ACS Medicinal Chemistry Letters

Letter

pubs.acs.org/acsmedchemlett

Development and Preliminary Evaluation of TFIB, a New Bimodal Prosthetic Group for Bioactive Molecule Labeling

Emilie M. F. Billaud,[§,∥,⊥] Aurélien Vidal,[†,§,∥,⊥] Amélie Vincenot,[∥,⊥] Sophie Besse,[∥,⊥] Bernadette Bouchon,[∥,⊥] Eric Debiton,[∥,⊥] Elisabeth Miot-Noirault,[∥,⊥] Imen Miladi,[∥,⊥] Latifa Rbah-Vidal,[‡,∥,⊥] Philippe Auzeloux,[∥,⊥,#] and Jean-Michel Chezal*[,∥,⊥,#]

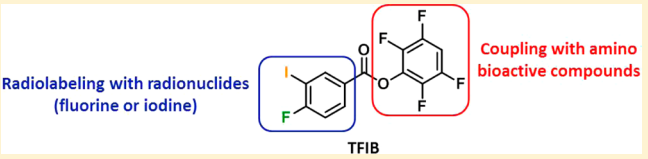
[∥]Clermont Université, Université d'Auvergne, Laboratoire d'Imagerie Moléculaire et Thérapie Vectorisée, BP 10448, F-63000 Clermont-Ferrand, France

[⊥]INSERM, U990, F-63005 Clermont-Ferrand, France

[#]Centre Jean Perrin, F-63011 Clermont-Ferrand, France

Supporting Information

ABSTRACT: The new readily available prosthetic group, tetrafluorophenyl 4-fluoro-3-iodobenzoate (**TFIB**), designed for both molecular imaging and targeted radionuclide therapy purposes was radiolabeled either with fluorine or iodine radionuclides with excellent radiochemical yields and purities. These radiolabeled tags were conjugated to *N*,*N*-diethylethylenediamine to give melanin-targeting radiotracers [^{125}**I**]**9** and [^{18}**F**]**9**, which were successfully evaluated by PET and gamma scintigraphic imaging in B16F0 pigmented melanoma-bearing C57BL/6J mice. Then, radiolabeled [^{125}**I**]/[^{18}**F**]**TFIB** was used to tag tumor-targeting peptides (i.e., $PEG_3[c(RGDyK)]_2$ and NDP-MSH targeting $\alpha_v\beta_3$ integrin and MC1R receptors, respectively) in mild conditions and with good radiochemical yields (47−83% d.c.) and purities (>99%). The resulting radiolabeled peptides were assessed both *in vitro* and by PET imaging in animal models.



KEYWORDS: *Bioconjugation, prosthetic group, radiochemistry, radiofluorination, radioiodination, molecular imaging*

Iodine radioisotopes are the radiohalides most widely used for the labeling of small organic compounds, peptides, antibodies, oligonucleotides, or nanoobjects. This is due to the remarkable physical properties of several iodine radioisotopes (e.g., ^{125}I, ^{123}I, ^{124}I, and ^{131}I), which allow a broad range of applications from biochemical research to nuclear medicine.[1] For the radioiodination of compounds with no aromatic group or that are sensitive to radiolabeling conditions, the most useful technique remains the use of iodine-containing prosthetic groups. Accordingly, a variety of radioiodinated bifunctional agents have been developed that exploit conjugation strategies such as acylation, hydrazone, imidate, or carbamate formation or thiol alkylation.[2−4] Among them, the best known and most extensively studied labeling agents are two activated esters, *N*-succinimidyl 3-(4-hydroxy-3-iodophenyl) propionate[5] (also called Bolton−Hunter reagent) and *N*-succinimidyl 3-iodobenzoate (**SIB**)[6] (Figure 1). In the growing field of personalized cancer medicine, tumor-targeted radioiodinated agents are promising tools for multimodal or theranostic purposes.

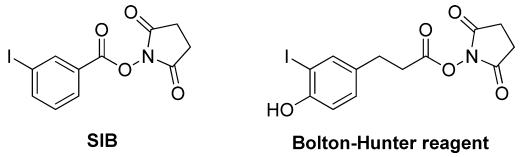


Figure 1. Examples of common iodinated aromatic prosthetic groups.

According to the iodine radioisotope used, they can enable imaging by single-photon emission computed tomography (SPECT, with ^{123}I) or positron emission tomography (PET, with ^{124}I) for the diagnosis and/or stratification of patients, followed by targeted radionuclide therapy with ^{131}I. PET, the most sensitive functional imaging technique, is preferred. However, the physical properties of ^{124}I present some limitations[7] ($t_{1/2}$ = 4.2 d; β^+ 1.5 and 2.1 MeV, 22%) that reduce the spatial resolution of PET images and so lead to high patient dosimetry.

We set out to develop an isostructural prosthetic group allowing both fluorine and iodine radiolabeling. Such a prosthetic reagent could facilitate clinical transfer of new tandem PET and therapeutic radiopharmaceuticals for the following reasons: first, ^{18}F ($t_{1/2}$ = 109.8 min) is currently the PET radionuclide of choice given its favorable nuclear and chemical properties, including its decay process (97%, β^+ emission) and low positron energy (635 keV). Second, ^{18}F and ^{131}I are inexpensive, do not suffer from production limitations, and are currently available in pharmaceutical grade, unlike other pairs of PET-therapy radioisotopes such as ^{64}Cu−^{67}Cu, ^{44}Sc−^{47}Sc, ^{86}Y−^{90}Y, or ^{71}As−^{77}As.[8] Finally, the radioiodinated or radiofluorinated conjugates will possess

Received: October 18, 2014
Accepted: November 24, 2014
Published: November 24, 2014

ACS Publications © 2014 American Chemical Society

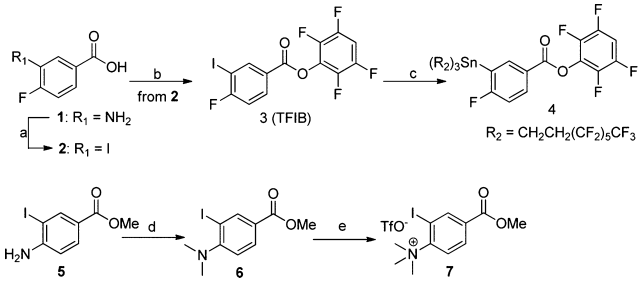
Petitioner GE Healthcare – Ex. 1050, p. 168

similar physicochemical properties (e.g., lipophilicity, steric hindrance, polarizability, etc.), a key parameter when developing tumor receptor-targeting ligands.

Nevertheless, the "ideal" prosthetic group has to meet certain criteria: (i) it should be a small organic compound with a low steric hindrance to avoid as far as possible any modifications of the pharmacological properties of the coupled bioactive compound; (ii) the radiolabeled prosthetic group precursors must be readily available for widespread use; they must be synthesizable in very few steps starting from commercially available reagents, with fast and reliable protocols; (iii) the radiolabeled prosthetic group must be obtainable promptly in high radiochemical yield, purity, and specific activity; (iv) the coupling reaction must be fast and use mild reaction conditions, with high selectivity, high yield, and easy purification methods; and (v) conjugates must be stable toward *in vivo* dehalogenation.

In this context and on the basis of our earlier work on the development of (hetero)aromatic benzamides for PET imaging and targeted radionuclide therapy of melanoma,[9,10] we designed the first bimodal fluorinated and iodinated prosthetic group, the tetrafluorophenyl 4-fluoro-3-iodobenzoate (**3** or **TFIB**, Scheme 1), as a suitable acylating agent for the labeling

Scheme 1. Preparation of TFIB and Radiolabeling Precursors[a]



[a]Reagents and conditions. (a) HCl, $NaNO_2$, KI, 0 °C, 0.5 h then RT 3 h, 86%; (b) 1. $(COCl)_2$, DMF, CH_2Cl_2, 0 °C, 0.5 h, then RT, 17 h; 2. TFP, NEt_3, CH_2Cl_2, RT 4 h, 63%; (c) 1. LiCl, Zn, THF, dibromoethane, Me_3SiCl, Xantphos, $CoCl_2$, 50 °C, 3 h; 2. tris(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl)tin bromide, THF, 50 °C, 72 h, 28%; (d) paraformaldehyde, $NaBH_3CN$, AcOH, RT, 4 h, 99%; (e) MeOTf, Et_2O, RT, 4 d, 49%.

of a broad variety of compounds bearing a primary amine function. Here we present preliminary results of the synthesis, radiolabeling, and biological applications of **TFIB**, highlighting its potential for both PET imaging and targeted radionuclide therapy applications.

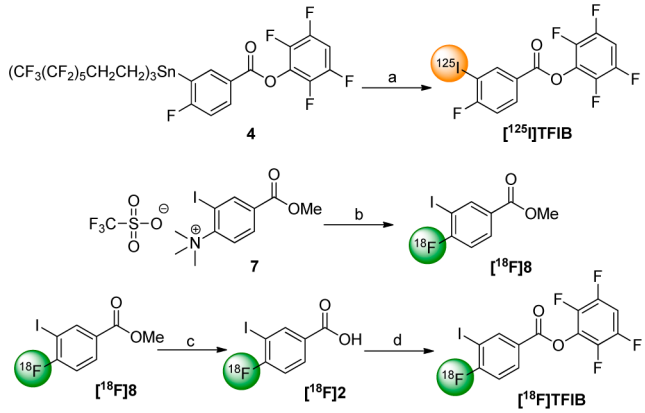
TFIB was prepared in two steps, starting from commercial 3-amino-4-fluorobenzoic acid (**1**, Scheme 1): diazotization–iodination giving compound **2** followed by esterification with 2,3,5,6-tetrafluorophenol (TFP) using an acyl chloride intermediate.

It should be noted that the tetrafluorophenyl ester group was chosen as the activated ester function for its high stability under basic conditions, frequently used for amide bond formation.[11,12] **TFIB** was thus obtained with 54% overall yield.

For radioiodination, we decided to use a (perfluoro)tin derivative as precursor (**4**, Scheme 1). It allows high specific activity radiolabeling under mild conditions and can be easily separated from radioiodinated **TFIB** using FluoroFlash solid-phase extraction cartridges (F-SPE), avoiding time-consuming purification by HPLC.[13] Precursor **4** was synthesized from **TFIB** by treating (perfluoro)tin bromide with an organozinc intermediate[14] generated *in situ* using a cobalt-Xantphos-catalyzed LiCl-mediated protocol.[15,16] Precursor **4** was thus prepared in a one-pot two-step procedure starting from **TFIB**, with 28% overall yield.

A three-step radiolabeling strategy starting from methyl ester **7** (Schemes 1 and 2) was carried out and based, for the

Scheme 2. Radiosyntheses of [^{125}I/^{18}F]TFIB[a]



[a]Reagents and conditions. (a) [^{125}I]NaI, AcOH, iodogen, MeOH, RT, 40 min, 71%; (b) [^{18}F]KF, K_{222}, MeCN, 80 °C, 4 min; (c) 1. NaOH 0.5 N, 50 °C, 2 min; 2. HCl 0.5 N; (d) DCC, TFP, 40 °C, 5 min, overall RCY 32% (d.c.).

radiofluorination precursor, on a trimethylammonium triflate salt as leaving group for fluorine-18 aromatic nucleophilic substitution. Indeed, this salt is highly soluble in organic solvents and tends to be more reactive than compounds with halogen or nitro leaving groups.[17,18] It can also be readily purified using a C18 solid-phase extraction cartridge (C18-SPE). Precursor **7** was prepared in two steps, starting from commercial methyl 4-amino-3-iodobenzoate (**5**): reductive amination, in the presence of paraformaldehyde and sodium cyanoborohydride,[19] yielded quantitatively the derivative **6**, which was then methylated with methyl trifluoromethanesulfonate to give the quaternary ammonium triflate salt **7** in 49% overall yield.

High specific activity radioiodination of **TFIB** was carried out by iodo-demetalation of organotin **4** with iodine-125 ($t_{1/2}$ = 59.9 d; γ 35 keV, Scheme 2).

It should be noted that this radioisotope was used as a radiolabeling model for iodine-131 ($t_{1/2}$ = 8.0 d; β^- 606 keV, 90%, γ 365 and 637 keV, 89%) for first preclinical developments. Precursor **4** was treated with [^{125}I]NaI in the presence of the gentle oxidizing agent iodogen and acetic acid, at RT. [^{125}I]**TFIB** was then purified by F-SPE (F-SPE separation depends on the fluorine content of each loaded compound and on the fluorophilicity of the solvents used) and formulated on a C18-SPE. Radiolabeled prosthetic group [^{125}I]**TFIB** was finally obtained in MeCN with 60 min total preparation time, 71% overall radiochemical yield (RCY), radiochemical purity (RCP) > 99%, and a specific activity (SA) of 30–35 GBq·μmol^{-1}. [^{125}I]**TFIB** in MeCN was radiochemically stable at RT for at least 4 d after preparation.

Fully automated three-step radiosynthesis of [^{18}F]**TFIB** was developed on a Raytest SynChromR&D bireactor module (Scheme 2). Nucleophilic substitution of compound **7** by the

dx.doi.org/10.1021/ml500423v | ACS Med. Chem. Lett. 2015, 6, 168–172

Petitioner GE Healthcare – Ex. 1050, p. 169

Scheme 3. General Synthesis Pathway for Coupling Reactions between TFIB and the Three Compounds of Interest Chosen

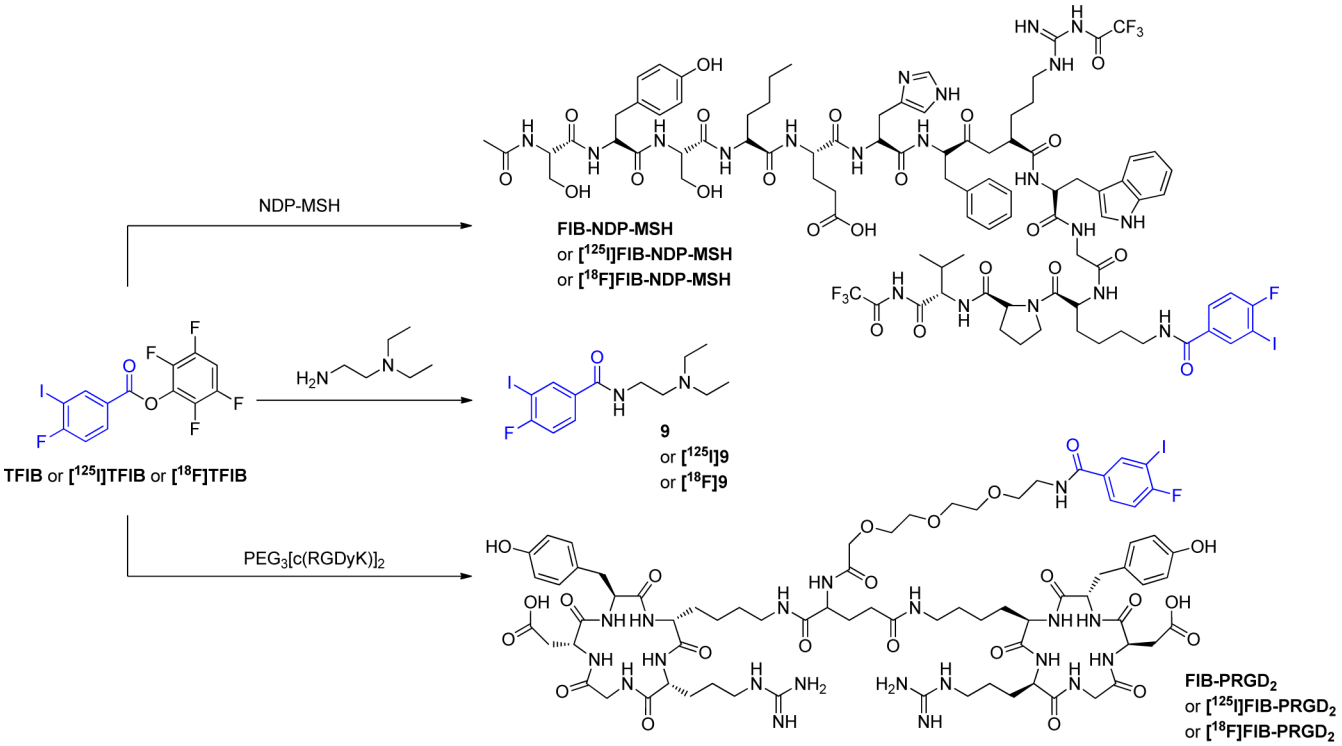


Table 1. Labeling Conditions and Radiochemical Data for Radiohalogenated 9, FIB-NDP-MSH, and FIB-PRGD$_2$

compd	volume of [X]TFIB/MeCN solution	amount of amine reagent (solvent/volume)	reaction temperature/time	purification	preparation time	RCY[a]	RCP[b]
[^{125}I]**9**	2 mL	10 μL	RT/10 min	C18-SPE	15 min	92%	>99%
[^{18}F]**9**	2 mL	10 μL (MeCN/1 mL)	RT/3 min	C18-SPE	8 min	90%	>99%
[^{125}I]**FIB-NDP-MSH**	33 μL	100 μg (borate buffer pH 8.0/100 μL)	45 °C/30 min	HPLC	60 min	83%	>99%
[^{18}F]**FIB-NDP-MSH**	1.2 mL	1.5 mg (borate buffer pH 8.0/1.5 mL)	50 °C/10 min	HPLC	25 min	57%	>99%
[^{125}I]**FIB-PRGD$_2$**	33 μL	100 μg (borate buffer pH 7.6/100 μL)	45 °C/30 min	HPLC	60 min	78%	>99%
[^{18}F]**FIB-PRGD$_2$**	1.2 mL	1.5 mg (borate buffer pH 7.6/1.5 mL)	50 °C/15 min	HPLC	31 min	47%	>99%

[a]Radiochemical yields are decay corrected. [b]Radiochemical purity determined by analytical HPLC.

dry [^{18}F]KF, K_{222} complex (5−10 GBq) at 80 °C in MeCN gave [^{18}F]**8** in 4 min (65−85% decay-corrected, d.c.). Subsequent saponification with a 0.5 N aqueous NaOH solution at 50 °C for 2 min provided [^{18}F]**2** as a sodium carboxylate salt (99% d.c.). Acidification of the media with a 0.5 N aqueous HCl solution was then necessary to trap intermediate [^{18}F]**2** on a C18-SPE cartridge. After elimination of hydrophilic and charged compounds with H_2O elution, [^{18}F]**2** was eluted in the second reactor with MeCN. The solution was further azeotroped at 100 °C for 5 min in the presence of Bu_4NOH (0.1 M/MeOH). Esterification of [^{18}F]**2** with *N*,*N*′-dicyclohexylcarbodiimide (DCC) and TFP in MeCN at 40 °C for 5 min then yielded [^{18}F]**TFIB** (68−78% d.c.). Semipreparative HPLC purification followed by formulation on a C18-SPE cartridge provided [^{18}F]**TFIB** in 61 min with 32% overall RCY (d.c.), RCP > 99%, and SA of 30−55 GBq·μmol^{-1}. [^{18}F]**TFIB** was obtained in MeCN ready for coupling, and the solution was radiochemically stable at RT for at least 3 h after preparation.

To validate our approach, we tested three [^{125}I/^{18}F]**TFIB** coupling reactions (Scheme 3 and Table 1). We first investigated the reaction between [^{125}I/^{18}F]**TFIB** and *N*,*N*-diethylethylenediamine, providing [^{125}I/^{18}F]**9**, a benzamide analogue of previously studied melanin-targeting ligands.[9] [^{125}I/^{18}F]**9** was obtained in a very short time (8−15 min), with excellent RCY (>90%, d.c.) and RCP (>99%). We then investigated coupling reactions between [^{125}I/^{18}F]**TFIB** and well-known tumor-targeting peptides NDP-MSH[20] (targeting melanocortin type 1 receptor (MC1R), overexpressed by most melanoma cells) and PEG3[c(RGDyK)]$_2$[21] (targeting $\alpha_v\beta_3$ integrin, overexpressed by numerous tumor cells), affording [^{125}I/^{18}F]**FIB-NDP-MSH** and [^{125}I/^{18}F]**FIB-PRGD$_2$**, respectively. For these reactions, carried out in mild conditions, many parameters were optimized, such as the pH, the temperature and duration of reaction, the total volume of solvent, and the ratio between the volumes of acetonitrile (**TFIB**) and buffer (peptide). These coupling reactions provided the radiolabeled peptides in a short time (25−60 min) with good RCY (47−83%, d.c.) and RCP (>99%). Chemical structures of conjugated

dx.doi.org/10.1021/ml500423v | ACS Med. Chem. Lett. 2015, 6, 168−172

Petitioner GE Healthcare – Ex. 1050, p. 170

peptides were confirmed by MALDI-ToF mass spectrometry for their nonradioactive counterparts.

The *in vivo* stability of [^{125}I/^{18}F]**9** and its affinity for melanin pigment were investigated *in vivo* by γ scintigraphic and PET imaging in primary melanoma-bearing mice. For [^{125}I/^{18}F]**9**, tumor uptakes and good clearances from nonpigmented tissues were noted (Tables 3 and S1, Supporting Information), giving high-contrast PET images ([^{18}F]**9**, Figure 2). Moreover, the

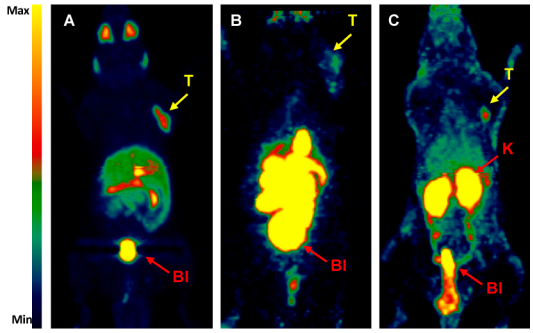


Figure 2. *In vivo* PET maximum-intensity-projection images at 1 h after injection. (A) [^{18}F]**9** biodistribution in B16F0 primary melanoma-bearing C57BL/6J mice. (B) [^{18}F]**FIB-NDP-MSH** biodistribution in B16F10 primary melanoma-bearing C57BL/6J mice. (C) [^{18}F]**FIB-PRGD$_2$** biodistribution in U87-MG primary glioblastoma-bearing nude mice. Arrows indicate the tumor (T), kidneys (K), and bladder (Bl).

tumor uptake values for [^{125}I]**9** and [^{18}F]**9** were quite similar and consistent at 1 and 3 h p.i. In addition, no radiodefluorination and only a slight radiodeiodination were observed, indicating that [^{125}I/^{18}F]**9** was quite stable *in vivo*. To investigate the influence of the prosthetic group on the biological properties of both peptides, *in vitro* competitive receptor-binding assays were carried out (see Table 2). The

Table 2. Receptor-Binding Affinities (IC$_{50}$) of Reference Peptides and FIB Conjugates

compd	MC1 receptor IC$_{50}$ (pM)[a]	$\alpha_v\beta_3$ receptor IC$_{50}$ (nM)[b]
[^{125}I]**NDP-MSH**	71 ± 4	
FIB-NDP-MSH	21 ± 1	
[^{125}I]**echistatin**		1.40 ± 0.01
FIB-PRGD$_2$		1.10 ± 0.60

[a]B16F10 murine melanoma cells. [b]U87-MG human glioblastoma cells.

calculated IC$_{50}$ value for the binding affinity to MC1R of **FIB-NDP-MSH** was found to be lower than for the reference NDP-MSH. The same trend was observed for the binding affinity to $\alpha_v\beta_3$ integrin of **FIB-PRGD$_2$** compared with the reference echistatin.

These results indicate that the 4-fluoro-3-iodobenzamide moiety does not interfere with the binding properties of the two bioactive peptides. *In vivo* PET imaging evaluation of [^{18}F]**FIB-NDP-MSH** and [^{18}F]**FIB-PRGD2** was conducted in primary melanoma-bearing mice and in primary glioblastoma-bearing mice, respectively. Considering tumor uptake (see Table 3), results with [^{18}F]**FIB-NDP-MSH** are similar to those described for NDP-MSH radiolabeled with [^{125}I]**SIB** or *para*-iodo *N*-succinimidyl 4-iodobenzoate [^{125}I]**PIB**.[22,23]

For [^{18}F]**FIB-PRGD2** we noted a slightly higher tumoral uptake in $\alpha_v\beta_3$ integrin positive tumors than in those determined with PRGD2 labeled with other ^{18}F-prosthetic groups.[24,25] No defluorination was observed for either radiopeptide between 1 and 3 h p.i. The observed predominant renal-urinary clearance route is also consistent with published results.[16,17] These preliminary *in vivo* PET imaging results suggest that the 4-fluoro-3-iodobenzamide moiety do not appear to interfere with the biological activity of both tested peptides.

In conclusion, we have developed a new prosthetic group that can be selectively radiofluorinated or radioiodinated with excellent radiochemical yields and purities. These radiolabeled acylating agents were first validated on a pigmented melanoma model through a melanin-binding benzamide approach and then successfully applied to the labeling and PET imaging of tumor-targeting peptides. These preliminary results encourage us to develop a more convenient procedure for the radiofluorination of **TFIB** (i.e., reducing the number of steps and/or the overall radiolabeling time) as well as to implement these findings in an extensive preclinical study involving PET imaging stratification and targeted radionuclide therapy applications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures for (radio)chemical synthesis, receptor-binding assays, *in vivo* imaging, compound characterization data, and HPLC (radio)chromatograms. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: j-michel.chezal@udamail.fr.

Present Addresses

[†]GIP Arronax, 1 rue aronnax, BP10112, F-44817 Saint-Herblain, France.

[‡]Université de Nantes, UFR sciences Pharmaceutiques et biologiques, 9 rue Bias, BP53508, F-44035 Nantes Cedex, France. Institut de Recherche en Santé IRS UN, Unité

Table 3. PET Quantitative Analysis of Tumors and Selected Tissues at 1 and 3 h after i.v. Injection of Radiotracer in Mice

compd	time	tumor[d]	muscle[d]	bone[d]	TMR[e]
[^{18}F]**9**[a]	1 h	6.8 ± 1.9	1.1 ± 0.6	0.9 ± 0.1	6.5 ± 2.4
	3 h	6.9 ± 0.5	0.4 ± 0.1	0.5 ± 0.1	15.5 ± 1.5
[^{18}F]**FIB-NDP-MSH**[b]	1 h	1.4 ± 0.9	0.3 ± 0.2	0.4 ± 0.2	4.0 ± 0.8
	3 h	0.7 ± 0.2	0.1 ± 0.0	0.2 ± 0.1	4.3 ± 1.2
[^{18}F]**FIB-PRGD2**[c]	1 h	7.3 ± 1.0	1.6 ± 0.7	1.7 ± 0.8	5.3 ± 2.5
	3 h	4.3 ± 1.0	0.9 ± 0.2	1.5 ± 0.9	4.9 ± 0.1

[a]B16F0 primary melanoma-bearing C57BL/6J mice. [b]B16F10 primary melanoma-bearing C57BL/6J mice. [c]U87-MG primary glioblastoma-bearing nude mice. [d]Data are expressed as percentage of injected dose per gram of tissue (%ID·g^{-1}) ± SD. [e]Tumor-to-muscle ratio.

Petitioner GE Healthcare – Ex. 1050, p. 171

INSERM 892-CNRS 6299 Centre de Recherche en Cancérologie Nantes-Angers, 8 quai Moncousu, BP70721, F-44007 Nantes Cedex, France.

Author Contributions

[§]These authors contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

This work was supported by CLARA (Cancéropôle Lyon Auvergne Rhône-Alpes), the French Directorate-General for Research and Innovation, the Auvergne Regional Council, the Puy-de-Dôme General Council, Clermont Community, the European Union, the European Regional Development Fund, the French Ligue contre le cancer, and the Bullukian Foundation.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Cyclopharma Laboratories for helpful technical supports and for radionuclide providing. The technical assistance of S. Tarrit is also gratefully acknowledged.

■ ABBREVIATIONS

HPLC, high pressure liquid chromatography; RT, room temperature; MALDI-TOF, matrix-assisted laser desorbtion/ionization time-of-flight; iodogen, 1,3,4,6-tetrachloro-3α,6α-diphenylglucoluril; Xantphos, 4,5-bis(diphenylphosphino)-9,9-dimethylxanthene; K_{222}, 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane

■ REFERENCES

(1) Adam, M. J.; Scott, D. J. Radiohalogens for imaging and therapy. *Chem. Soc. Rev.* **2005**, *34* (2), 153−163.

(2) Wilbur, D. S. Radiohalogenation of proteins: an overview of radionuclides, labeling methods, and reagents for conjugate labeling. *Bioconjugate Chem.* **1992**, *3* (6), 433−470.

(3) Dong, S.; Moroder, L.; Budisa, N. Protein iodination by click chemistry. *ChemBioChem* **2009**, *10* (7), 1149−1151.

(4) Avory, M. Iodine Radiolabelling Method. WO 2011070136, 2011.

(5) Bolton, A. E.; Hunter, W. M. The labeling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. *Biochem. J.* **1973**, *133* (3), 529−539.

(6) Vaidyanathan, G.; Zalusky, M. R. Preparation of N-succinimidyl 3-[*I]iodobenzoate: an agent for the indirect radioiodination of proteins. *Nat. Protoc.* **2006**, *1* (2), 707−713.

(7) Glaser, M.; Luthra, S. K.; Brady, F. Applications of positron-emitting halogens in PET oncology (Review). *Int. J. Oncol.* **2003**, *22* (2), 253−267.

(8) Zimmermann, R. G. Why are investors not interested in my radiotracer? The industrial and regulatory constraints in the development of radiopharmaceuticals. *Nucl. Med. Biol.* **2013**, *40* (2), 155−166.

(9) Chezal, J. M.; Dollé, F.; Madelmont, J. C.; Maisonial, A.; Miot-Noirault, E.; Moins, N.; Papon, J.; Kuhnast, B.; Tavitian, B. Labelled Analogues of Halobenzamides as Multimodal Radiopharmaceuticals and Their Precursors. WO 2009095872, 2009.

(10) Billaud, E. M. F.; Rbah-Vidal, L.; Vidal, A.; Besse, S.; Tarrit, S.; Askienazy, S.; Maisonial, A.; Moins, N.; Madelmont, J. C.; Miot-Noirault, E.; Chezal, J. M.; Auzeloux, P. Synthesis, radiofluorination, and *in vivo* evaluation of novel fluorinated and iodinated radiotracers for PET imaging and targeted radionuclide therapy of melanoma. *J. Med. Chem.* **2013**, *56* (21), 8455−8467.

(11) Wilbur, D. S.; Hamlin, D. K.; Srivastava, R. R.; Burns, H. D. Synthesis and radioiodination of *N*-Boc-*p*-(tri-n-butylstannyl)-L-phenylalanine tetrafluorophenyl ester: preparation of a radiolabeled phenylalanine derivative for peptide synthesis. *Bioconjugate Chem.* **1993**, *4* (6), 574−580.

(12) Lockett, M. R.; Phillips, M. F.; Jarecki, J. L.; Peelen, D.; Smith, L. M. A tetrafluorophenyl activated ester self-assembled monolayer for the immobilization of amine-modified oligonucleotides. *Langmuir* **2008**, *24* (1), 69−75.

(13) Zhang, W.; Curran, D. P. Synthetic application of fluorous solid-phase extraction (F-SPE). *Tetrahedron* **2006**, *62* (51), 11837−11865.

(14) Donovan, A.; Forbes, J.; Dorff, P.; Schaffer, P.; Babich, J.; Valliant, J. F. A new strategy for preparing molecular imaging and therapy agents using fluorine-rich (fluorous) soluble supports. *J. Am. Chem. Soc.* **2006**, *128* (11), 3536−3537.

(15) Jin, M. Y.; Yoshikai, N. Cobalt-Xantphos-catalyzed, LiCl-mediated preparation of arylzinc reagents from aryl iodides, bromides, and chlorides. *J. Org. Chem.* **2011**, *76* (7), 1972−1978.

(16) Krasovskiy, A.; Malakhov, V.; Gavryushin, A.; Knochel, P. Eficient synthesis of functionalized organozinc compounds by the direct insertion of zinc into organic iodides and bromides. *Angew. Chem., Int. Ed.* **2006**, *45* (36), 6040−6044.

(17) Banister, S.; Roeda, D.; Dollé, F.; Kassiou, M. Fluorine-18 chemistry for PET: A concise introduction. *Curr. Radiopharm.* **2010**, *3* (2), 68−80.

(18) Olberg, D. E.; Arukwe, J. M.; Grace, D.; Hjelstuen, O. K.; Solbakken, M.; Kindberg, G. M.; Cuthbertson, A. One step radiosynthesis of 6-[^{18}F]fluoronicotinic acid 2,3,5,6-tetrafluorophenyl ester [^{18}F]F-Py-TFP): a new prosthetic group for efficient labeling of biomolecules with fluorine-18. *J. Med. Chem.* **2010**, *53* (4), 1732−1740.

(19) Zhang, X.; He, Y.; Liu, S.; Yu, Z.; Jiang, Z. X.; Yang, Z.; Dong, Y.; Nabinger, S. C.; Wu, L.; Gunawan, A. M.; Wang, L.; Chan, R. J.; Zhang, Z. Y. Salicylic acid based small molecule inhibitor for the oncogenic Srchomology-2 domain containing protein tyrosine phosphatase-2 (SHP2). *J. Med. Chem.* **2010**, *53* (6), 2482−2493.

(20) Cheng, Z.; Zhang, L.; Graves, E.; Xiong, Z.; Dandekar, M.; Chen, X.; Gambhir, S. S. Small-animal PET of melanocortin 1 receptor expression using a ^{18}F-labeled alpha-melanocyte-stimulating hormone analog. *J. Nucl. Med.* **2007**, *48* (6), 987−994.

(21) Chin, F. T.; Shen, B.; Liu, S.; Berganos, R. A.; Chang, E.; Mittra, E.; Chen, X.; Gambhir, S. S. First experience with clinical-grade ([^{18}F]FPP(RGD$_2$): an automated multi-step radiosynthesis for clinical PET studies. *Mol. Imaging Biol.* **2012**, *14* (1), 88−95.

(22) Garg, P. K.; Alston, K. L.; Welsh, P. C.; Zalutsky, M. R. Enhanced binding and inertness to dehalogenation of alpha-melanotropic peptides labeled using *N*-succinimidyl 3-iodo benzoate. *Bioconjugate Chem.* **1996**, *7* (2), 233−239.

(23) Chen, J.; Giblin, M. F.; Wang, N.; Jurisson, S. S.; Quinn, T. P. In vivo evaluation of ^{99m}Tc/^{188}Re-labeled linear alpha-melanocyte stimulating hormone analogs for specific melanoma targeting. *Nucl. Med. Biol.* **1999**, *26* (6), 687−693.

(24) Wu, Z.; Li, Z. B.; Cai, W.; He, L.; Chin, F. T.; Li, F.; Chen, X. ^{18}F-labeled mini-PEG spacered RGD dimer (^{18}F-FPRGD2): synthesis and microPET imaging of $\alpha_v\beta_3$ integrin expression. *Eur. J. Nucl. Med. Mol. Imaging* **2007**, *34* (11), 1823−1831.

(25) Li, W.; Lang, L.; Niu, G.; Guo, N.; Ma, Y.; Kiesewetter, D. O.; Shen, B.; Chen, X. *N*-succinimidyl 4-[^{18}F]-fluoromethylbenzoate-labeled dimeric RGD peptide for imaging tumor integrin expression. *Amino Acids* **2012**, *43* (3), 1349−1357.

Petitioner GE Healthcare – Ex. 1050, p. 172

Title: Development and Preliminary Evaluation of TFIB, a New Bimodal Prosthetic Group for Bioactive Molecule Labeling**

Authors: Emilie M.F. Billaud, Aurélien Vidal, Amélie Vincenot, Sophie Besse, Bernadette Bouchon, Eric Debiton, Elisabeth Miot-Noirault, Imen Miladi, Latifa Rbah-Vidal, Philippe Auzeloux, Jean-Michel Chezal*

Table of Contents

1. Material and General Methods S2
2. Experimental Procedures and Spectroscopic Data for All Compounds S4
3. Radiochemistry S10
4. HPLC Conditions S15
5. Chromatograms for All Radiolabeled Compounds S16
6. In Vitro Receptor-Binding Assay S25
7. In Vivo Studies S25
8. References S27

Petitioner GE Healthcare – Ex. 1050, p. 173

1. Material and General Methods

Chemistry. All reagents and solvents were obtained from commercial sources and used as received. Peptides NDP-MSH and $PEG_3[c(RGDyK)]_2$ were purchased from Bachem AG and Peptides International, respectively. All solvents were dried using common techniques.[1] Unless otherwise noted, moisture-sensitive reactions were conducted under dry argon atmosphere. Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} plates and visualized with UV light and/or developed with iodine, ninhydrin or potassium permanganate. Flash chromatography was performed on silica gel 60A normal phase, 35–70 µm (SDS). Uncorrected melting points (mp) were recorded on Reichert-Jung-Koffler apparatus. NMR spectra (200 MHz for ^{1}H and 50 MHz for ^{13}C (J-MOD)) and 2D-NMR (^{1}H-^{1}H COSY, ^{1}H-^{13}C, HSQC, ^{1}H-^{13}C HMBC) were recorded for each compound on a Bruker Avance 200 instrument with chemical shift values expressed in ppm relative to TMS (δ_H 0.00 and δ_C 0.0) or residual chloroform (δ_H 7.26 and δ_C 77.2) as standard; ^{19}F NMR spectra (470 MHz) were recorded on a Bruker Avance 500 apparatus using α,α,α-trifluorotoluene as external reference (δ: –63.7 ppm). Infrared spectra (IR) were recorded on a FT Vector 22 or a FTIR Nicolet IS10 with attenuated total reflectance (ATR) accessory. Electron impact mode mass spectra (EI-MS) were obtained on a HP5890 series II chromatograph coupled to a HP5985B mass spectrometer (Agilent Technologies) or a Finnigan Trace DSQ spectrometer (Thermo Fischer). The analysis of samples was performed in MeCN or MeOH at a final concentration of 1 pmol.µL^{-1}. Electrospray ionization mass spectra (ESI-MS) were obtained on an Esquire-LC spectrometer (Bruker Daltonics). The analyses were performed in 50% aqueous MeCN or MeOH at a final concentration of 1 pmol.µL^{-1}. Microanalyses were performed by Analytical Laboratory of the CNRS (Vernaison, France) for the elements indicated.

Mass spectrometry analysis of the conjugated peptides. Conjugated peptides FIB-NDP-MSH and FIB-$PRGD_2$ were analyzed by MALDI-TOF MS on a Voyager-DE-Pro mass spectrometer (AB Sciex) using cyano-4-hydroxycinnamic acid as matrix in a positive reflector mode. Calibration was performed using a peptide calibration mixture (LaserBiolabs). Fragmentation data were obtained by MALDI-PSD-TOF analysis. Alternatively, peptides were analyzed by ESI-MS on an Esquire-LC mass spectrometer (Bruker Daltonics). MS and MS/MS analyses were performed at a final concentration of 1 pmol.µL^{-1} in aqueous MeCN solution containing 0.1% HCO_2H.

Radiochemistry. [^{125}I]NaI (3.6 GBq.mL^{-1}, 643.8 GBq.mg^{-1}), as a no-carrier-added solution in reductant free 10^{-5} M aqueous sodium hydroxide solution (pH 8-11), was purchased from PerkinElmer Life and Analytical Sciences. Iodo-Gen® and FluoroFlash® SPE cartridges were purchased from Sigma-Aldrich. Semi-preparative RP-HPLC purifications were performed on a Perkin Elmer system equipped with a Flexar LC autosampler, a Series 200 pump, a Peltier column oven, a vacuum degasser, a Photodiode Array Detector (PDA) and a gamma radioactivity flow through detector (GabiStar, Raytest). No-carrier-added fluorine-18 (half-life: 109.8 min) was produced *via* the [^{18}O(p, n)^{18}F] nuclear reaction by irradiation of a 2.8 mL [^{18}O]H_2O target (>97%-enriched, Bruce Technology) on a CPH14 cyclotron (14 MeV proton Beam, Cyclopharma laboratories). Typical production of [^{18}F]fluoride at the end of

Petitioner GE Healthcare – Ex. 1050, p. 174

bombardment for a 90 to 100 µA.h (120 min) irradiation: 185 GBq (5 Ci). The delivered radioactivity ranged from 3 and 11 GBq (delivery time). Radiochemistry syntheses and semi-preparative HPLCs were performed using a mono- or bi-reactor Synchrom R&D syntheses module (Raytest). SepPak® QMA and C18 were purchased from Waters or Macherey-Nagel. Radio-TLCs were run on Merck pre-coated neutral aluminium oxide 60 F_{254} or silica gel 60 F_{254} plates and measured on an AMBIS 400 (Scanalytics). Detection of K_{222} was achieved using the standard chloroplatinate color test.[2] Analytical HPLCs were performed on a Perkin Elmer system equipped with a Flexar LC autosampler, a Series 200 pump, a Peltier column oven, a vacuum degasser, a PDA and a GabiStar detector (Raytest). Unless otherwise indicated, analyses were performed at λ=254 nm. A delay time of 0.35 min was observed between the first detector (UV) and the second (gamma). Purity and specific activity were determined by analytical HPLC. For each biologically tested compound, radiochemical purity was over 99%. The identity of each radioiodinated or radiofluorinated compound was confirmed in radio-HPLC by co-elution with its non-radioactive counterpart.

Octanol-water distribution coefficient. Approximately 74 kBq of radiotracer was added to a mixture of PBS (1.0 mL, pH 7.4) and *n*-octanol (1.0 mL) in an Eppendorf microcentrifuge tube. The mixture was vigorously vortexed for 1 min, three times at room temperature. After centrifugation (3.000 rpm, 5 min), aliquots (100 µL) of both layers were sampled and the radioactivity was measured using a gamma counter equipped with a NaI(Tl) well type scintillation counter (Model 1480 Wizard, Perkin Elmer). Three independent experiments were performed in duplicates.

Cell cultures. The B16F0 syngenic melanoma cells were obtained from ATCC (no. CRL-6322). B16F0 cell cultures were maintained as monolayers in Dulbecco's Modified Eagle's Medium (DMEM)/Glutamax (Invitrogen, Fisher Scientific) supplemented with 10% fetal bovine serum (Invitrogen), and 4 $\mu g.mL^{-1}$ of gentamycin base (Invitrogen). The B16F10 syngenic melanoma cells were obtained from ATCC (no. CRL-6475). B16F10 cell cultures were maintained as monolayers in DMEM/Glutamax supplemented with 10% fetal bovine serum and 4 $\mu g.mL^{-1}$ of gentamicin base. The U87-MG human glioblastoma cells were obtained from ATCC (no. HTB-14). Cell cultures were maintained as monolayers in Modified Eagle's Medium (MEM)/Glutamax (Invitrogen, Fisher Scientific) supplemented with 10% fetal bovine serum and 4 $\mu g.mL^{-1}$ of gentamycin base. All cells were grown at 37°C in a humidified incubator containing 5% CO_2.

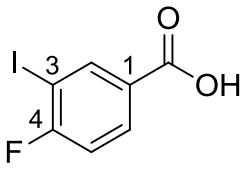
Primary melanoma and glioblastoma models. Animals were handled and cared for in accordance with the guidelines for the Care and Use of Laboratory Animals (National Research Council, 1996) and 2010/63/UE animal experiment European directive. Protocols were performed under the authorization of the local ethic C2E2A committee (authorization no. CE18-09 and CE86-12) and conducted under the supervision of authorized investigators in accordance with the institution's recommendations for the use of laboratory animals. Animals were handled and maintained at 21°C with a 12 h/12 h light/dark cycle. Primary melanoma models were induced by subcutaneous injection of B16F0 or B16F10 murine melanoma cells on 6- to 8-week old C57BL/6J male mice (Charles River). Primary glioblastoma model was induced by subcutaneous injection of U87-MG human cells on 6- to

Petitioner GE Healthcare – Ex. 1050, p. 175

8-week old NMRI nude male mice (Janvier Labs). For transplantations, cells in exponential growth phase were washed with phosphate buffer saline (PBS), trypsinized, centrifuged and resuspended in PBS. Mice anesthetized by isoflurane (2%) inhalation were inoculated with 3 x 10^5 melanoma B16F0 or B16F10 cells or 3 x 10^6 glioblastoma U87-MG cells in PBS (0.1 mL) by subcutaneous injection on the right shoulder.

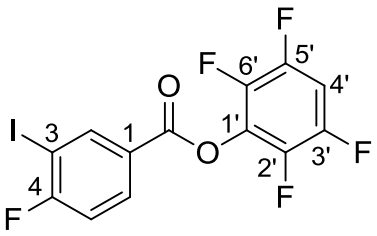
2. Experimental Procedures and Spectroscopic Data for All Compounds

4-Fluoro-3-iodobenzoic acid (2)



To a solution of commercial 3-amino-4-fluorobenzoic acid (**1**) (1.00 g, 6.45 mmol) in concentrated hydrochloric acid (37%, 8 mL), was added dropwise at -15°C, a solution of sodium nitrite (667 mg, 9.67 mmol) in water (10 mL), while maintaining the temperature below -10°C. The mixture was stirred at -10°C for 30 min, then a solution of potassium iodide (3.21 g, 19.34 mmol) in water (10 mL) was added dropwise, while maintaining the temperature below -5°C. The resulting solution was warmed to 0°C for 30 min and then to room temperature. After 3 h, the mixture was cooled to 0°C and quenched by slow addition of a 10 N aqueous sodium hydroxide solution until pH 3. 1 M aqueous sodium thiosulfate solution was then added (20 mL) and the solution was extracted with dichloromethane (3 x 50 mL). The organic fractions were pooled, washed with water (20 mL), dried on magnesium sulfate, filtered and evaporated to dryness to give 4-fluoro-3-iodobenzoic acid (**2**) (1.47 g, 5.53 mmol, 86%) as an orange solid which was used without further purification. Mp 169-171°C (lit.,[3] 173-174°C); ^{1}H NMR (500 MHz, $CDCl_3$) δ=7.16 (m, 1H, H-5), 8.10 (m, 1H, H-6), 8.54 (dd, 1H, ^4J(H,F)=6.0 Hz, ^4J(H,H)=2.1 Hz, H-2); ^{13}C NMR (50 MHz, $(CD_3)_2SO$) δ=83.0 (d, ^2J(C,F)=27 Hz, C-3), 116.5 (d, ^2J(C,F)=25 Hz, C-5), 129.5 (C-1), 132.4 (d, ^3J(C,F)=9 Hz, C-6), 140.9 (C-2), 164.5 (d, ^1J(C,F)=249 Hz, C-4), 165.7 (C=O); ^{19}F NMR (500 MHz, $CDCl_3$) δ=-86.0; IR (KBr) ν=626, 1039, 1291, 1426, 1484, 1585, 1686, 2500-3100 cm^{-1}; ESI-MS: *m/z* 264.94 ([M-H]$^-$ calc. 264.91).

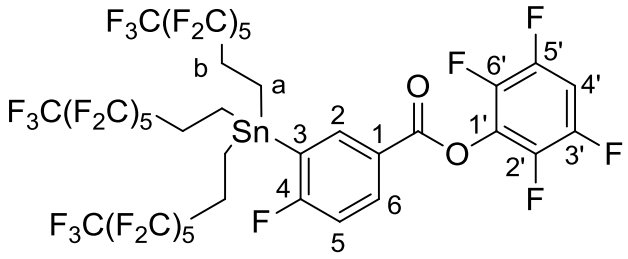
2,3,5,6-Tetrafluorophenyl 4-fluoro-3-iodobenzoate (3, TFIB)



To a solution of 4-fluoro-3-iodobenzoic acid (**2**) (1.00 g, 3.76 mmol) in anhydrous dichloromethane (9 mL), was added anhydrous *N*,*N*-dimethylformamide (29 µL, 0.38 mmol) under argon atmosphere. The reaction mixture was cooled to 0°C, and then a solution of

oxalyl chloride (636 µL, 7.52 mmol) in anhydrous dichloromethane (9 mL) was added dropwise. The mixture was stirred at 0°C for 30 min then at room temperature for 17 h. The solvent was removed under reduced pressure and the residue was co-evaporated twice with anhydrous toluene (2 x 5 mL). The crude acyl chloride was taken up under argon athmosphere in anhydrous dichloromethane (9 mL) before slowly adding a solution of 2,3,5,6-tetrafluorophenol (1.25 g, 7.52 mmol) and freshly distilled triethylamine (576 µL, 4.14 mmol) in anhydrous dichloromethane (9 mL). After stirring at room temperature for 4 h, the reaction mixture was poured into a solution of saturated aqueous sodium hydrogen carbonate (20 mL) and brine (20 mL). The aqueous phase was extracted with dichloromethane (2 x 20 mL). The combined organic extracts were dried on magnesium sulfate, filtered and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (cyclohexane/dichloromethane, 75:25, v/v) to give 2,3,5,6-tetrafluorophenyl 4-fluoro-3-iodobenzoate (**3**) (985 mg, 2.38 mmol, 63%) as a white solid. Mp=109-111°C; R_f=0.25 (SiO_2, cyclohexane/dichloromethane, 75:25, v/v); ^{1}H NMR (200 MHz, $CDCl_3$) δ=7.07 (m, 1H, H-4'), 7.21 (dd, 1H, ^3J(H,H)=8.5 Hz, ^3J(H,F)=7.6 Hz, H-5), 8.20 (m, 1H, H-6), 8.62 (dd, 1H, ^4J(H,H)=2.1 Hz, ^4J(H,F)=5.9 Hz, H-2); ^{13}C NMR (50 MHz, $CDCl_3$) δ=81.8 (d, ^2J(C,F)=27 Hz, C-3), 103.7 (t, ^2J(C,F)=23 Hz, C-4'), 116.2 (d, ^2J(C,F)=25 Hz, C-5), 125.1 (d, ^4J(C,F)=3 Hz, C-1), 133.1 (d, ^3J(C,F)=9 Hz, C-6), 140.9 (ddd, ^1J(C,F)=252 Hz, ^2J(C,F)=15 Hz, ^3J(C,F)=4 Hz, C-2',C-6'), 146.1 (ddd, ^1J(C,F)=243 Hz, ^2J(C,F)=11 Hz, ^3J(C,F)=4 Hz, C-3', C-5'), 148.6 (td, ^2J(C,F)=12 Hz, ^3J(C,F)=4 Hz, C-1'), 142.5 (d, ^3J(C,F)=3 Hz, C-2), 160.4 (C=O), 165.8 (d, ^1J(C,F)=256 Hz, C-4); ^{19}F NMR (500 MHz, $CDCl_3$) δ=-84.1 (F-4), -139.5 and -153.6 (4F, F-2', F-3', F-5', F-6'). IR (ATR accessory) ν=670, 1081, 1247, 1486, 1505, 1523, 1589, 1644, 1747, 3094 cm^{-1}; EI-MS: *m/z* 414.11 ([M+H]$^+$, 8%), 249.04 (100%), 221.10 (20%), 94.12 (10%); Anal. ($C_{13}H_4F_5IO_2$) found: C: 38.00, H: 0.94, F: 22.82, I: 30.61, calculated: C: 37.71, H: 0.97, F: 22.94, I: 30.65.

2,3,5,6-Tetrafluorophenyl 4-fluoro-3-[tris(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl)stannyl]benzoate (4)

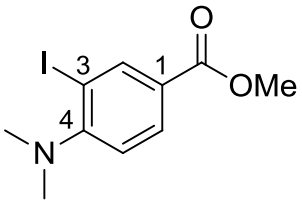


Anhydrous lithium chloride (41 mg, 0.97 mmol) was dried under vacuum at 150°C for 30 min. After cooling to room temperature under argon atmosphere, zinc powder (100 mesh, 95 mg, 1.45 mmol, 1.5 eq.) was added and the resulting mixture was dried under vacuum at 150°C for a further 20 min. After cooling to room temperature, anhydrous tetrahydrofuran (1.5 mL), 1,2-dibromoethane (4.2 µL, 48 µmol, 0.05 eq.) and chlorotrimethylsilane (1.2 µL, 10 µmol, 0.01 eq.) were successively added under argon atmosphere. The reaction mixture was stirred at room temperature for 5 min, then Xantphos (28 mg, 48 µmol, 0.05 eq.) and cobalt(II) chloride (10 mesh, 6.3 mg, 48 µmol, 0.05 eq.) were successively added under argon

Petitioner GE Healthcare – Ex. 1050, p. 177

atmosphere. After stirring for 5 min at room temperature, a solution of dry 2,3,5,6-tetrafluorophenyl 4-fluoro-3-iodobenzoate (**3**) (400 mg, 0.97 mmol) in anhydrous tetrahydrofuran (1 mL) was added. The mixture was heated at 50°C for 3 h under argon atmosphere. After cooling to room temperature, the corresponding organozinc solution was slowly added to a solution of tris(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl)tin bromide (212 µL, 0.32 mmol, 0.33 eq.) in tetrahydrofuran (2 mL) at 0°C under argon atmosphere. The mixture was heated at 50°C for 72 h. After cooling to room temperature, the solvent was evaporated, and the residue was taken up in FC-72 (2 mL) and methanol (4 mL). After decantation, the methanol phase was washed with FC-72 (2 mL). The perfluorous layers were combined and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (cyclohexane/dichloromethane 90:10→75:25, v/v) to give 2,3,5,6-tetrafluorophenyl 4-fluoro-3-[tris(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl)stannyl] benzoate (**4**) (128 mg, 88 µmol, 28%) as a colorless oil. R_f=0.33 (SiO_2, cyclohexane/ethyl acetate, 95/05, v/v); ^{1}H NMR (200 MHz, $CDCl_3$) δ=1.43 (t, 6H, ^3J(H,H)=8.2 Hz, CH_2-a), 2.37 (m, 6H, CH_2-b), 7.06 (m, 1H, CH-4'), 7.25 (dd, 1H, ^3J(H,H)=8.7 Hz, ^3J(H,F)=6.2 Hz, CH-5), 8.27 (m, 2H, CH-2, CH-6); ^{13}C NMR (50 MHz, $CDCl_3$) δ=0.4 (3C, ^1J(C,^{117}Sn/^{119}Sn)=380 Hz, C-a), 28.5 (t, 3C, ^2J(C,F)=23 Hz, C-b), 104.4 (t, ^2J(C,F)=23 Hz, C-4'), 116.5 (d, ^2J(C,F)=29 Hz, C-5), 109.8-126.8 (m, 20C, 15xCF_2, 3xCF_3, C-3, C-1), 136.1 (d, ^3J(C,F)=10 Hz, C-6), 140.8 (d, ^3J(C,F)=17 Hz, C-2), 141.7 (dd, 2C, ^1J(C,F)=250 Hz, ^2J(C,F)=17 Hz, C-2', C-6'), 147.1 (dd, 2C, ^1J(C,F)=244 Hz, ^2J(C,F)=12 Hz, C-3', C-5'), 149.5 (m, C-1'), 162.5 (C=O), 171.5 (d, ^1J(C,F)=244 Hz, C-4); ^{19}F NMR (500 MHz, $CDCl_3$) δ=-81.8 (9F, 3xCF_3), -85.9 (1F, F-4), -117.4 (6F, 3xCF_2), -122.9 (6F, 3xCF_2), -123.9 (6F, 3xCF_2), -124.5 (6F, 3xCF_2), -127.2 (6F, 3xCF_2), -139.8 and -153.9 (4F, F-2', F-3', F-5', F-6'); IR (ATR accessory) ν=1082, 1141, 1188, 1232, 1489, 1528, 1763 cm^{-1}; EI-MS: *m/z* 625.28 (18%), 407.14 (16%), 299.11 (39%), 289.23 (100%), 245.20 (42%), 239.29 (61%), 195.29 (31%), 77.16 (34%).

Methyl 4-(dimethylamino)-3-iodobenzoate (6)

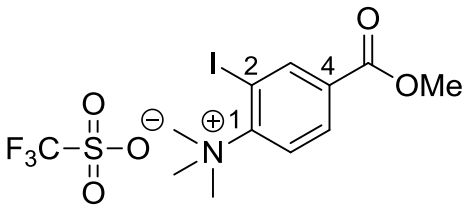


Paraformaldehyde (542 mg, 18.05 mmol) and sodium cyanoborohydride (567 mg, 9.02 mmol) were added portionwise to a solution of commercial methyl 4-amino-3-iodobenzoate (**5**) (500 mg, 1.80 mmol) in glacial acetic acid (10 mL) at 0°C. The mixture was stirred at room temperature for 4 h. After evaporation to dryness, the residue was taken up in ethyl acetate (20 mL), washed with a saturated aqueous sodium hydrogen carbonate solution (30 mL) and brine (30 mL). The organic phase was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (cyclohexane/ethyl acetate, 9:1, v/v) to give methyl 4-(dimethylamino)-3-iodobenzoate (**6**) (545 mg, 1.79 mmol, 99%) as a yellow oil. R_f=0.36 (SiO_2, cyclohexane/ethyl acetate, 9:1,

Petitioner GE Healthcare – Ex. 1050, p. 178

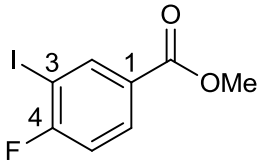
v/v); ^{1}H NMR (200 MHz, $CDCl_3$) δ=2.83 (s, 6H, $N(CH_3)_2$), 3.87 (s, 3H, OCH_3), 6.99 (d, 1H, ^{3}J(H,H)=8.4 Hz, H-5), 7.93 (dd, 1H, ^{3}J(H,H)=8.4 Hz, J=2.0 Hz, H-6), 8.47 (d, 1H, ^{4}J(H,H)=2.0 Hz, H-2). ^{13}C NMR (50 MHz, $CDCl_3$) δ=44.5 (2C, $N(CH_3)_2$), 52.2 (OCH_3), 93.7 (C-3), 119.2 (C-5), 125.5 (C-1), 130.6 (C-6), 142.1 (C-2), 159.0 (C-4), 165.7 (C=O). IR (NaCl) ν=607, 1246, 1289, 1383, 1433, 1491, 1548, 1591, 1718, 2868, 2948 cm^{-1}; ESI-MS: *m/z* 305.96 ($[M+H]^+$ calc. 306.00).

2-Iodo-4-(methoxycarbonyl)-*N*,*N*,*N*-trimethylanilinium trifluoromethanesulfonate (7)



Methyl trifluoromethanesulfonate (178 µL, 1.57 mmol) was added under argon atmosphere to a solution of methyl 4-(dimethylamino)-3-iodobenzoate (**6**) (200 mg, 0.66 mmol) in anhydrous diethyl ether (2 mL). The reaction mixture was stirred at room temperature for 4 d. The appearing precipitate was collected by filtration and washed with anhydrous diethyl ether (2 x 2 mL) to give 2-iodo-4-(methoxycarbonyl)-*N*,*N*,*N*-trimethylanilinium trifluoromethanesulfonate (**7**) (150 mg, 0.32 mmol, 49%) as a white solid. Mp 97-99°C; ^{1}H NMR (200 MHz, CD_3CN) δ=3.81 (s, 9H, $N(CH_3)_3$), 3.91 (s, 3H, OCH_3), 7.99 (d, 1H, ^{3}J(H,H)=8.9 Hz, H-6), 8.15 (dd, 1H, ^{3}J(H,H)=8.9 Hz, ^{4}J(H,H)=2.0 Hz, H-5), 8.80 (d, 1H, ^{4}J(H,H)=2.0 Hz, H-3); ^{13}C NMR (50 MHz, CD_3CN) δ=53.6 (OCH_3), 57.6 (3C, $N(CH_3)_3$), 85.9 (C-2), 124.5 (C-6), 131.6 (C-5), 134.2 (C-4), 147.8 (C-3), 149.8 (C-1), 164.8 (C=O); IR (ATR accessory) ν=1116, 1151, 1227, 1251, 1380, 1440, 1460, 1479, 1570, 1595, 1723, 2849, 2917, 3057 cm^{-1}; ESI-MS: *m/z* 319.97 ($[M]^+$ calc. 320.01). HR-MS: *m/z* 320.0141 (M^+ calc. 320.0142)

Methyl 4-fluoro-3-iodobenzoate (8)

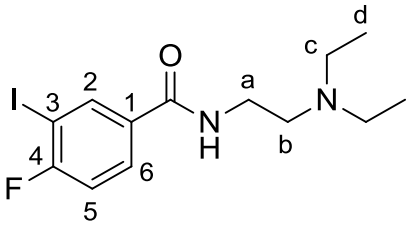


To a solution of 4-fluoro-3-iodobenzoic acid (**2**) (50 mg, 0.19 mmol) in anhydrous dichloromethane (1.5 mL) was added anhydrous *N*,*N*-dimethylformamide (1.5 µL, 19 µmol) under argon atmosphere. After cooling to 0°C, a solution of oxalyl chloride (32 µL, 0.38 mmol) in anhydrous dichloromethane (1 mL) was added dropwise. The mixture was stirred at 0°C for 1 h and then at room temperature for 16 h. The solvent was removed under reduced pressure and the residue was co-evaporated twice with anhydrous toluene (2 x 2 mL). The crude acyl chloride was taken up in anhydrous dichloromethane (1.5 mL) and a solution of anhydrous methanol (76 µL, 1.88 mmol) and distilled triethylamine (29 µL, 0.21 mmol) in anhydrous dichloromethane (1 mL) was slowly added under argon atmosphere. The mixture

Petitioner GE Healthcare – Ex. 1050, p. 179

was stirred at room temperature for 4 h. The reaction was quenched by addition of a saturated aqueous sodium hydrogen carbonate solution (15 mL) and brine (15 mL). After decantation, the aqueous phase was extracted with dichloromethane (2 x 10 mL) and the combined organic layers were dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (cyclohexane/dichloromethane, 75:25, v/v) to give methyl 4-fluoro-3-iodobenzoate (50 mg, 0.18 mmol, 95%) as a yellow oil. R_f=0.22 (SiO_2, cyclohexane/dichloromethane, 75:25, v/v); ^{1}H NMR (200 MHz, $CDCl_3$) δ=3.91 (s, 3H, CH_3), 7.10 (dd, 1H, ^3J(H,H)=8.6 Hz, ^3J(H,F)=7.6 Hz, H-5), 8.01 (m, 1H, H-6), 8.45 (dd, 1H, ^4J(H,F)=6.1 Hz, ^4J(H,H)=2.1 Hz, H-2); ^{13}C NMR (50 MHz, $CDCl_3$) δ=52.5 (CH_3), 81.2 (^2J(C,F)=26 Hz, C-3), 115.6 (^2J(C,F)=25 Hz, C-5), 128.1 (^4J(C,F)=3 Hz, C-1), 132.0 (^3J(C,F)=9 Hz, C-6), 141.3 (^3J(C,F)=2 Hz, C-2), 164.7 (^1J(C,F)=253 Hz, C-4), 164.9 (C=O). ^{19}F NMR (500 MHz, $CDCl_3$) δ=-87.7; IR (ATR accessory) ν=663, 1113, 1275, 1387, 1431, 1478, 1589, 1710, 2854, 2925 cm^{-1}; EI-MS: *m/z* 280.05 ($[M+H]^+$, 100%), 249.07 (61%), 221.01 (18%), 94.09 (8%).

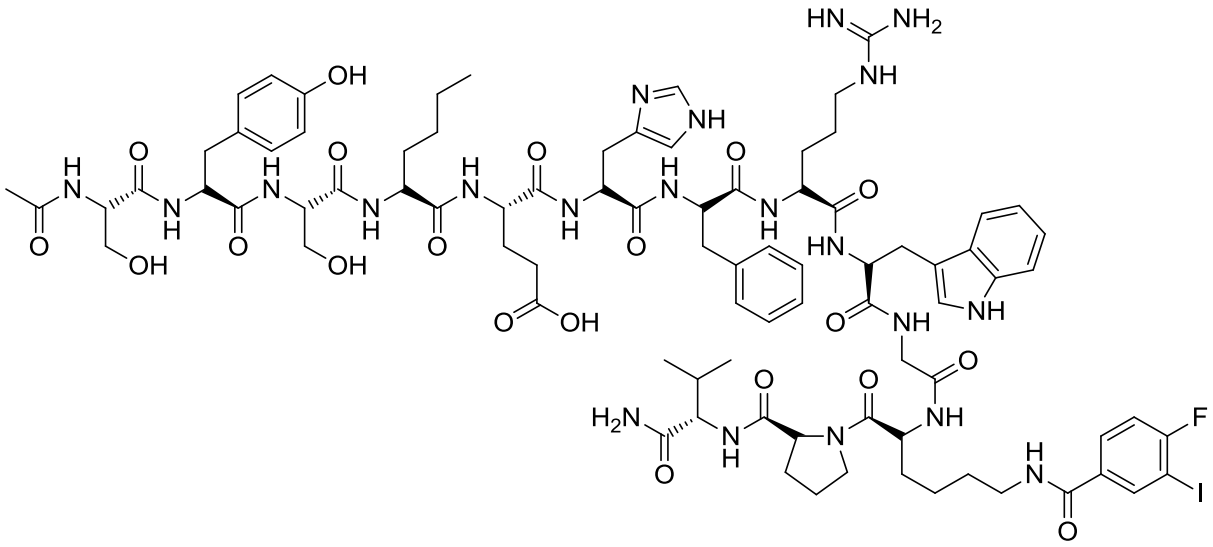
_N_-[2-(diethylamino)ethyl]-4-fluoro-3-iodobenzamide (9)



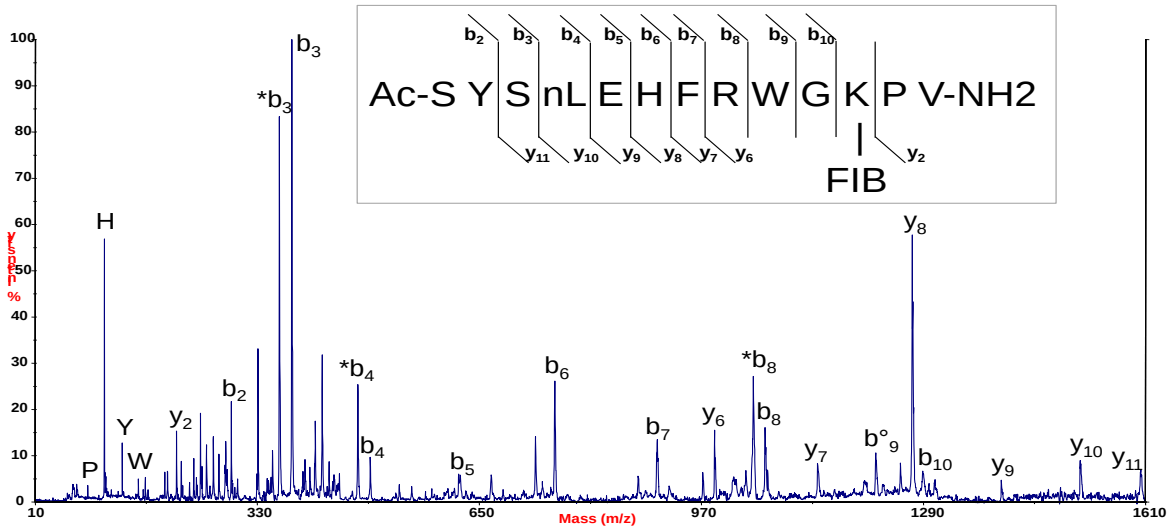
To a solution of **2** (500 mg, 1.88 mmol) in dry *N*,*N*-dimethylformamide (4 mL) were successively added *N*,*N'*-dicyclohexylcarbodiimide (427 mg; 2.07 mmol) and 1-hydroxybenzotriazole hydrate (280 mg; 2.07 mmol). The mixture was stirred under argon atmosphere at room temperature for 30 min, and then *N*,*N*-diethylethylenediamine (291 µL, 2.07 mmol) in distilled *N*,*N*-diisopropylethylamine (655 µL, 3.76 mmol) was added. The reaction mixture was stirred under argon atmosphere at room temperature for 16 h. The reaction was quenched by the addition of water (15 mL). The precipitate was filtered and washed with water (2 x 10 mL). The filtrate was extracted with dichloromethane (4 x 40 mL). The combined organic layers were washed with a 5% aqueous sodium hydrogen carbonate solution (2 x 15 mL), dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (dichloromethane/ethanol, 9:1→8:2, v/v) to give *N*-[2-(diethylamino)ethyl]-4-fluoro-3-iodobenzamide (**9,** 300 mg, 0.82 mmol, 44%) as a yellow oil. R_f=0.14 (SiO_2, dichloromethane/ethanol, 9:1, v/v); ^{1}H NMR (500 MHz, $CDCl_3$) δ=1.05 (t, 6H, ^3J(H,H)=7.1 Hz, 2xCH_3), 2.59 (q, 4H, ^3J(H,H)=7.1 Hz, CH_2-c), 2.66 (t, 2H, ^3J(H,H)=5.9 Hz, CH_2-b), 3.47 (q, 2H, ^3J(H,H)=5.8 Hz, CH_2-a), 7.08 (t, 1H, *J*=8.0 Hz, ^3J(H-F)=8.0 Hz, H-5), 7.11 (brs, 1H, NH), 7.74 (m, 1H, H-6), 8.22 (dd, 1H, ^4J(H-F)=5.9 Hz, ^4J(H,H)=2.1 Hz, H-2); ^{13}C NMR (126 MHz, $CDCl_3$) δ=11.8 (2C, 2x(CH_3)), 37.4 (C-a), 46.8 (2C, C-c), 51.3 (C-b), 81.4 (^2J(C-F)=26 Hz, C-3), 115.5 (^2J(C-F)=24 Hz, C-5), 128.9 (^3J(C-F)=8 Hz, C-6), 132.6 (C-

1), 138.8 (C-2), 163.5 (^{1}J(C-F)=251 Hz, C-4), 164.9 (C=O); ^{19}F NMR (500 MHz, $CDCl_3$) δ=-90.6; IR (ATR accessory) ν=663, 1038, 1308, 1376, 1480, 1544, 1575, 1592, 1636, 2814, 2933, 2968, 3081, 3200-3400 cm^{-1}; ESI-MS: *m/z* 365.05 ($[M+H]^+$ calc. 365.05).

FIB-NDP-MSH

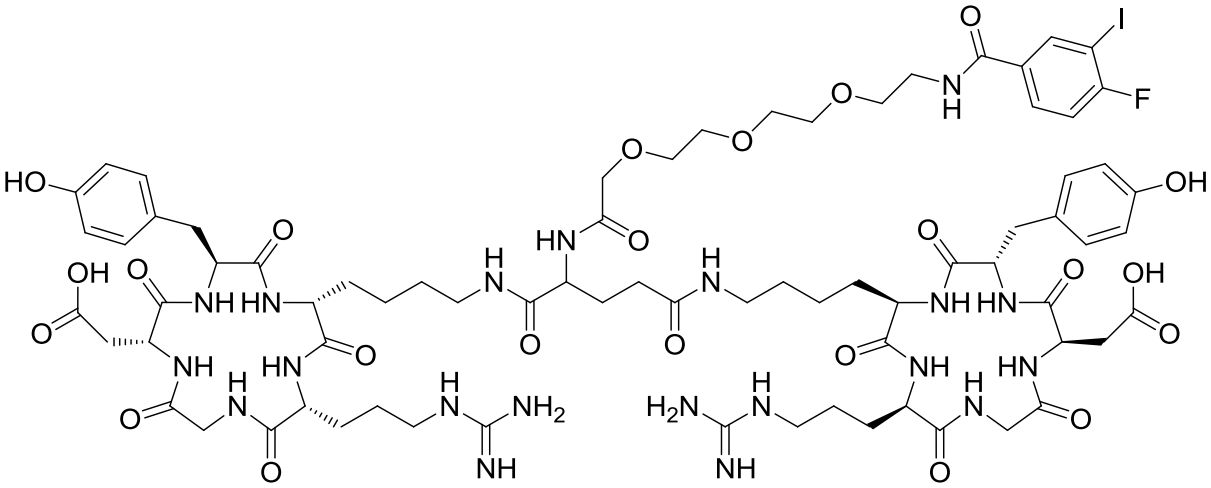


To a solution of NDP-MSH (1.0 mg) in borate buffer pH=8.6 (150 µL) was added a solution of **TFIB** in acetonitrile (100 µL, 1 $mg.mL^{-1}$). The reaction mixture was kept at room temperature for 1.5 h. **FIB-NDP-MSH** was then purified by semipreparative HPLC (Nucleodur C18 HTec column, 5 µm, 10 x 250 mm, Macherey-Nagel, equipped with a Nucleodur C18 HTec precolumn, 8 x 10 mm, Macherey-Nagel; flow: 4.0 $mL.min^{-1}$; λ=220 nm and 254 nm; acetonitrile/water (0.1% trifluoroacetic acid) 10:90→95:5, v/v, linear gradient in 12 min then 95:5 for 5 min then 10:90 for 8 min; tr=8.59 min). **FIB-NDP-MSH** was then lyophilized to give a white solid (0.18 mg). MALDI-TOF: *m/z* 1894.74 ($[M+H]^+$ calc. 1894.76).



MALDI-PSD mass spectrum of FIB-NDP-MSH peptide with the identified fragment ions localized on the corresponding peptide sequence. *b: b-H_2O

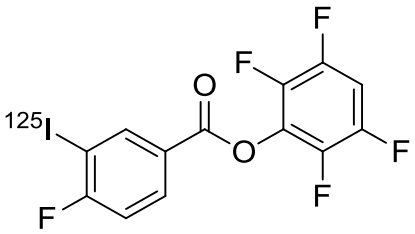
FIB-PRGD$_2$



To a solution of $PEG_3[c(RGDyK)]_2$ (2.1 mg) in borate buffer pH=8.0 (150 µL) was added a solution of **TFIB** in acetonitrile (210 µL, 1 mg.mL^{-1}). The reaction mixture was kept at room temperature for 1.0 h. **FIB-PRGD$_2$** was then purified by semi-preparative HPLC (Nucleodur C18 HTec column, 5 µm, 10 x 250 mm, Macherey-Nagel, equipped with a Nucleodur C18 HTec precolumn, 8 x 10 mm, Macherey-Nagel; flow: 4.0 mL.min^{-1}; λ=220 nm and 254 nm; acetonitrile/water (0.1% trifluoroacetic acid) 10:90→95:5, v/v, linear gradient in 12 min then 95:5 for 5 min then 10:90 for 8 min; tr=7.25 min). **FIB-PRGD$_2$** was then lyophilised to give a white solid (1.46 mg). MALDI-TOF: *m/z* 1787.67 ([M+H]$^+$ calc.1787.67). MALDI-TOF-PSD analysis as well as ESI-MS-MS analysis exhibited a fragmentation pattern with m/z 1169.0 as the major fragment. This m/z corresponds to the preferential fragmentation of one of the cyclic (RGDyK) peptides.

3. Radiochemistry

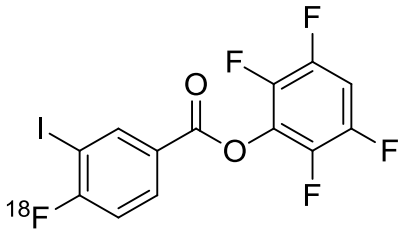
[^{125}I]2,3,5,6-Tetrafluorophenyl 4-fluoro-3-iodobenzoate ([^{125}I]TFIB)



To a solution of 2,3,5,6-tetrafluorophenyl 4-fluoro-3-[tris(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl)stannyl]benzoate (**4**) (100 µL, 1 mg.mL^{-1} in methanol) were successively added glacial acetic acid (20 µL), a solution of Iodo-Gen® (50 µL, 1 mg.mL^{-1} in methanol) and [^{125}I]NaI (30 MBq, 12 µL). The mixture was stirred at room temperature for 40 min. The reaction was quenched by the addition of aqueous sodium metabisulfite (25 µL, 1 mg.mL^{-1}), and the mixture was loaded onto a FluoroFlash® SPE cartridge (previously washed with

anhydrous *N*,*N*-dimethylformamide (1 mL) and preconditioned with methanol/water/acetic acid, 76:19:5, v/v/v, 20 mL)). The F-SPE cartridge was first eluted with water/acetic acid (95:5, v/v, 10 mL) to give fraction 1 containing salts and non-fluorous impurities, then with methanol/water/acetic acid (76:19:5, v/v/v, 15 mL) to give fraction 2 containing **[^{125}I]TFIB**, and finally with tetrahydrofuran (15 mL) to give fraction 3 containing fluorous-rich precursor **4**. Fraction 2 was then diluted with water (15 mL) and the radioactivity was trapped on a C18 cartridge (Chromafix® C18M, Macherey-Nagel). The cartridge was washed with water (10 mL) and compound **[^{125}I]TFIB** was eluted with acetonitrile (2 mL, formulation yield 98%). **[^{125}I]TFIB** was obtained in 60 min with 71% overall RCY and a specific activity of 30-35 GBq.µmol^{-1}. RCP>99% was determined by analytical HPLC (Method A described in section 4) ($Rt_{[^{125}I]TFIB}$=11.59 min). **[^{125}I]TFIB** was radiochemically stable in acetonitrile at room temperature for at least 4 d after preparation.

[^{18}F]2,3,5,6-Tetrafluorophenyl 4-fluoro-3-iodobenzoate ([^{18}F]TFIB)



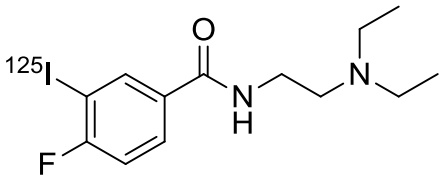
The activated [^{18}F]KF, K_{222} complex was obtained by an usual method in 16 min. Briefly, the aqueous [^{18}F]F$^-$ solution from target was flushed through an anion exchange resin (Sep-Pak Light Accell Plus QMA cartridge, Waters). The radioactivity was eluted to the first reactor with a K_2CO_3 (3.0 mg) and Kryptofix (K_{222}, 15.0 mg) solution (1 mL, acetonitrile/water, 70:30, v/v). Solvents were evaporated under reduced pressure and under a gentle stream of helium at 100°C for 3.5 min. The azeotropic drying was repeated by addition of anhydrous acetonitrile (1.0 mL) at 110°C for 3 min.

To the dry [^{18}F]KF, K_{222} complex was added a solution of compound **7** (10 mg) in anhydrous acetonitrile (1.0 mL), and the mixture was heated under stirring at 80°C for 4 min. After cooling to room temperature, 0.5 N aqueous sodium hydroxide (1.0 mL) was added and the mixture was heated at 50°C for 2 min. After cooling to room temperature, 0.5 N aqueous hydrochloric acid (1.2 mL) was added, the mixture was diluted with water (15 mL) and the radioactivity was trapped on a C18 cartridge (SepPak® Plus C18, Waters). The cartridge was washed with water (5 mL) and compound **[^{18}F]2** was eluted with acetonitrile (2.0 mL) in the second reactor containing a 0.1 M methanolic tetrabutylammonium hydroxide solution (100 µL). Solvents were evaporated under reduced pressure at 100°C for 5 min. After cooling to room temperature, a mixture of *N*,*N'*-dicyclohexylcarbodiimide (5.0 mg) and 2,3,5,6-tetrafluorophenol (8.0 mg) in acetonitrile (1.0 mL) was added and the reaction mixture was heated at 40°C for 5 min. After cooling to room temperature, the reaction mixture was diluted with HPLC eluent (3.0 mL) and purified by semi-preparative HPLC (Nucleodur C18 HTec column, 5 µM, 10 x 250 mm, Macherey-Nagel, equipped with a Nucleodur C18 HTec precolumn, 8 x 10 mm, Macherey-Nagel; flow: 4.5 mL.min^{-1}; acetonitrile/water (0.1% acetic

Petitioner GE Healthcare – Ex. 1050, p. 183

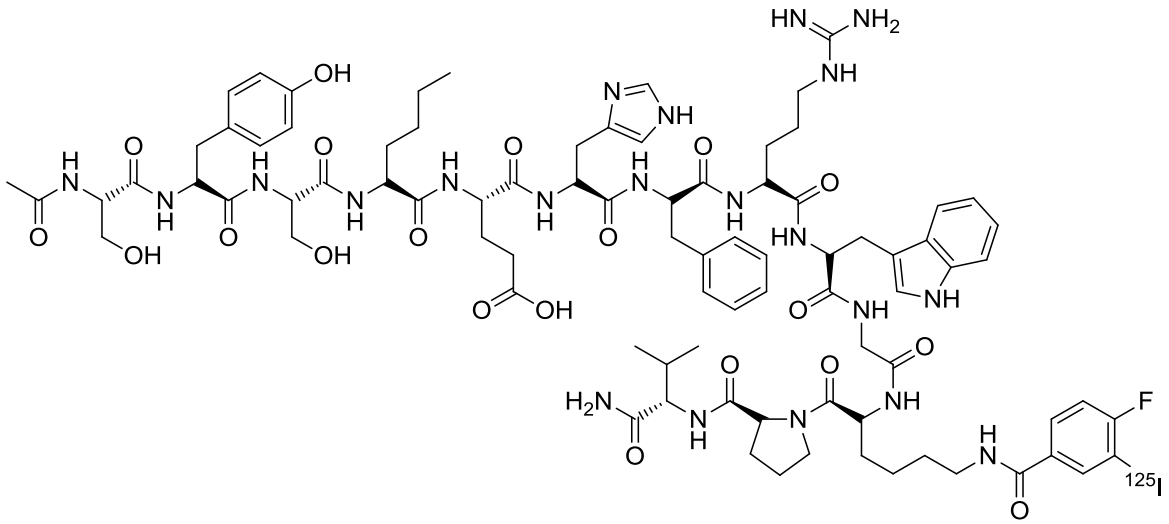
acid) 85:15, v/v; tr=10-11 min). The collected solution was diluted with water (20 mL) and the radioactivity was trapped on a C18 cartridge (Chromafix® C18, M, Macherey-Nagel). The cartridge was washed with water (5 mL) and compound **[^{18}F]TFIB** was eluted with acetonitrile (1.2 mL). **[^{18}F]TFIB** was obtained in 61 min with 32% overall RCY (decay-corrected) and a specific activity of 30-55 GBq.µmol^{-1}. RCP over 99% was determined by analytical HPLC (Method A described in section 4) ($Rt_{[^{18}F]TFIB}$=11.57 min). **[^{18}F]TFIB** was radiochemically stable in acetonitrile at room temperature for at least 3 h after preparation.

[^{125}I]*N*-[2-(diethylamino)ethyl]-4-fluoro-3-iodobenzamide ([^{125}I]9)



N,*N*-diethylethylenediamine (10 µL) was added to a solution of **[^{125}I]TFIB** (in 2 mL acetonitrile). The reaction mixture was kept at room temperature for 10 min, and then diluted with water (15 mL with 0.1% acetic acid). The radioactivity was trapped on a C18 cartridge (SepPak® Plus C18, Waters). The cartridge was washed with water (8 mL) and the radioactivity was eluted with ethanol (4 mL) and then saline. **[^{125}I]9** was obtained in 15 min with 92% overall RCY and a specific activity of 11-20 GBq.µmol^{-1}. RCP was determined by analytical HPLC (Method B described in section 4) ($Rt_{[^{125}I]9}$=9.46 min) and was >99%. **[^{125}I]9** was radiochemically stable at room temperature for at least 48 h after preparation (in ethanol/saline).

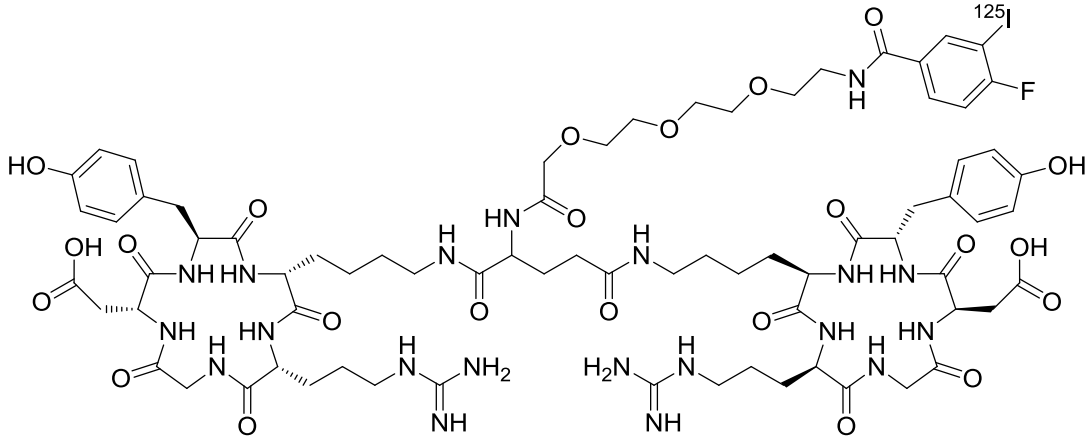
[^{125}I] [Nle^4, D-Phe^7, FIB-Lys^{11}]α-MSH ([^{125}I]FIB-NDP-MSH)



NDP-MSH (100 µL, 1 mg.mL^{-1} in borate buffer pH=8.0) was added to a solution of **[^{125}I]TFIB** in acetonitrile (33 µL). The reaction mixture was heated at 45°C for 30 min. After cooling to room temperature, **[^{125}I]FIB-NDP-MSH** was purified by semi-preparative HPLC (Nucleodur C18 HTec column, 5 µm, 10 x 250 mm, Macherey-Nagel, equipped with a

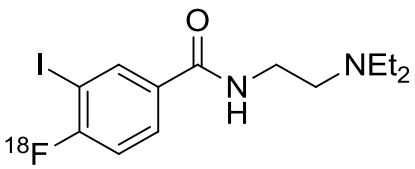
Nucleodur C18 HTec precolumn, 8 x 10 mm, Macherey-Nagel; flow: 4.0 mL.min^{-1}; λ=220 nm and 254 nm; acetonitrile/water (0.1% trifluoroacetic acid), 10:90→95:5, v/v linear gradient in 12 min then 95:5 for 5 min then 10:90 for 8 min; tr=8.90 min). Solvents were evaporated under reduced pressure, and the formulation was achieved in ethanol/saline (5:95, v/v,; formulation yield 95%). **[^{125}I]FIB-NDP-MSH** was obtained in 60 min with 83% overall radiochemical yield. RCP, determined by analytical HPLC (Method C described in section 4) ($Rt_{[^{125}I]FIB\text{-}NDP\text{-}MSH}$=9.01 min), was over 99%.

[^{125}I]FIB-PRGD$_2$



$PEG_3[c(RGDyK)]_2$ (100 µL, 1 mg.mL^{-1} in borate buffer pH=7.6) was added to a solution of **[^{125}I]TFIB** in acetonitrile (33 µL). The reaction mixture was heated at 45°C for 30 min. After cooling to room temperature, **[^{125}I]FIB-PRGD$_2$** was purified by semipreparative HPLC (Nucleodur C18 HTec column, 5 µM, 10 x 250 mm, Macherey-Nagel, equipped with a Nucleodur C18 HTec precolumn, 8 x 10 mm, Macherey-Nagel; flow: 4.0 mL.min^{-1}; λ=220 nm; acetonitrile/water (0.1% trifluoroacetic acid) 10:90→95:5, v/v linear gradient in 12 min then 95:5 for 5 min then 10:90 for 8 min; Rt=7.35 min. Solvents were evaporated under reduced pressure, and the formulation was achieved in ethanol/saline (5:95 v/v) **[^{125}I]FIB-PRGD$_2$** was obtained in 60 min with 78% overall radiochemical yield. RCP, determined by analytical HPLC (Method C described in section 4) ($Rt_{[^{125}I]FIB\text{-}PRGD2}$=8.39 min), was over 99%.

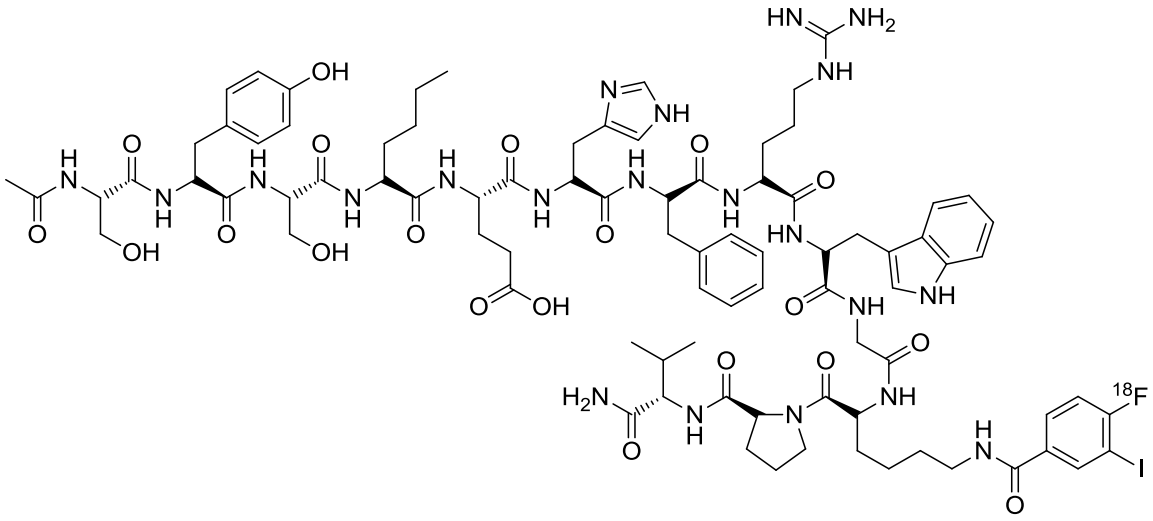
[^{18}F]*N*-[2-(diethylamino)ethyl]-4-fluoro-3-iodobenzamide ([^{18}F]9)



To a solution of **[^{18}F]TFIB** in acetonitrile (2.0 mL) was added a solution of *N*,*N*-diethylethylenediamine (10 µL) in acetonitrile (1.0 mL). The reaction mixture was kept at room temperature for 3 min, and then diluted with water (20 mL, 0.1% acetic acid). The radioactivity was trapped on a C18 cartridge (SepPak® Plus C18, Waters). The cartridge was washed with water (8 mL) and the radiotracer **[^{18}F]9** was eluted with ethanol (2 mL) and diluted with saline (formulation yield: 95%). **[^{18}F]9** was obtained in 8 min with 90% overall

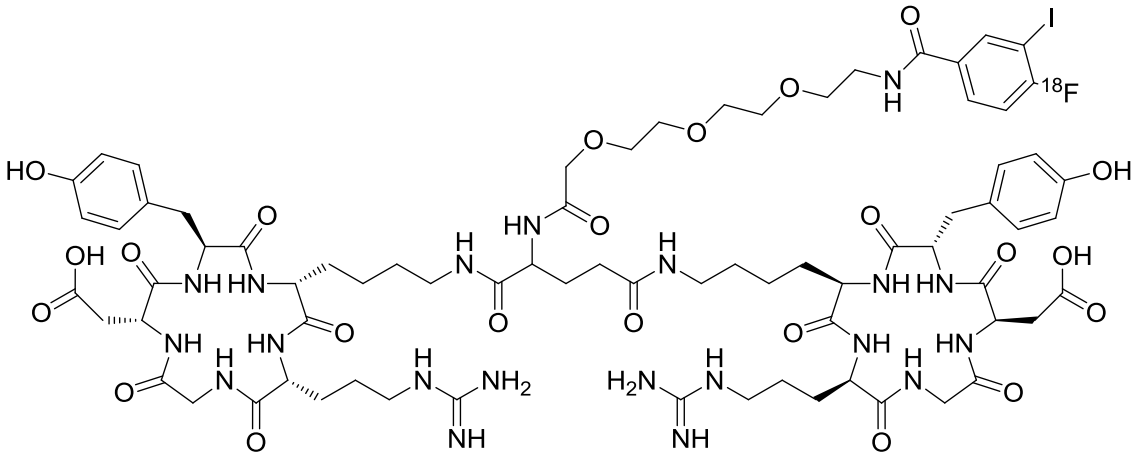
RCY (decay-corrected) and a specific activity of 23-25 GBq.µmol^{-1}. RCP over 99% was determined by analytical HPLC (Method B described in section 4) ($Rt_{[^{18}F]9}$=10.28 min). **[^{18}F]9** was radiochemically stable at room temperature for at least 2 h after preparation (in ethanol/saline 10:90, v/v).

[^{18}F][Nle^{4}, D-Phe^{7}, FIB-Lys^{11}]α-MSH ([^{18}F]FIB-NDP-MSH)



To a solution of **[^{18}F]TFIB** in acetonitrile (1.2 mL) was added a solution of NDP-MSH in borate buffer 0.05 N pH=8.0 (1.5 mL, 1 mg.mL^{-1}). The reaction mixture was heated at 50°C for 10 min. After cooling to room temperature, the radiotraceur **[^{18}F]FIB-NDP-MSH** was purified by semipreparative HPLC (Symmetry® Prep C18 column, 7 µm, 7.8 x 300 mm, Waters; flow: 3.0 mL.min^{-1}; acetonitrile/water (0.1% trifluoroacetic acid) 60:40, v/v, tr=5.50 min). The collected solution was diluted with water (20 mL) and the radioactivity was trapped on a C18 cartridge (SepPak® C18 Light, Waters). The cartridge was washed with water (5 mL) and the radiotracer **[^{18}F]FIB-NDP-MSH** was eluted with ethanol (3 mL) then saline (formulation yield: 99%). **[^{18}F]FIB-NDP-MSH** was obtained in 25 min with 57% overall RCY (decay-corrected). RCP, determined by analytical HPLC (Method C described in section 4) ($Rt_{[^{18}F]FIB\text{-}NDP\text{-}MSH}$=9.28 min), was over 99%.

[^{18}F]FIB-PRGD$_2$



To a solution of **[^{18}F]TFIB** in acetonitrile (1.2 mL) was added a solution of $PEG_3[c(RGDyK)]_2$ in borate buffer 0.05 N pH=7.6 (1.5 mL, 1 mg.mL^{-1}). The reaction mixture was heated at 50°C for 15 min. After cooling to room temperature, the radiotracer **[^{18}F]FIB-PRGD$_2$** was purified by semi-preparative HPLC (Symmetry® Prep C18 column, 7 µm, 7.8 x 300 mm, Waters; flow: 2.0 mL.min^{-1}; acetonitrile/water (0.1% trifluoroacetic acid) 60:40, v/v; tr=6.16 min). The collected solution was diluted with water (20 mL) and the radioactivity was trapped on a C18 cartridge (SepPak® C18 Light, Waters). The cartridge was washed with water (5 mL) and the radiotracer **[^{18}F]FIB-PRGD$_2$** was eluted with ethanol (3 mL) then saline (formulation yield: 99%). **[^{18}F]FIB-PRGD$_2$** was obtained in 31 min with 47% overall RCY (decay-corrected). RCP, determined by analytical HPLC (Method C described in section 4) ($Rt_{[^{18}F]FIB\text{-}PRGD2}$=8.46 min), was over 99%.

4. HPLC Conditions

The following HPLC conditions were used for the analysis of radiolabeled conjugates:

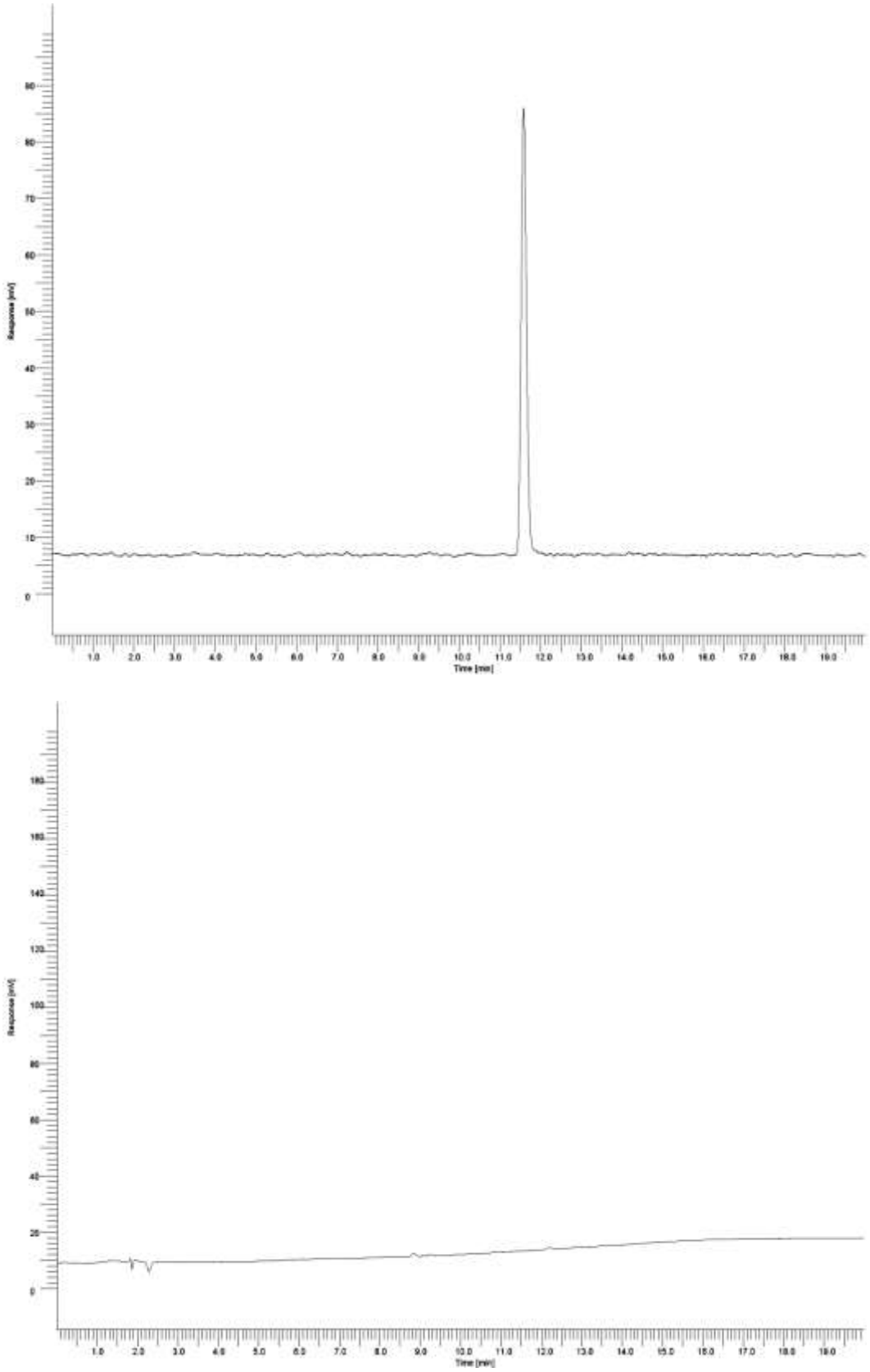
Method A. Column: Nucleodur C18 HTec, Macherey-Nagel, 5 µm, 4.6 x 250 mm; Flow: 1.0 mL.min^{-1}; UV detection wavelength: λ=254 nm; Elution conditions: acetonitrile/water (0.1% acetic acid) linear gradient in 10 min (70:30 to 100:0, v/v), followed by: isocratic (70:30) for 10 min (for ^{18}F-radiolabeled compound, Total time: 20 min) or by isocratic (100:0) for 22 min, then isocratic (70:30) for 8 min (for ^{125}I-radiolabeled compound, Total time: 40 min, $Rt_{[^{125}I]TFIB}$=11.59 min, $Rt_{\mathbf{4}}$=29.93 min).

Method B. Columns: Zorbax Extend-C18, Agilent, 5 µm, 4.6 x 150 mm (for **[^{125}I]9**), or Symmetry® C18, Waters, 5 µm, 4.6 x 150 mm (for **[^{18}F]9**); Flow: 0.9 mL.min^{-1}, UV detection wavelength: λ=254 nm; Elution conditions: methanol/water (0.2% NH_4OH) linear gradient for 10 min (60:40 to 90:10, v/v), then linear gradient for 7 min (60:40); Total time: 17 min.

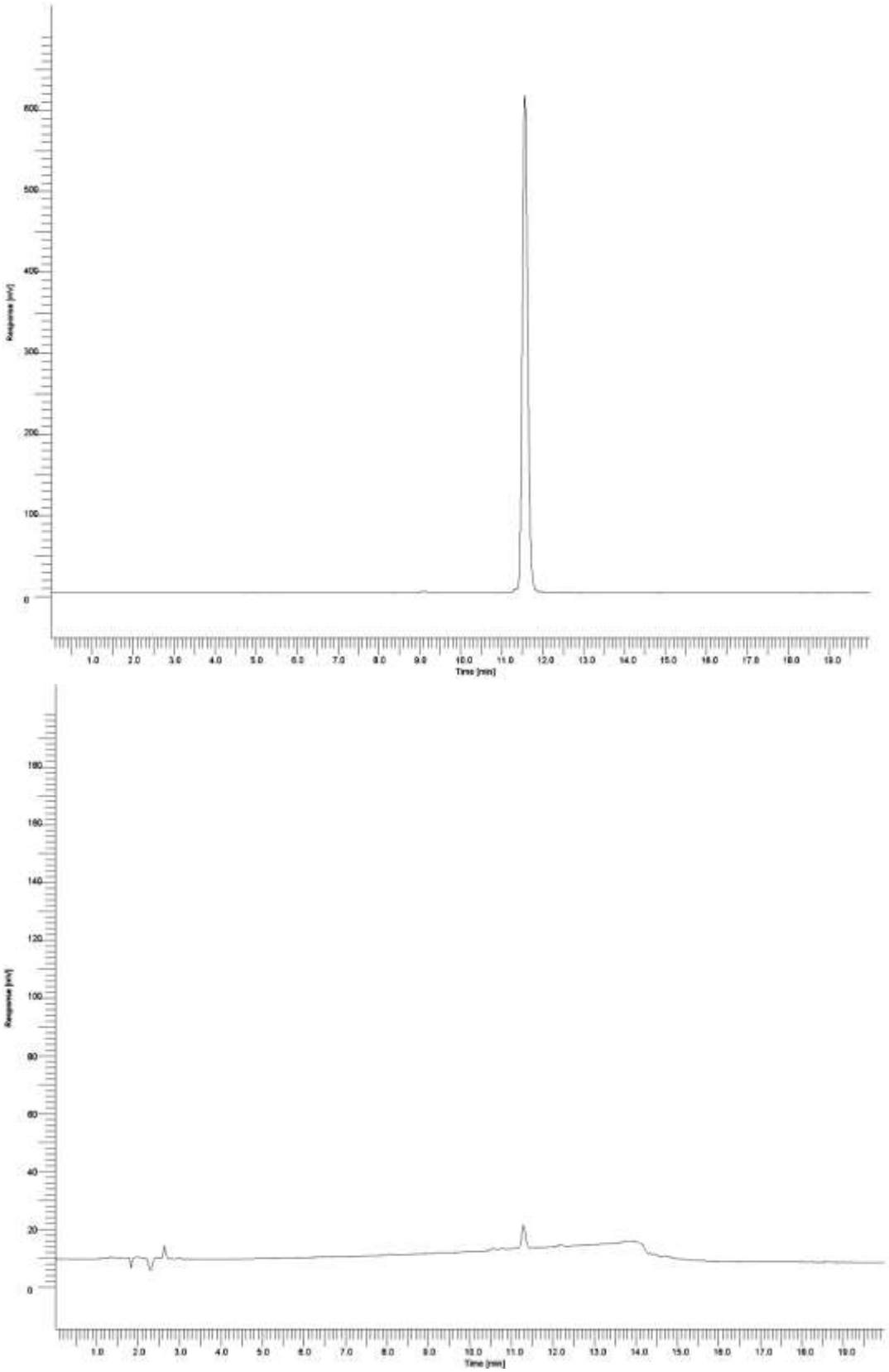
Method C. Column: Nucleodur C18 HTec, Macherey-Nagel, 5 µm, 4.6 x 250 mm; Flow: 1.0 mL.min^{-1}; UV detection wavelength: λ=220 nm; Elution conditions: acetonitrile/water (0.1% trifluoroacetic acid) linear gradient for 6 min (10:90 to 50:50, v/v), then linear gradient for 2 min (50:50 to 95:5) then isocratic for 5 min (95:5) followed by linear gradient for 6 min (10:90); Total time: 19 min.

Petitioner GE Healthcare – Ex. 1050, p. 187

5. Chromatograms for All Radiolabeled Compounds

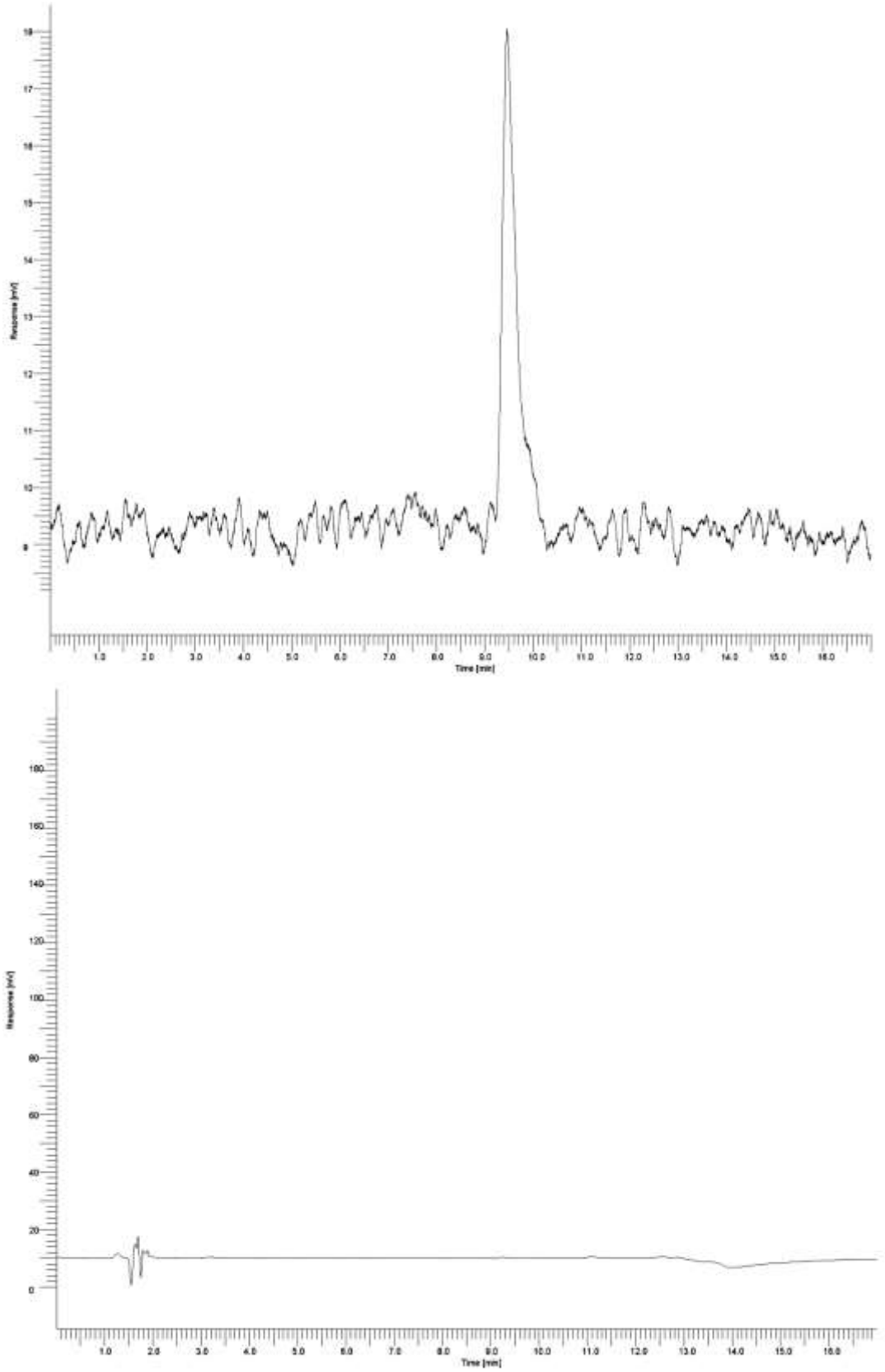


Radio(Top) and UV(Down)-chromatograms of **[^{125}I]TFIB** (method A)

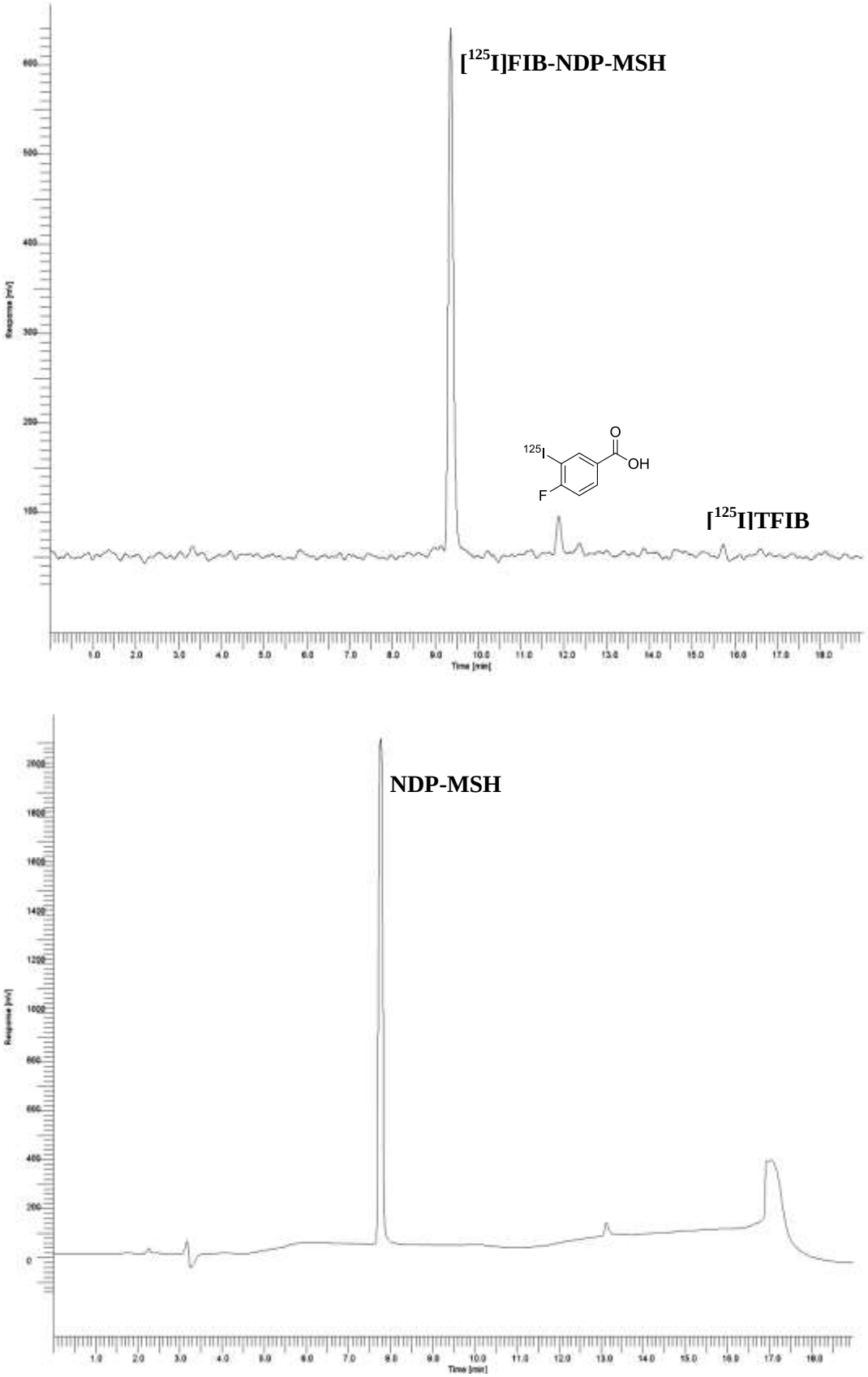


Radio(Top) and UV(Down)-chromatograms of **[^{18}F]TFIB** (method A)

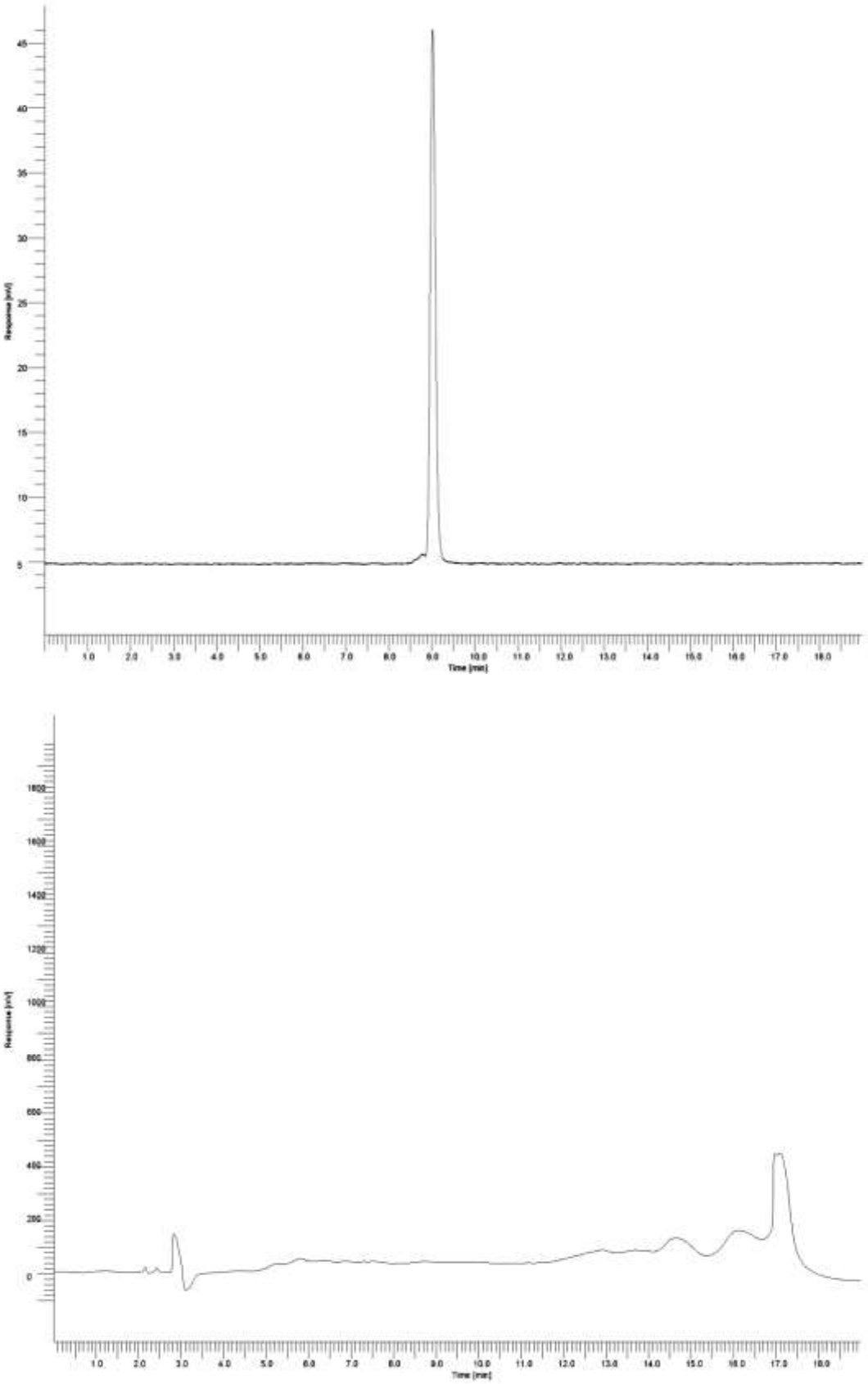
Petitioner GE Healthcare – Ex. 1050, p. 189



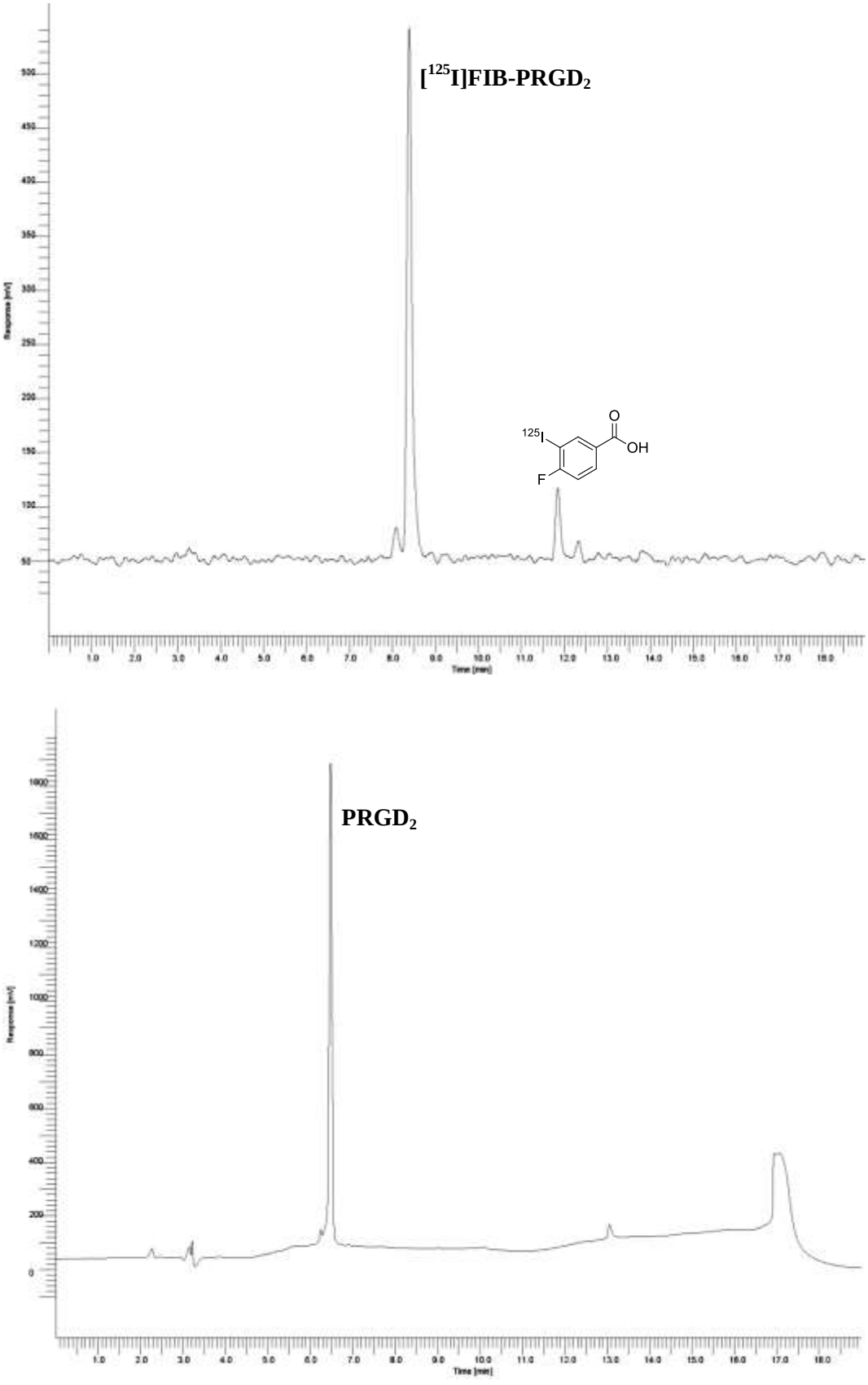
Radio(Top) and UV(Down)-chromatograms of **[^{125}I]9** (method B)



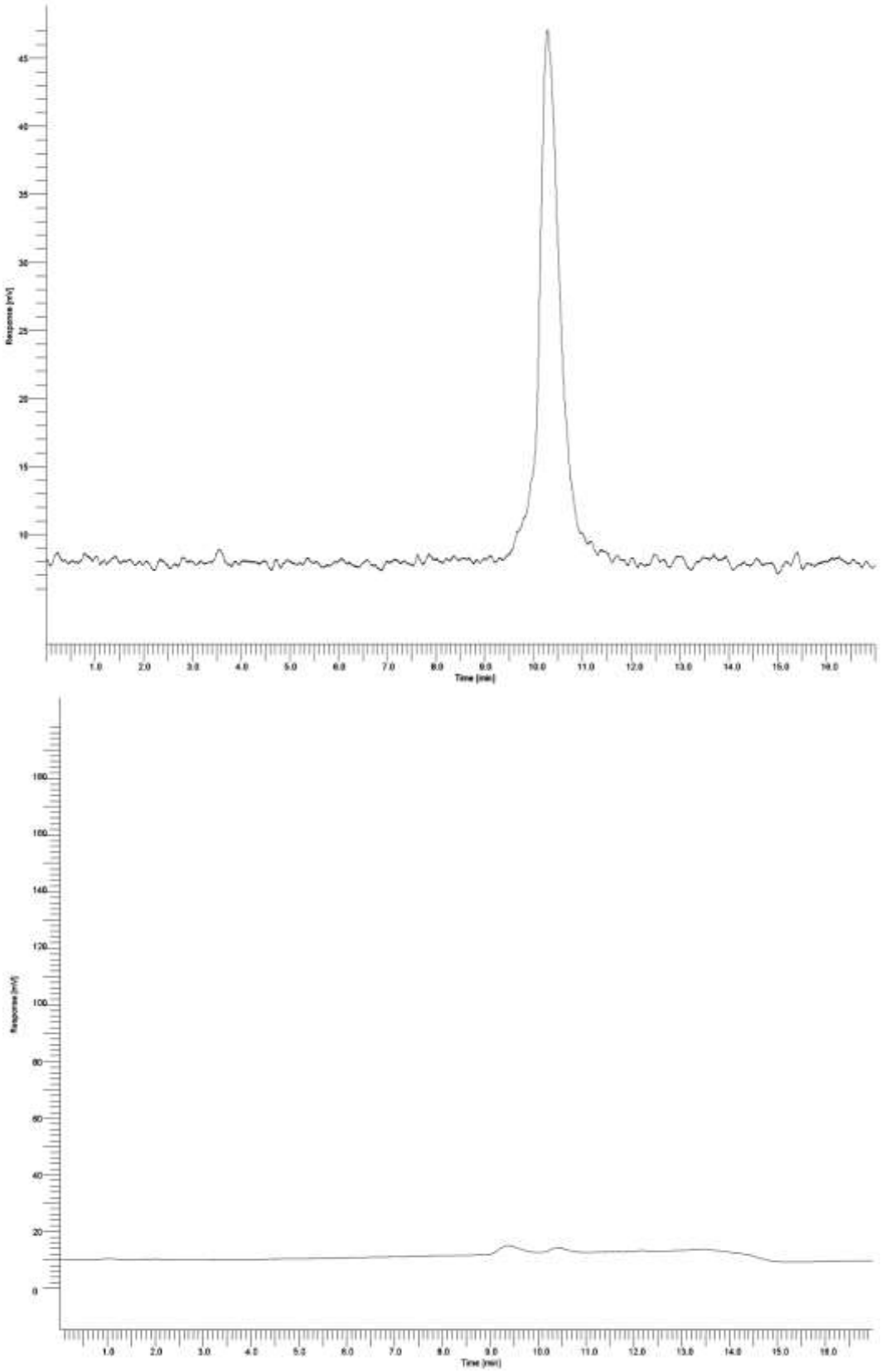
Radio (Top) and UV(Down)-chromatograms of crude **[^{125}I]FIB-NDP-MSH** (method C)



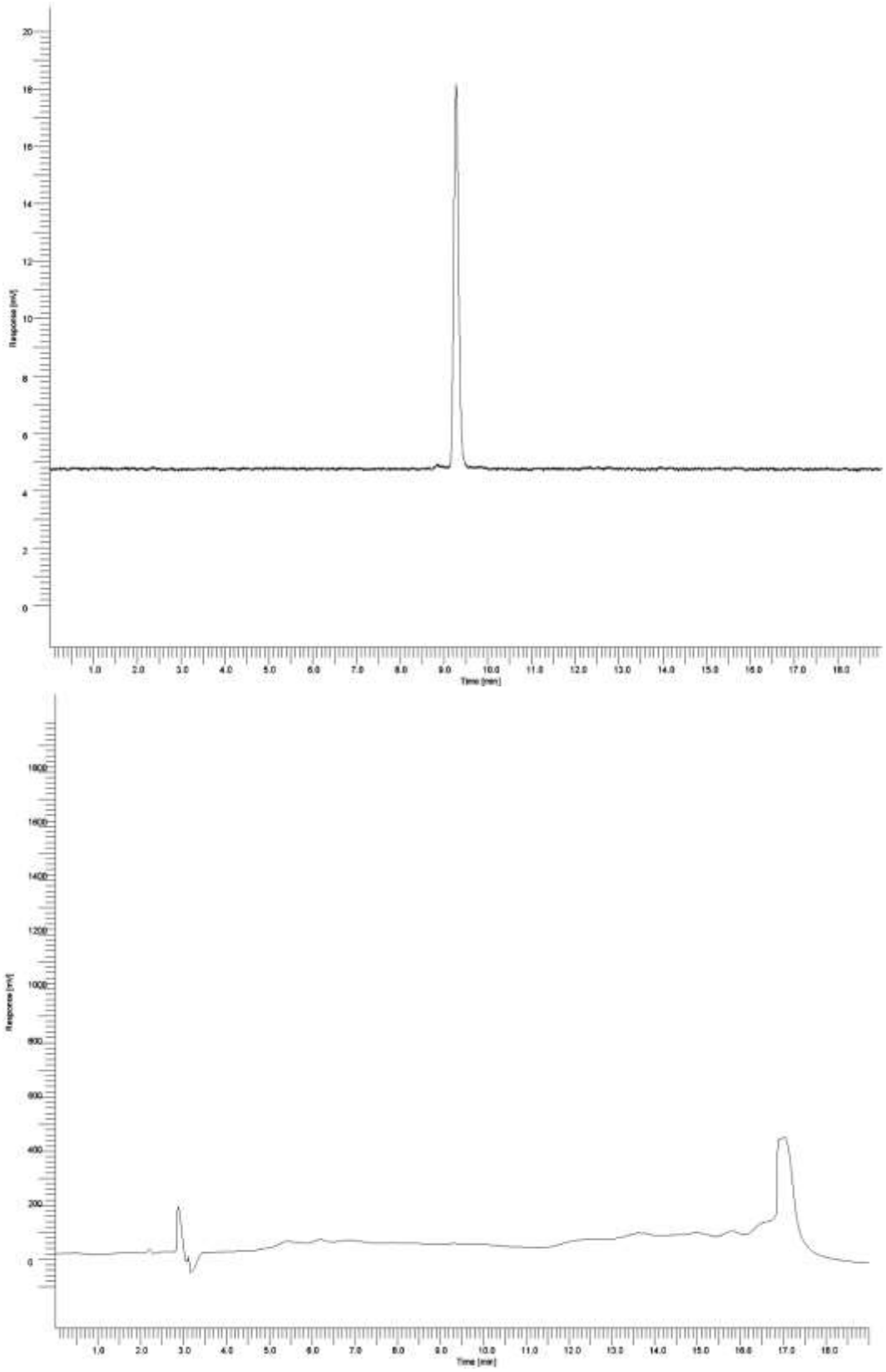
Radio (Top) and UV(Down)-chromatograms of purified **[^{125}I]FIB-NDP-MSH** (method C)



Radio (Top) and UV(Down)-chromatograms of crude **[^{125}I]FIB-PRGD$_2$** (method C)

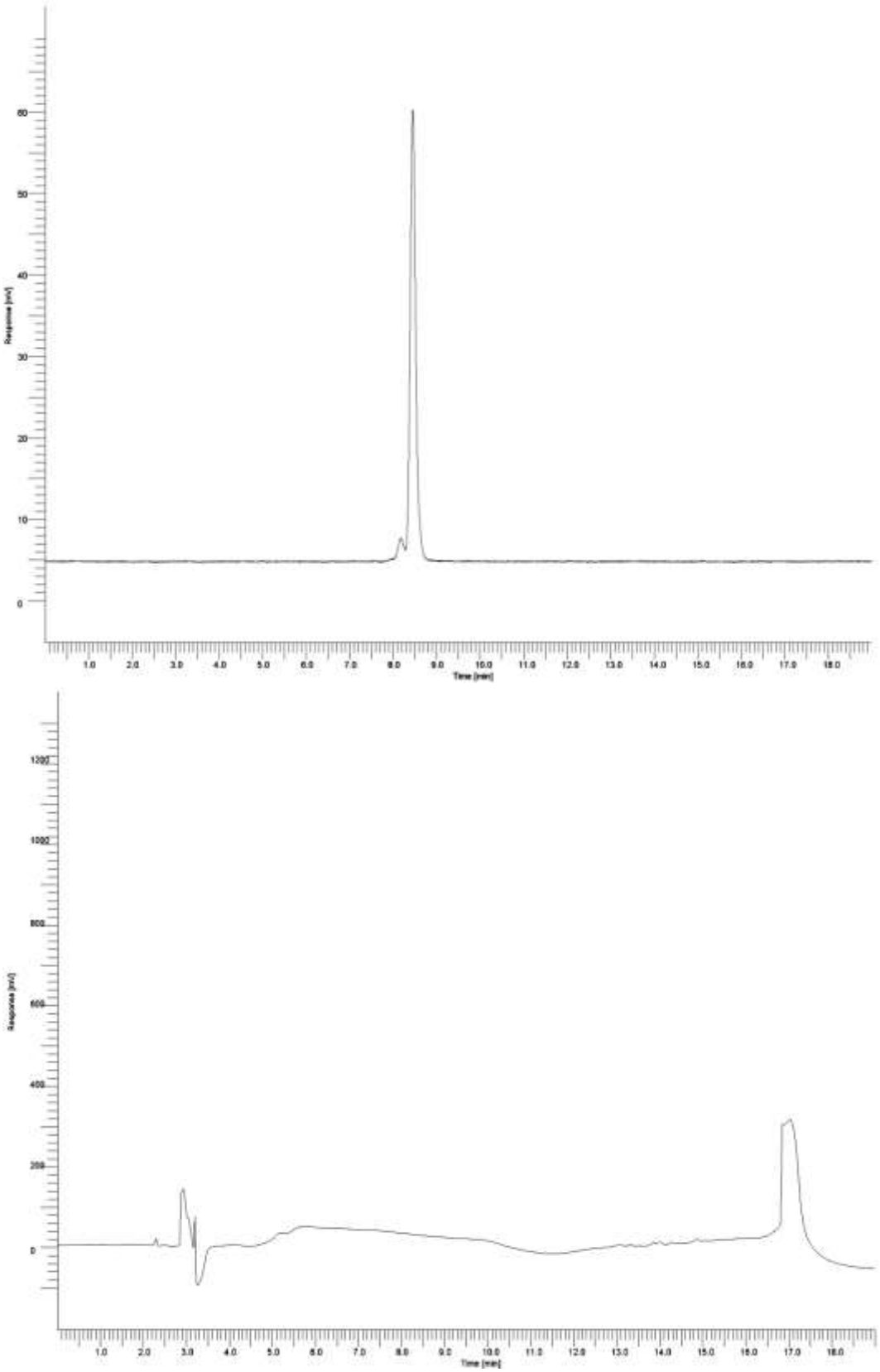


Radio (Top) and UV(Down)-chromatograms of **[^{18}F]9** (method B)



Radio (Top) and UV(Down)-chromatograms of **[^{18}F]FIB-NDP-MSH** (method C)

Petitioner GE Healthcare – Ex. 1050, p. 195



Radio (Top) and UV(Down)-chromatograms of **[^{18}F]FIB-PRGD$_2$** (method C)

6. *In Vitro* Receptor-Binding Assay

Receptor-binding affinity (inhibitory concentration of 50%; IC_{50}) of each compound was determined according to a slightly modified protocol developed by Guo *et al.*[4] B16F10 (containing Melanocortin 1 Receptors (mMC1-R)) or U87-MG (containing $\alpha_v\beta_3$ integrin) cells were maintained as monolayers in 24-well cell culture plates (5.10^5 cells/well) for 72 h before the assay. Assays were performed at room temperature and compounds were incubated with cells for 2 h. [^{125}I]Tyr-NDP-MSH or [^{125}I]Echistatin (81.4 TBq/mmol, PerkinElmer) was added to each well, simultaneously with a 1.10^{-12} to 5.10^{-5} M solution of each peptide (*i.e.* α-MSH (Sigma-Aldrich), NDP-MSH (ABBiotec) or Echistatin from *Echis carinatus* (Sigma-Aldrich)) in DMEM binding medium (0.4 mL; with *N*-(2-hydroxyl)piperazine-*N'*-(2-ethanesulfonic acid (25 mM), pH 7.4; 0.2% bovine serum albumin;1-10-phenantroline (0.3 mM)). Each compound was previously dissolved in DMSO and the final concentration was kept at 0.5% (v/v). The medium was discarded after incubation, and the cells were rinsed twice with ice-cold 0.2% bovine serum albumin in phosphate buffer saline pH 7.4 (0.5 mL). Finally, the cells were lysed in a 1 N aqueous sodium hydroxyde solution (0.5 mL) for 5 min and the radioactivity measured using the gamma counter. IC_{50} was calculated from the curve of concentration-dependent binding according to the formulae:

% of binding = [(Radioactivity in the well – Background Radioactivity)/(Radioactivity in the 0.5% DMSO control – Background Radioactivity)]*100

where Background Radioactivity is measured in a well with unlabeled NDP-MSH (1 µM) or unlabeled Echistatin (2.5 $\mu g.mL^{-1}$).

7. *In Vivo* Studies

In vivo experiments were performed when tumors were palpable and measurable, *i.e.* at day-11 for B16F10 melanoma, at day-12 for B16F0 melanoma and at day-29 for U87-MG glioblastoma models.

***[^{125}I]9** biodistribution*. On day-12 after B16F0 inoculation, **[^{125}I]9** was administered intravenously (5.4 ± 0.04 MBq/mouse) *via* the tail vein (4 animals). The injected activity was also assessed by counting the syringe with an activimeter (Capintec) before and after the injection of the radiotracer. At different times after administration (1 h, 3 h, 24 h, 72 h, 7 d) mice were anesthetized by intraperitoneal administration (200 µL per mouse) of a ketamine-xylazine mixture in saline (ketamine (Imalgene, Rhône Mérieux): 100 $mg.kg^{-1}$; xylazine (Rompun, Bayer): 10 $mg.kg^{-1}$). A 10-min duration image was acquired on anesthetized mice placed in the prone position over a parallel-hole collimator 1.8/0.2/20 (hole diameter/septum thickness/height in mm) of a gamma camera dedicated to small-animal imaging (γIMAGER®, Biospace Mesures). All acquisitions were performed with a 15% window centered on the 35-keV peak of ^{125}I. Quantitative analyses of scintigraphic scans were performed using the γVISION+ software (Biospace Mesures). For each animal and for each acquisition, regions of interest (ROI) were drawn around tumor uptake area and over controlateral paw background. For each ROI, total activity and surface average activity were determined and decay-

Petitioner GE Healthcare – Ex. 1050, p. 197

corrected. Tumor uptake was expressed in %ID.g^{-1} at each time point as previously described.[5] Thyroid uptake was determined for whole organ as percentage of injected dose. Tumor-to-muscle contrast was determined as follow: TMC = surface activity in tumor / surface activity in muscle.

Table S1: Scintigraphic scans quantitative analysis of tumors and selected organs at different time points after intravenous injection of **[^{125}I]9** (5.40 ± 0.04 MBq) in B16F0 primary melanoma-bearing C57BL/6J mice.

	1 h (n=4)	3 h (n=4)	24 h (n=4)	72 h (n=4)	7 d (n=4)
Tumor (%ID.g^{-1})	9.6 ± 2.6	9.6 ± 2.3	5.7 ± 0.8	2.4 ± 0.3	0.8 ± 0.2
Thyroid (%ID/organ)	3.5 ± 0.4	4.5 ± 0.2	3.2 ± 0.6	1.8 ± 0.3	0.9 ± 0.2
Ratio					
TMC*	2.8 ± 0.4	3.9 ± 0.7	11.8 ± 3.0	22.8 ± 10.4	16.5 ± 8.3

*Tumor-to-Muscle Contrast

In vivo PET imaging. Whole-body PET scans were acquired 1 h and 3 h after intravenous injection of [^{18}F]labeled compounds (15.3 ± 3.5 MBq per mouse for **[^{18}F]9**; 11.0 ± 0.4 MBq per mouse for **[^{18}F]FIB-NDP-MSH**; 9.33 ± 1.72 MBq per mouse for **[^{18}F]FIB-PRGD$_2$**) *via* the tail vein (2-3 animals/compound). The injected activity was assessed by counting the syringe with an activimeter before and after the injection of the radiotracer. For PET acquisitions, mice were anesthetized by intraperitoneal administration (200 µL per mouse) of a ketamine-xylazine mixture in saline (ketamine: 100 mg.kg^{-1}; xylazine: 10 mg.kg^{-1}). Whole-body acquisitions (20 min duration with 2 bed positions) were performed using a small-animal PET device (eXplore VISTA; GE Healthcare) with a 250–700 keV energy window set and 6 ns coincidence-timing window. The spatial resolution of this system was 1.4 mm full width at half maximum at the center of the field of view. Images were reconstructed using an ordered-subset expectation maximization (FORE/2D OSEM) method including corrections for scanner dead time, scattered radiations and randoms. No corrections were applied for partial volume or attenuation.

Regional radiotracer uptake was quantified by standard ROI analysis with eXplore VISTA software package (GE Healthcare) as previously described.[6] Data were analyzed based on regions of interest drawn over the tumors or tissue, with muscle being considered as background. The percentage of injected dose per gram (%ID/g) was calculated based on a calibration factor that was determined using a known quantity of radioactivity and by assuming a tissue density of 1 g.mL^{-1}. Tumor-to-muscle ratio was also determined to evaluate the image contrast.

Petitioner GE Healthcare – Ex. 1050, p. 198

8. References

(1) Armarego, W. L. F.; Perrin, D. D. in Purification of laboratory chemicals, 4th ed. (Eds.: Butterworth-Heinemann), Redd educational and professional publishing Ltd, Oxford, **1996**.

(2) Mock, B. H.; Winkle, W.; Vavrek, M. T. A color spot test for the detection of Kryptofix 2.2.2 in [^{18}F]FDG preparations. *Nucl. Med. Biol.* **1997**, *24* (2), 193–195.

(3) Chaikovski, V. K.; Filimonov, V. D.; Yagovkin, A. Y.; Kharlova, T. S. 2,4,6,8-tetraiodoglycoluril in sulfuric acid as new powerful reagent for iodination of deactivated arenes. *Tetrahedron Lett.* **2000**, *41* (47), 9101-9104.

(4) Guo, H.; Yang, J.; Gallazzi, F.; Miao, Y. Effects of the amino acid linkers on the melanoma-targeting and pharmacokinetic properties of ^{111}In-labeled lactam bridge-cyclized alpha-MSH peptides. *J. Nucl.Med.* **2011**, *52* (4), 608-616.

(5) Miot-Noirault, E.; Papon, J.; Gardette, M.; Bonnet-Duquennoy, M.; Labarre, P.; Maisonial, A.; Madelmont, J. C.; Maublant, J.; Chezal, J. M.; Moins, N. The use of [^{125}I] scintigraphic *in vivo* imaging in melanoma-bearing mice for a rapid prescreening of vectors to melanoma tissue. *Cancer. Biother. Radiopharm.* **2009**, *24* (5), 629-636.

(6) Rbah-Vidal, L.; Vidal, A.; Besse, S.; Cachin, F.; Bonnet, M.; Audin, L.; Askienazy, S.; Dollé, F.; Degoul, F.; Miot-Noirault, E.; Moins, N.; Auzeloux, P.; Chezal, J. M. Early detection and longitudinal monitoring of experimental primary and disseminated melanoma using [^{18}F]ICF01006, a highly promising melanoma PET tracer. *Eur. J. Nucl. Med. Mol. Imaging*, **2012**, *39* (9), 1449-1461.