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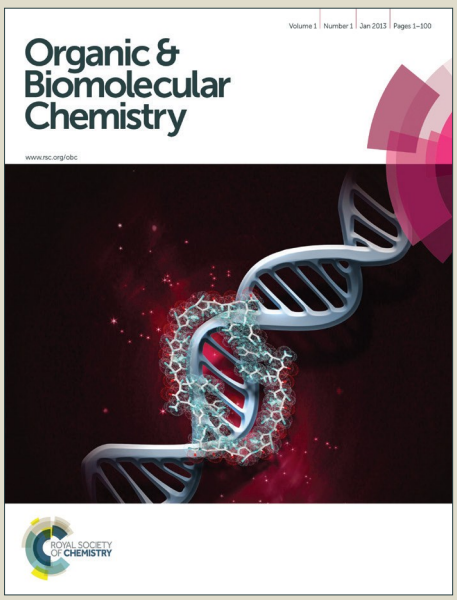

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Synthesis, Radiolabeling with Fluorine-18 and Preliminary *in vivo* Evaluation of a Heparan Sulphate Mimetic as Potent Angiogenesis and Heparanase Inhibitor for Cancer Applications

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Abstract

Heparan Sulfate (HS) mimetics are able to block crucial interactions of the components of the extracellular matrix in angiogenic processes and as such, represent a valuable class of original candidates for cancer therapy. Here we first report the synthesis and *in vitro* angiogenic inhibition properties of a conjugated, novel and rationally-designed octasaccharide-based HS mimetic. We also herein report its labeling with fluorine-18 and present the preliminary *in vivo* Positron Emission Tomography imaging data in rats. This constitutes one of the rare examples of labeling and *in vivo* evaluation of a synthetic, polysaccharide-based, macromolecule.

Communication

Heparan sulfates (HS) are glycosaminoglycans that are ubiquitously expressed as proteoglycans on cell surfaces and throughout the extracellular matrix (ECM) in most multicellular animals. HS proteoglycans play an important role in the regulation of several aspects of cancer biology, tumor progression, metastasis but also angiogenesis, *i.e.* the growth of new blood vessels from those already surrounding a tumor (*1, 2*).

While it is now widely recognized that tumors are critically dependent on angiogenesis (*3, 4*), its inhibition constitute one of the major therapeutic strategy for cancer treatment (*5*). Several growth factors, their receptors, extracellular matrix molecules and enzymes bind to specific sites on the HS sugar chain to facilitate cellular and biochemical responses associated with angiogenesis. HS mimetics, by blocking these interactions and inhibiting crucial processes to tumor progression, could therefore be considered as a new class of cancer therapeutics (*1, 6-8*).

Positron Emission Tomography (PET) is a nuclear-based imaging technique that permits assessment and quantification of specific and fundamental biochemical and physiological processes at the molecular level *in vivo*, both in animals and humans. As such, PET represents a tremendous tool in drug development due to the possibility to access *in vivo* and non-invasively to the ADMET parameters of new drug candidates, once labeled with a positron-emitter (*9*). From a chemical point of view, PET imaging relies indeed on the preparation and administration of a positron-emitting radiolabeled probe also called a radiotracer. For this purpose, fluorine-18 ($T_{1/2}$: 109.8 min) is one of the preferred radioisotope due to its adequate physical and nuclear characteristics (*i.e.* its short half-life and low positron energy) but also to the successful use in clinical oncology of 2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG), the currently most widely used PET-radiopharmaceutical and manifestly a motor behind the growing availability and interest for this positron emitter in radiopharmaceutical chemistry.

Fluorine-18 is also the radionuclide of choice when the labeling of high molecular weight compounds are concerned, such as HS mimetics, and this moreover when prosthetic labeling - one of the most efficient and reliable methodology to give access to fluorine-18-radiolabeled macromolecules - is envisaged. Prosthetic labeling consists in the preparation of a low molecular weight reagent bearing fluorine-18 followed by its conjugation with the desired macromolecule. This foreign-labeling strategy has the advantage of offering a flexibility in the choice of chemical routes, including those requiring drastic chemical conditions for the preparation of the labeled prosthetic reagent, while the conjugation of the latter with a macromolecule can then be done using milder

conditions needed to preserve the latter's integrity (*10*). Numerous prosthetic reagents have been designed among which [^{18}F]FPyME, a fluoropyridine-based maleimide reagent, that has demonstrated its efficiency for a selective conjugation with sulfhydryl functions borne by macromolecules (*11, 12*). Interestingly, during the timeframe of the present work, Ning *et al.* published the first radiolabeling of a maltohexaose derivative with fluorine-18 (*13*), based on a comparable two-step strategy, showing once more the efficacy of such prosthetic approaches.

With the aim of supporting the development of a novel class of HS mimetics as druggable entity in cancer therapy, we herein report the preparation of a fluoropyridinilated derivative of a rationally designed, fully synthetic, octasaccharide-based, lead candidate (Figure 1) and its *in vitro* preliminary characterization in a proliferation inhibition assay. We also describe its high yielding radiolabeling with fluorine-18 *via* a prosthetic approach using [^{18}F]FPyME and the *in vivo* preliminary results from PET-imaging studies in rats.

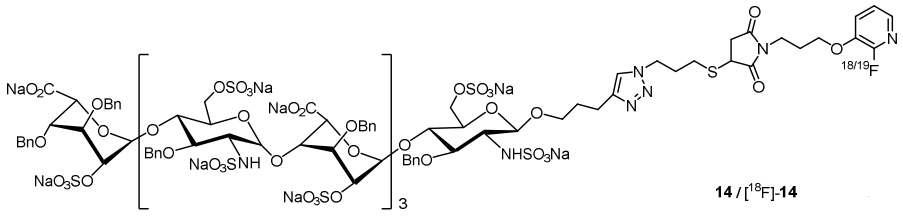
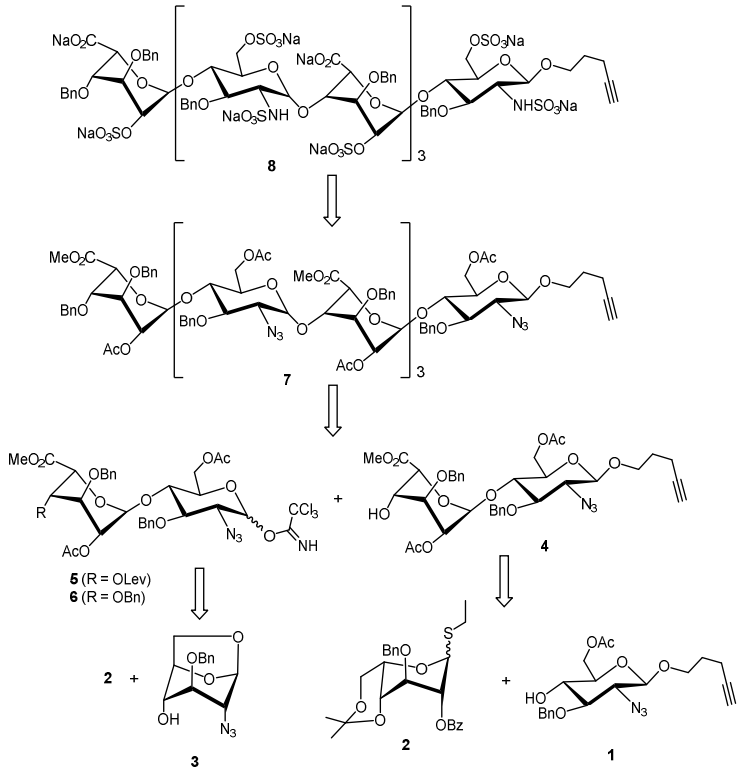


Figure 1. Chemical structure of the conjugated and labeled octasaccharide-based HS mimetic [$^{18/19}$F]-**14**.

The octasaccharide **8** (Scheme 1) is the key-intermediate for the preparation of **14**. Compound **8** has been prepared by functionalization of **7**, which has been assembled from 3 different disaccharide units (**4**, **5** and **6**) (*14*). Over the past few years, several approaches for the preparation of oligosaccharides have been documented in the literature. These strategies mainly differ in the protecting group patterns of the building blocks, timing of the oxidation step to access the uronic acid and the glycosylation procedure. One of the major efforts in the synthesis of **8** is the preparation of the three different disaccharides (**4**, **5** and **6**) that constitute respectively the reducing end of the chain, the repeating unit and the capping unit. The syntheses of these disaccharides, in sufficient quantities and with the appropriate protective groups (two mandatory requirements for the preparation of complex octasaccharide, *e.g.* **8**), were performed in a similar manner as described previously by Petitou and co-workers (*15*) starting from three different monosaccharides (**1**, **2** and **3**, Scheme 1) (*14, 16-18*).

Note that temporary protecting groups are required at the C4 position group of the building block **5** to allow for an easy deprotection in anticipation of chain elongation. Here, levulinoyl ester has been used (*19, 20*). Additionally, the hydroxyl groups that will be *O*-sulfated at a late stage and those that should remain free have to be protected differently. Commonly, acetate esters serve to mask hydroxyl groups to be orthogonally sulfated, while benzyl ethers mask hydroxyl groups that will not be modified. The amine group of D-glucosamine also requires the placement of different protecting groups. Azides have been used for these protections (*21-23*).



Scheme 1. Retrosynthetic scheme used in the preparation of the octasaccharide **8**.

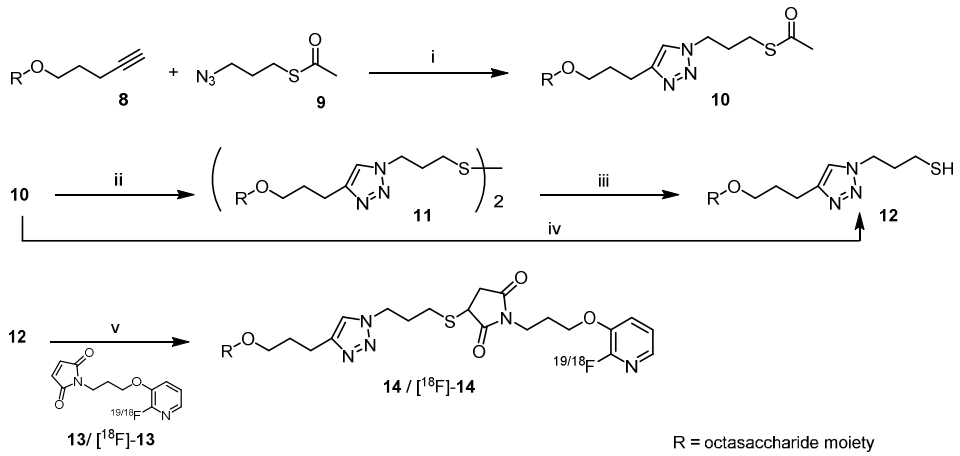
Compound **8** is expected to interfere with two major processes in tumor development : i) angiogenesis partly mediated *via* growth factors such as VEGF, FGF-2 and PDGF-β, or endothelial progenitor cells recruitment mediated *via* SDF-1α, and ii) metastic potential mediated *via* heparanase activity. The heparanase is indeed able to cleave HS in the ECM thereby releasing the above mentioned growth factors involved in angiogenesis. The binding affinities of **8** have been determined using the BIAcore technology for the proteins VEGF-A, FGF-2, PDGF-β and SDF1-α, and the inhibition potential in a heparanase activity assay (*14, 24, 25*). The determined IC_{50} values (Table 1) showed a strong affinity for the growth factors (all in the nmolar range) as well as an efficient

inhibition of the heparanase activity. These *in vitro* characterizations demonstrate the potential of the octasaccharide **8** as inhibitor of angiogenesis processes.

Table 1. Growth factor binding and heparanase inhibition assays for compound **8**.

	IC_{50} (nM)				
Compound #	VEGF-A	FGF-2	PDGF-β	SDF-1α	Heparanase
8	4.5±0.3	3.0±0.6	10.6±0.3	43±20	41±15

Derivatization of compound **8** and conjugation with **13** were carried out as illustrated in scheme 2. Briefly, copper-catalyzed Huisgen 1,3 dipolar cycloaddition of **8** (bearing an alkyne function at the anomeric-position of the reducing end of the octasaccharide) with **9** (bearing the azide counterpart) was thus performed leading to the formation of **10** in 92% yield. Deacetylation of the sulfhydryl moiety and subsequent reaction with the thiol-sensitive prosthetic reagent FPyME (**13**) afforded the target conjugated octasaccharide **14** in 63% yield. The thiol-containing oligosaccharide **12** was indeed produced and stored as its *S*-acylated form to avoid its dimerization as **11** by a disulfide bridge formation.



Reagents and conditions : i) $CuSO_4$, ascorbic acid, *t*-BuOH/H_2O, rt, 1 h; ii) LiOH, H_2O, rt, 12 h; iii) TCEP, H_2O, rt, 10 min; iv) hydroxylamine (50 mM) in PBS (100 mM, pH 7.4), rt, 45 min; v) FPyME or [^{18}F]FPyME in DMSO, PBS (100 mM, pH 7.4), rt, 15 min.

Scheme 2. Derivatization of compound **8** and subsequent conjugation with the prosthetic reagent [$^{19/18}$F]FPyME, leading to the target octasaccharide [$^{19/18}$F]-**14**.

The cycloaddition reaction between compounds **8** and **9** was conducted using standard conditions. A combination of *tert*-butanol and water, as reaction medium, was found optimal for the formation of

the triazole **10**. Then, conjugation with FPyME was first carried out in non-radioactive conditions to obtain and characterize **14**, which will also serve as authentic reference for the labeling with fluorine-18. For this, deprotection of the sulfhydryl function was performed according to two procedures. Firstly, treatment of compound **10** with lithium hydroxide in water over 12 h gave exclusively the dimer **11**, which has been identified by HPLC (HPLC B, conditions B_2 (see *supporting information* section) : *Rt*(**10**) = 13.4 min, *Rt*(**11**) = 14.1 min) and characterized by ESI/MS (Table 2). This dimer was then dissociated using a TCEP-buffered aqueous solution to restore **12** and allow for the *in situ* conjugation with FPyME (**13**). Alternatively, compound **10** was treated with a buffered aqueous solution of hydroxylamine for 45 min to afford **12** to which compound **13**, in solution in dimethyl sulfoxide (DMSO), was directly added. The deprotection of **10** was followed by HPLC analyses (HPLC B, conditions B_2 : *Rt*(**10**) = 13.4 min, *Rt*(**12**) = 11.8 min). Compound **14** has been purified by semi-preparative HPLC and characterized by MS (Table 2 and *supporting information* section).

Table 2. Mass spectrometry characterization of compounds **10**, **11**, **12** and **14**.

Compound #	**10**	**11**	**12**	**14**
Calculated	3361.4121 $C_{121}H_{147}N_7O_{78}S_{13}$	6636.7874 $C_{238}H_{288}N_{14}O_{154}S_{26}$	3319.4015 $C_{119}H_{145}N_7O_{77}S_{13}$	3569.4769 $C_{131}H_{156}FN_9O_{80}S_{13}$
Measured	671.7616 $[M-5H]^{5-}$	751.2876 $[M+DBA-10H]^{9-}$	663.4343 $[M-5H]^{5-}$	713.3445 $[M-5H]^{5-}$

The biological activity of **14** has been evaluated in a FGF-2 induced NHDF proliferation assay and compared to the one of the parent octasaccharide **8**. The results presented in figure 2 clearly demonstrated that **14** inhibits FGF-2-induced fibroblast proliferation at a concentration of 30 µM and this in a similar manner that **8**. This also demonstrated the relevance of the prosthetic approach selected for the fluorine-18-labeling of these macromolecules.

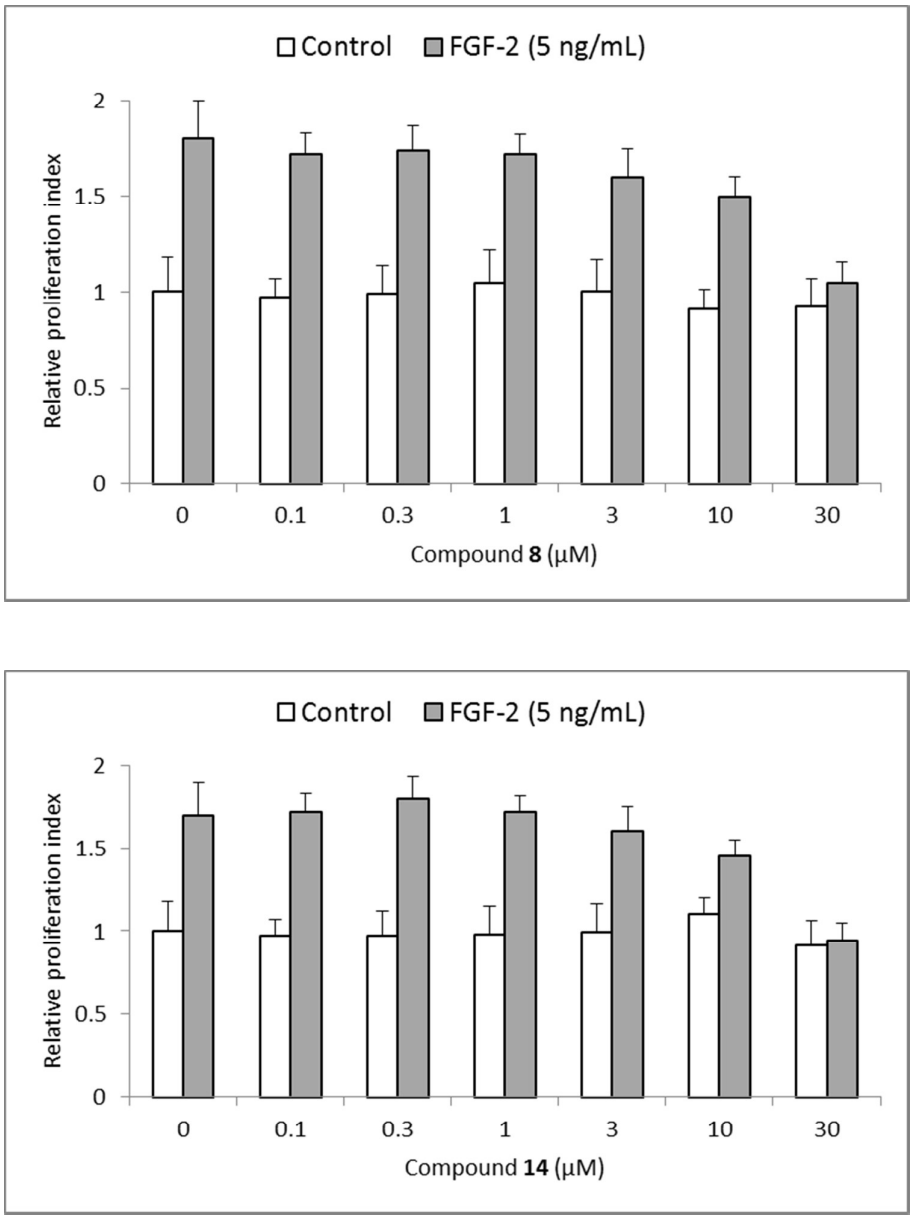


Figure 2. Inhibition of FGF-2-induced NHDF cell proliferation by **8** and **14**.

Fluorine-18-labeling of **14** was performed using [^{18}F]-**13**, the radiofluorinated version of FPyME (Scheme 2). [^{18}F]FPyMe was prepared in high yields (28 to 37 % decay-corrected yield based on starting fluoride) according to already published procedures (*11*) (details are given in the *supporting information* section). In parallel to the preparation of [^{18}F]-**13**, the *S*-acetyl protected octasaccharide **10** was treated with hydroxylamine for 45 min to obtain quantitatively compound **12** (as reported above). Conjugation of [^{18}F]-**13** with **12** was performed in mild conditions (DMSO/phosphate buffer saline) for a short reaction time of 15 min at room temperature. The yield of conjugation was evaluated to be > 50% according to radioTLC before purification. Rapid and efficient purification

using a commercially available pre-packed Sephadex NAP10™ cartridge allowed for a total separation of the conjugated fluorine-18-labeled octasaccharide [^{18}F]-**14** and the non-reacted reagent [^{18}F]-**13**. Chemical purity and identity of [^{18}F]-**14** were assessed by radioTLC and analytical radioHPLC. Co-injection of an aliquot of formulated [^{18}F]-**14** with an authentic sample of **14** showed identical retention time as well as the absence of [^{18}F]-**13** on the chromatogram (HPLC B, conditions B_1 : *Rt*(**10**) = 16.5 min, *Rt*(**12**) = 15.8 min, *Rt*([^{18}F]-**13**) = 17.0 min and *Rt*([^{18}F]-**14**) = 18.4 min).

The conjugation and purification steps lasted 20 to 25 min and allowed for the production of [^{18}F]-**14** in 55 to 60% isolated and decay-corrected yields, based on [^{18}F]FPyME. Typically, starting from a 4.8 to 6.7 GBq [^{18}F]FPyME production batch, 2.4 to 4.0 GBq of [^{18}F]-**14** was produced with an average decay-corrected specific radioactivity of 20 GBq/µmol.

The entire process of preparation of [^{18}F]-**14** (production of [^{18}F]-**13** + conjugation with **12**) lasted 130 to 135 min, a synthesis time that is compatible with the half-life of fluorine-18. The overall isolated and decay-corrected radiochemical yield of production of [^{18}F]-**14** ranged from 15 to 24%, based on starting [^{18}F]fluoride.

Note that once again, combination of the high yielding radiosynthesis of the reagent and the efficient conjugation of it with sulfhydryl containing macromolecules, would make it possible to prepare in parallel several oligosaccharide-based macromolecules starting from a unique batch of [^{18}F]FPyME (*12*).

Male Wister rats were injected with 15±2 MBq of [^{18}F]-**14** and imaged using a high resolution HRRT (Siemens) PET scanner. Projection images (Figure 3) showed a rapid distribution of the radioactivity in vascular systems (vascular tree and heart), followed by an accumulation in kidneys and liver in a lesser extent while vascular radioactivity concentration remained elevated. Times Activity Curves (TACs, see *supporting information* section Figure S2 A) showed similar patterns for abdominal arteries, heart and lung indicating that the detected radioactivity is of vascular origin. Blood clearance was slow ($T_{1/2}$ > 90 min). Urinary excretion is expected due to the presence of radioactivity in the bladder at late time points. Pharmacodistribution showed that the uptake of radioactivity in other organs (intestine, muscle, brain) remained low (see *supporting information* section Figure S2 B). [^{18}F]-**14** displayed a long-lasting residence-time in the vascular system and a progressive accumulation in elimination organs (liver, kidneys, bladder) (see *supporting information* section Figure S3). These observations are consistent the biodistribution of M402, another rationally engineered HS mimetic, tagged with a fluorescent dye *(26)*. These preliminary *in vivo* properties are favorable for the development of an HS mimetic drug candidate for cancer applications.

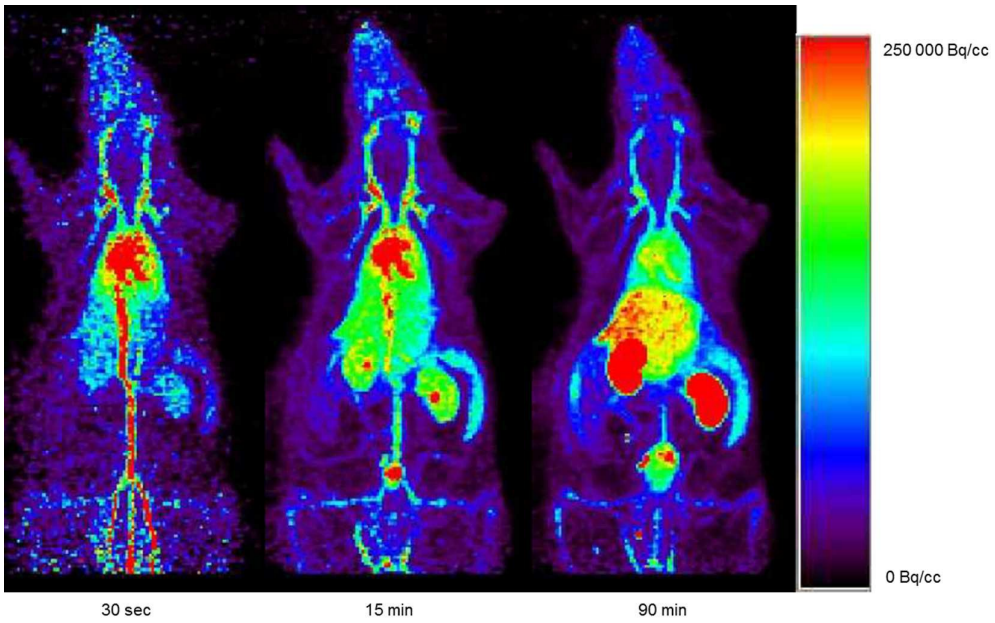


Figure 3. PET images (projection) following [^{18}F]-**14** *i.v.* injection in rats at 30 sec, 15 min and 90 min.

In conclusion, the octasaccharide **14**, as a conjugated analog of the HS mimetic inhibitor **8**, has been synthesized and characterized *in vitro*. Fluorine-18-labeling has been achieved permitting *in vivo* preliminary PET-imaging evaluation in healthy rodents. Evaluation of the radiotracer in a rat tumor-model will be the next step.

References

1. Sasisekharan, R., Shriver, Z., Venkataraman, G., and Narayanasami, U. (2002) Roles of heparan-sulphate glycosaminoglycans in cancer, *Nature Reviews Cancer 2*, 521-528.
2. Wegrowski, Y., and Maquart, F. X. (2004) Involvement of stromal proteoglycans in tumour progression, *Critical Reviews In Oncology Hematology 49*, 259-268.
3. Bergers, G., and Benjamin, L. E. (2003) Tumorigenesis and the angiogenic switch, *Nature Reviews Cancer 3*, 401-410.
4. Carmeliet, P., and Jain, R. K. (2000) Angiogenesis in cancer and other diseases, *Nature 407*, 249-257.
5. Herbst, R. S. (2006) Therapeutic options to target angiogenesis in human malignancies, *Expert Opinion on Emerging Drugs 11*, 635-650.
6. Duval, S., Sineriz, F., Arlicot, N., Barbier-Chassefière, V., Vercouillie, J., Delbé, J., Papy-Garcia, D., Guilloteau, D., and Barritault, D. (2012) Development of radiotracers for the molecular imaging of tumor matrix microenvironment (HIMIMT), *IRBM 33*, 86-91.
7. Lever, R., and Page, C. R. (2002) Novel drug development opportunities for heparin, *Nature Reviews Drug Discovery 1*, 140-148.

8. Presta, M., Leali, D., Stabile, H., Ronca, R., Camozzi, M., Coco, L., Moroni, E., Liekens, S., and Rusnati, M. (2003) Heparin derivatives as angiogenesis inhibitors, *Current Pharmaceutical Design 9*, 553-566.
9. Weissleder, R., and Mahmood, U. (2001) Molecular imaging, *Radiology 219*, 316-333.
10. Kuhnast, B., and Dollé, F. (2010) The challenge of labeling macromolecules with fluorine-18 : Three decades of research, *Current Radiopharmaceuticals 3*, 174-201.
11. de Bruin, B., Kuhnast, B., Hinnen, F., Yaouancq, L., Amessou, M., Johannes, L., Samson, A., Boisgard, R., Tavitian, B., and Dollé, F. (2005) 1-[3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione: Design, synthesis, and radiosynthesis of a new [^{18}F]fluoropyridine-based maleimide reagent for the labeling of peptides and proteins, *Bioconjugate Chemistry 16*, 406-420.
12. Denholt, C. L., Kuhnast, B., Dollé, F., Hinnen, F., Hansen, P. R., Gillings, N., and Kjaer, A. (2010) ^{18}F-labelling of a series of potential EGFRvIII targeting peptides with a parallel labelling approach using [^{18}F]FPyME, *Journal of Labelled Compounds & Radiopharmaceuticals 53*, 774-778.
13. Ning, X. H., Seo, W., Lee, S., Takemiya, K., Rafi, M., Feng, X. L., Weiss, D., Wang, X. J., Williams, L., Camp, V. M., Eugene, M., Taylor, W. R., Goodman, M., and Murthy, N. (2014) PET Imaging of Bacterial Infections with Fluorine-18-Labeled Maltohexaose, *Angewandte Chemie-International Edition 53*, 14096-14101.
14. Cabannes, E., Caravano, A., Lewandovski, D., Motte, V., Nancy-Portebois, V., Petitou, M., and Pierdet, P. Patent WO 2010/029185 (2010).
15. Tabeur, C., Mallet, J. M., Bono, F., Herbert, J. M., Petitou, M., and Sinay, P. (1999) Oligosaccharides corresponding to the regular sequence of heparin: Chemical synthesis and interaction with FGF-2, *Bioorganic & Medicinal Chemistry 7*, 2003-2012.
16. Duchaussoy, P., Jaurand, G., Driguez, P. A., Lederman, I., Gourvenec, F., Strassel, J. M., Sizun, P., Petitou, M., and Herbert, J. M. (1999) Design and synthesis of heparin mimetics able to inhibit thrombin. Part 1 - Identification of a hexasaccharide sequence able to inhibit thrombin and suitable for 'polymerisation', *Carbohydrate Research 317*, 63-84.
17. Petitou, M., Duchaussoy, P., Lederman, I., Choay, J., Sinay, P., Jacquinet, J. C., and Torri, G. (1986) Synthesis of heparin fragments, *Carbohydrate Research 147*, 221-236.
18. Tabeur, C., Machetto, F., Mallet, J. M., Duchaussoy, P., Petitou, M., and Sinay, P. (1996) L-iduronic acid derivatives as glycosyl donors, *Carbohydrate Research 281*, 253-276.
19. Basten, J., Jaurand, G., Oldehanter, B., Duchaussoy, P., Petitou, M., and Vanboeckel, C. A. A. (1992) Biologically -active heparin-like fragments with a non-glycosamine glycan structure.3. Ortho-alkylated-ortho-sulfated pentasaccharides, *Bioorganic & Medicinal Chemistry Letters 2*, 905-910.
20. Petitou, M., Imberty, A., Duchaussoy, P., Driguez, P. A., Ceccato, M. L., Gourvenec, F., Sizun, P., Herault, J. P., Perez, S., and Herbert, J. M. (2001) Experimental proof for the structure of a thrombin-inhibiting heparin molecule, *Chemistry -a European Journal. 7*, 858-873.
21. Haller, M., and Boons, G. J. (2001) Towards a modular approach for heparin synthesis, *Journal of the Chemical Society-Perkin Transactions 1*, 814-822.
22. Kovensky, J., Duchaussoy, P., Bono, F., Salmivirta, M., Sizun, P., Herbert, J. M., Petitou, M., and Sinay, P. (1999) A synthetic heparan sulfate pentasaccharide, exclusively containing L-iduronic acid, displays higher affinity for FGF-2 than its D-glucuronic acid-containing isomers, *Bioorganic & Medicinal Chemistry 7*, 1567-1580.
23. Lucas, R., Hamza, D., Lubineau, A., and Bonnaffé, D. (2004) Synthesis of glycosaminoglycan oligosaccharides - An unexpected inhibitory effect of a remote N-acetyl group upon trichloroacetimidate-mediated couplings, *European Journal of Organic Chemistry*, 2107-2117.

24. Cochran, S., Li, C. P., Fairweather, J. K., Kett, W. C., Coombe, D. R., and Ferro, V. (2003) Probing the interactions of phosphosulfomannans with angiogenic growth factors by surface plasmon resonance, *Journal of Medicinal Chemistry 46*, 4601-4608.
25. Cochran, S., Li, C. P., and Ferro, V. (2009) A surface plasmon resonance-based solution affinity assay for heparan sulfate-binding proteins, *Glycoconjugate Journal 26*, 577-587.
26. Zhou, H., Roy, S., Cochran, E., Zouaoui, R., Chu, CL., Duffner, J., Zhao, G., Smith, S., Galcheva-Gargova, Z., Karlgren, J., Dussault, N., Kwan, RYQ., Moy, E., Barnes, M., Long, A., Honan, C., Qi, YW., Ganguly, T., Schultes, B., Venkataraman, G., Kishimoto, TK. (2011) M402, a Novel Heparan Sulfate Mimetic, Targets Multiple Pathways Implicated in Tumor Progression and Metastasis. *Plos One, 6,* e21106.