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# Development and preclinical evaluation of [<sup>18</sup>F]FBVM as a new potent PET tracer for vesicular acetylcholine transporter

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## Abstract

Age-related neurodegenerative diseases have in common the occurrence of cognitive impairment, a highly incapacitating process that involves the cholinergic neurotransmission system. The vesicular acetylcholine transporter (VAcHT) positron emission tomography (PET) tracer [<sup>18</sup>F]fluoroethoxybenzovesamicol ((-)-[<sup>18</sup>F]FEOBV) has recently demonstrated its high value to detect alterations of the cholinergic system in Alzheimer's disease, Parkinson's disease and dementia with Lewy body. We present here the development of the new vesamicol derivative tracer (-)-(R,R)-5-[<sup>18</sup>F]fluorobenzovesamicol ((-)-[<sup>18</sup>F]FBVM) that we compared to (-)-[<sup>18</sup>F]FEOBV in the same experimental conditions. We show that: i) *in vitro* affinity for the VAcHT was 50-fold higher for (-)FBVM ( $K_i = 0.9 \pm 0.3$  nM) than for (-)FEOBV ( $K_i = 61 \pm 2.8$  nM); ii) *in vivo* in rats, a higher signal-to-noise specific brain uptake and a lower binding to plasma proteins and peripheral defluorination were obtained for (-)-[<sup>18</sup>F]FBVM compared to (-)-[<sup>18</sup>F]FEOBV. Our findings demonstrate that (-)-[<sup>18</sup>F]FBVM is a highly promising PET imaging tracer which could be sufficiently sensitive to detect in humans the cholinergic denervation that occurs in brain areas having a low density of VAcHT such as the cortex and hippocampus.

**Keywords :** Alzheimer's disease, Neuroimaging, Parkinson's disease, Radiopharmaceutical, Vesamicol

## 1. Introduction

Age-related neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and different types of dementia including Parkinson's disease dementia (PDD) and dementia with Lewy body (DLB) are a growing public health problem <sup>[1]</sup>. These diseases are complex and heterogeneous, involving the dysfunction of several distinct neuronal networks <sup>[2]</sup>, and to date no curative treatment is available. Therefore, a major challenge that could improve treatments would be to differentiate sub-phenotypes during the disease course and ideally from the early stages. In this context, positron emission tomography (PET) imaging is unquestionably a highly relevant tool. This method can localize and quantify longitudinally with high sensitivity various brain molecular targets able to reflect either broad mechanisms such as mitochondrial and synaptic dysfunctions and neuroinflammation, or more specific hallmarks such as abnormal protein aggregates and neurotransmission system alterations <sup>[3]</sup>.

These neurodegenerative diseases have in common the occurrence of cognitive impairment, a highly incapacitating process that involves various actors among which the cholinergic neurotransmission system [4-6]. The involvement of this system can be monitored through PET neuroimaging of different molecular targets, both at pre-synaptic and post-synaptic level. Regarding the post-synaptic acetylcholine receptors, several clinical studies have described a reduction in the nicotinic  $\alpha 4\beta 2$  subtype in AD [7-10] and PD [11-13]. At the pre-synaptic level, the brain uptake of radiolabelled substrates of acetylcholinesterase has been shown to decrease in several neurodegenerative disorders associated to cognitive deficits [14-17]. Another pre-synaptic marker, i.e. the vesicular acetylcholine transporter (VAcHT) had already been shown to be reduced using the SPECT tracer [ $^{123}\text{I}$ ]iodobenzovesamicol, [ $^{123}\text{I}$ ]IBVM in several neurodegenerative disorders [18-20] and a new interest of this index of cognitive impairment recently emerged in studies demonstrating that the VAcHT PET tracer [ $^{18}\text{F}$ ]fluoroethoxybenzovesamicol ([ $^{18}\text{F}$ ]FEOBV) described in 1998 [21] is of great value in detecting alterations of the cholinergic system in AD [22-24], PD [25,26], and DLB [27,28].

The organisation of the cholinergic system is highly complex and diffuse, with cell bodies localised in different brain structures such as the basal forebrain and brainstem, in the form of groups projecting into specific target areas such as the hippocampus, olfactory bulbs, amygdala, neocortex, and thalamus [29,30]. The case of striatal cholinergic interneurons is particular, with limited localisation in the structure. The VAcHT, which is responsible for the storage of acetylcholine in synaptic vesicles [31], is present both in cell bodies and neuron endings [32]. PET exploration of the VAcHT can therefore be highly informative regarding the integrity of cholinergic neurons, on condition that this method displays a sufficiently high resolution and sensitivity to detect and quantify low densities of this molecular target in small brain areas. This requires that the radioactive probe should have especially a high affinity for the target [33].

By comparison with the available tracer (-)-FEOBV, (-)-5-fluorobenzovesamicol ((-)-FBVM) was previously found to have a more than 5 times higher affinity for the VAcHT [34] (Fig. 1). We therefore hypothesised that its radioactive analogue could be a powerful PET imaging tool. We present here the synthesis and characterization of the racemic and enantiopure 5-FBVM derivatives which were then radiolabeled with fluor-18, purified, and compared *in vivo* to (-)-[ $^{18}\text{F}$ ]FEOBV in the rat.

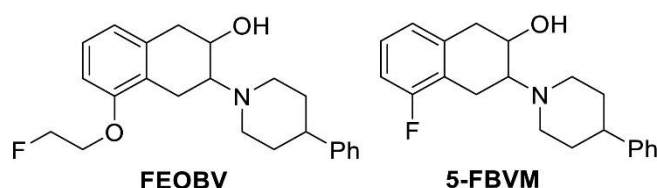
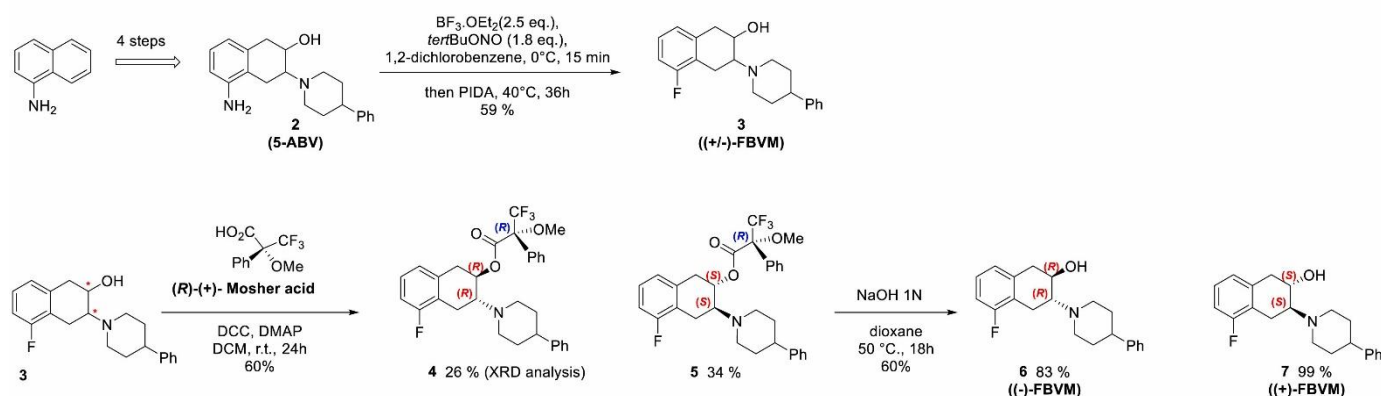


Fig. 1. FEOBV and FBVM

## 2. Results and discussion

### 2.1. Chemistry and radiochemistry

From our previous study [34], we demonstrated the high affinity of FBVM enantiomers to bind to VAcHT (around 4 nM for the (-)-FBVM). In a first attempt to radiolabel these compounds with fluorine-18 we used fluoro-de-triazenation, but unfortunately this approach did not lead to the expected radiolabelled compounds. In the last decade, significant improvements in  $S_NAr$  fluorine-18 radiolabelling were achieved even on a deactivated position [35-37]. Thus, after having successfully obtained a radiofluorinated COX-2 compound with a fluorine on the aromatic ring, we focused our radiolabelling strategy on the use of a pinacol borane precursor [38]. For the cold chemistry, we first focused our efforts on the synthesis of racemic 5-aminobenzovesamicol **2** in a large scale (multigram) from 1-aminonaphthalene **1** [39]. Preparation of **3** was achieved under a revised Balz-Schiemann procedure involving the use of *tert*-butyl nitrite and  $\text{BF}_3 \cdot \text{Et}_2\text{O}$  and PIDA as reagents [40]. This synthesis approach led to trans isomers and a chiral separation on Chiralpak IA led to the 2 pure stereoisomers **6** and **7** (retention time: 16 min and 21 min, respectively) in a near 50/50 ratio but at this step, no indication of the absolute configuration of each asymmetric carbon was available (Scheme 1).



Scheme 1. Synthesis of FBVM isomers and chiral Mosher diastereoisomers 4/5.

To determine formally the absolute configuration of each asymmetric carbon, we used the Mosher derivatization method. **3** and (*R*)-Mosher's acid under Steglich esterification led to the two diastereoisomer esters **4** and **5** which were easily separated by flash chromatography. The hydrochloride form of **4** was crystallized and gave single crystals suitable for X-ray diffraction analysis. **4** crystallizes in the non-centrosymmetric orthorhombic space group  $P2_12_12_1$  (Table S1, supporting information). The asymmetric unit contains one protonated molecule and a chloride anion (Fig. 1). The bond lengths are all in the expected range (1.369(10) – 1.405(8) for the C–C bonds of aromatic rings, 1.501(7) – 1.546(6) for C–C or C–N single bonds, 1.325(7) – 1.355(6) for C–F bonds, 1.439(8) – 1.462(6) for C–O single bonds and 1.214(6) and 1.329(6) for the carboxyl group corresponding respectively to the C=O double bond and C–O single bond with a partially delocalised doublet), aromatic rings are almost planar (max deviation: 0.008 Å for C4) and cyclohexane and piperidine moieties present the boat and chair conformation respectively. The cohesion of the network is ensured by charge assisted H-bonding between the ammonium group and the chloride anion, completed by weaker van der Waals interactions.

The absolute configuration of the crystal structure was assigned by analysis of the Bijvoet differences leading to a Hooft parameter of 0.00(2) and a Flack parameter of 0.02(2) [41–43]. According to these two parameters, both significantly very close to zero, it can be formally established that the three asymmetric carbons possess *R* absolute configurations. An ORTEP representation of the molecular structure is given in Fig. 2. To our knowledge, this is the first crystallization of a vesamicol derivative leading to a formal identification of asymmetric centres as well as the confirmation of the 3D relative conformation of each element of the pharmacophore.

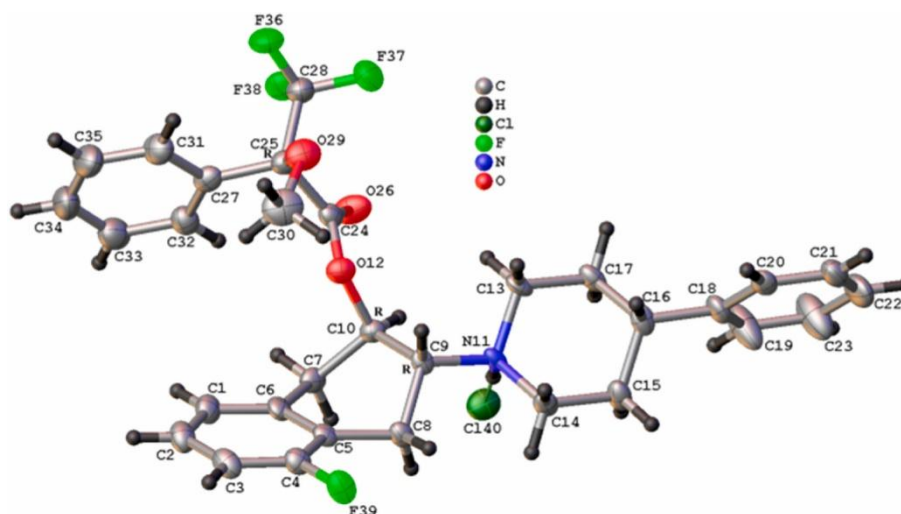
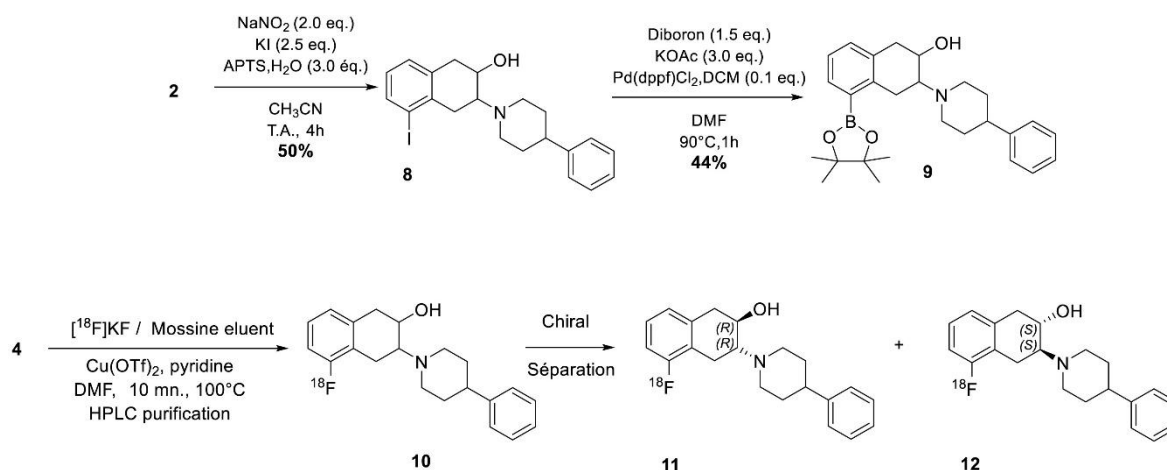


Fig. 2. ORTEP representation of the asymmetric unit of **4**·HCl and identification of the *R,R,R* absolute configurations of chiral carbons (C9, C10 and C25). The thermal ellipsoids are drawn at the 50% probability level.

In order to verify concordance between all the results, a saponification of **4** was performed and led to **6** which co-eluted under our HPLC conditions with (–)-FBVM and corresponded to the first peak observed after (*rac*)-FBVM chiral HPLC

analysis. Finally, saponification of **5** led to the enantiopure **7** which co-eluted with (+)-FBVM and the second peak observed after (*rac*)-FBVM chiral HPLC analysis.

The compound **2** led first to **8** after diazotation and reaction with KI in moderate yield (Scheme 2). A palladium catalysed reaction involving Pd(dppf)Cl<sub>2</sub> as catalyst and Bis(pinacolato)diboron led to the boronic ester **9** which was used to perform radiolabelling with radiofluoride ion. The low yield obtained for **7** is due to difficulties of purifying the boronic ester. The racemic [<sup>18</sup>F]**10** was obtained using Cu(OTf)<sub>2</sub> as catalyst, with pyridine in DMF followed by HPLC purification on a reverse phase column. An additional HPLC purification using a Chiralpak IA chromatography column (Fig. 3A) led to the two corresponding enantiopure probes **11** (–)-(R,R) and **12** (+)-(S,S) by order of elution (Fig. 3B).



Scheme 2. Synthesis of precursor **9** and its radiolabeling with <sup>18</sup>F up to chiral purification to get pure **11** (–)-(R,R) or **12** (+)-(S,S).

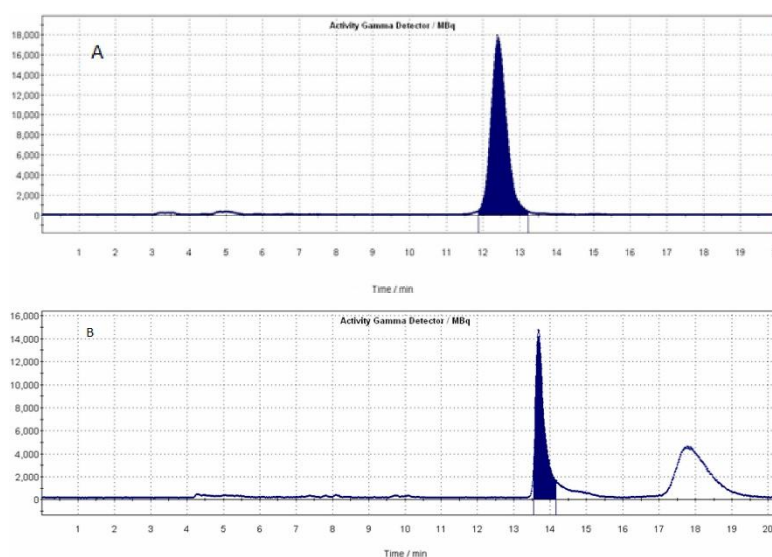


Fig. 3. Semi-preparative purification to obtain **10** (rac), **11** (–)-(R,R) or **12** (+)-(S,S). Only radioactive signals are reported: A) Purification on Luna PhenylHexyl to obtain **10**; B) Purification on Chiralpak IA to obtain **11** or **12** with time retention of 13.7 and 17.8 min, respectively.

The whole process leading to **10** (rac) was achieved in 60 ± 2 min with a radiochemical yield (RCY) up to 43% and a molar activity between 75 and 151 GBq/μmole. The production of **11** and **12** was achieved in 83 and 87 min, respectively, with a RCY up to 29% and molar activity in the 70–145 GBq/μmole range.

The (-)-[<sup>18</sup>F]FEOBV was synthesized using a method adapted from the original process described by Mulholland et al. [44]. The pure precursor (-)-TEBV was heated at 115 °C for 7.5 min, then the reaction was cooled and quenched with water. The following steps were similar to the process used for (+/-)-[<sup>18</sup>F]FBVM production. The radiosynthesis of (-)-[<sup>18</sup>F]FEOBV was achieved in 80 ± 3 min with a RCY of 71 ± 10.1% and a molar activity of 248.9 ± 56.4 GBq/μmole.

The chemical and plasmatic stability of **10** were checked up to 4h after the end of production and no degradation of the probe was observed.

## 2.2. In vitro experiments

The affinity properties for the VACHT and the enantioselectivity of new blocking agents derived from vesamicol were first described in an extensive structure-activity study that demonstrated the high potency of the chemical family of benzovesamicol [45]. At that time, the growing interest in imaging *in vivo* the VACHT as a relevant marker of cholinergic terminals led to several of these compounds such as [<sup>123</sup>I]IBVM [46] and [<sup>18</sup>F]FEOBV [21] being radiolabelled. In the present study, we achieved the synthesis and formal identification of pure FBVM **3** (*Rac*), **6** (-)-FBVM and **7** (+)-FBVM. This allowed us to measure the affinity of these compounds in comparison to the (+/-)-FEOBV and (-)-FEOBV enantiomer in the same *in vitro* experiments performed on a rat brain membrane preparation, in order to confront the results to those obtained in *in vivo* studies using the fluor-18-labelled derivatives. As shown in Table 1, the affinity for the VACHT of the **3** (*Rac*) was excellent ( $K_i = 1.3 \pm 0.2$  nM towards the reference probe [<sup>3</sup>H]vesamicol), slightly better for the **6** (-)-FBVM enantiomer ( $K_i = 0.9 \pm 0.3$  nM), and indisputably lower for the **7** (+)-FBVM enantiomer ( $K_i = 35 \pm 5$  nM). In comparison to our FBVM compounds, the affinity of the (+/-)-FEOBV and (-)-FEOBV enantiomer was 40- to 50-fold reduced ( $K_i = 61 \pm 2.8$  nM and  $50 \pm 10$  nM, respectively). These results agreed with the enantioselectivity of vesamicol derivatives, although we found for the FBVM compounds a better *in vitro* affinity for the VACHT than that previously published ( $K_i = 3.68$ , 6.95 and 10.9 nM for the (-) enantiomer, (+) enantiomer and rac, respectively), in different experimental conditions, i.e. cell extracts expressing the rat VACHT [34] instead of rat fresh tissue in our studies. However, it is noticeable that in both studies, the FBVM compounds had a better affinity than the FEOBV compounds, e.g., 50-fold for the (-) enantiomers in the present study (Table 1).

**Table 1**  
Affinity for the VACHT of FBVM and FEOBV racemic and enantiomers.

	$K_i$ (nM)
(+/-)-FBVM <b>3</b>	$1.3 \pm 0.2$
(-)-FBVM <b>6</b>	$0.9 \pm 0.3$
(+)-FBVM <b>7</b>	$35 \pm 5$
(+/-)-FEOBV	$61 \pm 2.8$
(-)-FEOBV	$50 \pm 10$

The  $K_i$  towards VACHT was measured on a membrane preparation from rat brain hemispheres according to a modified version of a previously described method [47] using [<sup>3</sup>H]vesamicol. The  $IC_{50}$  values were determined graphically for each compound and the  $K_i$  was calculated according to Cheng and Prussof [48]. The results are expressed as mean  $K_i \pm SEM$  from 3 independent experiments.

## 2.3. Ex vivo biodistribution and *in vivo* PET imaging in the rat brain

To date, (-)-[<sup>18</sup>F]FEOBV is the best characterized PET tracer useful for VACHT imaging in animal models [21,49-53] and humans, in physiological [54] and more recently pathological [55-61] conditions. Therefore, we compared our new FBVM compounds to this reference tracer in the rat model by performing *ex vivo* biodistribution experiments and *in vivo* PET imaging.

The accumulation of (+/-)-[<sup>18</sup>F]FBVM **10**, (-)-[<sup>18</sup>F]FBVM **11**, (+)-[<sup>18</sup>F]FBVM **12** and (-)-[<sup>18</sup>F]FEOBV was then studied at 2 h after i.v. injection in animals that received (Vesamicol groups) or not (Control groups) a pre-injection of the VACHT antagonist vesamicol.

In the control group receiving (-)-[<sup>18</sup>F]FEOBV, we observed a blood level accumulation of  $0.265 \pm 0.09\%$  ID/g, in agreement with previously published data [21], whereas it was significantly lower for (+/-)-[<sup>18</sup>F]FBVM **10**, (+)-[<sup>18</sup>F]FBVM **12** and (-)-[<sup>18</sup>F]FBVM **11** ( $0.016 \pm 0.001$ ,  $0.016 \pm 0.001$  and  $0.012 \pm 0.001\%$  ID/g, respectively). The bone accumulation, which is an index of *in vivo* defluorination, was also higher for (-)-[<sup>18</sup>F]FEOBV ( $0.254 \pm 0.011\%$  ID/g) than for FBVM derivatives ( $0.108 \pm 0.05$ ,  $0.105 \pm 0.007$  and  $0.096 \pm 0.06\%$  ID/g for (+/-)-[<sup>18</sup>F]FBVM **10**, (+)-[<sup>18</sup>F]FBVM **12** and (-)-[<sup>18</sup>F]FBVM **11**, respectively). Overall, these results can be explained by a higher binding to plasma proteins and a higher peripheral radio-defluorination for (-)-[<sup>18</sup>F]FEOBV than for the [<sup>18</sup>F]FBVM racemic and enantiomers, which could reduce the free plasmatic fraction available for brain uptake.

In the brain (Table 2), the uptake of (+/-)-[<sup>18</sup>F]FBVM **10**, (-)-[<sup>18</sup>F]FBVM **11** and (-)-[<sup>18</sup>F]FEOBV in control groups was significantly higher in the striatum and significantly lower in the cerebellum than in other brain regions (i.e., the frontal cortex, hippocampus and thalamus). However, for (+)-[<sup>18</sup>F]FBVM **12**, a statistically significant difference was reached exclusively between the striatum and cerebellum.

**Table 2**  
Brain biodistribution of (+/-)-[<sup>18</sup>F]FBVM 10, (+)-[<sup>18</sup>F]FBVM 12, (-)-[<sup>18</sup>F]FBVM 11, and (-)-[<sup>18</sup>F]FEOBV in control and vesamicol-injected rats.

	(+/-)-[ <sup>18</sup> F]FBVM 10		(+)-[ <sup>18</sup> F]FBVM 12		(-)-[ <sup>18</sup> F]FBVM 11		(-)-[ <sup>18</sup> F]FEOBV	
	Control (n = 6)	VES (n = 6)	Control (n = 6)	VES (n = 5)	Control (n = 8)	VES (n = 9)	Control (n = 6)	VES (n = 6)
<b>Cer</b>	0.185 ±0.003	0.062 ±0.003**	0.215 ±0.011	0.057 ±0.004**	0.232 ±0.017	0.095 ±0.008*	0.235 ±0.011	0.235 ±0.010
<b>Stri</b>	0.721 ±0.011††	0.379 ±0.015**	0.327 ±0.015†	0.105 ±0.009**	1.356 ±0.069††	0.736 ±0.045**	0.844 ±0.025††	0.594 ±0.030**
<b>Cx</b>	0.420 ±0.005††	0.166 ±0.009**	0.265 ±0.012	0.075 ±0.006**	0.723 ±0.052††	0.333 ±0.023**	0.450 ±0.013††	0.347 ±0.016**
<b>Hip</b>	0.402 ±0.008††	0.153 ±0.007**	0.296 ±0.011	0.073 ±0.007**	0.611 ±0.041††	0.349 ±0.045**	0.382 ±0.011†	0.326 ±0.014
<b>Thal</b>	0.368 ±0.005††	0.157 ±0.006**	0.258 ±0.014	0.084 ±0.007**	0.591 ±0.032††	0.312 ±0.018**	0.468 ±0.018††	0.370 ±0.018*

All rats were sacrificed at 2 h after i.v. injection of the tracer. In the vesamicol-injected groups (VES), animals received an i.v. injection of (-)vesamicol at the dose of 0.5 μmol/kg, 5 min before tracer injection. The results are expressed as mean % injected dose/g tissue ± SEM in different rat brain regions, i.e., the cerebellum (Cer), striatum (Stri), frontal cortex (Cx), hippocampus (Hip), and thalamus (Thal).

Values were compared using the two-way Anova, Bonferroni post-hoc test. In Control groups: †p < 0.01, ††p < 0.001 vs cerebellum. In VES groups: \*p < 0.01, \*\*p < 0.001 vs Control group in the same brain region.

A striatum-to-cerebellum uptake ratio of 3.90, 1.52, 5.84 and 3.59 was obtained for (+/-)-[<sup>18</sup>F]FBVM **10**, (+)-[<sup>18</sup>F]FBVM **12**, (-)-[<sup>18</sup>F]FBVM **11** and (-)-[<sup>18</sup>F]FEOBV, respectively. The comparison of the ratio between the extra-striatal areas, i.e., the frontal cortex, hippocampus, thalamus, and the cerebellum showed that it was always higher for (-)-[<sup>18</sup>F]FBVM **11** (3.12, 2.63, 2.55, respectively) than for (-)-[<sup>18</sup>F]FEOBV (1.91, 1.63, 1.99).

The PET images of each tracer recorded between 1 and 2 h post-injection were in perfect agreement with the above *ex vivo* biodistribution study in control animals, illustrating that a clearly higher signal-to-noise ratio was obtained for (-)-[<sup>18</sup>F]FBVM **11** compared to the other tracers, whereas a poorly contrasted image was obtained for (+)-[<sup>18</sup>F]FBVM **12** (Fig. 4).

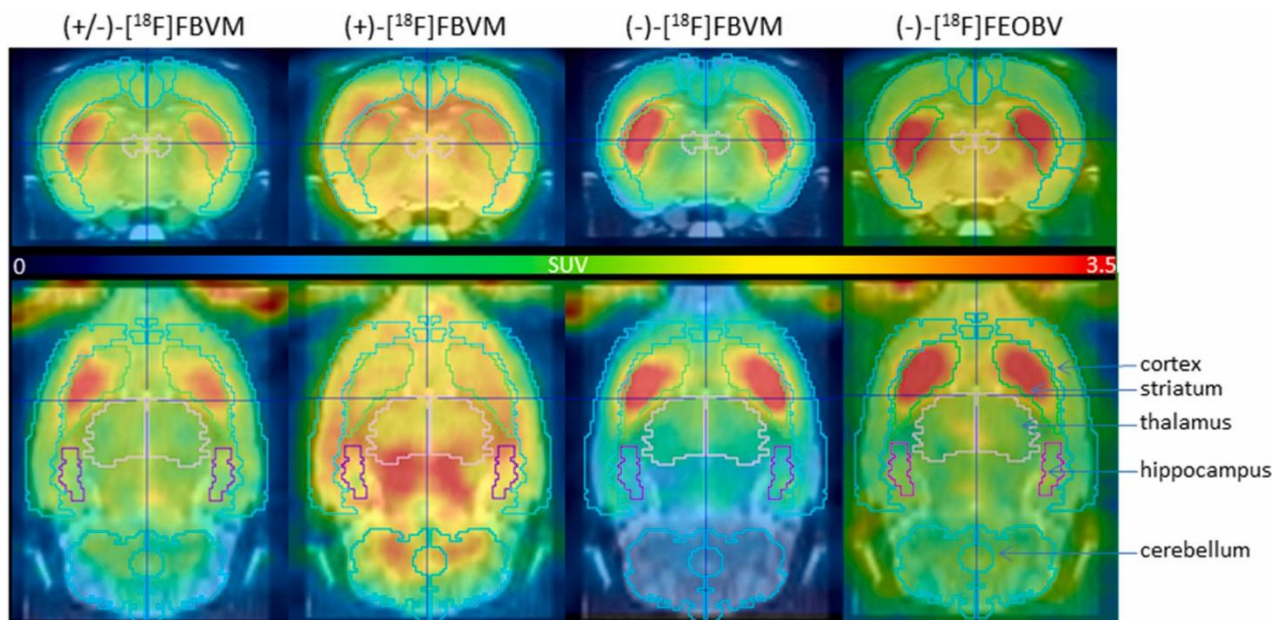


Fig. 4. PET studies in rat brain. Representative (+/-)-[<sup>18</sup>F]FBVM **10**, (+)-[<sup>18</sup>F]FBVM **12**, (-)-[<sup>18</sup>F]FBVM **11** and (-)-[<sup>18</sup>F]FEOBV images, 60–120 min p.i. Top: coronal view, bottom: horizontal view.

Overall, both *ex vivo* biodistribution and *in vivo* imaging in control rats showed that the regional brain distribution of (+/-)-[<sup>18</sup>F]FBVM **10**, (-)-[<sup>18</sup>F]FBVM **11** and (-)-[<sup>18</sup>F]FEOBV was in agreement with the known localisation of the VACht which is considered as an index of cholinergic innervation and can be explored by nuclear imaging methods using the cerebellum as a brain region of non-specific binding [21,46].

We also compared the *in vivo* kinetics of our best vesamicol derivatives (-)-[<sup>18</sup>F]FBVM **11** and (-)-[<sup>18</sup>F]FEOBV, and observed that both tracers had a rapid accumulation in the brain and then a progressive washout with a plateau between 60 and 120 min post-injection (Fig. 5), in agreement with previously reported data with [<sup>18</sup>F]FEOBV in rodent brain [21,52].

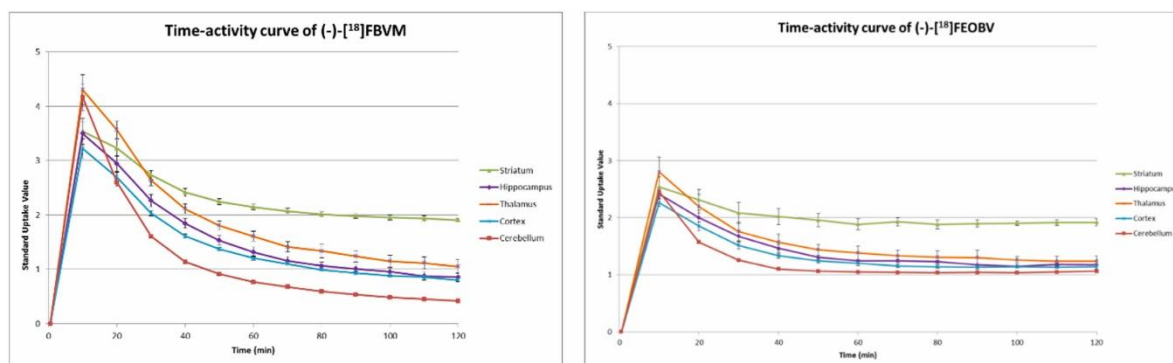


Fig. 5. Time-activity curves of (-)-[<sup>18</sup>F]FBVM **11** ( $n = 4$ ) and (-)-[<sup>18</sup>F]FEOBV ( $n = 2$ ) in different brain regions.

Our *ex vivo* competition experiments (i.e., VES groups) demonstrated that in agreement with their brain localisation in control groups, the uptake of the 4 tracers was mainly related to their binding to the VACht. The pre-administration of vesamicol induced a significant decrease of radioactivity in the striatum (-47%, -68%, -46% and -30% for (+/-)-[<sup>18</sup>F]FBVM **10**, (+)-[<sup>18</sup>F]FBVM **12**, (-)-[<sup>18</sup>F]FBVM **11** and (-)-[<sup>18</sup>F]FEOBV in comparison to control groups, respectively; Table 2). The uptake was also significantly reduced in other brain regions than the striatum in VES groups except for (-)-[<sup>18</sup>F]FEOBV in the cerebellum and hippocampus. The fact that the vesamicol pre-injection induced a reduction of (+/-)-[<sup>18</sup>F]FBVM **10**, (+)-[<sup>18</sup>F]FBVM **12**, and (-)-[<sup>18</sup>F]FBVM **11** but not (-)-[<sup>18</sup>F]FEOBV uptake in the cerebellum which has very poor cholinergic innervation, could be related to an *in vivo* binding of the FBVM compounds on other sites than the VACht. This characteristic had previously been observed for several imaging tracers with a benzovesamicol structure [62].

In order to look deeper into this point, we performed an *ex vivo* competition experiment comparing our best new tracer (-)-[<sup>18</sup>F]FBVM **11** with (-)-[<sup>18</sup>F]FEOBV using a pre-injection of haloperidol which is known to be a potent antagonist of



dopamine D2 and also sigma receptors [63]. As observed in control groups from the previous experiment, the uptake of (-)-[<sup>18</sup>F]FBVM **11** and (-)-[<sup>18</sup>F]FEOBV was significantly lower in the cerebellum than in the other brain regions studied (Table 3). In these groups, we also obtained similar striatum-to-cerebellum uptake ratios to previously, with values of 5.98 and 3.94 for (-)-[<sup>18</sup>F]FBVM **11** and (-)-[<sup>18</sup>F]FEOBV, respectively.

**Table 3**  
Brain biodistribution of (-)-[<sup>18</sup>F]FBVM **11** and (-)-[<sup>18</sup>F]FEOBV in control and haloperidol-injected rats.

	(-)-[ <sup>18</sup> F]FBVM <b>11</b>		(-)-[ <sup>18</sup> F]FEOBV	
	Control (n = 6)	HAL (n = 6)	Control (n = 6)	HAL (n = 6)
<b>Cer</b>	0.197 ±0.003	0.067 ±0.003*	0.269 ±0.009	0.248 ±0.006
<b>Stri</b>	1.178 ±0.046††	1.714 ±0.042**	1.06 ±0.061††	1.586 ±0.063**
<b>Cx</b>	0.512 ±0.023††	0.358 ± 0.01**	0.581 ±0.012††	0.504 ±0.025
<b>Hip</b>	0.444 ±0.019††	0.418 ± 0.03	0.486 ±0.012††	0.479 ±0.02
<b>Thal</b>	0.478 ±0.02††	0.353 ± 0.013*	0.484 ±0.013††	0.441 ±0.019

All rats were sacrificed at 2 h after i.v. injection of the tracer. In the Haloperidol groups (HAL), animals received an i.v. injection of haloperidol at the dose of 1 mg/kg (2.6 µmol/kg), 5 min before tracer injection.

Results are expressed as mean % injected dose/g tissue ± SEM in different rat brain regions, i.e., the cerebellum (Cer), striatum (Stri), frontal cortex (Cx), hippocampus (Hip), and thalamus (Thal).

Values were compared using the two-way Anova, Bonferroni post-hoc test. In Control groups: †p < 0.01, ††p < 0.001 vs cerebellum. In HAL groups: \*p < 0.01, \*\*p < 0.001 vs Control group in the same brain region.

We observed that the administration of haloperidol had no effect on the blood level of the two tracers at 2 h post-injection, with values similar to those obtained in the previous experiment ((-)-[<sup>18</sup>F]FBVM **11**: 0.011 ± 0.001 and 0.013 ± 0.001%ID/g in the HAL and control group, respectively; (-)-[<sup>18</sup>F]FEOBV: 0.274 ± 0.007 and 0.284 ± 0.01%ID/g in the HAL and control group, respectively). In contrast, it had previously been shown that the pre-injection of haloperidol was able to induce a reduction in blood activity and overall brain uptake of (-)-[<sup>18</sup>F]FEOBV in mice, but the dose was 10-fold higher than in the present study [21]. Our experiment also showed a rise in the uptake of both (-)-[<sup>18</sup>F]FBVM **11** (+46%) and (-)-[<sup>18</sup>F]FEOBV (+50%) specifically in the striatum of HAL groups compared to controls, as already observed in the above-mentioned study [21]. Such a result had also been observed with other tracers from the benzovesamicol chemical family and was related to the increased cholinergic activity induced by the blockage of dopamine D2 receptors by haloperidol [64,65]. The haloperidol pre-injection induced a significant reduction in the uptake of (-)-[<sup>18</sup>F]FBVM **11** in brain regions other than the striatum, i.e., the cerebellum (-66%), frontal cortex (-30%) and thalamus (-26%), whereas no modification was detected for (-)-[<sup>18</sup>F]FEOBV (Table 3). Such a reduction had already been observed with other benzovesamicol VAcHT tracers [65] and could be related to the blockade of σ receptors which are almost homogeneously localized in the rat brain with a lower expression in the striatum [66]. A reduced influx rate of (-)-[<sup>18</sup>F]FEOBV was also observed in the same brain regions with a high dose of haloperidol (10 mg/kg) [52]. Overall, our comparison study could be related to a potential higher *in vivo* binding of (-)-[<sup>18</sup>F]FBVM **11** than (-)-[<sup>18</sup>F]FEOBV to σ receptors.

### 3. Conclusions

We have described for the first time a new PET tracer useful for VAcHT imaging, (-)-[<sup>18</sup>F]FBVM **11**, and identified the absolute configuration of its chemical structure. We demonstrated the major importance of enantiomerism, with a highly improved *in vitro* affinity and *in vivo* signal-to-noise ratio regarding the binding to the VAcHT of the (-)-enantiomer in comparison to the racemic (+)-enantiomer. The comparison of this tracer to the reference tracer (-)-[<sup>18</sup>F]FEOBV in the rat model showed that both tracers had rather similar properties but that a significantly better signal-to-noise ratio was demonstrated for (-)-[<sup>18</sup>F]FBVM **11**, although a potential partial off-target binding cannot be excluded. Several characteristics of (-)-[<sup>18</sup>F]FBVM **11** could explain why a higher *in vivo* contrast was obtained for it than for (-)-[<sup>18</sup>F]FEOBV, such as the 50-fold better affinity measured in the same experimental conditions, and a lower binding to plasma proteins and peripheral defluorination. In view of these properties, (-)-[<sup>18</sup>F]FBVM **11** could be more sensitive to detect *in vivo* the cholinergic denervation that occurs particularly in AD in brain areas having a lower density

of VAcHT than the striatum, such as the cortex and hippocampus. The present study has several limitations and it will be necessary to explore more precisely the *in vivo* properties of (-)-[<sup>18</sup>F]FBVM **11**, in particular in experimental pathological models of cholinergic denervation in rodents, and in physiological and pathological conditions in non-human primates. However, this new tracer is highly promising for future clinical applications.

## 4. Experimental section

### 4.1. Chemistry

<sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded on a Bruker DPX 250 or 400 Mhz instrument using CDCl<sub>3</sub> and DMSO-*d*<sub>6</sub>. The chemical shifts are reported in parts per million ( $\delta$  scale), and all coupling constant (*J*) values are reported in hertz. The following abbreviations were used for the multiplicities: s (singlet), d (doublet), t (triplet), q (quartet), p (pentuplet), m (multiplet), sext (sextuplet), and dd (doublet of doublets). Melting points are uncorrected. IR absorption spectra were obtained on a PerkinElmer PARAGON 1000 PC, and the values are reported in inverse centimeters. HRMS spectra were acquired in positive mode with an ESI source on a Q-TOF mass by the "Fédération de Recherche" ICOA/CBM (FR2708) platform. Monitoring of the reactions was performed using silica gel TLC plates (silica Merck 60 F 254). Spots were visualized by UV light (254 nm and 356 nm). Column chromatography was performed using silica gel 60 (0.063–0.200 mm, Merck.). All reagents were purchased from commercial suppliers and were used without further purification.

#### 4.1.1. 5-Fluoro-3-(4-phenylpiperidin-1-yl)-1,2,3,4-tetrahydronaphthalen-2-ol **3**

To an oven-dried tube reactor equipped with stirrer bar and a Teflon screw cap was added compound **2** <sup>[34]</sup> (849 mg, 2.6 mmol), BF<sub>3</sub>.Et<sub>2</sub>O (818  $\mu$ l, 6.6 mmol, 2.5 eq.) and 1,2-dichlorobenzene (9 ml). The mixture was cooled to 0 °C then *tert*BuONO (629  $\mu$ l, 4.7 mmol, 1.8 eq.) was dropwise added *via* a syringe under argon atmosphere and 0 °C. The mixture was stirred at 0 °C for 15 min then a solution of PIDA (170 mg, 0.5 mmol, 0.2 eq.) in 1,2-dichlorobenzene (2 mL) was added. The reactor was sealed and heated to 40 °C for 36h. After cooling the mixture to r.t., solvent was removed under vacuum. The crude was solubilised in DCM (30 mL) and washed with a saturated aqueous solution of NaHCO<sub>3</sub> (30 mL), dried over MgSO<sub>4</sub>, filtered. After removing the solvent under reduced pressure, the resulting crude was purified by flash chromatography using EP/EtOAc (10/0 to 1/1) as eluent to afford the desired **3** (500 mg, 59%) as a pale beige solid. Mp: 147–148 °C. IR (ATR diamond,  $\nu$ , cm<sup>-1</sup>): 3434, 3079, 2931, 2910, 1579, 1492, 1462, 1411, 1376, 1278, 1141, 1003, 769, 711, 696. HRMS (ESI<sup>+</sup>): calc. for C<sub>21</sub>H<sub>25</sub>FNO [M+H]<sup>+</sup>: 326.19146 found: 326.19131.

<sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>)  $\delta$  7.37–7.17 (m, 5H, 5H–Ar), 7.11 (td, *J* = 7.9, 5.7 Hz, 1H, H–Ar), 6.95–6.80 (m, 2H, 2 H–Ar), 4.34 (brs, 1H, OH), 3.87 (td, *J* = 10.2, 5.6 Hz, 1H, CH), 3.33 (dd, *J* = 16.2, 5.7 Hz, 1H, CH<sub>2</sub>), 3.15–3.02 (m, 1H, CH<sub>2</sub>), 3.02–2.84 (m, 3H), 2.83–2.50 (m, 4H), 2.44 (td, *J* = 11.5, 2.5 Hz, 1H, CH<sub>2</sub>), 2.01–1.86 (m, 3H), 1.85–1.62 (m, 1H). <sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>)  $\delta$  161.1 (d, <sup>1</sup>*J* = 243.9 Hz, Cq), 146.2(Cq), 136.8 (d, <sup>3</sup>*J* = 4.3 Hz, Cq), 128.6 (2CH), 127.4 (d, <sup>3</sup>*J* = 8.7 Hz, CH), 127.0(2CH), 126.4 (CH), 124.7 (d, <sup>4</sup>*J* = 3.3 Hz, CH), 122.7 (d, <sup>2</sup>*J* = 17.6 Hz, Cq), 112.4 (d, <sup>2</sup>*J* = 21.9 Hz, CH), 66.2 (CH), 65.4(CH), 53.8(CH<sub>2</sub>), 45.1 (CH<sub>2</sub>), 43.0 (CH), 37.9 (d, <sup>4</sup>*J* = 2.2 Hz, CH<sub>2</sub>), 34.5 (CH<sub>2</sub>), 34.0 (CH<sub>2</sub>), 19.2 (d, <sup>4</sup>*J* = 3.3 Hz, CH<sub>2</sub>). <sup>19</sup>F NMR (235 MHz, CDCl<sub>3</sub>)  $\delta$  -117.3 (dd, *J* = 9.9, 5.5 Hz). HPLC PhenylHexyl-Phenomenex, 10 × 250 mm, ACN/ammonium acetate 0.1 M 70/30, 4 mL min<sup>-1</sup> flow, retention time 11 min. HPLC Chiralpak IA, Daicel, 10 × 250 mm, eluent ACN/ammonium acetate 0.1 M 85/15, 4 mL min<sup>-1</sup> flow, retention time Enantiomer 1: 18 min, retention time Enantiomer 2: 22 min, ratio 50/50.

#### 4.1.2. General procedure for synthesis of Mosher's esters

In an oven-dried round-bottom flask, compound **3** (650 mg, 2.0 mmol) was solubilised in DCM (5 mL). DCC (515 mg, 2.5 mmol, 1.25 eq.), DMAP (5 mg, 0.4 mmol, 0.2 eq.) and (+)-(R)-Mosher acid (561 mg, 2.4 mmol, 1.2 eq.) were added respectively under argon atmosphere. The reaction mixture was left under stirring at r.t for 24h. The reaction was stopped by adding a saturated aqueous solution of NaHCO<sub>3</sub> (30 mL) and then transferred to a separating funnel. The organic layer was collected and the aqueous layer was extracted with DCM (2 x 30 mL). Then organic layers were combined together, dried over MgSO<sub>4</sub> and filtered. Solvent was removed under reduced pressure. The resulting crude was purified by flash chromatography on silica gel column using EP/EtOAc (100/0 to 95:5) as eluent to afford the desired products **4** (276 mg, 26%) and **5** (369 mg, 34%) as white amorphous solids.

#### 4.1.3. (R)-(2R,3R)-5-fluoro-3-(4-phenylpiperidin-1-yl)-1,2,3,4-tetrahydronaphthalen-2-yl 3,3,3-trifluoro-2-methoxy-2-phenylpropanoate **4**

HRMS (ESI<sup>+</sup>): calc. for C<sub>31</sub>H<sub>32</sub>F<sub>4</sub>NO<sub>3</sub> [M+H]<sup>+</sup>: 542.23128 found: 542.23097. IR (ATR Diamond,  $\nu$ , cm<sup>-1</sup>): 3060.5, 3002.6, 2947.0, 1738.2, 1588.1, 1496.7, 1384.4, 1187.1, 1079.8, 1054.2793.5, 763.0, 745.8, 736.7. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>)  $\delta$  7.80 (dd,  $J = 6.5, 3.1$  Hz, 2H, 2H-Ar), 7.54–7.44 (m, 3H, 3 H-Ar), 7.41–7.31 (m, 2H, 2H-Ar), 7.31–7.21 (m, 3H, 3 H-Ar), 7.21–7.09 (m, 1H, 1 H-Ar), 7.02–6.80 (m, 2H, 2 H-Ar), 5.63 (td,  $J = 9.5, 5.9$  Hz, 1H, CH), 3.78 (s, 3H, OMe), 3.32–3.09 (m, 4H), 3.07–2.76 (m, 4H), 2.64–2.40 (m, 2H), 2.02–1.62 (m, 4H). <sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>)  $\delta$  166.0 (Cq), 160.7 (d, <sup>1</sup> $J = 244.5$  Hz, Cq), 146.5 (Cq), 135.7 (d, <sup>4</sup> $J = 4.4$  Hz, Cq), 132.6 (Cq), 129.8 (CH), 128.6 (4CH), 127.8 (2CH), 127.5 (d, <sup>3</sup> $J = 8.6$  Hz, CH), 126.9 (2CH), 126.3 (CH), 124.0 (d, <sup>3</sup> $J = 3.3$  Hz, CH), 123.5 (q, <sup>1</sup> $J = 288.9$ , CF<sub>3</sub>), 122.5 (d, <sup>2</sup> $J = 17.7$  Hz, Cq), 112.9 (d, <sup>2</sup> $J = 21.8$  Hz, CH), 85.0 (q, <sup>2</sup> $J = 27.4$  Hz, Cq), 71.2 (CH), 63.2 (CH), 55.70 (d, <sup>4</sup> $J = 1.5$  Hz, CH<sub>3</sub>), 52.8 (CH<sub>2</sub>), 46.8 (CH<sub>2</sub>), 42.9 (CH), 34.8 (d, <sup>4</sup> $J = 2.1$  Hz, CH<sub>2</sub>), 34.5 (CH<sub>2</sub>), 33.2 (CH<sub>2</sub>), 19.9 (d, <sup>4</sup> $J = 2.9$  Hz, CH<sub>2</sub>). <sup>19</sup>F NMR (235 MHz, CDCl<sub>3</sub>)  $\delta$  -71.1, -117.8 (dd,  $J = 9.4, 5.7$  Hz).

4.1.4. (R)-(2S,3S)-5-fluoro-3-(4-phenylpiperidin-1-yl)-1,2,3,4-tetrahydronaphthalen-2-yl 3,3,3-trifluoro-2-methoxy-2-phenylpropanoate **5**

HRMS (ESI<sup>+</sup>): calc. for C<sub>31</sub>H<sub>32</sub>F<sub>4</sub>NO<sub>3</sub> [M+H]<sup>+</sup>: 542.23128 found: 542.23125. IR (ATR Diamond,  $\nu$ , cm<sup>-1</sup>): 3025.6, 2978.2, 2497.9, 1746.9, 1619.3, 1584.5, 1493.1, 1397.5, 1293.4, 1166.1, 1012.2, 966.5, 786.5, 699.7. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>)  $\delta$  7.74–7.61 (m, 2H, 2H-Ar), 7.47–7.38 (m, 3H, 3H-Ar), 7.34–7.26 (m, 2H, 2H-Ar), 7.23–7.06 (m, 4H, H-Ar), 6.94–6.83 (m, 2H, H-Ar), 5.51 (td,  $J = 8.6, 5.5$  Hz, 1H, CH), 3.63 (q,  $J = 1.2$  Hz, 3H, OMe), 3.34 (dd,  $J = 16.2, 5.6$  Hz, 1H, CH<sub>2</sub>), 3.15–2.73 (m, 6H), 2.51 (td,  $J = 11.3, 2.7$  Hz, 1H, CH<sub>2</sub>), 2.42–2.22 (m, 2H), 1.67 (dtq,  $J = 28.9, 12.2, 4.0$  Hz, 4H, 2CH<sub>2</sub>). <sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>)  $\delta$  166.0 (Cq), 160.7 (d, <sup>1</sup> $J = 244.8$  Hz, Cq), 146.5 (Cq), 135.6 (d, <sup>4</sup> $J = 4.4$  Hz, Cq), 132.4 (Cq), 129.7 (CH), 128.5 (2CH), 128.5 (2CH), 127.8 (2CH), 127.5 (d, <sup>3</sup> $J = 8.6$  Hz, CH), 126.9 (2CH), 126.2 (CH), 124.0 (d, <sup>4</sup> $J = 3.4$  Hz, CH), 123.5 (q, <sup>1</sup> $J = 288.6$  Hz, CF<sub>3</sub>), 122.7 (d, <sup>2</sup> $J = 17.7$  Hz, Cq), 113.0 (d, <sup>2</sup> $J = 21.7$  Hz, CH), 84.7 (q, <sup>2</sup> $J = 27.5$  Hz, Cq), 72.3 (CH), 62.6 (CH<sub>2</sub>), 55.7 (CH<sub>3</sub>), 51.3 (CH<sub>2</sub>), 48.7 (CH<sub>2</sub>), 42.8 (CH), 34.7 (d, <sup>4</sup> $J = 2.2$  Hz, CH<sub>2</sub>), 34.2 (CH), 33.6 (CH<sub>2</sub>), 21.3 (d, <sup>4</sup> $J = 3.1$  Hz, CH<sub>2</sub>). <sup>19</sup>F NMR (235 MHz, CDCl<sub>3</sub>)  $\delta$  -71.84, -118.06 (dd,  $J = 9.5, 5.7$  Hz).

4.1.5. 1-((2R,3R)-8-fluoro-3-(((R)-3,3,3-trifluoro-2-methoxy-2-phenylpropanoyl)oxy)-1,2,3,4-tetrahydronaphthalen-2-yl)-4-phenylpiperidin-1-ium chloride **4•HCl**

In a round bottom flask, **4** (250 mg, 0.46 mmol) was solubilised in dry dioxane (3 mL) and the solution cooled to 0 °C. A 4 N HCl solution in dioxane (173  $\mu$ L, 0.69 mmol, 1.5 eq.) was added dropwise and was left under stirring at r.t for 24h. Solvent and excess of HCl were removed slowly under atmospheric pressure to afford crystals. The crude was then triturated with Et<sub>2</sub>O to obtain needles, which were collected by filtration and then dried under vacuum to obtain **4•HCl** in quantitative yield. Crystals were collected for XRD analysis. Mp: 168–169 °C. IR ( $\nu$  cm<sup>-1</sup>): 3028.3, 1748.3, 1590.4, 1470.3, 1245.0, 1163.4, 963.9, 845.0, 700.1, 664.1, 552.4. <sup>1</sup>H NMR (250 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  11.62 (bs, 1H, NH), 7.48 (s, 5H, H-Ar), 7.40–7.30 (m, 2H, H-Ar), 7.29–7.12 (m, 4H, H-Ar), 7.10–6.92 (m, 2H, H-Ar), 5.95 (d,  $J = 4.9$  Hz, 1H, CH), 3.99–3.83 (m, 1H, CH), 3.79–2.90 (m, 11H), 2.88–2.67 (m, 1H, CH), 2.27 (d,  $J = 14.0$  Hz, 2H, CH<sub>2</sub>), 2.07–1.86 (m, 2H, CH<sub>2</sub>). <sup>13</sup>C NMR (63 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  164.7 (Cq), 159.1 (d, <sup>1</sup> $J = 244.2$  Hz, Cq), 144.1 (Cq), 135.5 (d, <sup>4</sup> $J = 2.9$  Hz, Cq), 130.7 (Cq), 130.0 (CH), 128.8 (2CH), 128.6 (2CH), 128.2 (d, <sup>3</sup> $J = 8.2$  Hz, CH), 127.2 (2CH), 126.6 (3CH), 124.2 (CH), 123.1 (q, <sup>1</sup> $J = 290.4$  Hz, CF<sub>3</sub>), 119.7 (d, <sup>2</sup> $J = 16.5$  Hz, Cq), 113.4 (d, <sup>2</sup> $J = 20.9$  Hz, CH), 84.2 (q, <sup>2</sup> $J = 27.2, 26.6$  Hz, Cq), 71.9 (CH), 63.14 (CH), 55.6 (CH<sub>3</sub>), 50.6 (CH<sub>2</sub>), 49.3 (CH<sub>2</sub>), 39.2 (CH), 32.6 CH<sub>2</sub>, 29.8 (CH<sub>2</sub>), 29.6 (CH<sub>2</sub>), 20.7 (CH<sub>2</sub>). <sup>19</sup>F NMR (235 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  -70.9, -119.9 (dd,  $J = 9.1, 5.8$  Hz).

4.1.6. (2R,3R)-5-fluoro-3-(4-phenylpiperidin-1-yl)-1,2,3,4-tetrahydronaphthalen-2-ol ((-)-(R,R)-5-FBVM) **6**

In a round-bottom flask compound **4** (96 mg, 0.14 mmol) was solubilised in 1,4-dioxane (3 mL) then 1 M aqueous solution of NaOH (3 mL) was added and the reaction mixture was left under stirring at 50 °C for 18h. Solvents were removed under reduced pressure then the resulting crude was solubilised in water and extracted with EtOAc (3 x 5 mL). Organic layers were combined together and dried over MgSO<sub>4</sub>. Solvent was removed under reduced pressure to afford the desired product **6** as a pale beige solid (48 mg, 83%). [ $\alpha$ <sub>D</sub>]<sup>20</sup> = -82.0 ( $c = 0.01$  g/mL Chloroform). Mp: 148–149 °C. IR (ATR Diamond,  $\nu$ , cm<sup>-1</sup>): 3379, 3062, 3027, 2937, 2771, 1618, 1601, 1578, 1413, 1325, 1238, 1161, 1022, 1003, 977, 804, 758. HRMS (ESI<sup>+</sup>): calc. for C<sub>21</sub>H<sub>25</sub>FNO [M+H]<sup>+</sup>: 326.19146 found: 326.1913. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>)  $\delta$  7.37–7.17 (m, 5H, H-Ar), 7.11 (td,  $J = 7.9, 5.7$  Hz, 1H, H-Ar), 6.95–6.80 (m, 2H, H-Ar), 4.34 (brs, 1H, OH), 3.87 (td,  $J = 10.2, 5.6$  Hz, 1H, CH), 3.33 (dd,  $J = 16.2, 5.7$  Hz, 1H, CH<sub>2</sub>), 3.15–3.02 (m, 1H, CH<sub>2</sub>), 3.02–2.84 (m, 3H), 2.83–2.50 (m, 4H), 2.44 (td,  $J = 11.5, 2.5$  Hz, 1H, CH<sub>2</sub>), 2.01–1.86 (m, 3H), 1.85–1.62 (m, 1H). <sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>)  $\delta$  161.1 (d, <sup>1</sup> $J = 243.9$  Hz, Cq), 146.2 (Cq), 136.8 (d, <sup>4</sup> $J = 4.3$  Hz, Cq), 128.6 (2CH), 127.4 (d, <sup>3</sup> $J = 8.7$  Hz, CH), 127.0 (2CH), 126.4 (CH), 124.7 (d, <sup>4</sup> $J = 3.3$  Hz, CH), 122.7 (d, <sup>2</sup> $J = 17.6$  Hz, Cq), 112.4 (d, <sup>2</sup> $J = 21.9$  Hz, CH), 66.2 (CH), 65.4 (CH), 53.8 (CH<sub>2</sub>), 45.1 (CH<sub>2</sub>), 43.0 (CH), 37.9 (d, <sup>4</sup> $J = 2.2$  Hz, CH<sub>2</sub>), 34.5 (CH<sub>2</sub>), 34.0 (CH<sub>2</sub>), 19.2 (d, <sup>4</sup> $J = 3.3$  Hz, CH<sub>2</sub>). <sup>19</sup>F NMR (235 MHz, CDCl<sub>3</sub>)  $\delta$  -117.3 (dd,  $J = 9.9, 5.5$  Hz). HPLC

Chiralpak IA, Daicel, 10 × 250 mm, eluent ACN/ammonium acetate 0.1 M 85/15, 4 mL min<sup>-1</sup> flow, retention time 18 min.

#### 4.1.7. (2*S*,3*S*)-5-fluoro-3-(4-phenylpiperidin-1-yl)-1,2,3,4-tetrahydronaphthalen-2-ol ((+)-(*S,S*)-5-FBVM) **7**

In a round-bottom flask compound **5** (100 mg, 0.17 mmol) was solubilised in 1,4-dioxane (3 mL) then 1 M aqueous solution of NaOH (3 mL) was added and the reaction mixture was left under stirring at 50 °C for 18h. Solvents removed under reduced pressure then the resulting crude was solubilised in water and extracted with EtOAc (3 x 5 mL). Organic layers were combined together and dried over MgSO<sub>4</sub>. Solvent was removed under reduced pressure to afford the desired product **7** as pale beige solid (56 mg, 99%).  $[\alpha_D]^{20} = +82.8$  ( $c = 0.01$  g/mL Chloroform). Mp: 148–149 °C. IR ( $\nu$  cm<sup>-1</sup>): 3379, 3064, 2771, 1619, 1578, 1492, 1374, 1218, 1161, 1073, 791. HRMS (ESI<sup>+</sup>): calc. for C<sub>21</sub>H<sub>25</sub>FNO [M+H]<sup>+</sup>: 326.19146 found: 326.1913. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>)  $\delta$  7.37–7.17 (m, 5H, H-Ar), 7.11 (td,  $J = 7.9, 5.7$  Hz, 1H, H-Ar), 6.95–6.80 (m, 2H, H-Ar), 4.34 (brs, 1H, OH), 3.87 (td,  $J = 10.2, 5.6$  Hz, 1H, CH), 3.33 (dd,  $J = 16.2, 5.7$  Hz, 1H, CH<sub>2</sub>), 3.15–3.02 (m, 1H, CH<sub>2</sub>), 3.02–2.84 (m, 3H), 2.83–2.50 (m, 4H), 2.44 (td,  $J = 11.5, 2.5$  Hz, 1H, CH<sub>2</sub>), 2.01–1.86 (m, 3H), 1.85–1.62 (m, 1H, H of CH<sub>2</sub>). <sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>)  $\delta$  161.1 (d,  $^1J = 243.9$  Hz, Cq), 146.2 (Cq), 136.8 (d,  $^4J = 4.3$  Hz, Cq), 128.6 (2CH), 127.4 (d,  $^3J = 8.7$  Hz, CH), 127.0 (2CH), 126.4 (CH), 124.7 (d,  $^4J = 3.3$  Hz, CH), 122.7 (d,  $^2J = 17.6$  Hz, Cq), 112.4 (d,  $^2J = 21.9$  Hz, CH), 66.2 (CH), 65.4 (CH), 53.8 (CH<sub>2</sub>), 45.1 (CH<sub>2</sub>), 43.0 (CH), 37.9 (d,  $^4J = 2.2$  Hz, CH<sub>2</sub>), 34.5 (CH<sub>2</sub>), 34.0 (CH<sub>2</sub>), 19.2 (d,  $^4J = 3.3$  Hz, CH<sub>2</sub>). <sup>19</sup>F NMR (235 MHz, CDCl<sub>3</sub>)  $\delta$  -117.3 (dd,  $J = 9.9, 5.5$  Hz). HPLC Chiralpak IA, Daicel, 10 × 250 mm, eluent ACN/ammonium acetate 0.1 M 85/15, 4 mL min<sup>-1</sup> flow, retention time 22 min.

#### 4.1.8. 5-Iodo-3-(4-phenylpiperidin-1-yl)-1,2,3,4-tetrahydronaphthalen-2-ol (**5-IBVM**) **8**

In a round bottom flask, compound **2** (1.00 g, 3.10 mmol) was solubilised in CH<sub>3</sub>CN (18 mL) then PTSA.H<sub>2</sub>O (1.80 g, 9.32 mmol, 3.0 eq.) was added in one portion. The mixture was cooled down to 0 °C then a solution of NaNO<sub>2</sub> (428 mg, 6.2 mmol, 2.0 eq.) and KI (1.20 g, 7.75 mmol, 2.5 eq.) in water (5 mL) was added. After stirring at 0 °C for 15 min, the reaction mixture was allowed to warm up to r.t and left under stirring for 4h. Water (17 mL) and a NaHCO<sub>3</sub> saturated solution (35 mL) were added. The aqueous layer was extracted with EtOAc (3 x 25 mL) and the combined organic layers were dried over MgSO<sub>4</sub>, filtered then dried under reduced pressure. The residue was purified by flash chromatography on silica gel column (PE/EtOAc: 85/15) to afford the desired **8** as a yellowish solid (587 mg, 50%). <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>)  $\delta$  7.70 (d,  $J = 7.8$  Hz, 1H, H-Ar), 7.39–7.16 (m, 6H, H-Ar), 7.10 (d,  $J = 7.7$  Hz, 1H, H-Ar), 6.83 (t,  $J = 7.7$  Hz, 1H, OH), 3.83 (td,  $J = 10.2, 5.6$  Hz, 1H, CH), 3.24–2.33 (m, 12H).

#### 4.1.9. 3-(4-Phenylpiperidin-1-yl)-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1,2,3,4-tetrahydronaphthalen-2-ol **9**

In an oven-dried tube, **8** (100 mg, 0.23 mmol) was solubilised in degassed anhydrous DMF (2 mL) then B<sub>2</sub>Pin<sub>2</sub> (88 mg, 0.35 mmol, 1.5 eq.), KOAc (68 mg, 0.69 mmol, 3.0 eq.) and Pd(dppf)Cl<sub>2</sub>.DCM (19 mg, 0.02 mmol, 0.1 eq.) were added successively. The tube was sealed under argon and heated up to 160 °C for 1h30. The reaction mixture was cooled to r.t then DMF was removed under reduced pressure. The residue was washed with brine (20 mL) and filtered on a pad of celite. The filtrate was extracted by EtOAc (3 x 10 mL). Organic layers were combined, dried over MgSO<sub>4</sub> and filtered. After removing solvent under reduced pressure, the residue was purified by flash chromatography on silica gel column (PE/EtOAc 85/15 and 5% of Et<sub>3</sub>N) to afford the desired product **9** as a white solid (50 mg, 44%) which was readily used to prevent degradation. R<sub>f</sub>: 0.27 (PE/EtOAc 85/15). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  7.62 (dd,  $J = 7.1, 1.7$  Hz, 1H, HAr), 7.37–7.24 (m, 4H, 4H-Ar), 7.24–7.09 (m, 3H, 3xHAr), 3.87 (td,  $J = 10.2, 5.8$  Hz, 1H, CH), 3.45 (dd,  $J = 16.7, 4.7$  Hz, 1H, H of CH<sub>2</sub>), 3.29 (dd,  $J = 15.9, 5.8$  Hz, 1H, H of CH<sub>2</sub>), 3.04–2.71 (m, 6H, CH and CH<sub>2</sub> and 3H of CH<sub>2</sub>), 2.57 (tt,  $J = 11.9, 3.9$  Hz, 1H, CH), 2.39 (td,  $J = 11.5, 2.3$  Hz, 1H, H of CH<sub>2</sub>), 1.97–1.83 (m, 3H, H of CH<sub>2</sub> and CH<sub>2</sub>), 1.76 (m, 1H, H of CH<sub>2</sub>), 1.37 (s, 12H, 4xCH<sub>3</sub>). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  146.4 (Cq), 141.4 (Cq), 134.0 (CH), 133.8 (Cq), 132.4 (CH), 128.6 (2CH and Cq), 127.0 (2CH), 126.3 (CH), 125.5 (CH), 83.7 (2Cq), 67.0 (CH), 65.6 (CH), 53.9 (CH<sub>2</sub>), 45.4 (CH<sub>2</sub>), 43.1 (CH), 38.6 (CH<sub>2</sub>), 34.6 (CH<sub>2</sub>), 34.1 (CH<sub>2</sub>), 26.3 (CH<sub>2</sub>), 25.2 (2CH<sub>3</sub>), 25.0 (2CH<sub>3</sub>). IR ( $\nu$  cm<sup>-1</sup>): 3424, 3030, 2972, 2942, 2916, 2855, 2805, 1587, 1493, 1470, 1450, 1428, 1385, 1371, 1349, 1316, 1265, 1141, 1127, 1112, 1066, 1052, 1003, 986, 967, 858, 827, 794.4, 774, 756, 726, 699, 666, 635. HRMS (ESI<sup>+</sup>): calc. for C<sub>27</sub>H<sub>37</sub>BNO<sub>3</sub> [M+H]<sup>+</sup>: 434.2861, found: 434.2866.

## 4.2. Crystallography

Crystallographic data of compound **4** were collected at 150 K with an Bruker APEX-II diffractometer using monochromatic Mo-K $\alpha$  radiation (0.71073 Å). The collected data were reduced using the Saint software suite (Bruker

AXS Inc., Madison, Wisconsin, USA). Then absorption correction was performed using the SADABS software (SADABS, Bruker AXS Inc., Madison, Wisconsin, USA). The crystal structure was solved by direct methods using ShelXT and refined against  $F^2$  by full least square method within the OLEX2 program [67,68] also used for drawings. Full crystallographic results have been deposited at the Cambridge Crystallographic Data Centre (CCDC 2104956, [www.ccdc.cam.ac.uk/structures](http://www.ccdc.cam.ac.uk/structures)).

### 4.3. Radiochemistry

#### 4.3.1. General

No-carrier-added aqueous  $^{18}\text{F}$ -fluoride ion was produced on a cyclotron (PET trace, GE Healthcare) by irradiation of enriched  $^{18}\text{O}$   $\text{H}_2\text{O}$  with protons via the  $^{18}\text{O}(p,n)^{18}\text{F}$  nuclear reaction.  $^{18}\text{F}$  Fluoride was transferred to a modified TRACERlab FX-FN Pro (GE) synthesizer and passed through an anion-exchange resin (Waters Sep-Pak Accell Light QMA cartridge in the carbonate form).

#### 4.3.2. Synthesis of *(rac)*-[ $^{18}\text{F}$ ]-FBVM 10

Trapped  $^{18}\text{F}$ -fluoride was isolated by elution with 550  $\mu\text{L}$  of aqueous solution of eluent solution containing KOTf (5 mg) and  $\text{K}_2\text{CO}_3$  (50  $\mu\text{g}$ ). Azeotropic drying by addition of ACN (1 mL) was performed. The evaporation was performed at 90  $^\circ\text{C}$  under helium flow and vacuum, and the operation was repeated twice prior to nucleophilic substitution. Precursor **8** (2 mg) with  $\text{Cu}(\text{OTf})_2$  (3.6 mg) dissolved in DMF (960  $\mu\text{L}$ ) and pyridine (40  $\mu\text{L}$ ) were added to the reactive fluorine ions. The reaction was heated at 100  $^\circ\text{C}$  for 10 min, then cooled to 30  $^\circ\text{C}$  and diluted with water (8 mL). The crude medium was passed through a *t*C18 plus cartridge (Waters) and rinsed with additional water (4 mL) to trap the desired compound and remove most polar derivatives. Crude *(rac)*-[ $^{18}\text{F}$ ]-FBVM 10 was eluted from the cartridge by CAN (2 mL) and diluted with ammonium acetate 0.1 M (1 mL). The solution was loaded onto the HPLC loop of the synthesizer and purified on a semi-preparative column (Luna PhenylHexyl-Phenomenex, 10  $\times$  250 mm) using ACN/ammonium acetate 0.1 M 70/30 as mobile phase at a 4 mL  $\text{min}^{-1}$  flow. Under this condition the *(rac)*-[ $^{18}\text{F}$ ]-FBVM was collected around 11 min. The collected fraction was diluted with water (30 mL) and trapped on a *t*C18 light cartridge. *(rac)*-[ $^{18}\text{F}$ ]-FBVM was eluted from the cartridge by 0.8 mL of injectable ethanol and formulation was completed by the addition of 7.2 mL of saline.

#### 4.3.3. Synthesis of *(-)*-(*R,R*)-5-[ $^{18}\text{F}$ ]FVBM 11 and *(+)*-(*S,S*)-5-[ $^{18}\text{F}$ ]FBVM 12

The production of isolated enantiopure probes occurred following the radiosynthesis of *(+/-)*-5-[ $^{18}\text{F}$ ]FBVM 10 completed by a chiral HPLC purification. The collected HPLC fraction of pure *(+/-)*-5-[ $^{18}\text{F}$ ]FBVM 10 was loaded onto the loop of the synthesizer and purified on a semi-preparative chiral column (Chiralpak IA, Daicel, 10  $\times$  250 mm) using ACN/ammonium acetate 0.1 M 85/15 as mobile phase at a 4 mL  $\text{min}^{-1}$  flow. Under these conditions the *(-)*-[ $^{18}\text{F}$ ]FVBM 11 was selectively collected around 18 min and *(+)*-[ $^{18}\text{F}$ ]FBVM 12 around 22 min. The enantiomerically pure fractions *(-)* or *(+)* were diluted with water (35 mL) and trapped on a *t*C18 light cartridge. *(-)*-[ $^{18}\text{F}$ ]FVBM 11 and *(+)*-[ $^{18}\text{F}$ ]FBVM 12 was eluted from the cartridge by 0.8 mL of injectable ethanol and formulation was completed by the addition of 7.2 mL of saline. As we identified that the most active compound was *(-)*-[ $^{18}\text{F}$ ]FVBM 11 and thus the first eluted peak from the chiral purification, we modified the production to obtain it in a single HPLC purification. Alternatively, the use of ACN/ammonium acetate 0.1 M/MeOH:70/10/20 as mobile phase at a 4 mL  $\text{min}^{-1}$  flow led to *(-)* [  $^{18}\text{F}$  ]FVBM 11 with a retention time of 14 min. The formulation was achieved as previously reported. Using this method we were able to achieve *(-)*-[ $^{18}\text{F}$ ]FVBM 11 production and formulation in  $68 \pm 3$  min.

#### 4.3.4. Quality control

Radiotracers were controlled by analytical HPLC equipped with UV and radio detectors. The *(rac)*-[ $^{18}\text{F}$ ]-FBVM 10 purity was checked using an analytical column (Phenomenex Luna 5 $\mu$  Phenyl Hexyl 4,6  $\times$  250 mm) using ACN/Ac Am 0,1 M 70/30 as mobile phase at a 1 mL/min flow. Under these conditions, the time retention was 9 min. In the case of enantiomeric purification, additionally to the previous control, a HPLC control using a chiral column (Chiralpak IA 4,6  $\times$  250 mm 5  $\mu$ ) was used to evaluate the enantiomeric purity of either *(-)* [  $^{18}\text{F}$  ]FVBM 11 or *(+)* [  $^{18}\text{F}$  ]FBVM 12. For this control, ACN/Ac Am 0.1 M 85/15 was used as mobile phase at a 1 mL/min flow. Under these conditions the time retention of *(-)* [  $^{18}\text{F}$  ]FVBM 11 and *(+)* [  $^{18}\text{F}$  ]FBVM 12 were respectively 16.2 and 21.1 min. For all compounds,

radiochemical purity was better than 99%, molar activity higher than 100 GBq/ $\mu$ mole and no degradation was observed in the formulation media nor in serum for at least 4h.

#### 4.3.5. Synthesis of (-)-[<sup>18</sup>F]FEOBV

The tracer was prepared using an method adapted from the original process described by Mulholland et al. [21]. The corresponding enantiomerically pure precursor ((-)-TEBV, 1.5 mg) was used for the one step radiolabeling of (-)-[<sup>18</sup>F]FEOBV. The [<sup>18</sup>F]fluoride was eluted from the cartridge using an aqueous eluent solution (3 mg of K<sub>2</sub>CO<sub>3</sub> diluted in 230  $\mu$ L of pure water and 5.8 mg of Kryptofix<sub>222</sub> diluted in 570  $\mu$ L of acetonitrile). Azeotropic drying by addition of ACN (1 mL) was performed. The evaporation was performed at 90 °C under helium flow and vacuum, and the operation was repeated twice. The precursor (1.5 mg) dissolved in ACN (0.7 mL) was transferred to the reactor and heated at 115 °C for 7.5 min. The medium was cooled to 40 °C, diluted with water (8 mL) and passed through a t-C18 cartridge. Then, the cartridge was rinsed with water (5 mL), eluted with ACN (2 mL) and diluted with water (1 mL). The crude solution was injected to the loop and purified by HPLC using ACN/ammonium acetate 0.1 M (60/40) at a 4 mL/min flow. The (-)-[<sup>18</sup>F]FEOBV was collected with a time retention of 15 min, diluted with water (40 mL) and the solution was passed through a light t-C18 cartridge. The cartridge was rinsed with water (5 mL), eluted with 500  $\mu$ L of ethanol and the formulation was completed by the addition of 3.5 mL of 0.9% NaCl. The radiosynthesis was achieved in 80  $\pm$  3 min with a RCY of 71  $\pm$  10.1% and a molar activity of 248.9  $\pm$  56.4 GBq/ $\mu$ mole.

#### 4.3.6. Stability

The stability in plasma of the injectable solution ((*rac*)-[<sup>18</sup>F]-FBVM 10) was checked by HPLC up to 4 h after the end of preparation. To conduct this control, 100  $\mu$ L of the radiotracer solution was mixed with 1 mL of rat plasma and incubated at 37°C. Then, 500  $\mu$ L of acetonitrile were added, the organic layer was removed and filtered, 20  $\mu$ L was loaded onto the HPLC, and the stability was checked at a 4-h time point after production. The radiochemical stability was also checked up to 4h after the end of preparation. The HPLC conditions used to check the chemical and plasmatic stability are those reported above in quality control using the Luna column. No degradation was observed.

### 4.4. Biological studies

#### 4.4.1. Animals

Experiments were performed on male Wistar rats weighing 280–320 g (Centre d'Élevage R. Janvier, Le Genest St Isle, France). All animal procedures were conducted in accordance with the requirements of the European Community Council Directive 2010/63/EU for the care of laboratory animals and with the authorization of the Regional Ethical Committee (N° APAFIS#4208–2016022218004689 v3 and APAFIS#26811–2020073110598684 v2).

#### 4.4.2. In vitro binding studies

The K<sub>i</sub> towards VAcHT was measured for stable (+/-)-FBVM 3, (+)-FBVM 7, (-)-FBVM 6, (+/-)-FEOBV and (-)-FEOBV on a membrane preparation from rat brain hemispheres according to a modified version of a previously described method (Scheunemann et al., 2004). Stable L-(-)-vesamicol was obtained from Sigma Aldrich (Saint-Quentin-Fallavier, France) and [<sup>3</sup>H]vesamicol (molar activity 1705.7 GBq/mmol) from Perkin-Elmer (Courtaboeuf, France). The IC<sub>50</sub> values were determined graphically for each compound and the K<sub>i</sub> was calculated according to Cheng and Prussof [48]. The results were expressed as mean K<sub>i</sub>  $\pm$  SEM from 3 independent experiments.

#### 4.4.3. Biodistribution studies

Each tracer, (+/-)-[<sup>18</sup>F]FBVM 10, (+)-[<sup>18</sup>F]FBVM 12, (-)-[<sup>18</sup>F]FBVM 11 and (-)-[<sup>18</sup>F]FEOBV was evaluated in two groups of rats (one control group and one VES group, n = 5–9/group). Animals were injected i.v. under isoflurane gas anesthesia (Baxter, France; 4% in O<sub>2</sub>) in the penis vein with 3.61–8.18 MBq of one of these tracers dissolved in 0.3 mL of saline. In the VES groups, injection of the tracer was preceded (5 min) by i.v. injection of vesamicol (L-(-)-vesamicol, Sigma Aldrich, Saint-Quentin-Fallavier, France) at the dose of 0.5  $\mu$ mol/kg.

In a 2nd set of experiments, (-)-[<sup>18</sup>F]FBVM **11** and (-)-[<sup>18</sup>F]FEOBV were evaluated in two other groups of rats (one control group and one HAL group, n = 6/group). In the HAL group, injection of the tracer was preceded (5 min) by i.v. injection of haloperidol (Haldol®, Janssen, 2 mg/ml) at the dose of 1 mg/kg (2.6 μmol/kg).

All animals were killed by decapitation at 2 h post injection of the tracer. The whole brain was quickly removed and dissected into cortex, striatum, hippocampus, thalamus and cerebellum. A fraction of blood and bone were also removed. Samples were weighed and radioactivity was measured in a γ counter (2480 Gamma counter Wizard, Perkin Elmer), and the percentage of injected dose per gram of tissue (%ID/g) was calculated by comparison with samples to standard dilutions of the injected solution.

#### 4.4.4. PET acquisition and data analysis

The rats received an i.v. injection of around 37 MBq of (+/-)-[<sup>18</sup>F]FBVM **10**, (+)-[<sup>18</sup>F]FBVM **12**, (-)-[<sup>18</sup>F]FBVM **11** or (-)-[<sup>18</sup>F]FEOBV (n = 2–4 rats/tracer). Acquisitions were made on a microPET-CT SuperArgus system (Sedecal, Madrid, Spain), which has an effective axial/trans axial field of view (FOV) of 4.8/6.7 cm, a spatial resolution less than 2 mm and a sensitivity above 2.5% in the whole FOV. Animals were anesthetized with isoflurane, at 3% in O<sub>2</sub> for induction and then 1.5–2% during scanning. Each acquisition lasted 121 min and PET list-mode scans were rebinned into 1 frame of 1 min followed by 12 frames of 10 min. Each PET scan was corrected for random, scatter, and attenuation, and the images were reconstructed using a 2-D OSEM algorithm (GE Healthcare, France) into voxels of 0.3875 × 0.3875 × 0.775 mm<sup>3</sup>.

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Author contributions : JV: supervision of radiochemistry, radiochemistry experiments and analyses of radiotracers; FB: supervision of chemistry; SS: PET imaging experiments; NR, HM, RBO: chemical synthesis and analysis of compounds; AC, NP, FS: structural analysis; ZG: *in vitro* experiments; GC, AD: radiochemistry experiments and analyses of radiotracers; SB, JB: animal experiments; DG: conceptualization; MM: crystallography data; PE: conceptualization; SR & SC: conceptualization, co-supervision, writing.

*All authors contributed to the article and approved the submitted version.*

Declaration of competing interest : The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data : The following is the Supplementary data to this article: [Download](#) : Download Word document (4MB)

#### Abbreviations :

AD - Alzheimer's disease  
 PD - Parkinson's disease  
 PDD - Parkinson's disease dementia  
 DLB - dementia with Lewy body  
 PET - Positron emission tomography  
 VAcHT - vesicular acetylcholine transporter  
 FEOBV – fluoroethoxybenzovesamicol  
 FBVM - fluorobenzovesamicol

Data availability : No data was used for the research described in the article.

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