).** To a stirred solution of 6 (0.50 g, 0.67 mmol) in acetic anhydride (6.5 mL) was added $Cu(OTf)_2$ (25 mg, 0.067 mmol) at room temperature under Ar. After 48 h, the reaction was quenched with triethylamine and reaction mixture was co-evaporated with toluene under reduced pressure. The residue was purified by flash column chromatography (35% EtOAc-hexane) to provide 7 (0.54 g, 94%, a mixture of β/α (2:1, n/n)-L-gulopyranosyl anomers) as white foam.$^1$H NMR (400 MHz, $CDCl_3$) δ 1.92 (s, 3H), 1.99 and 2.07 (2 × s, 3H), 2.02 (s, 3H), 2.08 (s, 3H), 2.12 (s, 3H), 3.44 and 3.57 (dd, J = 3.6, 9.6 Hz and dd, J = 3.2, 9.6 Hz, 1H), 3.85 and 3.92 (2 × m, 1H), 4.04-4.33 (m, 5 H), 4.47 and 4.50 (2 × d, 1H, J = 12.2 Hz, both), 4.65 and 4.75 (2 × m, 1H), 5.05 – 5.17 (m, 2H), 5.19-5.22 (m, 1H), 5.44 and 5.48 (dd, J = 1.2, 4.0 Hz and bd, J = 3.6 Hz, 1H), 5.79 and 5.89 (dd, J = 3.6 Hz, both and dd, J = 3.6 Hz, both, 1H), 6.05 and 6.36 (d, J = 8.4 Hz and d, J = 4.4 Hz, 1H, H1β and H1α, 2:1, n/n), 6.93 – 7.15 (m, 3H), 7.21 – 7.23 (m, 2H), 7.47 – 7.72 (m, 6H), 8.07 (m, 2H), 8.14 – 8.26 (m, 2H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 20.66, 20.7, 20.8, 20.83, 20.85, 20.9, 21.0, 61.88, 61.91, 62.53, 62.56, 64.8, 66.1, 66.5, 66.9, 66.95, 67.1, 67.2, 68.3, 68.4, 69.5, 69.8, 70.2, 70.7, 71.1, 71.9, 73.1, 73.7, 89.5, 91.0, 95.6, 96.9, 127.6, 127.7, 127.8, 128.0, 128.2, 128.3, 128.6, 128.66, 128.7, 128.74, 128.8, 129.3, 129.94, 129.98, 130.1, 130.13, 133.8, 133.93, 133.98, 137.4, 164.79, 164.81, 164.88, 164.9, 168.8, 169.1, 169.5, 169.63, 169.66, 169.7, 170.4, 170.6; HRMS calcd for $C_{43}H_{46}O_{18}Na$ [M + Na]+: 873.2582, found: 873.2540.

**2-O-(2,4,6-tri-O-Acetyl-α-D-mannopyranosyl)-3,4-di-O-benzoyl-6-O-acetyl-α/β-L-gulopyranosyl acetate (8).** A suspension of 7 (0.53 g, 0.62 mmol), and $Pd(OH)_2$ (50 mg) in ethylacetate (10 mL) was stirred under $H_2$ for 12 h. The reaction mixture was filtered through a short bed of Celite, eluting with ethylacetate. The filtrate was evaporated to dryness and purified by flash column chromatography (50% EtOAc-hexane) to afford 8 (0.47 g, quant., a mixture of β/α (2:1, n/n)-L-gulopyranosyl anomers) as a white foam. $^1$H NMR (500 MHz, $CDCl_3$) δ 1.98, 2.00, 2.05, 2.07, 2.10, 2.10, 2.11, 2.17 (8 × s, 15H), 2.34 (bs, 1 H), 3.79 and 3.86 (dd, J = 3.0, 4.5 Hz and dd, J = 3.0, 5.0 Hz, 1H), 3.94 and 4.00 (2 × m, 1H), 4.06-4.35 (m, 5H), 4.61 and 4.74 (dd, 1H, J = 5.5 Hz, both and dd, J = 6.2, 6.3 Hz,

1H), 4.92-5.01 (m, 2H), 5.08 and 5.11 (2 × bs, 1H), 5.43 and 5.46 (d, J = 2.9 Hz and d, J = 3.2 Hz, 1H), 5.76 and 5.87 (dd, J = 3.1 Hz, both and dd, J = 3.6 Hz, both, 1H), 6.08 and 6.40 (d, J = 8.0 Hz and d, J = 4.0 Hz, 1H, H1β and H1α, 2:1, n/n), 7.45 – 7.51 (m, 4H), 7.60 – 7.62 (m, 2H), 8.04 – 8.17 (m, 4H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ 20.65, 20.69, 20.8, 20.9, 21.1, 61.9, 62.38, 62.41, 64.6, 66.2, 68.1, 68.1, 68.28, 68.30, 68.90, 68.94, 69.0, 69.3, 70.2, 71.3, 71.4, 71.8, 77.3, 89.6, 91.0, 95.1, 96.1, 128.6, 128.65, 128.69, 128.73, 128.76, 129.1, 129.93, 129.97, 130.0, 133.7, 133.9, 134.0, 164.70, 164.72, 164.86, 164.9, 168.9, 169.3, 170.0, 170.1, 170.5, 170.59, 170.62, 170.8, 171.2; HRMS calcd for $C_{36}H_{40}O_{18}K$ [M + K]+: 799.1852, found: 799.1833.

**2-O-[2,4,6-tri-O-Acetyl-3-O-(p-nitrophenylformyl)-α-D-mannopyranosyl)]-3,4-di-O-benzoyl-6-O-acetyl-L-gulopyranosyl acetate (9).** To a solution of **8** (1.4 g, 1.2 mmol) in pyridine (30 mL) was added N,N-dimethylamino pyridine (0.58 g, 4.7 mmol ) and p-nitrophenyl chloroformate (0.95 g, 4.7 mmol). The reaction mixture was stirred at 40 °C for 10 h and poured into a two-phase solution of EtOAc (50 mL) and $H_2O$ (50 mL) with vigorous stirring. The organic layer was subsequently washed with 2 N aqueous HCl (2 × 20 mL), saturated aqueous $NaHCO_3$ (50 mL), and saturated aqueous NaCl (50 mL). Organic layer was dried over anhydrous $Na_2SO_4$, and concentrated in vacuo. Flash column chromatographic (40% EtOAc-hexane) purification of residue provided 9 (1.51 g, 90%, a mixture of β/α (3:1, n/n)-L-gulopyranosyl anomers) as colorless syrup.$^1$H NMR (400 MHz, $CDCl_3$) δ 1.99, 2.00, 2.02, 2.02, 2.06, 2.09, 2.09, 2.10, 2.14, 2.18 (10 × s, 15H), 4.06-4.38 (m, 6H), 4. 63 and 4.81 (ddd, J = 6.3, 6.2, 0.9 Hz and dd, J = 6.5, 6.4 Hz, 1H), 4.85 and 4.98 (dd, J = 10.1, 3.2 Hz and dd, J = 10.0, 3.4 Hz, 1H), 5. 15 and 5.17 (d, J = 1.5 Hz and d, 1H, J = 1.6 Hz, 1H), 5.27-5.34 (m, 2H), 5.44 and 5.00 (dd, J = 1.2, 4.0 Hz and d, J = 3.6 Hz, 1H), 5.76 and 5.88 (dd, J = 3.6 Hz, both and dd, 1H, J = 3.6 Hz, both, 1H), 6.07 and 6.46 (d, J = 8.4 Hz and d, J = 4.4 Hz, 1H, H1β and H1α, 3:1, n/n), 7.27-7.33 (m, 2H), 7.43-7.50 (m, 4 H), 7.58-7.65 (m, 2H), 8.06-8.15 (m, 4 H), 8.20 – 8.24 (m, 2H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 20.62, 20.65, 20.67, 20.7, 20.8, 20.9, 21.0, 60.4, 61.8, 62.0, 62.1, 62.2, 64.2, 65.1, 65.6, 66.2, 66.4, 67.4, 67.9, 68.0, 68.3, 68.4, 69.3, 69.7, 70.3, 71.9, 74.1, 74.2, 77.3, 89.5, 90.9, 95.2, 95.7, 121.85, 121.87, 125.14, 125.18, 128.5, 128.6, 128.62, 128.66, 128.71, 128.74, 128.9, 129.92, 129.95, 130.1, 133.8, 133.9, 134.0, 145.48, 148.53, 151.17, 151.22, 155.3, 155.4, 164.9, 165.1, 165.2, 168.7, 169.5, 169.6, 169.7, 169.8, 170.4, 170.44, 170.48; HRMS calcd for $C_{43}H_{43}NO_{22}Na$ [M + Na]+: 948.2174, found: 948.2168.

**2-O-[2,4,6-tri-O-Acetyl-3-O-(p-nitrophenylformyl)-α-D-mannopyranosyl)]-3,4-di-O-benzoyl-6-O-acetyl-L-gulopyranose (10).** To a stirred solution of 9 (0.49 g, 0.53 mmol) and acetic anhydride (200 µL) in dry $CH_2Cl_2$ (10 mL) was added 30% solution of HBr in AcOH (1.5 mL) at 0 °C under nitrogen. The reaction mixture was stirred for 24 h at room temperature and poured then into ice-water. The aqueous portion was extracted with EtOAc. The combined organic layers were washed with saturated aqueous $NaHCO_3$ and brine, dried over $Na_2SO_4$, filtered and concentrated in vacuo. The crude residue was directly used for the next step. To a solution of crude product intermediate in aqueous (0.5% H2O) acetone 2,4,6-tri-tert-butyl pyrimidine (0.20 g, 0.79 mmol) and AgOTf (0.15 g, 0.58 mmol) were added. After stirring for 10 min. at ambient temperature, triethylamine was added. The reaction mixture was filtered through a Celite bed, and the bed was washed thoroughly with acetone. The filtrate was evaporated to dryness and purified by flash column chromatography (50% EtOAc-hexane) to furnish 10 (0.45 g, 96%, a mixture of β/α (1:13, n/n)-L-gulopyranosyl anomers)) as colorless syrup.$^1$H NMR (500 MHz, $CDCl_3$) δ 1.99, 2.00, 2.03, 2.04, 2.09, 2.12, 2.14 (7 × s, 12H), 4.00 (dd, 1H, J = 3.5, 8.0 Hz), 4.08-4.31 (m, 5H), 4.38-4.41 (m, 1H), 4.51 and 4.80 (dd, 1H, J = 6.2, 6.1Hz and dd, J = 5.8, 5.7Hz, 1H), 5.05 (dd, 1H, J = 3.5, 10.0 Hz), 5.12 and 5.14 (2 × s, 1H), 5.21 (dd, 1H, J = 6.0, 7.5 Hz), 5.29-5.33 (m, 2 H), 5.39 and 5.47 (d, J = 2.9 Hz and d, J = 3.7 Hz, 1H), 5.77 and 5.81 (dd, J = 3.4, 3.5 Hz and d, J = 3.5, 3.4 Hz, 1H), 7.23-7.32 (m, 2H), 7.39-7.48 (m, 4H), 7.56-7.63

(m, 2H), 8.03-8.09 (m, 4H), 8.21-8.26 (m, 2H); $^{13}$C NMR (125 MHz, CDCl3) δ 20.72, 20.74, 20.8, 29.6, 29.7, 62.2, 62.4, 65.6, 66.6, 68.1, 68.7, 68.8, 71.0, 73.1, 74.5, 77.3, 93.4, 95.6, 145.5, 151.3, 155.4, 165.0, 165.1, 169.7, 169.9, 170.7, 171.0; HRMS calcd for $C_{41}H_{41}NO_{21}Na$ [M + Na]+: 906.2069, found: 906.2055.

**5-Hexyn-1-yl-2-O-[2,4,6-tri-O-acetyl-3-O-(p-nitrophenylformyl)-α-D-mannopyranosyl)]-3,4-di-O-benzoyl-6-O-acetyl-α/β-L-gulopyranoside (11).** Trifluoromethanesulfonic anhydride (0.12 mL, 0.69 mmol) was added to a suspension of 10 (0.44 g, 0.50 mmol), diphenyl sulfoxide (0.28 g, 1.4 mmol), 2,4,6-tri-tert-butyl pyrimidine (0.37 g, 1.5 mmol) and 4 Å molecular sieves in dichloromethane (10 mL) at -70 °C under argon. The reaction mixture was stirred at this temperature for 5 min and then at -40 °C for 1.5 h. 5-hexyn-1-ol (66 µL, 0.60 mmol) was added at -40 °C. The solution was stirred at this temperature for 1 h, and then temperature was allowed to increase slowly to room temperature during 3 h. The reaction was quenched with an excess of triethylamine and filtered through a Celite bed. The bed was washed with dichloromethane (20 mL) and the filtrate was washed sequentially with saturated aqueous solution of sodium bicarbonate solution (50 mL) and sodium chloride (50 mL). The organic layer was dried over sodium sulfate and concentrated. The residue was purified by silica gel flash column chromatography (36% EtOAc-hexane) to afford first an anomeric mixture (α/β ≈ 3:2, n/n) (11) that could be re-purified (several times using the same eluent system) to give finally α-anomer (0.28 g, 58%) as a white foam and β-anomer (0.18 g, 25%) as colorless syrup. The α/β-anomeric configuration of the products was verified by C1-H1 coupling constants according to Bock and Pedersen.[25] α-anomer:$^{1}$H NMR (500 MHz, $CDCl_3$) δ 1.65-1.69 (m, 2 H), 1.83-1.87 (m, 2 H), 1.95 (t, 1H, J = 3.0 Hz), 2.02 (s, 3 H), 2.07 (s, 3 H), 2.11 (s, 3 H), 2.13 (s, 3 H), 2.19-2.23 (m, 2 H), 3.53 (m, 1H), 3.95 (m, 1H), 4.07-4.13 (m, 2 H), 4.20-4.23 (m, 2 H), 4.28 (dd, 1H, J = 7.5, 11.5 Hz), 4.30 (dd, 1H, J = 5.0, 12.5 Hz), 4.67 (m, 1 H), 5.03 (dd, 1H, J = 3.0, 10.0 Hz), 5.06 (d, 1H, J = 4.0 Hz), 5.15 (d, 1H, J = 2.0 Hz), 5.30 (dd, 1H, J = 10.0 Hz, both), 5.37 (dd, 1H, J = 1.5, 3.0 Hz), 5.43 (dd, 1H, J = 1.5, 3.5 Hz), 5.69 (dd, 1H, J = 3.5 Hz, both), 7.30-7.34 (m, 2 H), 7.43-7.50 (m, 4 H), 7.56-7.64 (m, 2 H), 8.06-8.08 (m, 2H), 8.12-8.14 (m, 2H), 8.22-8.26 (m, 2 H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ18.1, 20.74, 20.75, 20.77, 20.78, 25.12, 28.70,62.3, 62.5, 64.3, 65.7, 66.3, 68.37, 68.40, 68.9, 9.1, 69.2, 70.8, 71.2, 74.2, 77.3, 84.0, 97.1 (C-1', $J_{C-H}$ = 175.1 Hz), 97.4 (C-1, $J_{C-H}$ = 169.3 Hz), 121.8, 125.2, 128.3, 128.7, 128.9, 129.4, 129.9, 130.2, 133.4, 133.8, 145.5, 151.1, 155.3, 165.0, 165.4, 169.6, 169.7, 170.48, 170.54; HRMS calcd for $C_{47}H_{49}NO_{21}Na$ (M + Na)+: 986.2695, found: 986.2648. β-anomer: $^{1}$H NMR (500 MHz, $CDCl_3$) δ 1.62-1.67 (m, 2 H), 1.72-184 (m, 2 H), 1.95 (t, 1H, J = 3.0 Hz), 2.04 (s, 3 H), 2.09 (s, 3 H), 2.10 (s, 3 H), 2.16 (s, 3 H), 2.24 (m, 2H), 3.60 (m, 1H), 4.03 (m, 1H), 4.05 (dd, 1H, J = 3.5, 7.5 Hz), 4.16 (dd, 1H, J = 2.0, 12.5 Hz), 4.24 (dd, 1H, J = 6.0, 11.5 Hz), 4.30 (dd, 1H, J = 4.5, 12.5 Hz), 4.33 (dd, 1H, J = 7.0, 12.0 Hz), 4.40 (m, 1 H), 4.47 (m, 1 H), 4.85 (d, 1H, J = 7.5 Hz), 5.06 (dd, 1H, J = 3.5, 10.0 Hz), 5.14 (d, 1H, J = 2.0 Hz), 5.30 (dd, 1H, J = 1.5, 3.5 Hz), 5.35 (dd, 1H, J = 10.0 Hz, both), 5.38 (dd, 1H, J = 1.5, 4.0 Hz), 5.79 (dd, 1H, J = 3.5 Hz, both), 7.31-7.35 (m, 2 H), 7.42-7.50 (m, 4H), 7.60-7.66 (m, 2H), 8.06-8.12 (m, 4H), 8.23-8.31 (m, 2H); $^{13}$C NMR (125 MHz, CDCl3) δ 18.2, 20.74, 20.77, 20.8, 25.0, 28.7, 61.8, 62.1, 65.4, 66.2, 68.0, 68.6, 68.7, 68.9, 69.4, 70.78, 70.81, 74.7, 83.9, 94.8 (C-1', $J_{C-H}$ = 175.6 Hz), 99.2 (C-1, $J_{C-H}$ = 161.4 Hz), 121.9, 125.2, 128.6, 128.7, 128.9, 130.0, 130.03, 133.8, 133.9, 145.5, 151.4, 155.4, 165.07, 165.10, 169.6, 169.7, 170.5, 170.6; HRMS calcd for $C_{47}H_{49}NO_{21}Na$ (M + Na)+: 986.2695, found: 986.2648.

**Synthesis of [18F]bleomycin disaccharide (13).** Prior to radiosynthesis, global deprotection/carbamoyl formation of 11 to gain 12 was carried out as follows: 7N solution of ammonia in methanol (8 mL) was added to a solution of 11 (53 mg, 55 µmol, as an anomeric mixture α/β ≈ 3:2, n/n) in methanol (1.0 mL). The mixture was stirred for 12 h at rt. and evaporated to dryness.

The residue was dissolved in water and eluted through a short RP C-18 column using water-acetonitrile (7:3, v/v) to give quantitatively (25 mg) of 12 as colorless sticky oil (HRMS: 488.1744 calcd for $C_{19}H_{31}NO_{12}Na$ [M + Na]+, found 488.1725). Radiosynthesis was performed following previously described protocols with slight modifications.[8-10] In brief, 18F-fluoride was produced using the $^{18}O$ (p,n)$^{18}F$ nuclear reaction on $^{18}O$-enriched water (98 % isotopic pure, Rotem Industries Ltd, Israel). Following irradiation with 17 MeV protons of 10 µA beam current using the MGC-20 cyclotron (NIIEFA, Russia), the aqueous solution of $^{18}F$-fluoride (800 µL, 6-7 GBq) was collected into a borosilicate glass reaction vessel containing 1.7 mg potassium carbonate and 3.8 mg Kryptofix® 222. After evaporation of water, 0.7 mg (1.9 µmol) tosylate precursor (14) dissolved in 500 µL DMSO (4 Å) was added to $^{18}F$-fluoride–Kryptofix complex. The reaction mixture was heated to 80 °C for 5 min, and the resulting amber colored solution was diluted with 1.5 mL water before application on Jupiter C12 (10 µm, 90 Å, 250 x 10 mm) from Phenomenex (Værløse, Denmark). The $^{18}F$-fluorinated prosthetic agent **15** ([18F]FATG) was isolated following preparative RP-HPLC using a methanol gradient in water with a flow rate of 5.0 mL/min. A Hitachi LaChrom 7000 pump (Merck, Germany) was used and radioactivity was monitored using a NaI(Tl) scintillation detector (Bicron Corporation, USA). The collected peak fraction was diluted in 20 mL water and concentrated on Oasis HLB (30 mg) SPE cartridge (Waters, USA). **15** was eluted with 250 µL THF and collected into a Protein LoBind tube (Eppendorf, Germany) containing 80 µg (172 nmol) 12 in 80 µL water. To this solution, freshly prepared aqueous catalyst mixture containing 0.6 µg copper(II) sulfate (pentahydrate) and 2.4 µg sodium ascorbate was added. The reaction proceeded for 15 min at 60 °C, and subsequently diluted with 1.5 mL water. Facilitated by a column switcher, the product [18F]bleomycin disaccharide **13** was isolated on a second Jupiter C12 (10 µm, 90 Å, 250 x 10 mm) using acetonitrile gradient in water with a flow rate of 5.0 mL/min. The collected fraction of 13 was diluted with 20 mL water, concentrated on Sep-Pak light C18 cartridge (Waters, USA), and subsequently eluted with 0.5 mL ethanol. Product was characterized by RP-HPLC using a Phenomenex Luna C18 column. Before use in biological studies, the end product was formulated in phosphate-buffered saline to a maximal ethanol concentration of 10 % v/v.

**Cell lines and tumor model.** Human ER+ MCF-7 breast cancer cells were cultured in RPMI, supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine, penicillin/streptomycin (Gibco®, by Life Technologies, MA, USA) and 1 µg/ml insulin (Sigma-Aldrich Chemie GMBH, Schnelldorf, Germany). Cell cultures were maintained in incubators at 37°C and 5% CO2 / 95% air. Female BALB/c nu/nu mice (n = 6, 22.5 ± 1.08 g, 3-4 weeks old at arrival) were purchased from Janvier Labs (Cedex, France). The mice were housed in sterile cages in a temperature-controlled room with 12-h light/12-h dark schedule and were fed with autoclaved chow and water ad libitum. After 1 week of acclimatization, 5 x 106 MCF-7 cells were injected bilaterally into the fourth mammary fat pad under isoflurane anesthesia. A 60-day release 17β-estradiol pellet (0.72 mg/pellet, Innovative Research of America, Sarasota, FL) was subcutaneously inserted between the scapulae of each mice.

**Uptake of [18F]bleomycin disaccharide radiotracer (13) into tumor bearing mice**. At 9 weeks after implantation mice were intravenously injected via the tail vein with 13 (4.98 ± 0.38 MBq) followed by 100 µl of 0.9% NaCl to flush the catheter. One mice was positioned in a PET/CT scanner (Siemens Medical Solutions Inc, Knoxville, TN, USA) for an 8 minute CT acquisition and a 120 min long dynamic PET list mode scan (energy window of 350 – 650 keV). Immediately after the scan the mice, as well as the remaining 5 mice, were euthanized (120 min post injection) and the absolute **18**F-radioactivity uptake was determined from blood, muscle and tumor using a well counter (3”× 3” NaI (Tl) crystal, Bicron 3MW3/3P; Bicron Inc. OH, USA). Tissues were weighed, counted for radioactivity, and corrected for background radioactivity and radioactivity decay. The quantity of

radioactivity was expressed as the percentage of injected dose per gram of tissue (% ID/g). PET data was divided into 31 time frames (6 ×10 s, 4 × 15 s, 2 × 30 s, 2 × 120 s, 1 × 180 s, 10 × 300 s and 6 × 600 s) and reconstructed with use of 3D-OSEM. The voxel size was 0.8 × 0.8 × 0.79 mm3. During the scan, the body temperature of the mouse was kept at 37ºC with a heating pad on the scanner bed. Images were analyzed using the Inveon Research Workplace software (Siemens Medical Solutions). Regions of interest were drawn manually around the left cardiac ventricle (blood), liver, kidney, intestine, muscle and tumor. The uptake of 13 was expressed as time-activity curves (TACs).

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**Notes**

The project followed the guidelines of good scientific practice set by the National Advisory Board on Research Ethics in Finland. Animals were cared for in accordance with the Directives 2012/707/EU, 2014/11/EU Act 497/2013 and Decree 564/2013 and of the European Parliament and of the Council for the Care and Use of Laboratory Animals. License ESAVI2329/04.10.07/2017 was obtained from the State Provincial Office of Finland, and it covered all animal procedures within this project.

**Keywords:** bleomycin • bleomycin disaccharide • carbohydrate synthesis • PET imaging • 18F radiotracer

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