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Synthesis and preliminary characterization of a new radioactive ligand for dopamine transporter - *N*-(3-iodoprop-2*E*-enyl)-2β-carbo-[^{3}H]methoxy-3β-(4'-methylphenyl)nortropane ([^{3}H]PE2I)

Thesis for Master's Degree

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TABLE OF CONTENTS

ABBREVIATIONS 3

1. INTRODUCTION 4

2. THEORETICAL PART 6

2.1 Dopamine transporter: structure and function 6
2.2 DA_T and disease 10
2.3 PET and SPECT 11
2.4 Dopamine re-uptake inhibitors 12

3. EXPERIMENTAL PART 15

3.1 Materials and methods 15
3.2 Synthetic procedures 17
3.3 Results and discussion 23

4. CONCLUSIONS 27

5. KOKKUVÕTE 28

6. ACKNOWLEDGEMENTS 29

REFERENCES 30

APPENDIX 37

ABBREVIATIONS

ADHD – attention deficit hyperactivity disorder
β-CFT – 2β-carbomethoxy-3β-(4-fluorophenyl)tropane
DA_T – dopamine transporter
DEEP - 1-(2-(diphenylmethoxy)ethyl)-4-(2-(4-azido-3-iodophenyl)ethyl)piperazine
EtOAc – ethyl acetate
Et_2O – diethyl ether
GABA – γ-aminobutyric acid
GA_T - γ-aminobutyric acid transporter
GBR12909 - 1-(2 (bis(4-fluorophenyl)methoxy)ethyl)-4-(3-phenylpropyl)piperazine
MeOH - methanol
MPP^+ - 1-methyl-4-phenylpyridinium
PE – petroleum ether
PET – positron emission tomography
PE2I - *N*-(3-iodoprop-2*E*-enyl)-2β-carbomethoxy-3β-(4'-methylphenyl)nortropane
TEA – triethylamine
TFA – trifluoroacetic acid
TM – transmembrane segment
Tris – tris(hydroxymethyl)aminoethane
SER_T – serotonin transporter
SPA – scintillation proximity assay
SPECT – single photon emission computed tomography
RTI-82 - 3-(p-chlorophenyl)-tropane-2-carboxylic acid 4'-azido-3'-iodophenyl ethyl ester

1. INTRODUCTION

Recent achievements in life sciences have significantly advanced our understanding of the role of neurotransmitter systems in brain functioning, including the phenomena involving dopaminergic nerve terminals. Dopamine and signal transduction pathway revolving around it has been of particular interest to medical community, as disturbances in functioning of dopaminergic system are linked to numerous pathologies, including schizophrenia, ADHD, Parkinson's disease and others neurodegenerative disorders (Leriche *et al*. 2004; Carlsson *et al*. 2004; Jenner *et al*. 2003; DiMaio *et al*. 2003). The main molecular components of this neurotransmitter system are dopamine receptors and dopamine transporters. If particular advances have been made in description of dopamine receptor families (Menelas & Davies 2001; Dearry *et al*. 1990; Sunahara *et al*. 1991, Sokoloff *et al*. 1990, Van Tol *et al*. 1991), much less is still known about the molecular structure, mechanism and specificity of action of dopamine transporter protein, although its implication in a wide range of medical conditions and disorders have been recently well documented (Weintraub *et al*. 2005; Storch *et al*. 2004; Seeman *et al*. 1990; Hitri *et al*. 1994, Ginovart et al., 1997). Therefore, in parallel with the receptors, the transporter proteins have also been used as targets for positron emission tomography tracer ligands, used for quick and non-invasive data acquisition about components of the dopaminergic system *in vivo*, and opening new diagnostic perspectives in this area (van Waarde 2000; Jucaite *et al*. 2006; Maziere & Halldin 2004).

These recent advancements in new diagnostic applications of dopaminergic ligands have widened needs for more thorough characterisation of their interaction with the components of dopaminergic system, including also experiments *in vitro.* Especially this concerns studies of the transporter protein, for which there is distinct lack of data concerning such aspects as mechanism and kinetics of transporter-ligand interaction, as the list of available selective ligands for this component of the dopaminergic system is rather short. In this study we have added into this list a new tritium-labelled specific DA_T ligand *N*-(3-iodopropyl-2*E*-enyl)-2β-carboxy-[^{3}H]methyl-3β-(4'-tolyl)nortropane, which unlabelled form is known as efficient DA_T inhibitor PE2I. After synthesis, the initial investigation into applicability of this new radioligand for *in vitro* studies was also

undertaken, using the striatal membrane fragments from mice brain. As PE2I is currently used for animal and human PET investigations, use of the same ligand for *in vitro* research can be quite worthwhile as the results can be compared directly with other data obtained from *in vivo* experiments, as the ligand used in both is the same.

Part of the results presented in this work has been presented in the following papers:

1. Stepanov V., Järv J. Interaction of tritium-labelled dopamine transporter PE2I with mice striatal membrane preparation. *Proc. Est. Acad. Sci. Chem*. (2006) (accepted for publication)
2. Stepanov V. Synthesis of tritium labelled *N*-(3-iodoprop-2E-enyl)-2β-carbomethoxy-3β-(4'-methylphenyl)nortropane. Abstracts of 29th Estonian Chemistry Days (2005) Tallinn, p. 107
3. Stepanov V., Magnus Schou M., Järv J., Halldin C. Synthesis of 3H-labeled N-(3-iodoprop-2E-enyl)-2β-carbomethoxy-3β-(4-methylphenyl) nortropane (PE2I) and its interaction with mice striatal membrane fragments. *Appl Rad Isot*. submitted for publication

2. THEORETICAL PART

2.1 Dopamine transporter: structure and function

Dopamine transporter (further in the text referred to as DA_T) is a multi-domain trans-membrane protein that belongs to Na^+/Cl^- dependent transporter family (SCDNT), with other members of the group being 5-HT transporter (SER_T), GABA transporter [GA_T(1-3)], norepinephrine transporter (NE_T) and some others (Newman *et al*. 2002; Chen *et al*. 2000; Masson *et al*. 1999). Prime function of dopamine transporter is to remove dopamine from synaptic cleft after it has been released from presynaptic neuron. Rapid removal of signalling molecule (dopamine) significantly reduces synapse "recycle time" and speeds up "firing rate" of the neuron.

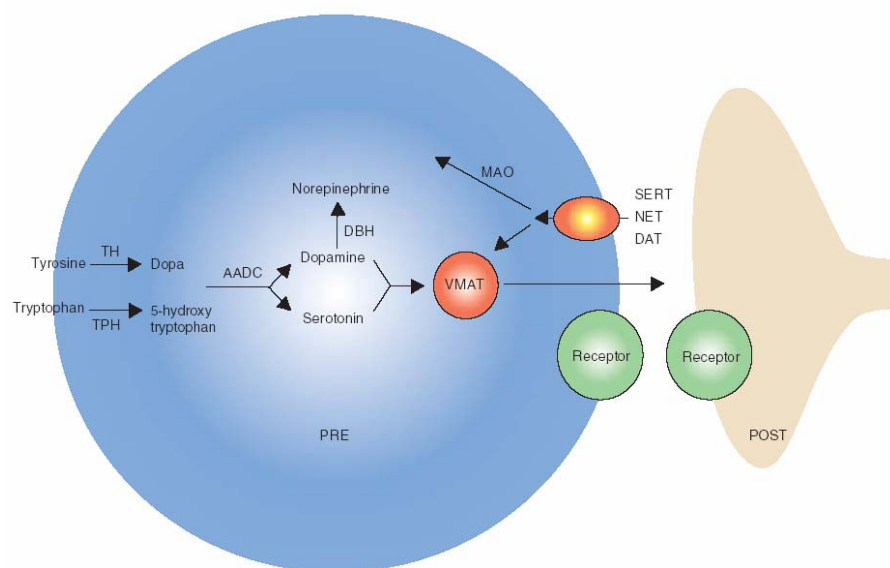

Fig 1.

From Glatt & Reus, 2003

AACD: aromatic amino acid decarboxylase; DAT: dopamine transporter; SERT: serotonin transporter; NET: norepinephrine transporter; VMAT: vesicular monoamine transporter; MAO: monoamine oxidase

Comparison techniques and hydropathy analysis done in analogue with G protein-coupled receptors and other membrane proteins indicate that the transporter has 12 hydrophobic transmembrane (TM) segments (see Fig. 2), connected by 11 alternating intracellular and intracellular loops, with both C- and N-terminals located in the cytosol inside the cell, with total of approximately 620 amino acids residues (the exact number

depends on the species in question (Chen *et al*. 2000). There are also limited structural data available for DA_T derived from comparison between DA_T and *Escherichia Coli* Na^+/H^+ antiporter (Ravna *et al*. 2003). The recently published crystallographic data for leucine transporter $LeuT_{Aa}$ (belonging to SCDNT family) from *Aquifex aeolicus* confirms those predictions and provides reasonable high-resolution structure analogue for DA_T, supplemented by structural data for glutamate transporter analogue from *Pyrococcus horikoshii* (Yamashita *et al*. 2005; Yernool *et al*. 2004) (See Fig. 2).

DA_T has been cloned from several mammalian species, including human, monkey, rat, mouse and bovine (for review on cloning of neurotransmitter transporters see Blakely, 1992), and also from *C. elegans* and *Eloria noyesi,* among other species (Usdin *et al*. 1991; Giros *et al*. 1991; Giros *et al*. 1992; Kilty *et al*. 1991; Chen *et al*. 2006). The latest result is rather interesting due the fact that *E. noyesi* actually feeds on the leaves of coca plants containing significant amounts of DA_T inhibitor cocaine, while cloned dopamine transporter has similar affinity for cocaine compared to other species (Chen *et al*. 2006).

Dopamine transporters from different mammalian species display significant homology (>80%), homology between DA_T of different organisms is smaller, for example DA_T from *C. elegans* shares only 43% of its sequence with human dopamine transporter. Dopamine transporters in general also exhibit significant homology reaching 50% with other membrane monoamine transporters with numerous segments conserved, such as SER_T, GA_T and NE_T (Chen *et al*. 2000; Usdin *et al*. 1991; Miller *et al*. 2001). Despite, until very recently, the lack of crystallographic structural data, numerous site-directed mutagenesis studies were conducted and allowed certain insight into functions of DA_T. One of the most important facts site-directed mutagenesis established is that numerous binding sites exist within DA_T for various types of ligands, both that are transported by DA_T and also DA_T inhibitors (Lin *et al*. 1999; Ukairo *et al*. 2005; Uhl & Lin 2003). While information following is a compilation of mutagenesis data from various mammal species, the findings can be applied to group in general due to extremely high homology between species, especially in cross-species conserved fragments (Chen *et al*. 2000).

The NH_2 terminal plays significant role in the Na^+ and Cl^- exchange process in

catecholamine transporters, partially being responsible ionic dependence of dopamine uptake, the carboxyl-terminal tail part also plays some role in the transport process as it's truncation from Pro^{597} down results in bi-phasic dopamine uptake kinetics and decreased affinity for dopamine, also significantly reducing high-affinity components of β-CFT binding in rDA_T and hDA_T (Syringas *et al*. 2001; Lee *et al*. 1996). In TM domains substitutions following substitutions of corresponding amino acids to alanine generally result in binding affinity decrease for cocaine-analogues while leaving dopamine affinity for hDA_T relatively intact: TM2: Phe^{98}, Pro^{101}, Phe^{105}; TM3: Phe^{154}; TM4: Tyr^{251}; TM5: Tyr^{273}; TM6: Thr^{315}, Gln^{316}, Phe^{331}; TM7: Phe^{361}; TM8: Trp^{406}, Phe^{410}; TM9: Thr^{455}, Ser^{459}, Thr^{464}; TM1: Trp^{519}, Trp^{523}, Phe^{530}, while similar effects are less pronounced or absent in extracellular loops (Uhl & Lin 2003). Mutation at aforementioned residues also influence dopamine transport in mDA_T, the Phe^{105}→Ala/Leu/Ile/Ser replacement creates transporters with low transport activity, while Phe^{105}→Tyr/Trp retained 75% of dopamine transport activity and high affinity for cocaine (Wu & Gu 2003). It is generally recognized that phenylalanine residues play significant role in selective influence of dopamine transport and cocaine analogs recognition: point mutations Phe→Ala in positions 76, 98, 390 and 361 result in normal (wild-type) dopamine affinity for rDA_T but reduce affinity of β-CFT (cocaine analogue) 3- to 8-fold; Phe155→Ala substitution reduced more than 10-fold affinity of dopamine in rDA_T, the affinity decrease for β-CFT was significantly less pronounced (Lin *et al*. 1999). Tryptophan residues in positions 162, 255 and 310 also play some role in dopamine transport process, as their substitution for alanine reduced dopamine affinity in uptake process, while replacement Trp→Ala in positions 406, 496 and 523 reduced affinity for β-CFT without affecting dopamine uptake (Lin *et al*. 2000). Affinity and uptake velocity are not affected in the same way by mutations introduced in DA_T: while affinity of DA_T for transportable substance MPP^+ can be reduced via Tyr^{533}→Phe, the transport velocity of the corresponding substance the mutant transporter is increased; the same residue also considered to play important role in the differential sensitivity mechanism of rDA_T and hDA_T for cocaine and MPP^+, highlighting again the issue of multiple binding sites for different agents (Mitsuhata *et al*. 1998). Residues that are conserved throughout all Na^+/Cl^- dependent monoamine transporters are considered to be of special interest in relevance to molecular mechanism

of substance recognition and transport, mutations at Asp^{79} in DA_T (conserved throughout aforementioned transporter family) differentiate mechanism of action of benztropine and other common DA_T inhibitors of cocaine/mazindol type (Ukairo *et al*. 2005). Other recently published data supports topologically different or partially overlapping sites for different inhibitors of mDA_T (or DA_T in general for that matter), particularly for cocaine/MPP^+ and amphetamine-like (Chen *et al*. 2005). Other groups have pointed out that some of the amino acid resides, such as conserved cysteines (positions 180 and 189 in particular), while not affecting binding and transport in a significant way, have considerable influence on full (functional) transporter expression, indicating that they might be actively involved in process of DA_T protein transportation and incorporation into the cellular membrane (Wang *et al*. 1995). Tyr^{335} has been shown to be involved in the regulation of the conformational states during transport phase (Loland *et al*. 2002). It was also shown that not the entire protein (DA_T) is needed to archive at least some (or in some cases "wild-type") affinity for ligands, as separate part of the protein incorporated into cell membrane are capable of binding radioactively labeled compounds such as [^{125}I]DEEP and [^{125}I]RTI-82, with binding shown to be specific and reversible (Vaughan & Kuhar, 1996). Some evidence has been put forward concerning the particular conformational changes that take place upon ligand binding to the transporter and also relating to arrangement of single DA_T units within cell membrane (Torres *et al*. 2003; Norregaard *et al*. 2003; Hastrup *et al*. 2003), however those rather broad areas lie somewhat outside the scope of this work and their in-depth discussion would be irrelevant to the current work.

2.2 DA_T and disease

Dopaminergic system dysfunctions has been implicated in a variety of pathological conditions in humans, ranging from mild forms of attention deficit hyperactivity disorder (ADHD) (Krause *et al*. 2005; Madras *et al*. 2005) to severely debilitating conditions like drug addiction, schizophrenia, Huntington's and Parkinson's diseases (Riddle *et al*. 2005; Morice *et al*. 2005; Laakso *et al*. 2001; Ginovart *et al*. 1997; Nutt *et al*. 2004). DA_T has also been shown to play important role in processes of memory and memory impairment with age (Erixon-Lindroth *et al*. 2005). Monoamine transporters in general have been a target for drug development for several decades (Glatt & Reus 2003). Compelling evidence shows that DA_T protein possesses multiple non-overlapping binding domains (see previous section) those different pharmacological profiles can be used to develop drugs that selectively block modulation of DA_T by inhibitors like cocaine while allowing dopamine transport continue in a normal fashion (Uhl *et al*. 2003). In view of large amount of data accumulated implicating DA_T in a variety of pathological neurological conditions and emerging data shedding light on its detailed structure, DA_T presents a noteworthy target for drug development and further research.

2.3 PET and SPECT

PET and SPECT are excellent methods for *in vivo* imaging, their major drawback is relatively low resolution – 1 to 10 mm for both PET and SPECT and slow data acquisition (Garcia-Alloza *et al*. 2004). Both techniques rely on short-lived radioactive isotopes that emit either γ-photon, or positron (e^+) that upon annihilation with electron from the tissue produces two γ-photons. SPECT uses short-lived radionuclide that emit a single γ-photon, the most common nuclides being ^{123}I and ^{99}Tc, while PET uses neutron-deficient positron-emitting isotopes like ^{11}C, ^{13}N, ^{15}O, ^{18}F, ^{76}Br. The resolution of PET is influenced by length of positron free-path in the tissue, the shorter the path the greater resolution is, and therefore isotopes emitting low-energy positrons (like ^{18}F) are preferred. SPECT systems have greater intrinsic resolution, as high-energy photon is emitted directly from nucleus, not via positrons' annihilation event; however SPECT systems sensitivity is currently inferior to PET (Kessler 2003). The unique advantage of PET over SPECT is that isotopes used for PET are mostly identical in biological sense to non-radioactive isotopes and therefore have no disruptive effect on biological processes being studied. Currently SPECT systems are more widely available than PET due to simplicity and lower cost, however PET remains superior method in terms of diversity of approaches, number of ligands used, sensitivity and spatial resolution (Kessler 2003).

PET can be used to detect and quantitize ligands and corresponding receptors and transporters from nanomolar to picomolar range (Kessler 2003; Garcia-Alloza *et al*. 2004; van Waarde 2000). The prerequisite demands that must be fulfilled for a ligand to be used in PET studies are: it must easily cross blood-brain barrier, it must possess high affinity and selectivity for the target – receptor or transporter in question and thirdly, its metabolism must proceed in a way that metabolites do not interfere with measurements (Maziere & Halldin 2004). Furthermore, it must be possible to perform labelling and purification of labelled ligand rapidly in order to obtain high specific radioactivity as half-lives of the isotopes are short. Several ligands have been synthesised for PET imaging of DA_T, most of them are either tropane derivates based around naturally occurring DA_T ligand cocaine, or rimcazole and GBR12909 analogues (Newman & Kulkarni 2002). The ligands most widely used for DA_T are tropane-derived cocaine analogues 3-aryl tropanes (Clarke *et al*. 1973; Goodman *et al*. 1994; Swahn *et al*. 1992).

2.4 Dopamine re-uptake inhibitors

Dopamine re-uptake inhibitors (also "dopamine transporter inhibitors") do not belong to any particular "class" of compounds. There is a wide range of structurally different compound classes that display affinity for DA_T, many (if not all) also display significant affinity for other monoamine transporters (Glatt & Reus, 2003; Rothman & Baumann 2003). It should be mentioned that there are two primary ways in which ligands modulate DA_T: dopamine uptake inhibitors – that is cocaine and cocaine-analogues (both in structural and physiological term), disrupt dopamine re-uptake from synaptic cleft after its release from presynaptic neuron by docking with transporter but not being transported and therefore effectively stopping dopamine uptake, while amphetamine-like compounds are in effect "releasers" – they disrupt VMA_T and DA_T not only preventing dopamine re-uptake, but also making them work in the opposite direction contributing to increase of dopamine in the synaptic cleft (Rothman & Baumann, 2003; Riddle *et al*. 2005). Probably one of the oldest dopamine uptake inhibitor known is cocaine, a natural compound found in the leaves of *Erythroxylum coca* - scrub bush native to South America, whose leaves reinforcing properties were discovered by South American Indians in pre-Columbian era, the compound itself was isolated in 1860. For structure of cocaine and other compounds mentioned in the text see Fig. 3 in the Appendix.

Systematic work with cocaine analogs that affect CNS has begun in the 70's, starting with the work of Clarke and co-workers, where a phenyl group has been attached directly to tropane cycle at C-3 position and this elimination of cocaine ester fragment at C-3 has resulted in significant increase of biological activity (Clarke *et al*. 1973). Since then tropane ring with (multi)-substituted aromatic group at C-3 has become one of the "corner-stones" for dopamine re-uptake inhibitor design (Ametamey *et al*. 1995; Goodman *et al*. 1994; Kim *et al*. 2003; Newman & Kulkarni 2002). Two other classes of compounds which exhibit high selectivity and affinity for DA_T are benztropines and piperidine derivatives (Newman & Kulkarni 2002; Foulon *et al*. 1992; van der Zee *et al*. 1980; Horn *et al*. 1971). While all three groups can loosely be loosely considered structural analogs of each other (along with rimcazole-type compounds (Izenwasser *et al*. 1993)) and tend to follow similar trends in term of affinity change with introduction of halogens and other substituents at aromatic fragments, their structure-activity relationship

regarding affinity towards DA_T can differ greatly. For 3-aryl tropanes the main requirement for high-affinity for DA_T is that stereochemistry at C-2 and C-3 is β and the overall stereochemistry must be R(-)- (Carroll *et al*. 1992; Wang *et al*. 1993). DA_T also appears to tolerate significant variation in the substituent at C-2 position; however no substituent at C-2 makes 3-aryl tropanes inactive at DA_T (Carroll *et al*. 1992; Davies *et al*. 1993; Kozikowsky *et al*. 1998; Kozikowsky *et al*. 1995). Quite in the opposite, the active stereoisomers of benztropine must have α-configuration at C-3 and S(+)-stereochemistry, otherwise they are inactive (see review Newman & Kulkarni 2002; Horn *et al*. 1971). Introduction of substituents (like methyl/ethyl groups and halogens) into aromatic fragments of both 3-aryl tropanes and benztropines generally improves affinity for monoamine transporters and affects selectivity for specific transporters and receptors (Newman & Kulkarni 2002; van der Zee *et al*. 1980; Kozikowsky *et al*. 1998; Carroll *et al*. 1991). The general rules for tropane-based cocaine analogues are as follows: the addition of (un)substituted aromatic cycle at C-3 with β-stereochemistry greatly improves ligand affinity towards monoamine transporters in general, while alkyl chain with substituents attached to basic nitrogen has significant impact on ligand specificity for a particular transporter (Kozikowsky *et al*. 1998; Carroll *et al*. 1991; Milius *et al*. 1991). However while many of the 3-aryl tropanes are very potent dopamine re-uptake inhibitors, in their majority they are relatively unselective and possess significant affinity towards other monoamine transporters. In view of the considerations above numerous systematic studies have been undertaken in order to optimize structure for both affinity and selectivity with hope of finding better ligands for DA_T that could be used in both *in vitro* and *in vivo* applications (Kozikowsky *et al*. 1998; Milius *et al*. 1991; Emond *et al*. 1997; Bülow *et al*. 2005). Among numerous ligands that were evaluated for this purpose by several groups (see above) one in particular found expensive application for quantification of DA_T density in *in vivo* applications – *N*-(3-iodoprop-2*E*-enyl)-2β-carbomethoxy-3β-(4'-methylphenyl)nortropane, originally synthesized by Emond and co-workers (Emond *et al*. 1997). The ligand acquired code-name PE2I and its behavior was studied extensively in both animal models and humans, labeled with Iodine-123 and 125, and also with carbon-11 (Emond *et al*. 1997; Guilloteau *et al*. 1998; Kuikka *et al*. 1998; Hall *et al*. 1999; Page *et al*. 2002; Jucaite et al. 2005).

The compound *N*-(3-iodoprop-2E-enyl)-2β-carbomethoxy-3β-(4'-methylphenyl) nortropane (PE2I) synthesized by Emond and co-workers possesses both high affinity and selectivity for dopamine transporter (K_d=17 nM, 5-HT_T/DA_T=32 vs 0.1 for β-CIT), and as seen from number of articles received considerable attention. Due to its structure, PE2I can be labeled by several isotopes without altering its structure in any way: labeling with carbon-11, oxygen-15 (although there is no particular reason to label PE2I with ^{15}O due to very short half-life of that isotope) and tritium are possible at the methoxy-group and labeling with iodine-123/125 is possible at the 3-iodoprop-2*E*-enyl fragment (Guilloteau *et al*. 1998; Jucaite *et al*. 2005). While carbon-11 and iodine-123 are useful isotopes for PET and SPECT imaging, they are hardly usable for *in vitro* studies, and the use of iodine-125 can also be problematic due to high biological risks associated with exposure to iodine-125, high penetration factor of the radiation given off and relatively short half-life, however high specific activity of iodine-125 is a definite plus. The fact that for typical *in vitro* applications specific activity in order of 70 Ci/mmol is often quite sufficient and tritium label is considered much less hazardous in terms of radiation (very low-penetrating β^+ emissions compared to other isotopes often used), and long half-life of tritium ($t_{0.5}$=13.1 years) made labeling of PE2I with tritium at carboxyl-terminal a viable proposition for obtaining selective DA_T ligand for *in vitro* use.

3. EXPERIMENTAL PART

3.1 Materials and methods

Cocaine hydrochloride was obtained from MacFarland Ltd, UK. *p*-tolylmagnesium bromide was purchased from Aldrich, Germany. Ethyl ether for Grignard reactions was distilled from sodium benzophenone immediately prior to use. Other chemicals (of at least analytical grade) were purchased from Fluka, Riedel-Hädel and Aldrich and were used without further purification, unless stated otherwise. Analytical TLC was run on pre-coated glass plates of silica gel 60 F_{254} (Merck). Compounds were visualized either under 254 nm UV-lamp or in an iodine chamber. Flash chromatography was conducted on 60-230 or 260-420 mesh silica gel (60 A) (Merck). NMR spectra were recorded on a Bruker 400 MHz DRX or Bruker AC-200 instrument. Chemical shifts (δ) are reported in ppm, downfield from TMS (s, d, t, m, bd, bt for singlet, doublet, multiplet, broad doublet and broad multiplet respectively). The mass spectra were measured on QTOF Ultima API instrument, IP=70 eV (Waters Corp. Milford, Mass., USA). The HPLC system used in the ^{3}H-labeling experiments consisted of an interface module (D-7000, Hitachi), an isocratic pump (L-7110, Hitachi), an injector (model 7725i with a 1.0 ml loop; Rheodyne) equipped with a μ-Bondapak-C18 column (300 x 7.8 mm, 10 μm; Waters) and an absorbance detector (L-7400; 254 nm; Hitachi) in series with a radiation detector (Radiomatic 150TR; Packard) equipped with a ^{3}H cell (178.2 μl, Packard).

Binding studies were performed using striatal membrane fragments of 3-month-old female white mice. Striatum was chosen as it is shown that it has the highest concentration of DA_T in the brain and is readily dissected form the mice brain, also [^{125}I]PE2I was shown to bind specifically to this area of brain (Ciliax *et al*. 1995; Hall *et al*. 1999). Mice were decapitated, their brains removed and striatum tissue rapidly dissected, snap-frozen in liquid nitrogen and stored at -83 °C. All experiments were conducted using 70 mM Tris buffer (pH 7.4) containing 50 mM NaCl and 5 mM KCl. [^{3}H]PE2I binding assay was performed as follows: 150 μl of suspension of striatal membranes (33 μg wet tissue), 100 μl of [^{3}H]PE2I solution of different concentration, ranging from 0.9 to 31 nM, and 50 μl of buffer or 60 μM PE2I solution to determine non-

specific binding of [^{3}H]PE2I, were mixed and incubated at 25 °C during 60 min. Then the samples were rapidly filtered on Whatman GF/B filters, washed twice with 5 ml of ice-cold assay buffer and filter-bound radioactivity was measured on a LKB Wallac 1219 Rackbeta liquid scintillation counter (57% ^{3}H counting efficiency with OptiPhase 'HiSafe' 3 liquid scintillation cocktail from Fisher Chemicals, UK). The filters were equilibrated in scintillation cocktail for 12 hours before counting. The specific binding was determined as difference between total and non-specific binding of [^{3}H]PE2I. Displacement of [^{3}H]PE2I from striatal membranes by unlabelled PE2I and GBR12935 were conducted as follows: 150 µl of suspension of striatal membranes (33 µg wet tissue), 50 µl of [^{3}H]PE2I (final concentration 10 nM), and 50 µl of solutions of unlabeled PE2I and GBR12935, correspondingly, at the appropriate final concentrations, were mixed and incubated at 25 °C during 60 min. Samples were rapidly filtered on Whatman GF/B filters, washed twice with 5 ml of ice-cold assay buffer and filter-bound radioactivity was measured.

Kinetic analysis of [^{3}H]PE2I binding with DA_T was performed using scintillation proximity assay with PVT PEI treated wheatgerm agglutinin-coupled SPA beads Type B (Amersham Biosciences) (Gobel *et al*. 1999; Herm, 2005). SPA beads (0.5 mg) were loaded with striatal membranes as follows: 0.5 mg of SPA beads were mixed with 1 ml of membrane suspension containing 50 µg of protein and incubated at 1 hour at room temperature, centrifuged at 4° C for 10 min (6 *g*), the supernatant was discarded and SPA pellet was re-suspended in 450 µl of incubation buffer. The kinetic assay was initiated by addition of 50 µl of 100 nM [^{3}H]PE2I solution. Time-course of radioligand binding to membranes, associated with SPA beads, was followed by counting the sample every 30 seconds on a LKB Wallac 1219 Rackbeta counter. Time-course of the non-specific binding was determined under similar conditions with excess (10 µM) of unlabeled PE2I present and total binding was taken as difference between total and non-specific binding.

Data processing was performed using software package GraphPad Prism 4 (San Diego, USA).

3.2 Synthetic procedures (Scheme 1)

Ecgonine hydrochloride (**2,** Scheme 1). Cocaine hydrochloride (**1**) (10.0 g, 29.42 mmol) was dissolved in concentrated hydrochloric acid (35% w/w, 150 ml) and heated to reflux for 24 hours. After reflux the reaction mixture was cooled to 4°C and left to stand for several hours. Precipitated benzoic acid was removed by filtration and the residue was washed three times with 5 ml of ice-cold concentrated hydrochloric acid. The filtrate was collected, evaporated to dryness, then redissolved in methanol (25 ml) and evaporated to dryness (three times), affording ecgonine hydrochloride (6.0 g, 27.36 mmol, 93% yield) as white crystals (R_f ($MeOH/CH_2Cl_2$, 20:80) 0.5). Crude product was not characterized and used without purification for the next step (Swahn *et al*. 1996).

Anhydroecgonine (**3**). Ecgonine hydrochloride (**2**) (4.0 g, 18.08 mmol) was dissolved in 100 ml of a freshly prepared saturated methanolic solution of HCl (~13 M), prepared by bubbling gaseous hydrogen chloride through methanol, and left stirring at room temperature (Swahn *et al*. 1996). After three days, solvents were removed *in vacuo* and water (20 ml) was added to the residue, the aqueous solution was made alkaline (pH > 8) with 3 M NaOH and extracted several times with diethyl ether (4x50 ml). The organic fractions were combined and dried over anhydrous $MgSO_4$. After the drying agent was removed by filtration, the ether was evaporated, affording 3.1 g of crude anhydroecgonine. The crude product was purified by vacuum distillation (63-65°C, 0.62 mbar), affording (2.46 g, 13.56 mmol, 75%) of anhydroecgonine as transparent oil. (R_f (CH_2Cl_2/MeOH 80:20) 0.6-0.4) MS: 182.12 (M^{+}+1) - 100%, 181.49 – 13%, 182.62 – 20%, 183.12 – 20%. ^{1}H-NMR (400 MHz, $CDCl_3$): 6.81 (tr. 1H), 3.77 (d. 1H), 3.70 (s. 3H), 3.23 (t. 1H), 2.64-2.59 (v. br. d. tr. 1H), 2.34 (s. 3H), 2.15 (m. 2H), 1.85 (m, 2H), 1.51 (m, 1H). An alternative method for obtaining anhydroecgonine using $POCl_3$, resulted in an inferior yield of anhydroecgonine compared to method described above (Goodman et al. 1994).

N-methyl-2β-carbomethoxy-3β-(4-methylphenyl)nortropane **(4)** (Clarke et al. 1973). All glassware for the Grignard reaction was dried for 3 hours at 120 °C, then cooled to room temperature, assembled and flushed with dry nitrogen for 20 min to remove air. To anhydrous ethyl ether (150 ml), *p*-tolylmagnesium bromide solution in anhydrous ethyl ether (1 M, 47 ml) was added and cooled to -60°C while stirred. Anhydroecgonine (**3**)

(2.20 g, 12.14 mmol) was dissolved in anhydrous ethyl ether (40 ml) and slowly added to the stirred solution of *p*-tolylmagnesium bromide over the course of 30 minutes, not allowing temperature to exceed -55°C. The reaction mixture was then stirred for 3.5 hours at -40 to -50°C, after which it was cooled to -76°C and 4.8 ml (5.34 mmol) of TFA in 20 ml of anhydrous ether were added. The resulting solution was stirred for another 15 minutes at -76°C, then the cooling bath was removed and the reaction mixture was allowed to warm to 0°C. Thereafter water (150 ml) was carefully added into the reaction mixture, the aqueous layer was acidified with conc. HCl ($pH<1$) and washed with ethyl ether, basified with conc. ammonia solution ($pH>8$) and extracted with ethyl ether (5x100ml). The combined ether fractions were dried over anhydrous $MgSO_4$ and concentrated to dryness, affording a crude mixture of *N*-methyl-2β-carbomethoxy-3β-(4-methylphenyl)nortropane and *N*-methyl-2β-carbomethoxy-3α-(4-methylphenyl) nortropane. *N*-methyl-2β-carbomethoxy-3β-(4-methylphenyl)nortropane (**4**) was purified by flash chromatography (silica gel, Et_2O/TEA 90:10), affording 1.56 g (5.70 mmol, 47%) of desired compound (R_f (Et_2O/TEA 90:10) 0.85-0.9) as transparent yellowish oil that solidifies upon standing at -18 °C. (MS: 274.13 (M^++1) – 100%, 273.16 – 40%, 275.18 – 45%. ^{1}H-NMR (400 MHz, $CDCl_3$) 7.16 (d, 2H), 7.07 (d, 2H), 3.51 (s, 3H, CO_2CH_3), 3.43 (m, 1H, H-1), 3.6 (m, 1H, H-5), 3.11 (m, 2H), 2.34 (s, 3H, N-CH_3), 2.26 (s, 3H, ar CH_3), 1.95 (m, 1H), 1.90-1.43 (m, 3H), 1.43 (m, 1H), 1.26 (m, 1H); ^{13}C-NMR (50 MHz, $CDCl_3$): 21, 25.2, 26, 33.4, 34.1, 42, 51, 52.8, 62.4, 65.4, 127.2, 128.7, 135.2, 139.9, 172.2. 0.36 g (1.33 mmol, 11%) of *N*-methyl-2β-carbomethoxy-3α-(4-methylphenyl)nortropane were also isolated (R_f (Et_2O/TEA 90:10) 0.6-0.65). Optimization of this step involved variation in the amount of p-tolylmagnesium bromide used in the reaction, temperature regime and reaction time. The yields were variable, but best results were obtained when anhydroecgonine was added very slowly at -55…-60° C and reaction is conducted for 3…4 hours (after finishing addition) at -35° C. With careful optimization it is possible to increase yield of 2β-3β isomer to 63% (for yields of similar compounds see Goodman *et al*. 1994; Carroll *et al*. 1991; Swahn *et al*. 1996). The isomer ratio and yield are also heavily dependent on the solvent – replacement of diethyl ether by tetrahydrofurane resulted in exclusive formation of 2β-3α isomer, with no 2β-3β detected by TLC analysis.

2β-carbomethoxy-3β-(4-methylphenyl)nortropane (**5**). 1.40 g (5.12 mmol) of *N*-methyl-2β-carbomethoxy-3*β*-(4-methylphenyl)nortropane (**4**) was dissolved in 4 ml (28.3 mmol) of 2,2,2-trichloroethyl chloroformate, and heated under nitrogen atmosphere at 120º-130º C for two hours (Goodman *et al*. 1994). The excess of chloroformate was distilled from crude carbamate under reduced pressure, the resulting yellow oil was dissolved in 95% acetic acid (30 ml), and zinc dust (3.5 g, 54 mmol) was added and the mixture was stirred for 15 hours at room temperature. Celite (~6 g) was then added and the mixture was filtered. Solid residue remaining of filter was washed twice with 5 ml of 95% acetic acid. Water (15 ml) was added to the filtrate and solution was extracted with chloroform (4x50 ml). Chloroform fractions were combined and washed with 10% NaOH aqueous solution until the aqueous layer remained basic (pH>9). The aqueous fractions combined were then extracted once with 50 ml of chloroform. Resulting chloroform fractions were combined (total of 250 ml) were washed with water (50 ml), dried over anhydrous $MgSO_4$, and solvent was removed. Crude product (~1.2 g, yellow oil) was purified using flash chromatography (silica gel, Et_2O/TEA 90:10), affording 980 mg (3.79 mmol, 74%) of 2β-carbomethoxy-3β-(4-methylphenyl)nortropane as slightly yellowish oil that crystallizes on standing into white crystals. (R_f (Et_2O/TEA 90:10) 0.2-0.35) MS: 260.16 (M^++1) – 100%, 260.19 – 70%, 260.80 – 70%, 261.19 – 45%. ^{1}H-NMR (400 MHz, $CDCl_3$) 7.0 (s, 4H), 3.7 (m, 2H), 3.4 (s, 3H, CO_2CH_3), 3.2 (m, 1H), 2.9 (m, 1H), 2.7 (m, 1H), 2.4 (m, 1H), 2.3 (s, 3H, ar CH_3), 2.2-1.9 (m, 2H), 1.8-1.6 (m, 3H)); ^{13}C-NMR (50 MHz, $CDCl_3$): 21, 27.8, 29.2, 34, 35.4, 51, 51.4, 53.8, 56.4, 127.2, 129, 136, 139.4. Alternative method for desmethylation using 1-chloroethylchloroformate in 1,2-dichloromethane was also tested (Swahn et al. 1996). The yield was inferior to method described above and crude product contained significant amount of impurities and also some unreacted starting material (complete reaction was not observed even after increasing amount of 1-chloroethylchloroformate and increasing reaction time), so method using 2,2,2-trichloroethyl chloroformate was adopted as method of choice. The work-up can be changed to omit extraction of acidic solution with dichloromethane and started with neutralization of acetic acid with concentrated aqueous ammonia solution to pH>8 and resulting solution extracted with dichloromethane. The yields were independent of the work-up procedure, but amount of work and solvents used is smaller

in the last case.

1-chloro-(E)-3-(tri-n-butylstannyl)-propen-1-yl chloride (**6^{A}**) (Goodman *et al*. 1994; Jung & Light 1982). Propargyl alcohol (40 mmol, 2.3 ml) was mixed with tributyltin hydride (52 mmol, 14.5 ml) and 1,1'-azobis(cyclohexanecarbonitrile) (10 mg). The reaction mixture was stirred under nitrogen atmosphere at 100°-105° C overnight. The desired (*E*)-3-tributylstannanyl-prop-2-en-1-ol was purified using flash chromatography (silica gel, Et_2O/TEA 90:10), affording 4.86 g (14.0 mmol, 35%) of desired compound as light colorless oil (R_f (Et_2O/TEA 90:10) 0.2-0.3) MS: 177 – 100%, 41 – 30%, 121 – 60%, 137 – 60%, 235 – 60%, 291 – 30%. ^{1}H-NMR (400 MHz, $CDCl_3$): 6.1 (m, 2H, CH=CH), 4.1 (m, 2H, CH2OH), 1.6-1.2 (m, 21H, butyl groups). ^{13}C-NMR (100 MHz, $CDCl_3$): 145, 126, 64, 27, 25, 12, 8 ppm. Significant amounts of trans- and gem- isomers were also isolated. Increasing the amount of initiator (1,1'-azobis(cyclohexanecarbonitrile)) results in increase in the yield of unwanted gem- isomer.

The obtained (*E*)-3-tributylstannanyl-prop-2-en-1-ol (4.5 g, 13.0 mmol) was dissolved in 25 ml of tetrachloromethane and converted to (E)-tributyl-(3-chloro-propenyl)-stannane by heating with triphenylphosphine (4.0 g, 15.1 mmol) at 60 °C for 48 hours. Crude product was purified using flash chromatography (silica gel, petroleum ether/TEA 98:2), affording 3.1 g (8.4 mmol, 65%) of (*E*)-tributyl-(3-chloro-propenyl)-stannane (**6^{A}**). (R_f (PE/TEA 98:2) 0.8). MS: 273.21 – 100%, 215.17 – 80%, 228.17 – 20%, 274.23 – 20%, (M+1) 365.17 – 15%, 413.34 – 40%, 414.35 – 10%. ^{1}H-NMR (200 MHz, DMSO-d6): 6.37-6.28 (m, 1H, C**H**=CH), 6.12-5.97 (m, 1H, CH=C**H**), 4.2 (m, 2H, CH_2Cl) 1.6-1.2 (m, 21H, butyl groups). ^{13}C-NMR (50 MHz, DMSO-d_6): 143, 133, 47, 28, 26, 13, 9 ppm.

N-(3-tributylstannanylprop-2E-enyl)-2β-carbomethoxy-3β-(4-methylphenyl)nortropane (**6**). (*E*)-tributyl-(3-chloro-propenyl)-stannane (**6^{A}**) (1.48 g, 4.04 mmol) and 2β-carbomethoxy-3β-(4-methylphenyl)nortropane (**5**) (950 mg, 3.67 mmol) were dissolved in 40 ml of absolute ethanol and 565 µl of triethylamine and 7.0 mg of potassium iodide. The solution was refluxed under nitrogen for 16 hours (Goodman *et al*. 1994; Emond *et al*. 1997). After solvent removal the crude product was purified by flash chromatography (silica gel, PE/EtOAc/TEA 85:15:1) affording 1.84 g (3.12 mmol, 85%) of *N*-(3-tributylstannanylprop-2*E*-enyl)-2β-carbomethoxy-3β-(4-methylphenyl)nortropane as thick, colorless oil, crystallizing into white crystals upon standing. MS: 590.3 – 100%

(significant amount of isotope lines around m/z 590). ^{1}H-NMR (200 MHz, $CDCl_3$): 0.84 (br m, 9H), 1.2…1.5 (double br m, 18H), 1.5…1.66 (m, 4H), 2.0 (m, 2H), 2.28 (s, ar-CH3), 2.61 (m, 1H), 2.89 (m, 2H), 3.12 (m, 1H), 3.4 (br. m. 1H), 3.49 (s. 3H), 3.67 (br. m. 1H). ^{13}C-NMR (50 MHz, $CDCl_3$): 9.5, 13.6, 20.9, 26, 26.1, 27.3, 29.1, 34, 34.3, 51, 52.9, 60.5, 61.2, 61.9, 127.3, 128.6, 130.1, 135.1, 140.1, 146.9, 172, 218 ppm.

N-(3-iodoprop-2E-enyl)-2β-carbomethoxy-3β-(4-methylphenyl)nortropane (**7**) (Goodman *et al*. 1994; Emond *et al*. 1997). *N*-(3-tributylstannanylprop-2*E*-enyl)-2β-carbomethoxy-3β-(4-methylphenyl)nortropane (0.97 g, 1.65 mmol) was dissolved in 15 ml of trichloromethane, cooled to 4 °C and 18 ml of 0.10 M iodine solution in trichloromethane was added drop wise during 3.5 hours. The resulting solution was washed with 10% aqueous sodium sulphite solution; the aqueous phase was extracted with 30 ml of chloroform. The organic fractions were combined and dried over anhydrous $MgSO_4$. After removal of the solvent the crude product was purified by flash chromatography (silica gel, hexane/Et_2O/TEA 20:10:1) affording 618 mg (1.45 mmol, 88%) of *N*-(3-iodoprop-2*E*-enyl)-2β-carbomethoxy-3β-(4-methylphenyl)nortropane as white waxy crystals (R_f (hexane/Et_2O/TEA 20:10:1) 0.7). MS: m/z 426.2 (100%), 427.2 (10%). ^{1}H-NMR (200 MHz, DMSO-d6): 1.45…2.0 (series of br m, 5H), 2.23 (s, ar-CH3), 2.41 (m, 1H), 2.82 (m, 2H), 2.97 (m, 2H), 3.29 (br. m. 1H), 3.38 (s. 3H), 3.49 (br. m. 1H), 6.35 (m, 2H), 7.0…7.15 (m, 4H). ^{13}C-NMR (50 MHz, DMSO-d6): 20.5, 25.1, 25.6, 32.7, 33.5, 50.3, 51.7, 57.1, 57.1, 60.7, 62.0, 78.2, 126.9, 128.1, 134.0, 140.2, 144.4, 170.9 ppm. Using strictly equivalent amount of iodine and adding it very slowly (over the course of 3.5 hours) results in significant improvement of the yield of this reaction: up to 97% compared to 55% (from Emond *et al*. 1997)

N-(3-iodoprop-(2E)-enyl)-2β-carboxy-3β-(4'-methylphenyl)nortropane (**8**). *N*-(3-iodoprop-2*E*-enyl)-2β-carbomethoxy-3β-(4-methylphenyl)nortropane precursor was prepared as follows: 390 mg (0.92 mmol) of *N*-[3-iodoprop-(2E)-enyl]-2β-carboxy-3β-(4'-methylphenyl)nortropane (**7**) was dissolved in 20 ml of 1:1 water/dioxane mixture and refluxed for 150 hours. Crude product was purified by flash chromatography (silica gel, chloroform/MeOH 90:10) affording 304 mg (0.74 mmol, 80%) of *N*-(3-iodoprop-2*E*-enyl)-2β-carbomethoxy-3β-(4-methylphenyl)nortropane as white crystals (R_f (chloroform/MeOH 90:10) 0.3). MS (HCl salt): m/z 412.1 (100%), 434.1 (10%). ^{1}H-

NMR (200 MHz, DMSO-d6): 1.45…2.13 (series of br m, 5H), 2.23 (s, ar-CH3), 2.52 (m, 1H), 3.0...3.2 (br m. 4H), 3.53 (br. m. 2H), 6.35 (m, 2H), 7.0…7.1 (m, 4H), 15.3 (br s, 1H). ^{13}C-NMR (50 MHz, DMSO-d6): 20.5, 24.5, 25.0, 33.9, 33.9, 52.0, 55.5, 59.1, 61.6, 82.3, 127.4, 128.4, 135.0, 138.4, 140.8, 172.4 ppm.

*Tritium-labeled PE2I (**9**)*. [^{3}H]PE2I was synthesized according to Scheme 2. [^{3}H]PE2I acid precursor (**8**) (0.0022 mmol, 0.9 mg) was dissolved in the mixture of 250 µl of dry dimethylformamide and 3 µl of freshly prepared tetrabutylammonium hydroxide (0.4 M). [^{3}H]-methyl iodide in toluene (150 µl) was added into the reaction mixture, which was thoroughly mixed for 30 seconds and heated at 80° C for 5 min in a sealed vial and cooled to room temperature. Almost complete (>99%) incorporation of tritium into labeled compound was achieved as confirmed by HPLC analysis. Acetonitrile (600 µl) was added to the resulting reaction mixture and **9** was eluted at 7.5 to 8 minutes and purified using reverse phase HPLC C-18 column (300 x 7.8 mm, 10 µm; Waters) with 25/75 ACN/0.01 M H_3PO_4 as the HPLC mobile phase. The mobile phase was evaporated at 45 °C to near dryness and the product was reconstituted in acetonitrile. The product had specific radioactivity of 73.4 Ci/mmol. The labeled product eluted from the column at time identical to unlabeled PE2I (as shown in Fig. 4).

*[^{11}C]PE2I (**10**)*. The synthesis of [^{11}C]PE2I was carried out as described in detail elsewhere (Halldin *et al*. 2003). The obtained [^{11}C]PE2I eluted from the HPLC C-18 column (300 x 7.8 mm, 10 µm; Waters) with the retention time identical to that of the standard reference sample (obtained from Karolinska Hospital, Department of Clinical Neuroscience) and determination of its specific radioactivity gave result of 8200 Ci/mmol.

3.3 Results and discussion

In this paper presented summary results of the expensive work undertaken with purpose of synthesizing N-(3-iodoprop-2E-enyl)-2β-carbomethoxy-3β-(4-methylphenyl) nortropane (PE2I) and its precursors for medical applications with view of obtaining new tritium-labeled tracer for DA_T *in vitro* and, in parallel, developing optimized working procedure for synthesizing other similar compounds based on tropane cycle for further projects involving synthesis of DA_T inhibitors. Summary yield of PE2I after optimization of procedure was about 18% in 6 steps starting from cocaine (as compared to approximately 9% from same starting material in literature.

The general procedure applied in synthesis of the PE2I precursor and the tritium-labeled PE2I was started from cocaine (**1**) and follows the synthetic pathway shown in Scheme 1. Separate steps of this synthesis have been elaborated by different authors and were combined into the joint synthetic procedure by Emond and co-workers (Emond *et al.* 1997). The procedure was futher optimized during work.

As described previously, the synthesis of anhydroecgonine (**3**) was rather straightforward and proceeded with a relatively high yield (75%), without purification of the ecgonine intermediate (**2**). The following Grignard reaction of anhydroecgonine (**3**) with *p*-tolylmagnesium bromide was performed at low temperature, as recommended by Clarke to increase the yield of *N*-methyl-2β-carbomethoxy-3β-(4-methylphenyl)nortropane (**4**) (Clarke et al. 1973). This reaction was found to be very sensitive upon the presence of small amounts of impurities in the starting material, but after distillation of **3** the yield of the 2β - 3β isomer reached 63% with careful optimization. Similar (slightly smaller) yields were reported for related compounds by other authors (Goodman *et al*. 1994; Carroll *et al*. 1991).

Demethylation of **4** was performed by refluxing in 2,2,2-trichloroethyl chloroformate and then reduction of the resulting carbamate with zinc under acidic conditions produced 2β-carbomethoxy-3β-(4-methylphenyl)nortropane (**5**) in 84% yield (compared with 80% for similar compounds as published by Goodman and co-workers (Goodman *et al*. 1994)). Compound **5** was then alkylated with tributyl-(3-chloro-propenyl)-stannane in absolute ethanol (Goodman *et al*. 1994; Jung & Light 1982), affording *N*-(3-tributylstannanylprop-2E-enyl)-2β-carbomethoxy-3β-(4-methylphenyl)

nortropane (**6**) (87% yield), which was thereafter iodinated to obtain the free base *N*-(3-iodoprop-2E-enyl)-2β-carbomethoxy-3β-(4-methylphenyl)nortropane (**7**) (95% yield), known as the dopamine transporter inhibitor PE2I . For tritium labeling as well as for synthesis of the appropriate ^{11}C labeled PET ligand, compound **7** must be demethylated and then radiomethylated to introduce the radioactive methyl group.

For demethylation, **7** was refluxed in a 1:1 mixture of dioxane and water for 5 days, affording desmethyl-PE2I (**8**) in 80% yield. The yield of the 7-step synthesis of desmethyl-PE2I, which is the precursor for [^{3}H]PE2I (**9**), was 18%.

The demethylated compound **8** was used for tritium-labelling with [^{3}H]methyl iodide. The methylation reaction was carried out in dimethylformamide as recommended previously (see Scheme 2) (Swahn *et al*. 1996). To improve the radiochemical yield of the [^{3}H]PE2I, the product **8** was not converted into a hydrochlorine or hydrobromine salt, but was kept as an internal salt, which was stable if stored below -15°C in the dark. The results of HPLC analysis of the radiolabeled product **9** and its precursor shown in Fig. 1.

To confirm the absence of unlabelled PE2I (**7**) in the precursor material (**8**), reaction of **8** with [^{11}C]methyl triflate was carried out as descried elsewhere (see Scheme 3) (Halldin *et al*. 2003). This procedure yielded the product [^{11}C]PE2I (**10)** with a specific radioactivity of 8200 Ci/mmol that confirmed the absence of unlabeled PE2I (**7**) in the starting material (**8**).

After labeling of precursor with [^{3}H]methyl iodide novel tritium-based ligand for DA_T quantification *in vitro* was obtained, with specific activity of 73.4 Ci/mmol and radiochemical purity in excess of 95% (Fig. 4).

N-(3-iodoprop-2E-enyl)-2β-carbomethoxy-3β-(4-methylphenyl)nortropane and two precursors for labeling with different isotopes (^{11}C, $^{125/123}I$ and ^{3}H) were synthesized with synthetic procedure optimized, resulting in yields higher than previously published for the corresponding compounds (up to 50% higher). Labeling of precursor with carbon-11 yielded compound with very high specific activity (SA=8200 Ci/mmol), leading to conclusion that precursor is virtually carrier-free and thus suitable for PET application.

Specific binding of the tritium-labeled PE2I with mice striatal membranes (Fig. 5) was described by a conventional binding isotherm (Eq. 1):

$$B_{eq} = \frac{B_{max} \cdot [L]}{K_D + [L]} \qquad \text{Eq. 1}$$

where B_{eq} stands for specifically bound [^{3}H]PE2I, B_{max} is the maximal observed radioligand binding with the membrane fragments and K_D is the dissociation constant. Non-specific binding was linearly dependent on radioligand concentration. The apparent K_D value calculated for [^{3}H]PE2I from the binding data was 11±4 nM (B_{max} = 46±8 pmol/mg of tissue). Relatively high levels of specific binding were observed, however, high non-specific binding was also observed by other authors (Page *et al*. 2002; Guilloteau *et al*. 1998). The obtained K_D value is similar to K_D values obtained by Emond and co-workers: 17±7 nM (using [^{3}H]GBR-12935 as a reporter ligand in rat striatum membranes (Emond *et al*. 1997). *In vitro* studies with [^{125}I]PE2I and mice striatum membranes and DAT expressed in COS cells give variable values for K_D ranging from 0.1 to 4 nM (*Page et al*. 2002; Guilloteau *et al*. 1998). Functional assays give mixed results ranging from pEC_{50} 7.3 to 8.7 in COS rDA_T cDNA transfected cells (Page *et al*. 2002).

The radioligand [^{3}H]PE2I was also used in displacement study with unlabelled PE2I and another DA_T inhibitor GBR12935. The results of this study are shown in Fig 6. The pIC50 values, calculated from these displacement curves for PE2I and GBR12935, were 7.1 ± 0.3 and 6.9 ± 0.3, respectively, with both ligands capable of displacing bound [^{3}H]PE2I to the non-specific binding level, indicating same target for ligand binding.

The time-course of [^{3}H]PE2I binding to DA_T was investigated using SPA method. The time-course of specific binding of 10 nM [^{3}H]PE2I with the membranes was measured by SPA assay as shown in Fig. 7. The process followed an exponential rate equation (Eq. 2):

$$B_t = B_{eq} \cdot \exp(-k_{obs} \cdot t) \qquad \text{(Eq. 2)}$$

where B_t stands for the specific radioligand binding at time moment t, B_{eq} is the maximal specific binding at equilibrium (at the end of the binding process) and k_{obs} is the observed rate constant of ligand binding. The observed rate constant k_{obs}= (4.0±0.5) 10^{-3} s^{-1} was obtained at 10 nM radioligand concentration (no other data concerning rate of association has been published before). This means that during 11.5 min above 96% of the binding sites were occupied by the ligand under the used conditions and the incubation time 60

min should be sufficient to reach the equilibrium state at as low as 2 nM [^{3}H]PE2I concentration. However, it must be noted that despite attempts for optimization, SPA method was cumbersome, time consuming and suffered inexplicable failures on several occasions, therefore it might not be very practical method for data acquisition in further experiments.

The fact that PE2I can be labeled with two different long-lived isotopes (^{3}H and ^{125}I), that can be diffentiated by counting, without changing ligand biological properties of the ligand, opens possibility for designing new type of direct ligand binding/competitive kinetics experiments with two reporter ligands instead of one - which can lead to significant improvement in the quality of data obtained form the experiment.

As iodine-labeled compounds have much shorter shelf-life and are somewhat hazardous to use due to formation of volatile radioactive iodine that is absorbed specifically in the thyroid, the tritium-labeled version of the compound might be a good alternative for quantification of DAT in vitro, given the data obtained.

In the future detailed studies of DA_T interaction with substrates and inhibitors belonging to different structural classes conducted using the developed [^{3}H]PE2I as reporter ligand will be undertaken to clarify important aspects of dopamine transporter protein function.

4. CONCLUSIONS

Procedure for synthesis of potent and selective DA_T inhibitor *N*-(3-iodoprop-2*E*-enyl)-2β-carbomethoxy-3β-(4-methylphenyl) nortropane has been optimized at several steps and the product was obtained in 6-step procedure with summary yield of 18%. Overall yield for desmethylated precursor for synthesis of carbon-11 and tritium labeled inhibitor was 15%. In summary, efficiency of the synthesis was improved more than two times.

Desmethylated precursor for radiolabelled PE2I was prepared as free base from parent compound and labeled with carbon-11 via O-methylation procedure to specific activity of 8200 Ci/mmol with radiochemical purity in excess of 98%. This indicates that the precursor was virtually "carrier-free" deeming it to be fit for use in PET applications. Labeling with tritium yielded [^{3}H]PE2I with specific activity of 73.4 Ci/mmol and radiochemical purity in excess of 95%.

[^{3}H]PE2I has been tested in preliminary evaluation experiments for activity at DA_T in mice striatum membrane preparation, yielding K_D=11±4 nM (B_{max} = 46±8 pmol/mg of tissue), which is in agreement with data published for [^{125}I]PE2I. The [^{3}H]PE2I was displaced from striatum membranes by both unlabelled PE2I (pIC_{50}= 7.1 ± 0.3) and GBR12935 (pEC_{50}= 6.9 ± 0.3) to the level of non-specific binding, indicating reversibility of the binding process. Measurement of the association rate constant of [^{3}H]PE2I with mDA_T yielded k_{obs}= (4.0±0.5) 10^{-3} s^{-1} at 10.0 nM [^{3}H]PE2I, indicating that the process is relatively rapid. From results of this preliminary evaluation it was concluded that [^{3}H]PE2I is a promising candidate for use as reporter ligand in *in vitro* investigations of DA_T and its kinetics.

5. KOKKUVÕTE

Dopamiin transporteri uue radioaktiivse ligandi *N*-(3-iodoprop-2*E*-enüül-2β-karbo-$[^3H]$metoksü-3β-(4'-metüülfenüül)nortropaani ($[^3H]$PE2I) süntees ja esialgne iseloomustamine.

Töös on esitatud kokkuvõte dopamiini transporteri selektivse ligandi *N*-(3-iodoprop-2*E*-enüül)-2β-karbometoksü-3β-(4-metüülfenüül)nortropaan − koodnimetusega (PE2I) ja selle ligandi radioaktiivset märkimist võimaldava prekursori sünteesi kohta. Kasutades süntesitud prekursorit valmistati ka triitiumiga märgistatud ligand, mis laiendab seni kasutusel olevate dopamiini transportvalkude uurimiseks kasutatavate ligandide nimistut ning on hetkel ilmselt üheks kõige selektiivsemalt toimivaks radioligandiks selles valdkonnas. Töö käigus optimeeriti sünteesitee mitmeid etappe, pidades silmas samade protsesside rakendatavust ka teiste sarnaste ühendite saamiseks. Selle töö tulemusena õnnestus suurendatda enamuse etappide saagiseid võrreldes varem kirjanduses avaldatus saagistega ning need ulatusid üle 50%.

Desmetüülitud PE2I prekursor saadi vaba alusena ning seda märgisttii testimise eesmärgil süsinik-11 isotoobiga, saades $[^{11}C]$PE2I preparaadi eriaktiivsuseks 8200 Ci/mmol. Selline tulemus näitas, et saadud prekursor ei sisalda O-metüüleeritud lõpproduktі (nn "carrier-compound") ning sobiv kasutamiseks PET uuringuteks.

Töös sünteesitud prekursori märgistamisel $[^3H]$metüüljodiidiga saadi ka uus triitiumiga märgistatud ligand DA_T uurimiseks in vitro tingimustes. Selle radioligandi eriaktiivsus oli 73.4 Ci/mmol, mis on sobiv uringute teiostamiseks. Kuna saadud radioligandi $[^3H]$PE2I sidumisomadused transportervalgu suhtes ei olnud eelnevalt kirjeldatud, testiti seda ligandi hiire juttkeha membraanipreparaati kasutades. Tulemusena saadud K_D väärtus 11±4 nM (B_{max} = 46±8 pmol/mg of tissue) on kooskõlas andmetega mis olid varem avaldatud $[^{125}I]$PE2I jaoks. Samuti leiti, et $[^3H]$PE2I sidumine on pöörduv ning spetsiifiline ning seda ligandi saab sidumiskompleksist välja tõrjuda märgitamata PE2I ning DA_T inhibiitori GBR12935 poolt. Radioligandi sidumise kiiruskonstandi mõõtmine näitas, et PE2I assotsiatsioon on suhteliselt kiire: 10.0 nM $[^3H]$PE2I kontsentratsiooni korral on näiv assotsiatsiooni kiiruskonstant k_{obs}= (4.0±0.5) 10^{-3} s^{-1}. Seega avab sünteesitud radioligand uusi võimalusi DA_T-i ja temaga toimivate ligandide sidumisprotsessi mehhanismi kineetiliseks uurimiseks.

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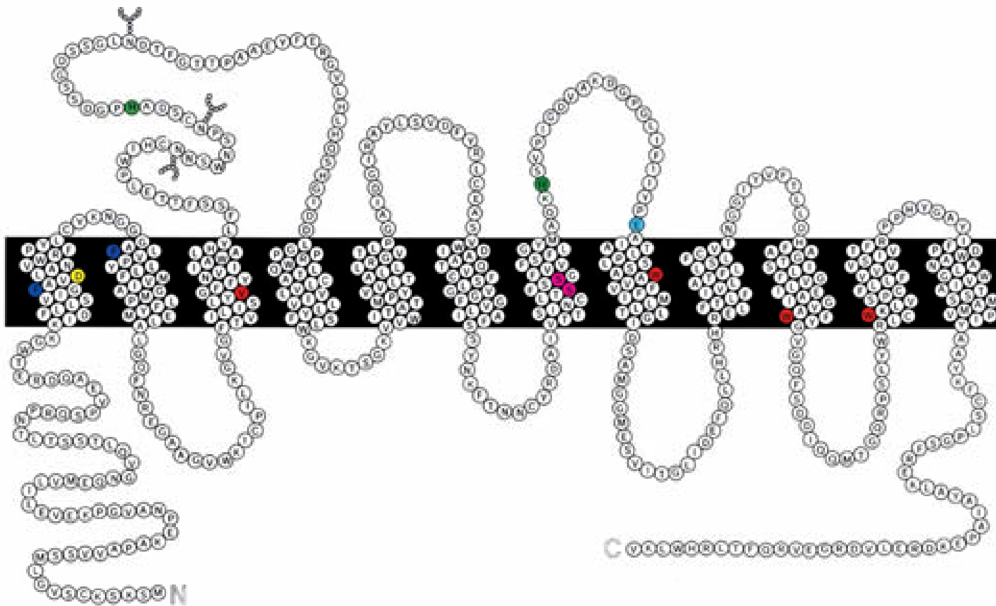
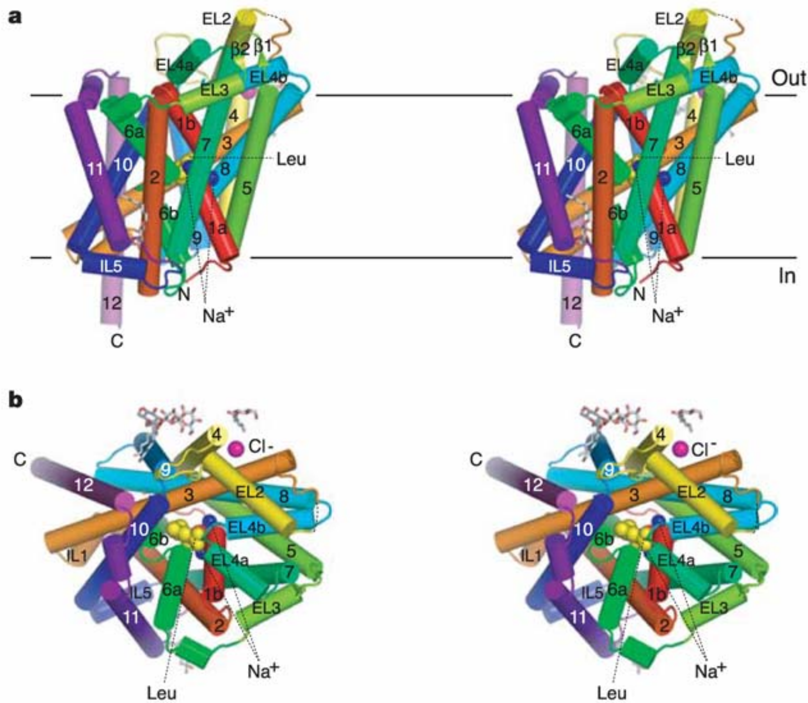
APPENDIX

Fig. 2 DA_T structure.



DAT structure. From Newman & Kulkarni, 2002.



LeuT$_{Aa}$ (belonging to SCDNT family) from *Aquifex aeolicus* from Yamashita *et al*., 2005. Leu shown as CPK model in yellow, Cl^- in magenta and $2Na^+$ in blue.
a – stereoview in plane of membrane, **b** – stereoview from the extracellular side.

Fig. 3. Common DA_T inhibitors and substrates.

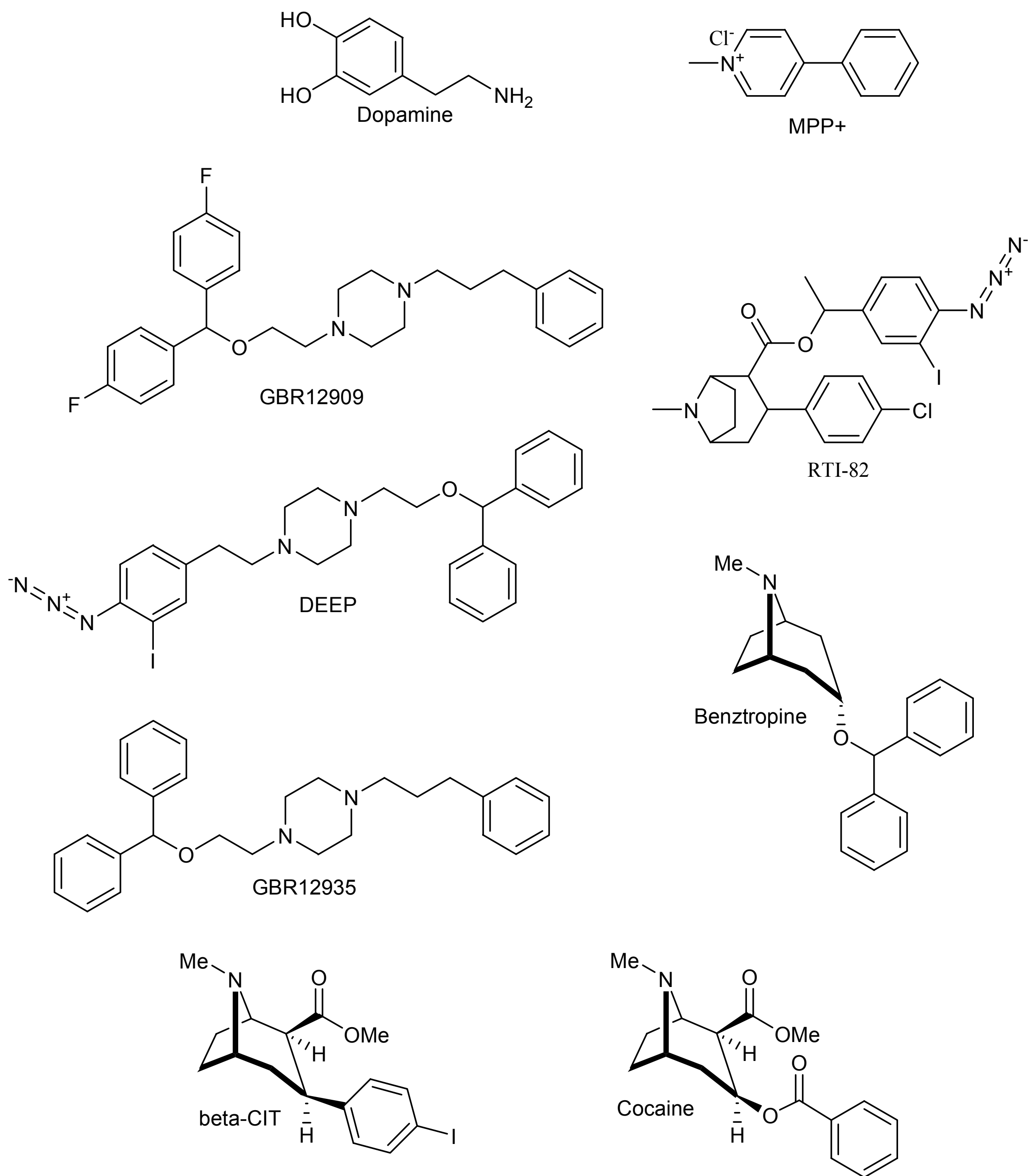
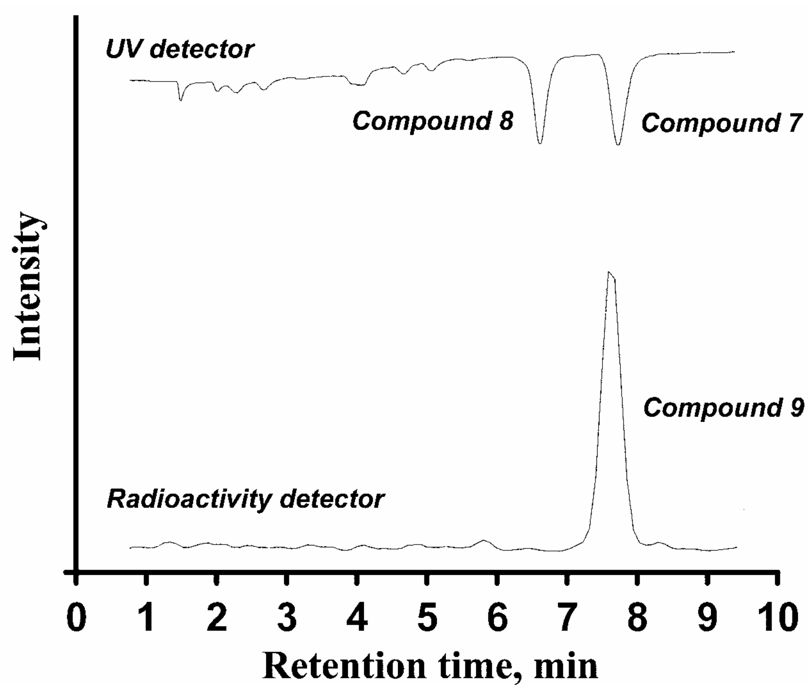


Fig. 4. Tritium-labeled PE2I.



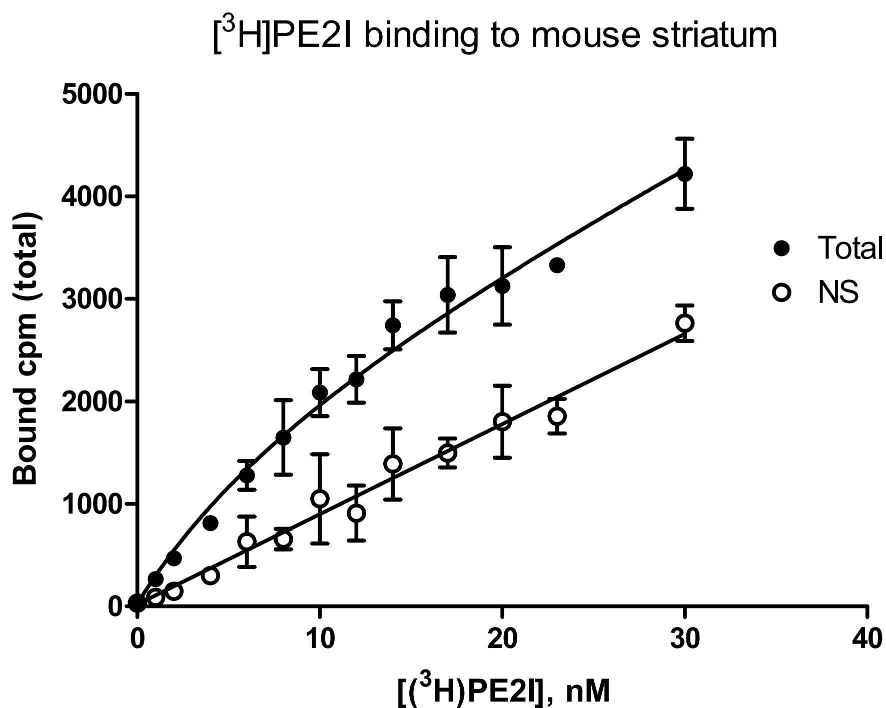
Compound 7 - unlabeled PE2I (UV channel)

Compound 9 - [3H]PE2I (radioactivity detector)

Compound 8 - PE2I acid precursor (UV channel)

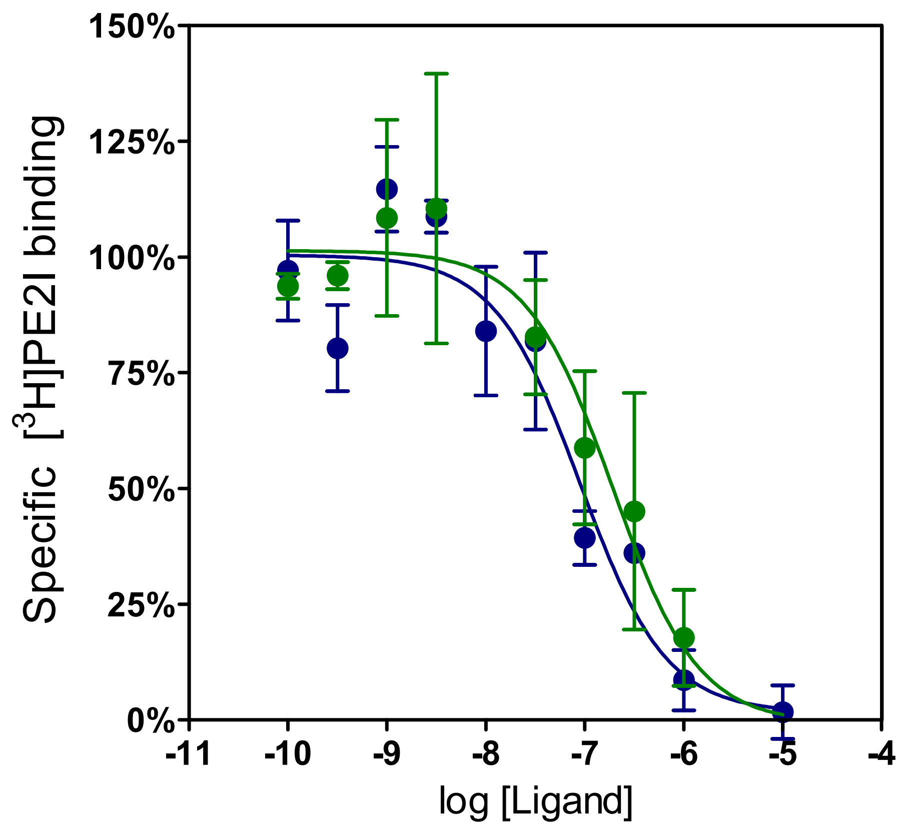
HPLC C-18 column (300 x 7.8 mm, 10 µm; Waters) with 25/75 ACN/0.01 M H_3PO_4

Fig. 5. Equilibrium binding of [^{3}H]PE2I with mice striatal membranes.



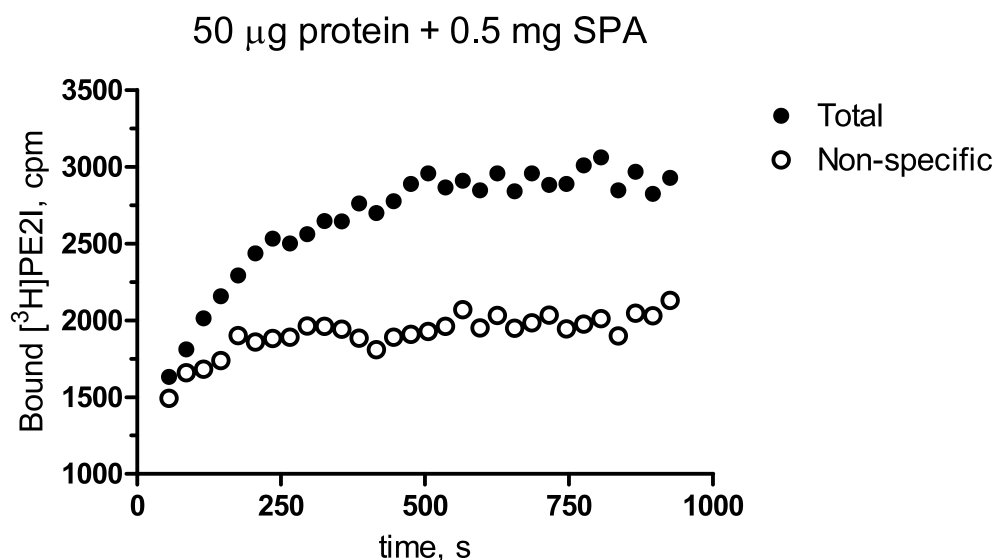
Specific binding of [^{3}H]PE2I with mice striatal membrane fragments (33 μg of wet tissue per sample, assay medium 70 mM Tris/HCl buffer, pH 7.4, 50 mM NaCl and 5 mM KCl, 25 ^{o}C).

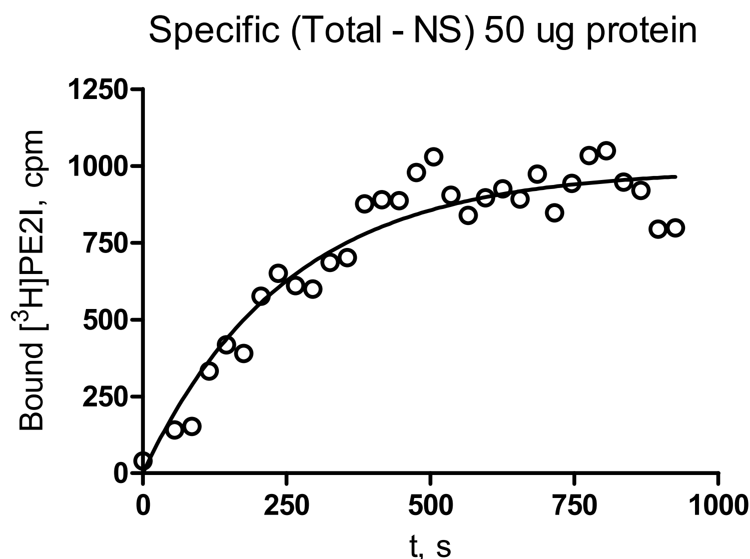
Fig. 6. Displacement of [^{3}H]PE2I from mice striatal membranes by unlabelled PE2I and GBR12935.



Displacement of [^{3}H]PE2I from its binding sites on mice striatal membrane fragments (33 µg of wet tissue per sample, assay medium 70 mM Tris/HCl buffer, pH 7.4, 50 mM NaCl and 5 mM KCl, 25 °C) by unlabelled PE2I (blue, pIC_{50}= 7.1 ± 0.3) and GBR 12935 (green, pEC_{50}= 6.9 ± 0.3).

Fig. 7. Time-course of [^{3}H]PE2I association with mice striatal membranes.



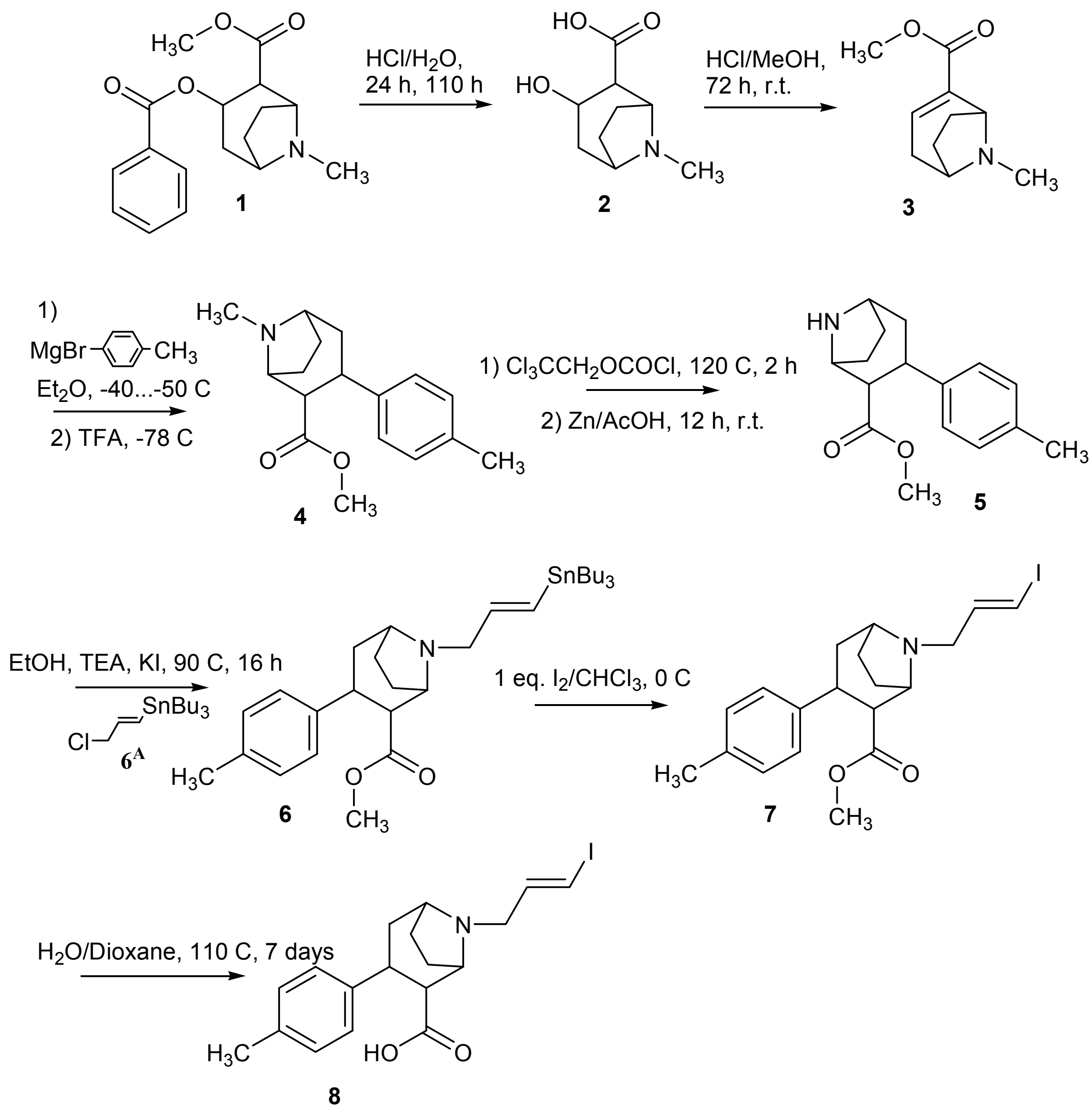


$$B_t = B_{eq} \cdot \exp\left(-k_{obs} \cdot t\right)$$

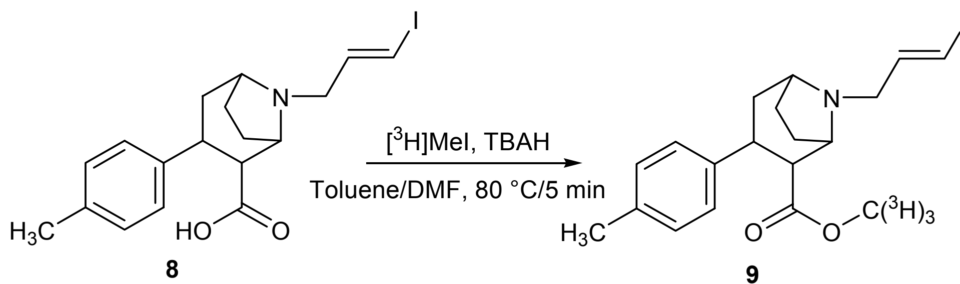
k_{obs}= (4.0±0.5) 10^{-3} s^{-1}

0.5 mg of SPA + 1 ml of membrane suspension (50 μg of protein) t_{inc}=1 hour at room temperature, centrifuged at 4° C for 10 min (6 *g*), re-suspended in 450 μl of incubation buffer. Assay was initiated by addition of 50 μl of 100 nM [^{3}H]PE2I solution. Time-course of radioligand binding to membranes, associated with SPA beads, was followed by counting the sample every 30 seconds on a LKB Wallac 1219 Rackbeta counter.

Scheme 1. General procedure for *N*-(3-iodoprop-2*E*-enyl)-2β-carbomethoxy-3β-(4'-methylphenyl)nortropane synthesis.



Scheme 2. Tritium-labeling of PE2I acid precursor via O-methylation reaction.



Scheme 3. Carbon-11 labeling of PE2I acid precursor via O-methylation reaction.