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Structure-activity relationship study towards non-peptidic positron emission tomography (PET) radiotracer for gastrin releasing peptide receptors: Development of [^{18}F] (*S*)-3-(1*H*-indol-3-yl)-*N*-[1-[5-(2-fluoroethoxy)pyridin-2-yl]cyclohexylmethyl]-2-methyl-2-[3-(4-nitrophenyl)ureido]propionamide

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ABSTRACT

Gastrin-releasing peptide receptors (GRP-Rs, also known as bombesin 2 receptors) are overexpressed in a variety of human cancers, including prostate cancer, and therefore they represent a promising target for *in vivo* imaging of tumors using positron emission tomography (PET). Structural modifications of the non-peptidic GRP-R antagonist PD-176252 ((*S*)-**1a**) led to the identification of the fluorinated analog (*S*)-3-(1*H*-indol-3-yl)-*N*-[1-[5-(2-fluoroethoxy)pyridin-2-yl]cyclohexylmethyl]-2-methyl-2-[3-(4-nitrophenyl)ureido]propionamide ((*S*)-**1m**) that showed high affinity and antagonistic properties for GRP-R. This antagonist was stable in rat plasma and towards microsomal oxidative metabolism *in vitro*. (*S*)-**1m** was successfully radiolabeled with fluorine-18 through a conventional radiochemistry procedure. [^{18}F] (*S*)-**1m** showed high affinity and displaceable interaction for GRP-Rs in PC3 cells *in vitro*.

1. Introduction

The gastrin-releasing peptide receptor (GRP-R) (also known as bombesin receptor 2, BB2) is a member of the mammalian bombesin receptor family [1] and is receiving increasing attention because of its high expression in several human tumors, including prostate cancer, breast cancer, small cell lung cancer, ovarian cancer, endometrial cancer, and gastrointestinal stromal tumors [2–4].

Consequently, bombesin, a 14-amino acid peptide that binds with high affinity to GRP-Rs, has been widely studied for the development of positron emission tomography (PET) diagnostics [5,6]. Currently, two different classes of radiolabeled bombesin analogs are under investigation. 1) Radiolabeled GRP-R agonists that internalize into tumor cells by endocytosis upon activation of the receptor. These radiolabeled GRP-R agonists have been studied on the assumption that radioactivity is accumulated with higher efficiency in tumor cells, thus enhancing tumor-to-background ratio [7]. 2) Radiolabeled GRP-R antagonists that do not internalize into tumor cells but exhibit a persistent binding to the receptor resulting in an overall increased accumulation of radioactivity in tumor tissue [8]. Even though the exact mode of action of the GRP-R antagonists has not been fully elucidated yet, they represent an interesting new class of peptidic vectors for the development of radiotracers [9].

Apart from a strong and specific interaction with the target receptor and reliable radionuclide introduction, an important requisite of a radiotracer is high stability *in vivo* over the course of PET imaging. However, radiolabeled peptides can be prone to considerable degradation after administration *in vivo* [10,11]. As a consequence, an insufficient accumulation of the peptidic radiotracer

Abbreviations: GRP, gastrin-releasing peptide; CNS, central nervous system; PET, positron emission tomography.

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or accumulation of its fragments in target tissues combined with high background radioactivity levels can occur. Thus, high stability of the radiolabeled peptide is a crucial property for efficient *in vivo* target visualization. A number of radiolabeled bombesin peptide analogs have been developed for PET imaging of GRP-R positive tumors (for recent reviews see Refs. [5,6]) but they showed quick degradation *in vivo* [12–14]. Thus, the limitations imposed by peptide pharmacokinetics with respect to binding and clearance demonstrate the need of significant improvements of such radiolabeled probes [15].

To overcome the above limitations, we have reasoned on the possibility to develop small-molecule radiotracers targeting GRP-Rs. Small molecules offer various advantages over peptides since they can be suitably designed to modulate potency, selectivity, lipophilicity, and cell permeability and therefore, to avoid poor tissue penetration, poor serum stability, and quick elimination [16].

One additional advantage of targeting non-peptidic radiotracers for GRP-Rs is the opportunity to access a radioligand labeled with fluorine-18 which shows good nuclear characteristics for PET such as low positron (β^+) energy (0.64 MeV) and appropriate physical half-life (109.7 min) [17]. In general, radiolabeling of biologically active peptides with fluorine-18 might represent a considerable challenge. Procedures for the direct radiolabeling with fluorine-18 normally require harsh reaction conditions (strong bases, high temperatures) not compatible with heat-sensitive biomolecules [18]. Usually, site-specific radiolabeling of peptides with fluorine-18 is achieved using suitable radiolabeled intermediates or prosthetic groups [19–22]. This approach includes multistep reaction procedures and is, therefore, time-consuming. Thus, the aim of the present study is to identify a non-peptidic fluorine-18 PET radioligand, endowed with antagonist properties, for visualization of GRP-R overexpression in tumor tissues. A search of the literature revealed that the compound PD-176252 ((*S*)-**1a**, Fig. 1) is the only non-peptidic GRP-R antagonist reported to date [23]. This antagonist was selected as starting point of the present study.

2. Results and discussion

The GRP-R antagonist (*S*)-**1a** was identified by researchers at Parke Davis in mid 1990's via the application of a so-called "peptoid" drug design strategy [23]. The original paper reported that (*S*)-**1a** displayed very high affinity for human cloned BB2 receptors stably expressed in CHO cells (K_i = 0.17 nM). To further assess the validity of (*S*)-**1a** as starting point for our study, we tested the affinity of (*S*)-**1a** for GRP-Rs expressed in PC3 cells, a human prostate cancer cell line with very high expression of GRP-Rs used in xenograft models to test prospective PET radiotracers. Despite (*S*)-**1a** is commercially available, its affinity for GRP-Rs in PC3 cells has never been assessed until now.

Thus, the affinities of (*S*)-**1a** and of reference compounds hGRP and bombesin were determined by measuring their ability to displace [^{125}I-Tyr4]bombesin from GRP-Rs in PC3 cells. The data reported in Table 1 indicated that hGRP and bombesin displayed K_i values comparable to those reported in the literature [24], whereas (*S*)-**1a** showed a K_i value of 50 nM in PC3 cells quite different from that reported (K_i = 0.17 nM) [23]. In addition, we evaluated the affinity of (*S*)-**1a** also for human cloned BB2 receptors and we found a K_i value of 61 nM which was again different from that originally reported. Also another study has reported affinity values of (*S*)-**1a** at GRP-Rs lower than K_i = 0.17 nM (IC_{50} = 230 nM in Balb 3T3 cells; IC_{50} = 170 nM in HuTU-80 cells) [25]. These differences might be due to the different cell lines used for the experiments. In any case, our binding data on GRP-Rs in PC3 cells supported the decision to select (*S*)-**1a** as the starting point for our studies.

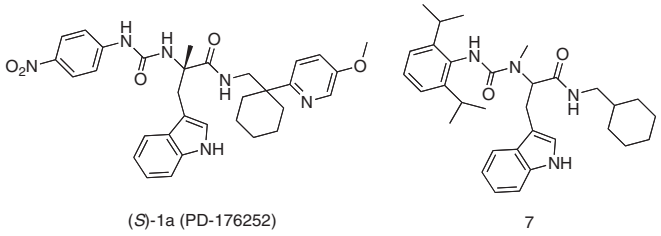


Fig. 1. Formula structures of reference compounds.

Subsequently, we analyzed the synthetic pathway to obtain (*S*)-**1a** as originally disclosed in a patent application [26]. Apart from 4-nitrophenylisocianate, the synthetic procedure required the amine **2a** (Fig. 2) and (*S*)-α-methyltryptophan ((*S*)-**12**). The synthesis of the amine **2a** requires a time-consuming eight-step sequence, characterized by very low overall yield (1.8%) [27]. As for (*S*)-**12**, it is commercially available but the cost represents a limit to the preparation of a large number of compounds. Actually, procedures for the asymmetric synthesis of (*S*)-**12** are reported in the literature [28,29] but, at least in our hands, they did not yield the desired amino acid in gram scale and/or with enantiomeric excess value of over 98%. In the light of these considerations, the synthetic procedure of (*S*)-**1a** did not seem ideal to explore the structure-activity relationships on a large number of (*S*)-**1a** analogs. Consequently, we initially designed derivatives of (*S*)- and (*R*)-tryptophan instead of (*S*)-**12**, because they were more affordable. Also, we evaluated the bicyclic amines **2b–e** (Fig. 2) as alternatives to **2a** because they are more synthetically accessible than **2a** or commercially available. Thus, the first set of compounds of the present study included derivatives (*R*)- and (*S*)-**6b–d**, (*R*,*S*)-**6e**, (*S*,*S*)-**6e**, and for comparative purposes (*S*)-**6a** (Table 1).

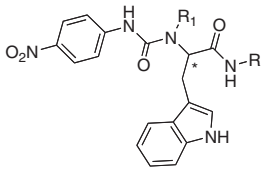
The synthesis of the target compounds (*S*)-**6a**, (*R*)- and (*S*)-**6b–d**, (*R*,*S*)-**6e**, (*S*,*S*)-**6e** is depicted in Scheme 1: Boc-protected *R*- or *S*-tryptophan ((*R*)- or (*S*)-**3**) was first condensed with amine **2a–e**, to give derivatives (*R*)-**4b–d**, (*S*)-**4a–d**, (*R*,*S*)-**4e**, and (*S*,*S*)-**4e**. The latter compounds were deprotected under acidic conditions to afford amines (*R*)-**5b–d**, (*S*)-**5a–d**, (*R*,*S*)-**5e**, and (*S*,*S*)-**5e**. Condensation of these latter with 4-nitrophenylisocyanate gave the desired target compounds.

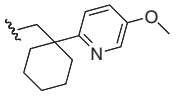
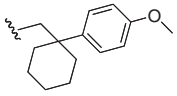
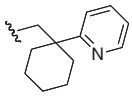
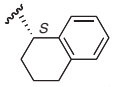
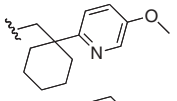
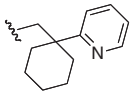
The binding data were very disappointing because the new compounds were devoid of GRP-R affinity (Table 1). Only compounds (*S*)-**6a**, (*R*)-**6b**,**d** displayed measurable binding affinities for GRP-Rs in PC3 cells (K_i = 4084–7039 nM), yet >80-fold lower than (*S*)-**1a**. The difference of affinity between (*S*)-**1a** and (*S*)-**6a** indicated that the α-methyltryptophan core is essential for affinity at GRP-Rs of this class of compounds.

Taking a closer look at the original studies [23,30], we noted that compound **7** (Fig. 1) was different from the GRP-R ligands analogs of (*S*)-**1a** because of the presence of the *N*-methyltryptophan fragment in place of the α-methyltryptophan one. Nonetheless, compound **7** displayed low but sizeable binding affinity for GRP-R (K_i = 1500 nM). Thus, we combined the structural features of **7** and (*S*)-**1a** by replacing the 2,6-diisopropylphenyl of **7** with the 4-nitrophenyl group (compounds (*R*)- and (*S*)-**11f**, Table 1). Then, the cyclohexylmethyl group in (*R*)- and (*S*)-**11f** was replaced by a bicyclic system as in compound (*S*)-**1a** (compounds (*S*)-**11a**,**d**, Table 1).

The synthesis of final compounds (*S*)-**11a**,**d**, (*R*)- and (*S*)-**11f** is depicted in Scheme 2. (*R*)- or (*S*)-*N*-benzyl-*N*-methyltryptophan ((*R*)- or (*S*)-**8**) [31] was condensed with amine **2a**,**d**,**f** to give amides (*S*)-**9a**,**d**, and (*R*)- and (*S*)-**9f** that were debenzylated by catalytic hydrogenation to give *N*-methyl derivatives (*S*)-**10a**,**d**, (*R*)- and (*S*)-**10f**. Condensation of these latter with 4-nitrophenylisocyanate gave the desired target compounds.

Table 1
Binding affinities of tryptophan derivatives.



Compound	R_1	R	GRP-Rs in PC3 cell membranes K_i ± S.E.M. [nM][a]	hBB2 receptors transfected in CHO cells K_i ± S.E.M. [nM][a]
(*S*)-**1a**	–	–	50 ± 3	61 ± 5
(*S*)-**6a**	H		7039 ± 300	1724 ± 149
(*R*)-**6b**	H		4084 ± 200	NT[b]
(*S*)-**6b**	H		>10,000 (26%)[c]	>5000 (30%)
(*R*)-**6c**	H		>10,000 (1%)	NT
(*S*)-**6c**	H		>10,000 (1%)	NT
(*R*)-**6d**	H		4133 ± 40	NT
(*S*)-**6d**	H		>10,000 (18%)	>5000 (46%)
(*R*,*S*)-**6e**	H		>10,000 (6%)	NT
(*S*,*S*)-**6e**	H		>10,000 (12%)	NT
(*S*)-**11a**	CH_3		>10,000 (6%)	>5000 (39%)
(*S*)-**11d**	CH_3		>10,000 (15%)	>5000 (18%)
(*R*)-**11f**	CH_3		>10,000 (30%)	>5000 (35%)
(*S*)-**11f**	CH_3		>10,000 (11%)	>5000 (39%)
hGRP	–	–	0.14 ± 0.04	0.025 ± 0.003
Bombesin	–	–	0.63 ± 0.06	0.058 ± 0.005

[a] Data from three independent experiments.
[b] Not tested.
[c] K_i not determined. Percentage of inhibition measured at 10 μM is given in parentheses.

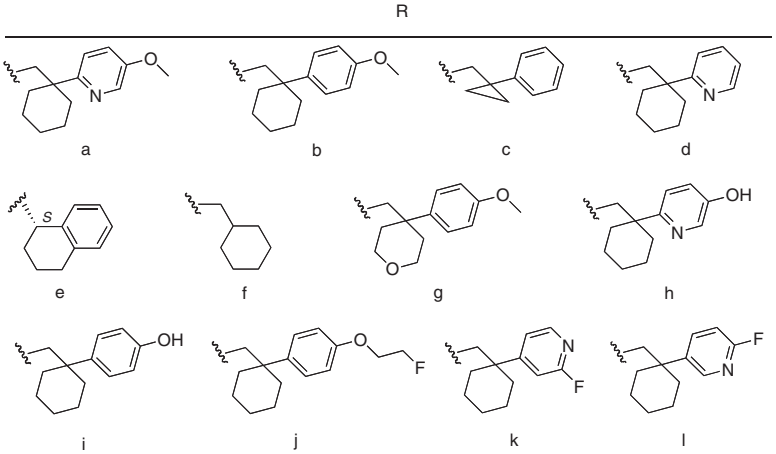
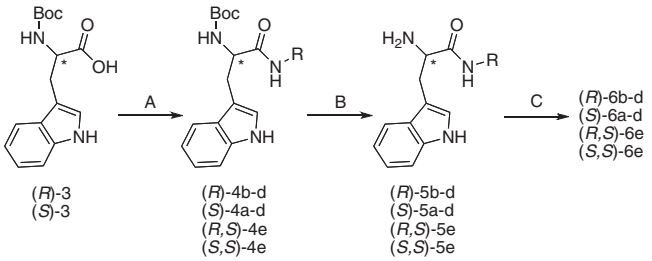
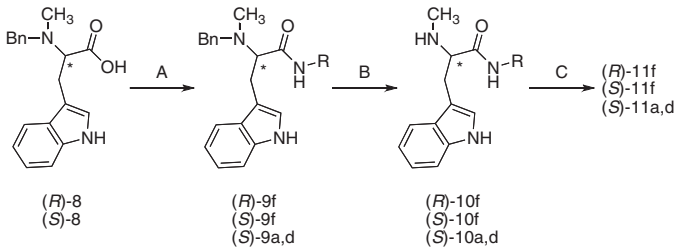


Fig. 2. Formula structures of amines **2a–l** (R-NH_2).



Scheme 1. Synthesis of tryptophan-based target compounds (*R*)-**6b–d**, (*S*)-**6a–d**, (*R*,*S*)-**6e**, and (*S*,*S*)-**6e**. Reagents and Conditions: (A) *N*,*N*′-carbonyldiimidazole, amine **2a–e**, anhydrous THF, 24 h, r.t.; (B) trifluoroacetic acid, CH_2Cl_2, 5 h, r.t.; (C) 4-nitrophenylisocianate, anhydrous THF, 15 h, r.t.



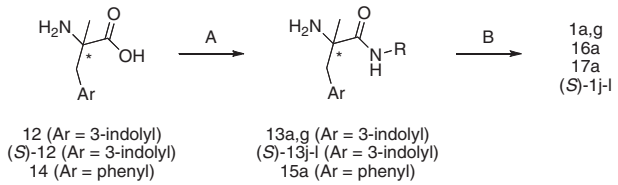
Scheme 2. Synthesis of *N*-methyl-tryptophan-based target compounds (*R*)- and (*S*)-**11f**, (*S*)-**11a**,**d**. Reagents and Conditions: (A) *N*,*N*′-carbonyldiimidazole, amine **2a**,**d**,**f**, (B) H_2 (5 atm), 10% Pd/C, ethanol; (C) *N*,*N*′-carbonyldiimidazole, 4-nitroaniline, anhydrous THF, 24 h, r.t.

The binding affinity data listed in Table 1 indicated that compounds (*S*)-**11a**,**d**, (*R*)- and (*S*)-**11f** were completely devoid of GRP-R affinity (K_i >10,000 nM), indicating the failure of the hypothesis that combining the structural features of **7** and (*S*)-**1a** would give GRP-R ligands.

Consequently, we modified our strategy to bypass the use of (*S*)-**12** by synthesizing the new target compounds as racemic mixtures starting from racemic **12**, and then separating the desired optically active target compounds by semi-preparative HPLC enantioseparation using a chiral stationary phase. Thus, keeping in mind the lesson learned from the set of compounds listed in Table 1, the backbone of the α-methyl amino acid was left unchanged and minimal structural variations with respect to (*S*)-**1a** were introduced. In particular, the commercially available amine **2g** was evaluated as possible replacement of the amine **2a**. Thus, we synthesized the target racemic compound **1g** which contains a tetrahydropyran ring in place of the cyclohexane ring. Racemate **1a** was prepared for comparative purposes. Also, α-methyltryptophan in compound **1a** was replaced by α-methylphenylalanine leading to compound **16a**, because (*S*)-α-methylphenylalanine is more affordable than (*S*)-**12**. The third modification was the replacement of the nitro group of **1a** with a methoxy group (compound **17a**) in order to verify the introduction of a site for possible radiolabeling.

The synthesis of compounds **1a**,**g**, **16a**, and **17a** is reported in Scheme 3: the appropriate racemic aminoacid **12** or **14** was reacted with the amine **2a**,**g** to give the corresponding derivatives **13a**,**g** and **15a**. Condensation of these latter with the appropriate phenylisocyanate gave the desired target compounds.

Racemic mixtures **1a**,**g**, **16a**, and **17a** were initially tested in radioligand binding assays in order to obtain preliminary indications of the effect of these structural modifications (Table 2). Among the four racemic mixtures, the highest affinity was shown by **1a**, again suggesting that even minor modifications on this framework could negatively affect binding properties. Then, we separated the enantiomers by semi-preparative HPLC using a cellulose-based chiral stationary phase. Although the separation of the



Scheme 3. Synthesis of α-methyltryptophan-based target compounds **1a**,**g**, **16a**, **17a**, and (*S*)-**1j–l**. Reagents and Conditions: (A) PyBOP, *N*-methylmorpholine, amine **2a**,**g**,**j–l**, anhydrous THF, r.t., overnight; (B) 4-methoxy- or 4-nitrophenylisocianate, anhydrous THF, 24 h, r.t.

enantiomers with an analytical column proceeded with the desired selectivity (α >0.8), the semi-preparative enantioseparation of **1a**,**g** and **17a** was suboptimal (e.e. 90%). In the case of **16a** the enantioseparation was not even possible. Nonetheless, in order to have indications of possible enantiopreference of the interaction of (*S*)-**1a** analogs with GRP-R binding site, the separated enantiomers of compounds **1a**,**g** and **17a** were submitted to radioligand binding assays (Table 2). The data clearly indicated that the later eluting enantiomer of all the three pairs possessed the highest binding affinity. Since the later eluting enantiomer of **1a** (compound **1aE2**) displayed the same retention time of the enantiomerically pure specimen of (*S*)-**1a**, the binding data suggested a very high eudismic ratio of **1a** enantiomers, showing an unprecedentedly reported role of stereochemistry in the interaction of (*S*)-**1a** with GRP-R.

Collectively, the binding data discussed until now indicated that minor changes in the structure of (*S*)-**1a** were not tolerated. Thus, the quest for more synthetically accessible analogs of (*S*)-**1a** remained open. A study towards this aim is being published elsewhere [32].

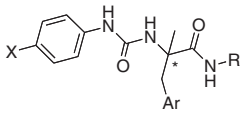
In order to achieve the aim of the study, we went back to the original structure-activity relationship study on (*S*)-**1a** [23], where it was reported that minor modifications on the aryl ring of the "right end" of the molecule might be tolerated. Therefore, we synthesized ^{19}F fluorinated analogs of (*S*)-**1a** that could be easily obtained in the ^{18}F radiolabeled form through conventional radiochemistry procedures. To this end, the most simple modification was the replacement of (*S*)-**1a** methoxy group with the 2-fluoroethoxy ((*S*)-**1m**) (Table 2). In addition, we replaced the 5-methoxy-2-pyridyl ring of (*S*)-**1a** with 4-(2-fluoroethoxy)phenyl, 2-fluoro-4-pyridyl, and 2-fluoro-5-pyridyl rings (compounds (*S*)-**1j–l**) (Table 2).

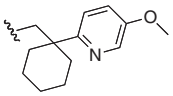
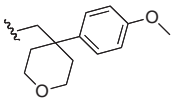
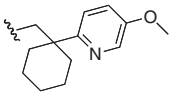
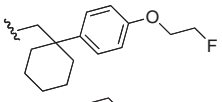
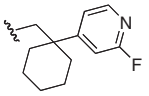
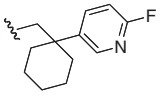
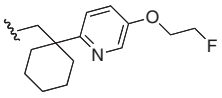
The synthesis of target compounds (*S*)-**1j–l** required the key amine intermediates **2j–l** (Fig. 2). Amine **2j** was synthesized according to Scheme 4: **2b** was first demethylated with 48% aqueous HBr to phenol **2i**, then Boc protected to give derivative **18**. This phenolic derivative was alkylated with 2-fluoroethyltosylate to give the intermediate **19**, that, after deprotection, afforded amine **2j**.

The synthesis of the key amines **2k**,**l** (Scheme 4) paralleled the published procedure for the synthesis of the amine **2a** [27]. Nitriles **20k**,**l** [33,34] were reacted with 1,5-dibromopentane in the presence of NaH to give the cyclohexane derivatives **21k**,**l**. After hydrogenation of the nitrile function of these latter compounds over Nickel-Raney, the desired amines **2k**,**l** were obtained. The final compounds (*S*)-**1j–l** were prepared starting from amines **2j–l** and the amino acid (*S*)-**12**, as already illustrated in Scheme 3.

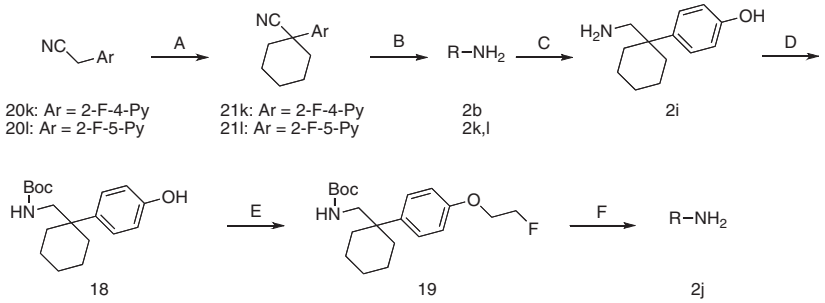
The synthesis of the final compound (*S*)-**1m** is depicted in Scheme 5. Compound **2a** was demethylated with 48% aqueous HBr to give compound **2h**. This latter amine was condensed with (*S*)-**12** to give the intermediate (*S*)-**13h** which, after reaction with 4-nitrophenylisocyanate, gave the phenolic derivative (*S*)-**1h**. Then, this latter phenol was reacted with 2-fluoroethyl tosylate to give the target compound (*S*)-**1m**.

Table 2
Binding affinities of α-methyl-tryptophan derivatives.



Compounds	X	Ar	R	GRP-Rs in PC3 cell membranes K_i ± S.E.M. [nM][a]
1a	NO_2	3-Indolyl		129 ± 5
1aE1[b]	NO_2	3-Indolyl		7874 ± 300
(*S*)-**1a**	NO_2	3-Indolyl		50 ± 3
1g	NO_2	3-Indolyl		1384 ± 60
1gE1	NO_2	3-Indolyl		>10,000 (8%)[d]
1gE2[c]	NO_2	3-Indolyl		4621 ± 110
16a	NO_2	Phenyl		1102 ± 55
17a	OCH_3	3-Indolyl		2466 ± 80
17aE1	OCH_3	3-Indolyl		>10,000 (7%)
17aE2	OCH_3	3-Indolyl		837 ± 50
(*S*)-**1j**	NO_2	3-Indolyl		300 ± 70
(*S*)-**1k**	NO_2	3-Indolyl		409 ± 65
(*S*)-**1l**	NO_2	3-Indolyl		160 ± 19
(*S*)-**1m**	NO_2	3-Indolyl		38 ± 2

[a] Data from three independent experiments.
[b] E1: faster eluting enantiomer.
[c] E2: later eluting enantiomer.
[d] K_i not determined. Percentage of inhibition measured at 10 μM is given in parentheses.



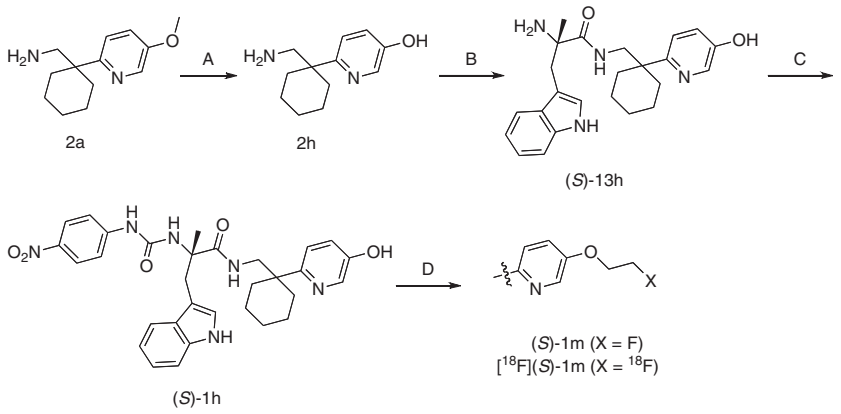
Scheme 4. Synthesis of intermediate amines **2j–l**. Reagents and Conditions: (A) NaH (60% oil dispersion), 1,5-dibromopentane, Et_2O/DMSO; r.t., 24 h; (B) H_2 (5 atm), Nickel-Raney, 2 M ethanolic NH_3; (C) 48% aqueous HBr, reflux, 4–5 h; (D) $(Boc)_2O$, Et_3N, H_2O/THF, r.t., overnight; (E) 2-fluoroethyl tosylate, Cs_2CO_3, DMF, reflux, 24 h; (F) 3 N HCl, dioxane, r.t., overnight.

These modifications finally succeeded (Table 2): compounds (*S*)-**1l** and (*S*)-**1m** retained high affinity for GRP-R, while (*S*)-**1j** and (*S*)-**1k** were about 10-fold less potent than the parent (*S*)-**1a**.

On the basis of the binding affinities, compounds (*S*)-**1l** and (*S*)-**1m** were subjected to further studies. Calcium flux studies showed that compounds (*S*)-**1a**,**l**,**m** did not induce any calcium flux in PC3 cell culture. Instead, these compounds were able to reduce dose-dependently the Ca^{2+} mobilization induced by bombesin, indicating competitive antagonism at GRP-R (see Figs. S1–S3, Supplementary Materials). The rank order of potency was (*S*)-**1m** > (*S*)-**1l** > (*S*)-**1a** (Table 3).

Then, we evaluated if (*S*)-**1a**,**l**,**m** were stable in rat plasma for, at least, the timeframe of a PET scan. All compounds showed the desired stability having a degradation half-life ($t_{1/2}$) longer than 2 h (Table 3). *In vitro* metabolism of compounds (*S*)-**1a**,**l**,**m** was evaluated after incubation with rat hepatic microsomes in the presence of an NADPH-generating system [35]. Compounds (*S*)-**1a**,**l** underwent significant oxidative metabolism, whereas (*S*)-**1m** was significantly more stable (Table 3).

Taken together, compound (*S*)-**1m** showed high stability in rat plasma and towards oxidative metabolism *in vitro* in addition to its high affinity and potency towards GRP-R, indicating its



Scheme 5. Synthesis of the target compound (*S*)-**1m** and radiosynthesis of [^{18}F](*S*)-**1m**. Reagents and Conditions: (A) 48% aqueous HBr, reflux, 4–5 h; (B) PyBOP, *N*-methylmorpholine, (*S*)-**12**, anhydrous THF, r.t., overnight; (C) 4-nitrophenylisocianate, anhydrous THF, 24 h, r.t.; (D) 2-fluoroethyl tosylate, Cs_2CO_3, acetone, reflux, 8 h; or 2-[^{18}F]fluoroethyl tosylate, NaH, DMF, 5 min, 125 °C.

Table 3
Further characterization of compounds of (*S*)-**1a**,**l**,**m**.

Compound	IC_{50}, nM[a]	Rat plasma stability ($t_{1/2}$, h)	Rat liver microsomal stability ($t_{1/2}$, min)
(*S*)-**1a**	290 ± 77	>2	4.4[b]
(*S*)-**1l**	201 ± 30	>2	2.5
(*S*)-**1m**	121 ± 13	>2	20

[a] EC_{50} of bombesin was 1.09 ± 0.19 nM.
[b] Data taken from Ref. [27].

suitability as potential PET tracer. Of note, (*S*)-**1m** showed IC_{50} value (Table 3) in the same range as other ^{19}F-bombesin analogs such as Al^{19}F-NOTA-4,7-lanthionine-BBN (114 ± 3 nM) and Al^{19}F-NOTA-2,6-lanthionine-BBN (15 ± 2 nM) which have shown good properties as PET tracers *in vitro* as well as *in vivo* [36].

[^{18}F](*S*)-**1m** was synthesized by reaction of 2-[^{18}F]fluoroethyl tosylate with the corresponding phenolic precursor (*S*)-**1h** in presence of NaH followed by HPLC purification (Scheme 5). The total synthesis time was 121 min. The obtained specific activity was 6.0 ± 4.7 GBq/μmol. In contrast to other [^{18}F]fluoroethylation reactions performed in our laboratories using the same purification method for 2-[^{18}F]fluoroethyl tosylate [37], the final radiochemical yield was quite low (<5%) because of substantial defluorination. In the HPLC-radiochromatogram, besides the peak of [^{18}F](*S*)-**1m**, a large peak was found at the void volume, most likely due to [^{18}F] fluoride. The formation of other byproducts can be ruled out as the similar reaction under 'cold' conditions yielded only (*S*)-**1m**. The radiochemical purity of the 2-[^{18}F]fluoroethyl tosylate intermediate was checked by TLC and UPLC and was >95%. However, when reacting with the phenolic precursor (*S*)-**1h** in the presence of NaH, the major part (>90%) of 2-[^{18}F]fluoroethyl tosylate was degraded to ^{18}F-fluoride as it was concluded from HPLC analysis. Nonetheless, [^{18}F](*S*)-**1m** was obtained with a radiochemical yield (RCY) of 3.4 ± 0.3% (40–90 MBq) and a radiochemical purity >95%. The stability of [^{18}F](*S*)-**1m** was confirmed with UPLC within the normal PET imaging time (2 h), revealing a radiochemical purity >95% within 2 h (see Supplementary Materials, Fig. 4S), allowing us to proceed with the *in vitro* experiments in PC3 cells.

The binding affinity of [^{18}F](*S*)-**1m** for GRP-R was assessed in whole PC-3 (GRPR +) cells (Fig. 3A). Results are plotted as sigmoid curves for the displacement of [^{18}F](*S*)-**1m** as a function of increasing concentrations of the GRP-R probe Glu[Aca-BN(7–14)]$_2$, previously characterized in our laboratories in competitive binding studies in the same PC-3 (GRPR +) cells using the reference radioligand ^{124}I-[Tyr4]-BN(1–14) [38]. In those experiments, Glu[Aca-BN (7–14)]$_2$ showed an IC_{50} value of 20 nM, which was comparable to that of bombesin(1–14) (IC_{50} = 14 nM). In displacement binding assays against [^{18}F](*S*)-**1m**, Glu[Aca-BN(7–14)]$_2$ showed the IC_{50} value of 0.10 nM. Thus, by comparing the IC_{50} values showed by Glu[Aca-BN(7–14)]$_2$ in the two experiments, we concluded that [^{18}F](*S*)-**1m** binds at GRP-R with lower affinity than bombesin(1–14), yet in the nanomolar range. Thus, [^{18}F](*S*)-**1m** may display favourable binding properties as a PET radiotracer for imaging GRP-Rs.

Cell binding and dissociation kinetics of [^{18}F](*S*)-**1m** were studied in PC3 cells. As depicted in Fig. 3B, the uptake of [^{18}F](*S*)-**1m** in PC3 cells gradually increased within 60 min of incubation, reaching a plateau after this time point. This binding kinetics of [^{18}F](*S*)-**1m** is similar to the binding kinetics of other ^{18}F-bombesin analogs [36]. The maximum cellular associated radioactivity was found to be 7% of the total added amount of radioactivity. The blocking studies with Glu[Aca-BN(7–14)]$_2$ revealed a decreased uptake to 4%. The dissociation kinetics of [^{18}F](*S*)-**1m** was rapid, remaining only 6% of the tracer within 120 min (Fig. 3C). This loosely bound activity decreased exponentially with a half-life of 13 min. Thus, considering the reversible high affinity binding and fast kinetics shown by [^{18}F](*S*)-**1m**, it is likely that the radioligand will show high accumulation and fast washout of radioactivity during the period of PET scan *in vivo*.

3. Conclusions

During the years, GRP-R has emerged as a target for cancer detection and treatment because of the different expression levels in healthy and tumor tissues. To date, numerous radiolabeled bombesin analogs have been reported as imaging tools or tumor targeting vectors. However, the fast metabolic degradation of bombesin-based radiotracers impacts their *in vivo* targeting capabilities and, thus, represents a limitation for medical applications. Here we have reported on the identification of the first non-peptidic PET radiotracer for GRP-Rs [^{18}F](*S*)-**1m**. Starting from the GRP-R antagonist (*S*)-**1a**, structure-activity relationships were explored in order to find synthetically accessible non-peptidic GRP-R ligands. The study showed that small changes in the backbone of the structure of (*S*)-**1a** translated into a loss in affinity. Only small changes on the pyridyl ring of (*S*)-**1a** were tolerated. In fact, 2-fluoroethoxy derivative (*S*)-**1m** was identified as a high affinity GRP-R antagonist, endowed with stability in rat plasma and toward microsomal oxidative metabolism *in vitro*. [^{18}F](*S*)-**1m** was successfully synthesized through conventional radiochemistry procedure, albeit in low radiochemical yield. [^{18}F](*S*)-**1m** confirmed high affinity for

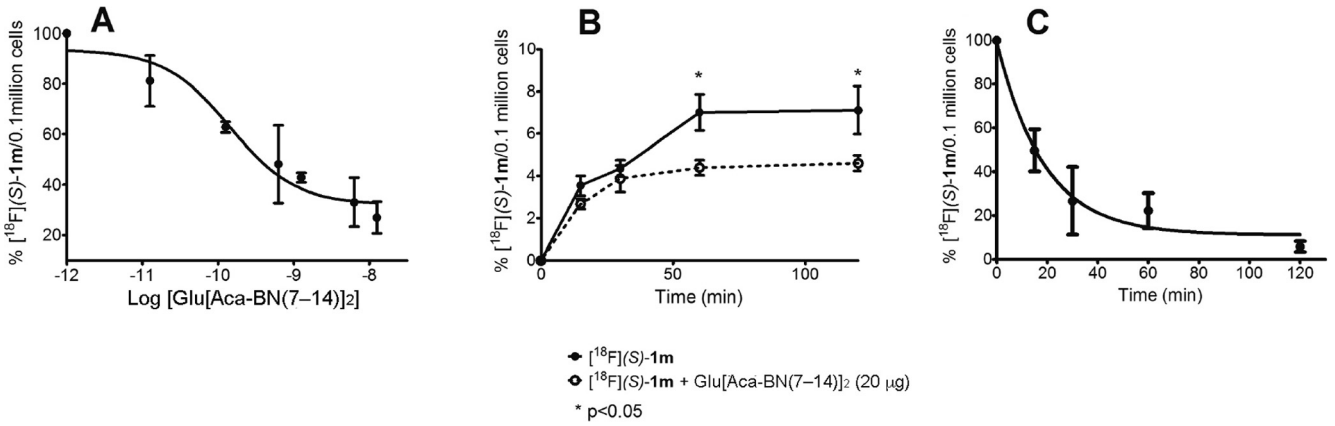


Fig. 3. A) Inhibition of [^{18}F](*S*)-**1m** binding to GRP-Rs on PC3 cells by Glu[Aca-BN(7–14)]$_2$. B) Cellular uptake of [^{18}F](*S*)-**1m** in PC3 prostate cancer cells. C) Dissociation kinetics of [^{18}F](*S*)-**1m** in PC3 cells.

GRP-Rs in whole PC3 cells and showed displaceable interaction with GRP-R *in vitro*. Interestingly, [^{18}F](*S*)-**1m**, compared to fluorine-18-labeled bombesin agonists, showed similar cellular associated radioactive uptake and faster efflux kinetics. The promising *in vitro* properties of [^{18}F](*S*)-**1m** open new perspectives in the field of GRP-R radiotracers suggesting that non-peptidic tracers might have very different properties in terms of cell uptake and dissociation kinetics as compared to peptidic tracers. Future studies with [^{18}F](*S*)-**1m** or with optimized fluorinated analogs will allow to compare *in vitro* and *in vivo* properties of peptidic and non-peptidic GRP-R PET radiotracers.

4. Experimental section

4.1. Chemistry

The purity of the target compounds (*R*)-**6b–d**, (*S*)-**6a–d**. (*R*,*S*)-**6e**, (*S*,*S*)-**6e**, (*R*)-**11f**, (*S*)-**11f**, (*S*)-**11a**,**d**, **1a**,**g**, **16a**, **17a**, (*S*)-**1j–l**, (*S*)-**1m** was assessed by RP-HPLC and combustion analysis. All compounds showed ⩾95% purity. Column chromatography was performed with 1:30 Merck silica gel 60A (63–200 μm) as the stationary phase. Melting points were determined in open capillaries on a Gallenkamp electrothermal apparatus. Elemental analyses (C, H, N) were performed on a Eurovector Euro EA 3000 analyzer. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. ^{1}H NMR spectra were recorded at 300 MHz on a Varian Mercury-VX spectrometer. All spectra were recorded on free bases. All chemical shift values are reported in ppm (δ). Recording of mass spectra was done on an HP6890-5973 MSD gas chromatograph/mass spectrometer; only significant *m*/*z* peaks, with their percentage of relative intensity in parentheses, are reported. ESI-MS/MS analysis were performed with an Agilent 1100 Series LC-MSD trap System VL workstation. All spectra were in accordance with the assigned structures. RP-HPLC analysis was performed on a Perkin-Elmer series 200 LC instrument using a Phenomenex Gemini C-18 column, (250 × 4.6 mm, 5 μm particle size) and equipped with a Perkin-Elmer 785A UV/VIS detector setting λ = 254 nm. Compounds (*R*)-**6b–d**, (*S*)-**6a–d**. (*R*,*S*)-**6e**, (*S*,*S*)-**6e**, (*R*)-**11f**, (*S*)-**11f**, (*S*)-**11a**,**d**, **1a**,**g**, **16a**, **17a**, (*S*)-**1j–l**, (*S*)-**1m** were eluted with $CH_3OH/H_2O/Et_3N$, 7:3:0.01, v/v at a flow rate of 1.0 mL/min.

The following compounds were prepared as described in the literature: 1-aminomethyl-1-(4-methoxypyridin-2-yl)cyclohexane (**2a**) [27]; 1-aminomethyl-1-(4-methoxyphenyl)cyclohexane (**2b**) [39]; 1-(1-phenyl)cyclopropylmethylamine **(2c**) [40]; 1-aminomethyl-1-(pyridin-2-yl)cyclohexane (**2d**) [41]; (*S*)- and (*R*)-*N*-benzyl-*N*-methyl-tryptophan ((*S*)-**8** and (*R*)-**8**) [31]; 4-cyanomethyl-2-fluoropyridine (**19k**) [33]; 5-cyanomethyl-2-fluoropyridine (**19l**) [34]; (*S*)-1,2,3,4-tetrahydro-1-naphthalenamine (**2e**) and cyclohexylmethylamine (**2f**) were purchased from Sigma-Aldrich (Milan, Italy), [4-(4-methoxyphenyl)tetrahydro-2*H*-pyran-4-yl] methylamine (**2g**) was purchased from Life Chemicals Europe GmbH (Munich, Germany), (*S*)-α-methyltryptophan ((*S*)-**12**) was purchased from Acros Organics (Geel, Belgium).

4.1.1. General procedure for the preparation of compounds *2h* and *2i*

A mixture of the amine **2a** (0.20 g, 0.91 mmol) or **2b** (0.21 g, 0.96 mmol) and 48% aqueous HBr (5 mL) was refluxed for 4–5 h (TLC monitoring). Then, the solvent was removed in vacuo and the residue was washed several times with absolute ethanol, to give the final hydrobromide salt as a white solid that was not further purified (quantitative yield).

*4.1.1.1. 1-Aminomethyl-1-(4-hydroxypyridin-2-yl)cyclohexane Hydrobromide (**2h**).* ^{1}H NMR (CD_3OD): δ 1.34–1.41 (m, 2H), 1.46–1.50 (m, 2H), 1.52–1.58 (m, 2H), 1.71–1.94 (m, 2H), 2.33 (app d, 2H), 3.29 (s, 2H), 3.30 (s, 3H, D_2O exchanged), 8.02–8.03 (m, 2H), 8.32–8.33 (m, 1H), 10.07 (s, 1H); ESI-MS *m*/*z* 207 $(M+H)^+$, ESI-MS/MS *m*/*z*: 190 (100).

*4.1.1.2. 1-Aminomethyl-1-(4-hydroxyphenyl)cyclohexane Hydrobromide (**2i**).* ^{1}H NMR (CD_3OD): δ 1.32–1.46 (m, 4H), 1.53–1.58 (m, 4H), 1.99–2.05 (m, 2H), 3.18 (app d, 2H), 3.30 (br s, 4H, D_2O exchanged), 6.82 (d, 2H, *J* = 8.5 Hz), 7.18 (d, 2H, *J* = 8.5 Hz); ESI+/MS *m*/*z*: 206 $(M+H)^+$, ESI-MS/MS *m*/*z*: 189 (100).

4.1.2. N-Boc-1-aminomethyl-1-(4-hydroxyphenyl)cyclohexane (*18*)

Amine **2i** (0.14 g, 0.5 mmol) was dissolved in mixture of H_2O/THF (8 mL, 1:3, v/v,) then triethylamine (0.15 mL, 1.5 mmol) and $(Boc)_2O$ (0.15 g, 0.5 mmol) were added. The reaction mixture was stirred at room temperature overnight. Then, THF was removed under reduced pressure and the aqueous residue was extracted with AcOEt (2 × 20 mL). The combined organic layers were dried over Na_2SO_4 and evaporated in vacuo. The crude residue was chromatographed (*n*-hexane/AcOEt, 4:1) to yield the title compound as a semisolid (0.10 g, 70% yield). ^{1}H NMR ($CDCl_3$): δ 1.32–1.46 (m + s, 13H), 1.53–1.58 (m, 4H), 1.99–2.05 (m, 2H), 3.10 (br s, 1H, D_2O exchanged) 3.18 (d, 2H, *J* = 6.3 Hz), 4.11–4.16 (m, 1H), 6.82 (d, 2H, *J* = 8.5 Hz), 7.18 (d, 2H, *J* = 8.5 Hz); ESI-MS *m*/*z* 304 $(M-H)^-$, ESI-MS/MS *m*/*z*: 204 (100).

*4.1.3. N-Boc-1-aminomethyl-1-(4-(2-fluoroethoxyphenyl)cyclohexane (**19**)*

Freshly prepared 2-fluoroethyltosylate (0.23 g, 1.00 mmol) was added to a suspension of **18** (0.13 g, 0.43 mmol) and Cs_2CO_3 (0.56 g, 1.72 mmol) in DMF. The reaction mixture was refluxed for 24 h, then the solvent was removed in vacuo, the residue was taken up with H_2O (10 mL) and extracted with EtOAc (2 × 10 mL). The combined organic phases were washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude residue was chromatographed (*n*-hexane:EtOAc, 4:1 as eluent) to afford pure **19** (0.05 g, 35% yield). ^{1}H NMR ($CDCl_3$): δ 1.31–1.42 (m + s, 13H), 1.44–1.58 (m, 4H), 2.01–2.04 (m, 2H), 3.19 (d, 2H, *J* = 6.3 Hz), 4.10–4.18 (m, 2H), 4.24–4.27 (m, 1H), 4.66–4.69 (m, 1H), 4.82–4.85 (m, 1H), 6.91 (d, 2H, *J* = 8.8 Hz), 7.24 (d, 2H, *J* = 8.8 Hz); ESI-MS *m*/*z* 374 $(M+Na)^+$, ESI-MS/MS *m*/*z*: 318 (100).

*4.1.4. 1-Aminomethyl-1-(4-(2-fluoroethoxyphenyl)cycloexane Hydrochloride (**2j**)*

Compound **19** (0.44 g, 1.3 mmol) was solubilized in a mixture of dioxane (5 mL) and 3 N HCl (5 mL) and stirred overnight. Then the reaction mixture was evaporated under reduced pressure to give a solid that was treated several times with absolute ethanol and again concentrated by evaporation in order to remove residual H_2O to afford title compound (0.37 g, quantitative yield). ^{1}H NMR (CD_3OD): δ 1.40–1.42 (m, 2H), 1.50–1.62 (m, 9H, 3H D_2O exchanged), 2.15–2.25 (m, 2H), 3.00 (s, 2H), 4.17–4.19 (m, 1H), 4.26–4.29 (m, 1H), 4.63–4.65 (m, 1H), 4.78–4.81 (m, 1H), 7.01–7.05 (m, 2H), 7.35–7.39 (m, 2H); ESI-MS *m*/*z* 252 $(M+H)^+$, ESI-MS/MS *m*/*z*: 235 (100), 153 (30).

*4.1.5. General procedure for the synthesis of compounds **21k–m***

To a stirred suspension of NaH (60% dispersion in mineral oil, 3.3 mmol) in DMSO (5 mL), under nitrogen at −15 °C, a solution of the nitrile **19k–m** (1.0 mmol) and 1.5-dibromopentane (1.0 mmol) in ether and DMSO (4:1, v/v) was added dropwise over 1 h. The mixture was then allowed to warm at room temperature and stirred for 24 h under nitrogen. Then, the reaction mixture was carefully quenched by adding dropwise isopropanol (3 mL) and water (5 mL). The reaction mixture was diluted with EtOAc (30 mL) and washed first with H_2O (20 mL), then with brine (20 mL). The separated organic layer was dried (Na_2SO_4) and concentrated in vacuo. The crude residue was chromatographed (petroleum ether/EtOAc, 7:3 as eluent) to give the target compound as a colorless oil.

*4.1.5.1. 1-Cyano-1-(2-fluoropyridin-4-yl)cyclohexane (**21k**).* 51% Yield. ^{1}H NMR ($CDCl_3$): δ 1.26–1.32 (m, 1H), 1.70–1.90 (m, 7H), 2.14 (app d, 2H), 7.05 (d, 1H, *J* = 1.4 Hz), 7.31 (dt, 1H, *J* = 2.3, 1.7 Hz), 8.25 (d, 1H, *J* = 5.5 Hz). GC/MS *m*/*z* 205 (M^++1, 7) 204 (M^+, 49), 149 (100).

*4.1.5.2. 1-Cyano-1-(2-fluoropyridin-5-yl)cyclohexane (**21l**).* 70% Yield. ^{1}H NMR ($CDCl_3$): δ 1.25–1.33 (m, 1H), 1.70–1.90 (m, 7H), 2.15 (app d, 2H), 6.97 (dd, 1H, *J* = 8.5, 3.0 Hz), 7.92 (dt, 1H, *J* = 7.4, 2.5 Hz), 8.34 (d, 1H, *J* = 2.8 Hz). ESI^+/MS *m*/*z*: 227 $(M+Na)^+$, ESI-MS/MS *m*/*z*: 227 (44), 145 (100).

*4.1.6. General procedure for the synthesis of amines **2k–m***

To a solution of the nitrile **20k–m** (1.0 mmol) in ethanolic NH_3 (20 mL, 2 M), Nickel-Raney was added and the mixture was saturated with H_2 at 5 atm pressure. The mixture was stirred at 40 °C for 22 h. Then, the reaction mixture was filtered through a Celite pad and the filtrate was concentrated in vacuo to afford the target amine (colorless oil) in quantitative yield.

*4.1.6.1. 1-Aminomethyl-1-(2-fluoropyridin-4-yl)cycloexane (**2k**).* ^{1}H NMR ($CDCl_3$): δ 1.18–1.37 (m, 4H), 1.52–1.65 (m, 6H), 2.10 (app d, 2H), 2.73 (s, 2H), 7.05 (d, 1H, *J* = 1.4 Hz), 7.31 (dt, 1H, *J* = 2.3, 1.7 Hz), 8.25 (d, 1H, *J* = 5.5 Hz). ESI^+/MS *m*/*z* 209 $(M+H)^+$, ESI-MS/MS *m*/*z* 192 (100).

*4.1.6.2. 1-Aminomethyl-1-(2-fluoropyridin-5-yl)cycloexane (**2l**).* ^{1}H NMR ($CDCl_3$): δ 1.21–1.41 (m, 4H), 1.55–1.67 (m, 6H), 2.11 (app d, 2H), 2.73 (s, 2H), 6.92 (dd, 1H, *J* = 8.5, 3.0 Hz), 7.75 (dt, 1H, *J* = 8.5, 2.5 Hz), 8.17 (d, 1H, *J* = 2.2 Hz). ESI^+/MS *m*/*z* 209 $(M+H)^+$, ESI-MS/MS *m*/*z* 192 (100), 136 (22).

*4.1.7. General procedure for the preparation of compounds (R)-**4b–d**, (S)-**4a–d**, (R,S)-**4e**, and (S,S)-**4e***

N,N′-Carbonyldiimidazole (1.1 mmol) was added to a solution of (*R*)- or (*S*)-**3** (1.0 mmol), in anhydrous THF (10 mL) under N_2. The reaction mixture was stirred at room temperature for 8 h, then a solution of the appropriate amine (1.0 mmol) in anhydrous THF was added. The reaction mixture was stirred at room temperature for 24 h, then the solvent was removed in vacuo and the residue was partitioned between in EtOAc (20 mL) and H_2O (20 mL). The aqueous phase was washed with EtOAc (2 × 20 mL) and the combined organic layers were dried over Na_2SO_4 and evaporated in vacuo. The crude was chromatographed on silica gel ($CHCl_3$/EtOAc, 9:1, as eluent) to afford pure target compound.

*4.1.7.1. (R)-t-Butyl-N-[1-(1H-indol-3-ylmethyl)-2-[1(4-methoxyphenyl)-cyclohexylmethyl]-2-oxoethyl]carbamate ((R)-**4b**).* Colorless solid wax. 80% Yield. ^{1}H NMR ($CDCl_3$): δ 1.23–1.61 (m + s, 17H), 1.78–1.85 (m, 1H), 2.98–3.12 (m, 2H), 3.20–3.31 (m, 2H), 3.75 (s, 3H), 4.27–4.32 (m, 1H), 5.10 (br d, 2H), 6.64 (br d, 2H), 6.76 (br d, 2H), 6.96 (d, 1H, *J* = 2.5 Hz), 7.12–7.24 (m, 3H), 7.38 (d, 1H, *J* = 8.0 Hz), 7.64 (d, 1H, *J* = 8.0 Hz), 8.03 (br s, 1H). ESI-MS *m*/*z* 504 $(M-H)^-$, ESI-MS/MS *m*/*z* 430 (100), 301 (69).

*4.1.7.2. (R)-t-Butyl-N-[1-(1H-indol-3-ylmethyl)-2-[1-(1-phenyl)cyclopropylmethyl]-2-oxoethyl]carbamate ((R)-**4c**).* Colorless solid wax. 50% Yield. ^{1}H NMR ($CDCl_3$): δ 0.62–0.74 (m, 4H), 1.40 (s, 9H), 1.61–1.69 (m, 2H), 3.10 (dd, 1H, *J* = 14.0, 6.0 Hz), 3.31 (dd, 1H, *J* = 14.0, 6.0 Hz), 4.32–4.38 (m, 1H), 5.13 (br s, 1H), 5.64 (br s, 1H), 6.93 (d, 2H, *J* = 2.5 Hz), 7.11–7.17 (m, 3H), 7.22 (dt, 1H, *J* = 6.0, 2.1 Hz), 7.32–7.40 (m, 2H), 7.41–7.45 (m, 1H), 7.65 (d, 1H, *J* = 8.0 Hz), 8.03 (br s, 1H). ESI-MS *m*/*z* 432 $(M-H)^-$, ESI-MS/MS *m*/*z* 358 (100), 229 (47).

*4.1.7.3. (R)-t-Butyl-N-[1-(1H-indol-3-ylmethyl)-2-[1-(2-pyridyl)cyclohexylmethyl]-2-oxoethyl]carbamate ((R)-**4d**).* Colorless solid wax. 97% Yield. ^{1}H NMR ($CDCl_3$): δ 1.30–1.50 (m, 17H), 1.72–1.85 (m, 1H), 1.89–2.00 (m, 1H), 3.11 (dd, 1H, *J* = 14.4, 7.6 Hz), 3.25 (dd, 1H, *J* = 14.3, 5.2 Hz), 3.30 (d, 2H, *J* = 5.5 Hz), 4.39 (br s, 1H), 5.12 (br s, 1H), 6.44 (br s, 1H), 6.95–7.05 (m, 3H), 7.12 (dt, 1H, *J* = 7.0, 1.1 Hz), 7.19 (dt, 1H, *J* = 7.0, 1.1 Hz), 7.33 (d, 1H, *J* = 8.0 Hz), 7.49 (br t, 1H), 7.63 (d, 1H, *J* = 8.0 Hz), 8.01 (br s, 1H), 8.33 (br s, 1H). ESI-MS *m*/*z* 475 $(M-H)^-$, ESI-MS/MS *m*/*z* 401 (100), 272 (62).

*4.1.7.4. t-Butyl-N-[1-(1H-indol-3-ylmethyl)-2-[(1S)-1-(1,2,3,4-tetrahydronaphthalen-1-yl)]-(2R)-2-oxoethyl]carbamate ((R,S)-**4e**).* White powder. 65% Yield. ^{1}H NMR ($CDCl_3$): δ 1.42 (s, 9H), 1.63 (br s, 3H), 1.82 (br s, 1H), 2.64 (t, 2H, *J* = 6.0 Hz), 3.16 (dd, 1H, *J* = 14.3, 7.7 Hz), 3.40 (dd, 1H, *J* = 14.4, 5.3 Hz), 4.42 (br s, 1H), 5.01–5.08 (m, 1H), 5.17 (br s, 1H), 5.87 (br d, 1H), 6.87 (br s, 1H), 7.07 (d, 2H, *J* = 1.1 Hz), 7.10–7.23 (m, 4H), 7.37 (d, 1H, *J* = 9.6 Hz), 7.70 (d, 1H, *J* = 8.0 Hz), 8.14 (br s, 1H). ESI–MS *m*/*z* 434 $(M+H)^+$, ESI-MS/MS *m*/*z* 378 (100), 334 (25), 248 (14).

*4.1.7.5. (S)-t-Butyl-N-[1-(1H-indol-3-ylmethyl)-2-[1-(5-methoxypyridin-2-yl)cyclohexylmethyl]-2-oxoethyl]carbamate ((S)-**4a**).* Colorless solid wax. 94% Yield. ^{1}H NMR ($CDCl_3$): δ 1.20–1.52 (m, 17H), 1.71–1.75 (m, 1H), 1.87–1.94 (m, 1H), 3.11–3.14 (m, 1H), 3.22–3.29 (m, 2H), 3.80 (s, 3H), 4.37–4.39 (m, 1H), 5.14–5.17 (m, 1H), 6.32 (br s, 1H), 6.85–6.99 (m, 4H), 7.11 (dt, 1H, J = 7.0, 1.1 Hz), 7.16 (dt, 1H, J = 8.0, 1.1 Hz), 7.33 (d, 1H, J = 8.0 Hz), 7.63 (d, 1H, J = 8.0 Hz), 8.01 (br s, 1H), 8.07 (br s, 1H). ESI-MS m/z 505 $(M-H)^-$, ESI-MS/MS m/z 431 (100), 302 (65).

*4.1.7.6. (S)-t-Butyl-N-[1-(1H-indol-3-ylmethyl)-2-[1-(4-methoxyphenyl)cyclohexylmethyl]-2-oxoethyl]carbamate ((S)-**4b**).* Colorless solid wax. 83% Yield. ^{1}H NMR ($CDCl_3$): δ 1.39–1.60 (m, 18H), 1.79–1.86 (m, 1H), 2.99–3.12 (m, 2H), 3.20–3.26 (m, 2H), 3.75 (s, 3H), 4.25–4.35 (m, 1H), 5.06 (br s, 1H), 6.64 (d, 2H, J = 8.0 Hz), 6.75 (d, 2H, J = 8.0 Hz), 6.96 (d, 1H, J = 2.2 Hz), 7.15 (dt, 1H, J = 7.0, 1.1 Hz), 7.23 (dt, 1H, J = 7.0, 1.1 Hz), 7.38 (d, 1H, J = 7.0 Hz), 7.63 (d, 1H, J = 7.0 Hz), 8.10 (br s, 1H). ESI-MS m/z 504 $(M-H)^-$, ESI-MS/MS m/z 430 (100), 301 (73).

*4.1.7.7. (S)-t-Butyl-N-[1-(1H-indol-3-ylmethyl)-2-[1-(1-phenyl)cyclopropylmethyl]-2-oxoethyl]carbamate ((S)-**4c**).* Colorless solid wax. 50% Yield. ^{1}H NMR ($CDCl_3$): δ 0.62–0.74 (m, 4H), 1.40 (s, 9H), 1.61–1.69 (m, 2H), 3.10 (dd, 1H, J = 14.0, 6.0 Hz), 3.31 (dd, 1H, J = 14.0, 6.0 Hz), 4.32–4.38 (m, 1H), 5.13 (br s, 1H), 5.64 (br s, 1H), 6.93 (d, 2H, J = 2.2 Hz), 7.10–7.17 (m, 3H), 7.22 (dt, 1H, J = 6.0, 2.1 Hz), 7.32–7.40 (m, 2H), 7.41–7.45 (m, 1H), 7.65 (d, 1H, J = 8.0 Hz), 8.10 (br s, 1H). ESI-MS m/z 432 $(M-H)^-$, ESI-MS/MS m/z 358 (100), 229 (47).

*4.1.7.8. (S)-t-Butyl-N-[1-(1H-indol-3-ylmethyl)-2-[1-(2-pyridyl)cyclohexylmethyl]-2-oxoethyl]carbamate ((S)-**4d**).* Colorless solid wax. 40% Yield. ^{1}H NMR ($CDCl_3$): δ 1.30–1.50 (m, 17H), 1.72–1.85 (m, 1H), 1.89–2.00 (m, 1H), 3.09–3.30 (m, 2H), 3.33 (d, 2H, J = 5.0 Hz), 4.39 (br s, 1H), 6.45 (br s, 1H), 6.95–7.05 (m, 3H), 7.11 (dt, 1H, J = 1.1, 7.0 Hz), 7.20 (dt, 1H, J = 7.0, 1.1 Hz), 7.33 (d, 1H, J = 8.0 Hz), 7.49 (br t, 1H, J = 7.4 Hz), 7.63 (d, 1H, J = 8.0 Hz), 8.02 (br s, 1H), 8.32 (br s, 1H). ESI-MS m/z 475 $(M-H)^-$, ESI-MS/MS m/z 401 (100), 272 (59).

*4.1.7.9. t-Butyl-N-[1-(1H-indol-3-ylmethyl)-2-((1S)-1,2,3,4-tetrahydronaphthalen-1-yl)-(2S)-2-oxoethyl]carbamate ((S,S)-**4e**).* White powder. 70%. Yield ^{1}H NMR ($CDCl_3$): δ 1.41 (s, 9H), 1.64 (br s, 3H), 1.91 (br s, 1H), 2.66 (app t, 2H, J = 6.2 Hz), 3.18 (dd, 1H, J = 14.3, 8.0 Hz), 3.35 (dd, 1H, J = 14.3, 5.5 Hz), 4.42 (br s, 1H), 5.00 (br s, 1H), 5.17 (br s, 1H), 5.87 (br d, 1H), 6.68 (br s, 1H), 6.94–7.15 (m, 4H), 7.12 (dt, 1H, J = 7.2, 1.4 Hz), 7.34–7.37 (m, 1H), 7.69 (d, 1H, J = 7.7 Hz), 8.10 (br s, 1H). ESI-MS m/z 434 $(M+H)^+$, ESI-MS/MS m/z 378 (100), 334 (24), 248 (14), 204 (9).

*4.1.8. General procedure for the preparation of compounds (R)-**5b–d**, (S)-**5a–d**, (R,S)-**5e**, and (S,S)-**5e***

Trifluoroacetic acid (5 mL) was added to a solution of (*R*)-**4b–d**, (*S*)-**4a–d**, (*R,S*)-**4e**, and (*S,S*)-**4e** in CH_2Cl_2 (20 mL). The reaction mixture was stirred at room temperature for 5 h, basified with aqueous 1 M NaOH, and extracted with EtOAc (3 × 20 mL). The combined organic layers were dried (Na_2SO_4) and concentrated in vacuo to give a crude residue that was purified by column chromatography ($CHCl_3/CH_3OH$, 19:1, as eluent) to afford the pure compound.

*4.1.8.1. (R)-2-Amino-3-(1H-indol-3-yl)-N-[1-(4-methoxyphenyl)cyclohexylmethyl]propanamide ((R)-**5b**).* Colorless solid wax. 99% Yield. ^{1}H NMR ($CDCl_3$): δ 1.32–1.56 (m, 10H), 1.92–1.97 (m, 2H), 2.84 (dd, 1H, J = 14.3, 8.5 Hz), 3.25–3.31 (m, 3H), 3.62 (dd, 1H, J = 8.5, 4.4 Hz), 3.80 (s, 3H), 6.80–6.84 (m, 3H), 6.99 (d, 1H, J = 2.2 Hz), 7.11–7.18 (m, 3H), 7.21 (dt, 1H, J = 8.0, 1.0 Hz), 7.38 (d, 1H, J = 8.0 Hz), 7.63 (d, 1H, J = 8.0 Hz), 8.09 (br s, 1H). ESI-MS m/z 404 $(M-H)^-$, ESI-MS/MS m/z 273 (39), 216 (100), 130 (18).

*4.1.8.2. (R)-2-Amino-3-(1H-indol-3-yl)-N-(1-phenylcyclopropylmethyl)propanamide ((R)-**5c**).* Colorless solid wax. 72% Yield. ^{1}H NMR ($CDCl_3$): δ 0.81–0.89 (m, 4H), 1.50 (br s, 2H), 2.86 (dd, 1H, J = 14.0, 8.0 Hz), 3.30 (dd, 1H, J = 14.0, 4.0 Hz), 3.32–3.51 (m, 2H), 3.65 (dd, 1H, J = 14.0, 4.0 Hz), 7.00 (d, 1H, J = 2.5 Hz), 7.11–7.44 (m, 9H), 7.64–7.67 (m, 1H), 8.10 (br s, 1H). ESI-MS m/z 334 $(M+H)^+$, ESI-MS/MS m/z 317 (100), 130 (30).

*4.1.8.3. (R)-2-Amino-3-(1H-indol-3-yl)-N-[1-(pyridin-2-yl)cyclohexylmethyl]propanamide ((R)-**5d**).* Colorless solid wax. 90% Yield. ^{1}H NMR ($CDCl_3$): δ 1.21–1.66 (m, 10H), 2.11–2.18 (m, 2H), 2.80 (dd, 1H, J = 14.6, 9.1 Hz), 3.29 (dd, 1H, J = 14.3, 4.2 Hz), 3.48 (d, 2H, J = 5.8 Hz), 3.62 (dd, 1H, J = 8.8, 4.1 Hz), 7.02 (d, 1H, J = 2.5 Hz), 7.08–7.12 (m, 2H), 7.19 (dt, 1H, J = 8.1, 1.1 Hz), 7.24 (d, 1H, J = 8.0 Hz), 7.35 (d, 1H, J = 8.0 Hz), 7.43 (br t, 1H), 7.58–7.65 (m, 2H), 8.17 (br s, 1H), 8.54–8.56 (m, 1H). ESI-MS m/z 377 $(M+H)^+$, ESI-MS/MS m/z 360 (100), 332 (14), 191 (30).

*4.1.8.4. (2R)-2-Amino-3-(1H-indol-3-yl)-N-((1S)-1,2,3,4-tetrahydronaphtalen-1-yl)propanamide ((R,S)-**5e**).* Colorless solid wax. 50% Yield. ^{1}H NMR ($CDCl_3$): δ 1.52 (br s, 2H), 1.66–1.82 (m, 3H), 1.98–2.06 (m, 1H), 2.68–2.81 (m, 2H), 3.06 (dd, 1H, J = 14.6, 8.5 Hz), 3.42 (dd, 1H, J = 13.5, 4.4 Hz), 3.78 (app q, 1H), 5.12–5.18 (m, 1H), 6.94–6.97 (m, 1H), 7.05–7.11 (m, 4H), 7.14 (dt, 1H, J = 6.0, 1.0 Hz), 7.22 (dt, 1H, J = 6.0, 1.0 Hz), 7.37–7.41 (m, 2H), 7.73 (d, 1H, J = 7.0 Hz), 8.13 (br s, 1H). ESI-MS m/z 334 $(M+H)^+$, ESI-MS/MS m/z 317 (10), 204 (100), 187 (46).

*4.1.8.5. (S)-2-Amino-3-(1H-indol-3-yl)-N-[1-(5-methoxypyridin-2-yl)cyclohexylmethyl]propanamide ((S)-**5a**).* Colorless solid wax. 83% Yield. ^{1}H NMR ($CDCl_3$): δ 1.22–1.58 (m, 10H), 2.07–2.12 (m, 2H), 2.81 (dd, 1H, J = 14.0, 9.0 Hz), 3.29 (dd, 1H, J = 14.0, 9.0 Hz), 3.41 (d, 2H, J = 6.0 Hz), 3.65 (dd, 1H, J = 14.0, 9.0 Hz), 3.83 (s, 3H), 7.02 (d, 1H, J = 2.5.0 Hz), 7.11–7.22 (m, 4H), 7.29–7.37 (m, 2H), 7.64 (d, 1H, J = 8.0 Hz), 8.12 (br s, 1H), 8.24 (dd, 1H, J = 1.2, 0.8 Hz). ESI-MS m/z 407 $(M+H)^+$, ESI-MS/MS m/z 390 (100), 221 (34), 204 (26).

*4.1.8.6. (S)-2-Amino-3-(1H-indol-3-yl)-N-[1-(4-methoxyphenyl)cyclohexylmethyl]propanamide ((S)-**5b**).* Colorless solid wax. 85% Yield. ^{1}H NMR ($CDCl_3$): δ 1.36–1.55 (m, 10H), 1.92–1.96 (m, 2H), 2.84 (dd, 1H, J = 14.6, 8.5 Hz), 3.25–3.31 (m, 3H), 3.61 (dd, 1H, J = 8.8, 4.7 Hz), 3.78 (s, 3H), 6.80–6.84 (m, 3H), 6.99 (d, 1H, J = 2.2 Hz), 7.09–7.15 (m, 3H), 7.18–7.24 (dt, 1H, J = 8.0, 1.0 Hz), 7.37 (d, 1H, J = 8.2 Hz), 7.64 (d, 1H, J = 7.7 Hz), 8.07 (br s, 1H). ESI-MS m/z 406 $(M+H)^+$, ESI-MS/MS m/z 389 (100), 260 (23), 250 (23).

*4.1.8.7. (S)-2-Amino-3-(1H-indol-3-yl)-N-(1-phenylcyclopropylmethyl)propanamide ((S)-**5c**).* Colorless solid wax. 90% Yield. ^{1}H NMR ($CDCl_3$): δ 0.80–0.91 (m, 4H), 1.42 (br s, 2H), 2.85 (dd, 1H, J = 14.0, 8.0 Hz), 3.30 (dd, 1H, J = 14.0, 4.0 Hz), 3.36–3.51 (m, 2H), 3.65 (dd, 1H, J = 14.0, 4.0 Hz), 7.00 (d, 1H, J = 2.5 Hz), 7.11–7.44 (m, 9H), 7.65 (br d, 1H, J = 3.9, 0.5 Hz), 8.11 (br s, 1H). ESI-MS m/z 334 $(M+H)^+$, ESI-MS/MS m/z 317 (100), 130 (24).

*4.1.8.8. (S)-2-Amino-3-(1H-indol-3-yl)-N-[1-(pyridin-2-yl)cyclohexylmethyl]propanamide ((S)-**5d**).* Colorless solid wax. 95% Yield. ^{1}H NMR ($CDCl_3$): δ 1.22–1.68 (m, 10H), 2.10–2.19 (m, 2H), 2.80 (dd, 1H, J = 14.0, 8.8 Hz), 3.29 (dd, 1H, J = 14.0, 4.1 Hz), 3.48 (d, 2H, J = 9.0 Hz), 3.65 (dd, 1H, J = 9.1, 4.1 Hz), 7.02 (d, 1H, J = 2.2 Hz), 7.08–7.17 (m, 2H), 7.19 (br t, 1H), 7.24 (d, 1H, J = 8.0 Hz), 7.35 (d, 1H, J = 8.0 Hz), 7.43 (br t, 1H), 7.58–7.65 (m,

2H), 8.17 (br s, 1H), 8.53–8.56 (m, 1H). ESI-MS *m/z* 377 $(M+H)^+$, ESI-MS/MS *m/z* 360 (100), 246 (15), 191 (25).

*4.1.8.9. (2S)-2-Amino-3-(1H-indol-3-yl)-N-((1S)-1,2,3,4-tetrahydronaphtalen-1-yl)propanamide ((S,S)-**5e**).* White powder. 97% Yield. ^{1}H NMR ($CDCl_3$): δ 1.50 (br s, 2H), 1.66–1.79 (m, 3H), 1.97–2.04 (m, 1H), 2.75 (app d, 2H), 2.98 (dd, 1H, *J* = 14.0, 6.0 Hz), 3.43 (dd, 1H, *J* = 14.0, 6.0 Hz), 3.78 (q app, 1H), 5.13–5.18 (m, 1H), 7.06–7.15 (m, 6H), 7.21 (dt, 1H, *J* = 7.0, 1.0 Hz), 7.37–7.44 (m, 2H), 7.70 (d, 1H, *J* = 7.0 Hz), 8.16 (br s, 1H). ESI-MS *m/z* 332 $(M-H)^-$, ESI-MS/MS *m/z* 332 (100), 202 (40), 130 (31).

*4.1.9. General procedure for the synthesis of the final compounds (R)-**6b–d**, (S)-**6a–d**, (R,S)-**6e**, and (S,S)-**6e***

To a solution of the amine (*R*)-**5b–d**, (*S*)-**5a–d**, (*R,S*)-**5e**, and (*S,S*)-**5e** (1.0 mmol) in anhydrous THF, a solution of 4-nitrophenylisocyanate (1.2 mmol) in the same solvent (10 mL) was added and the reaction mixture was stirred at room temperature for 15 h. After removing the solvent in vacuo, the residue was taken up in EtOAc (20 mL) and washed with H_2O (2 × 20 mL). The separated organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The crude residue was chromatographed as detailed below. The obtained solid was further purified by crystallization from EtOAc/petroleum ether to give the final compound as a yellow powder.

*4.1.9.1. (R)-3-(1H-indol-3-yl)-N-[1-(4-methoxyphenyl)cyclohexylmethyl]-2-[(4-nitrophenyl)-carbamoylamino]propanamide ((R)-**6b**).* 34% Yield. ^{1}H NMR (DMSO-d_6): δ 1.12–1.18 (m, 3H), 1.29–1.43 (m, 4H), 1.83–1.97 (m, 2H), 2.83–2.90 (m, 2H), 2.97–3.04 (m, 1H), 3.21–3.28 (m, 2H), 3.62 (s, 3H), 4.49 (q, 1H, *J* = 7.0 Hz), 6.52 (d, 1H, *J* = 8.0 Hz), 6.71 (d, 2H, *J* = 8.8 Hz), 6.94 (t, 1H, *J* = 7.4 Hz), 7.01–7.11 (m, 4H), 7.30 (d, 1H, *J* = 8.0 Hz), 7.41 (t, 1H, *J* = 5.9 Hz), 7.49–7.58 (m, 3H), 8.12 (d, 2H, *J* = 9.1 Hz), 9.39 (s, 1H), 10.82 (s, 1H). ESI-MS *m/z* 570 $(M+H)^+$, ESI-MS/MS *m/z* 406 (100), 389 (14). Anal. ($C_{32}H_{35}N_5O_5$) C, H, N.

*4.1.9.2. (R)-3-(1H-indol-3-yl)-N-(1-phenylcyclopropylmethyl)-2-[(4-nitrophenyl)carbamoylamino]propanamide ((R)-**6c**).* 37% Yield. ^{1}H NMR (DMSO-d_6): δ 0.69 (q, 2H, *J* = 9.7 Hz), 0.81–0.84 (m, 2H), 2.84–2.91 (m, 1H), 2.99–3.06 (m, 1H,) 2.20–2.27 (m, 1H), 3.36–3.43 (m, 1H), 4.51 (q, 1H, *J* = 6.8 Hz), 6.51, (d, 1H, *J* = 7.9 Hz), 6.89–7.05 (m, 3H), 7.08–7.23 (m, 5H), 7.29 (d, 1H, *J* = 8.2 Hz), 7.48–7.56 (m, 3H), 8.11 (app d, 3H), 9.40 (s, 1H), 10.79 (s, 1H). ESI-MS *m/z* 498 $(M+H)^+$, ESI-MS/MS *m/z* 360 (12), 334 (100). Anal. ($C_{28}H_{27}N_5O_4$) C, H, N.

*4.1.9.3. (R)-3-(1H-indol-3-yl)-N-[1-(pyridin-2-yl)cyclohexylmethyl]-2-[(4-nitrophenyl)-carbamoylamino]propanamide ((R)-**6d**).* 33% Yield. ^{1}H NMR (DMSO-d_6): δ 1.12–1.21 (m, 3H). 1.29–1.44 (m, 4H), 2.47–2.48 (m, 2H), 2.81–2.89 (m, 1H), 2.97–3.10 (m, 2H), 3.27–3.32 (m, 2H), 4.48 (q, 1H, *J* = 6.8 Hz), 6.51 (d, 1H, *J* = 8.0 Hz), 6.93 (t, 1H, *J* = 7.3 Hz), 7.00–7.12 (m, 3H), 7.19 (d, 1H, *J* = 8.0 Hz), 7.29 (d, 1H, *J* = 8.0 Hz), 7.49–7.57 (m, 4H), 7.66 (t, 1H, *J* = 5.6 Hz), 8.10 (d, 2H, *J* = 9.1 Hz), 8.47 (d, 1H, *J* = 3.6 Hz), 9.38 (s, 1H), 10.82 (s, 1H). ESI-MS *m/z* 541 $(M+H)^+$, ESI-MS/MS *m/z* 403 (12), 377 (100). Anal. ($C_{30}H_{32}N_6O_4$) C, H, N.

*4.1.9.4. 3-(1H-Indol-3-yl)-N-((1S)-1,2,3,4-tetrahydronaphthalen-1-yl)-(2R)-2-[(4-nitrophenyl)carbamoylamino]propanamide ((R,S)-**6e**).* 12% Yield. ^{1}H NMR (DMSO-d_6): δ 1.49–1.72 (m, 4H), 2.67–2.68 (m, 2H), 3.02–3.09 (m, 2H), 4.55 (q, 1H, *J* = 6.6 Hz), 4.91 (br s, 1H), 6.67 (d, 1H, *J* = 7.7 Hz), 6.93 (t, 2H, *J* = 7.0 Hz), 7.01–7.09 (m, 6H), 7.31 (d, 1H, *J* = 8.3 Hz), 7.55–7.59 (m, 3H), 8.13 (d, 2H, *J* = 9.4 Hz), 8.47 (d, 1H, *J* = 8.5 Hz), 9.49 (s, 1H), 10.84 (s, 1H). ESI-MS *m/z* 496 $(M-H)^-$, ESI-MS/MS *m/z* 358 (100), 229 (6). Anal. ($C_{28}H_{27}N_5O_4$) C, H, N.

*4.1.9.5. (S)-3-(1H-Indol-3-yl)-N-[1-(5-methoxypyridin-2-yl)cyclohexylmethyl]-2-[(4-nitrophenyl)-carbamoylamino]propanamide ((S)-**6a**).* 32% Yield. ^{1}H NMR (DMSO-d_6): δ 1.05–1.42 (m, 7H), 1.84–2.14 (m, 2H), 2.84–2.91 (m, 1H), 2.99–3.06 (m, 2H), 3.25–3.27 (m, 2H), 3.69 (s, 3H), 4.47 (q, 1H, *J* = 6.9 Hz), 6.60 (d, 1H, *J* = 8.0 Hz), 6.91–7.09 (m, 5H), 7.30 (d, 2H, *J* = 8.0 Hz), 7.50–7.61 (m, 4H), 8.10–8.17 (m, 3H), 9.46 (s, 1H), 10.83 (s, 1H). ^{13}C NMR (DMSO-d_6): 171.8; 162.4; 158.9; 156.9; 152.1; 150.9; 146.7; 145.7; 141.3; 132.4; 130.4; 128.8; 126.1; 123.7; 123.4; 121.9; 116.4; 114.8; 58.7; 55.1; 53.8; 50.2; 45.3; 37.5; 37.4; 33.8; 30.8; 26.3; 22.9. ESI-MS *m/z* 571 $(M+H)^+$, ESI-MS/MS *m/z* 407 (100), 390 (23), 221 (14). Anal. ($C_{31}H_{34}N_6O_5$) C, H, N.

*4.1.9.6. (S)-3-(1H-Indol-3-yl)-N-[1-(4-methoxyphenyl)cyclohexylmethyl]-2-[(4-nitrophenyl)-carbamoylamino]propanamide ((S)-**6b**).* 10% Yield. ^{1}H NMR (DMSO-d_6): δ 1.13–1.25 (m, 3H), 1.35–1.44 (m, 4H), 1.80–1.97 (m, 2H), 2.71–2.90 (m, 2H), 2.97–3.04 (m, 1H), 3.21–3.28 (m, 2H), 3.62 (s, 3H), 4.48 (q, 1H, *J* = 6.9 Hz), 6.61 (d, 1H, *J* = 8.2 Hz), 6.72 (d, 2H, *J* = 8.8 Hz), 6.94 (t, 1H, *J* = 7.0 Hz), 7.01–7.11 (m, 4H), 7.30 (d, 1H, *J* = 8.0 Hz), 7.39 (br t, 1H), 7.49–7.58 (d, 3H), 8.12 (d, 2H, *J* = 9.4 Hz), 9.46 (s, 1H), 10.82 (s, 1H). ^{13}C NMR (DMSO-d_6): 176.8; 158.9; 156.9; 152.1; 150.9; 146.7; 145.7; 141.3; 132.6; 130.4; 128.8; 126.1; 123.7; 123.4; 123.1; 121.9; 116.4; 114.8; 58.7; 53.8; 50.2; 45.3; 37.5; 37.4; 33.8; 30.8; 27.1. ESI-MS *m/z* 568 $(M-H)^-$, ESI-MS/MS *m/z* 430 (100), 301 (5). Anal. ($C_{32}H_{35}N_5O_5$) C, H, N.

*4.1.9.7. (S)-3-(1H-Indol-3-yl)-N-(1-phenylcyclopropylmethyl)-2-[(4-nitrophenyl)-carbamoylamino]propanamide ((S)-**6c**).* 30% Yield. ^{1}H NMR (DMSO-d_6): δ 0.66–0.75 (q, 2H, *J* = 9.0 Hz), 0.84 (br s, 2H), 2.84–2.94 (m, 1H), 2.99–3.06 (m, 1H), 3.20–3.26 (m, 1H), 3.36–3.43 (m, 1H), 4.51 (q, 1H, *J* = 6.8 Hz), 6.51, (d, 1H, *J* = 8.0 Hz), 6.92–7.05 (m, 3H), 7.09–7.31 (m, 6H), 7.48–7.56 (m, 3H), 8.09–8.12 (m, 3H), 9.40 (s, 1H), 10.79 (s, 1H). ^{1}ESI-MS *m/z* 498 $(M+H)^+$, ESI-MS/MS *m/z* 360 (129), 334 (100), 317 (19). Anal. ($C_{28}H_{27}N_5O_4$) C, H, N.

*4.1.9.8. (S)-3-(1H-Indol-3-yl)-N-[1-(pyridin-2-yl)cyclohexylmethyl]-2-[(4-nitrophenyl)-carbamoylamino]propanamide ((S)-**6d**).* 32% Yield. ^{1}H NMR (DMSO-d_6): δ 1.07–1.50 (m, 7H), 2.00–2.20 (m, 2H), 2.82–2.90 (m, 1H), 2.97–3.14 (m, 2H), 3.28–3.35 (m, 2H), 4.48 (q, 1H, *J* = 7.0 Hz), 6.53 (d, 1H, *J* = 8.0 Hz), 6.90–7.12 (m. 4H) 7.20 (d, 1H, *J* = 8.0 Hz), 7.30 (d, 1H, *J* = 8.0 Hz), 7.49–7.59 (m, 4H), 7.65 (br t, 1H), 8.10 (d, 2H, *J* = 9.4 Hz), 8.47 (q, 1H, *J* = 2.0 Hz), 9.40 (s, 1), 10.81 (s, 1H). ^{13}C NMR (DMSO-d_6): 171.9; 157.4; 154.2; 147.4; 140.9; 136.5; 136.3; 128.3; 127.8; 125.7; 123.9; 121.3; 118.9; 118.7; 117.1; 113.9; 111.7; 110.1; 55.1; 53.9; 49.0; 42.2; 33.5; 33.1; 29.0; 26.3; 22.1. ESI-MS *m/z* 541 $(M+H)^+$, ESI-MS/MS *m/z* 403 (13), 377 (100), 360 (30). Anal. ($C_{30}H_{32}N_6O_4$) C, H, N.

*4.1.9.9. 3-(1H-Indol-3-yl)-N-((1S)-1,2,3,4-tetrahydronaphthalen-1-yl)-(2S)-[(4-nitrophenyl)-carbamoylamino]propanamide ((S,S)-**6e**).* 35% Yield. ^{1}H NMR (DMSO-d_6): δ 1.59–1.66 (m, 2H), 1.80–1.83 (m, 2H), 2.66 (br s, 2H), 3.02–3.18 (m, 2H), 4.56 (q, 1H, *J* = 7.2 Hz), 4.88 (br s, 1H), 6.60 (d, 1H, *J* = 7.7 Hz), 6.67 (d, 1H, *J* = 7.7 Hz), 6.90–7.12 (m, 5H), 7.16 (d, 1H, *J* = 1.7 Hz), 7.34 (d, 1H, *J* = 8.0 Hz), 7.55–7.62 (d, 3H, *J* = 9.0 Hz), 8.12 (d, 2H, *J* = 9.4 Hz), 8.38 (d, 1H, *J* = 8.5 Hz), 9.45 (s, 1H), 10.88 (s, 1H). ESI-MS *m/z* 498 $(M+H)^+$, ESI-MS/MS *m/z* 368 (100), 351 (27), 204 (21). Anal. ($C_{28}H_{27}N_5O_4$) C, H, N.

*4.1.10. General procedure for the preparation of compounds (R)- or (S)-**9f**, (S)-**9a**,**d***

N,*N*′-Carbonyldiimidazole (1.1 mmol) was added to a solution of (*R*)- or (*S*)-**8** (1.0 mmol), in anhydrous THF (10 mL), under N_2. The reaction mixture was stirred at room temperature for 8 h, then the solution of the appropriate amine (1.0 mmol) in anhydrous THF was added. The reaction mixture was stirred at room temperature for 24 h, then the solvent was removed in vacuo and the residue was partitioned between EtOAc (20 mL) and H_2O (20 mL). The aqueous layer was extracted with EtOAc (2 × 20 mL) and the collected organic layers were dried (Na_2SO_4) and evaporated in vacuo. The crude residue was chromatographed ($CHCl_3$/EtOAc, 9:1, as eluent) to give pure target compound as a pale yellow solid.

*4.1.10.1. (S)-2-[Benzyl(methyl)amino-3-(1H-indol-3-yl)-N-[1-(5-methoxypyridin-2-yl)cyclohexylmethyl]propanamide ((S)-**9a**).* 55% Yield. ^{1}H NMR ($CDCl_3$): δ 1.30–1.62 (m, 8H), 1.91–2.23 (m, 2H), 2.04 (s, 3H), 3.10 (dd, 1H, *J* = 14.5, 5.5 Hz), 3.37–3.62 (m, 6H), 3.81 (s, 3H), 7.03–7.10 (m, 7H), 7.14–7.21 (m, 4H), 7.34 (d, 1H, *J* = 8.0 Hz), 7.55 (d, 1H, *J* = 8.0 Hz), 8.03 (br s, 1H), 8.20–8.21 (m, 1H). ESI-MS *m*/*z* 511 $(M+H)^+$, ESI-MS/MS *m*/*z* 390 (100), 380 (73), 263 (50).

*4.1.10.2. (S)-2-[Benzyl(methylamino-3-(1H-indol-3-yl)-N-[1-(pyridin-2-yl)cyclohexylmethyl]propanamide ((S)-**9d**).* 75% Yield. ^{1}H NMR ($CDCl_3$): δ 1.28–1.60 (m, 8H), 1.95–2.09 (m, 2H), 2.23 (s, 3H), 3.07 (dd, 1H, *J* = 14.5, 5.5 Hz), 3.39–3.61 (m, 6H), 7.02–7.10 (m, 5H), 7.11–7.20 (m, 6H), 7.34 (d, 1H, *J* = 8.0 Hz), 7.53–7.60 (m, 2H), 8.02 (br s, 1H), 8.51–8.53 (m, 1H). ESI-MS *m*/*z* 481 $(M+H)^+$, ESI-MS/MS *m*/*z* 360 (100), 350 (92), 263 (61).

*4.1.10.3. (R)-2-[Benzylmethylamino-3-(1H-indol-3-yl)-N-(cyclohexylmethyl)]propanamide ((R)-**9f**).* 70% Yield. ^{1}H NMR ($CDCl_3$): δ 0.78–0.89 (m, 2H), 1.07–1.18 (m, 3H), 1.29–1.38 (m, 1H), 1.60–1.67 (m, 5H), 2.39 (s, 3H), 3.05 (t, 3H, *J* = 6.3 Hz), 3.15 (dd, 1H, *J* = 14.5, 5.5 Hz), 3.46 (dd, 1H, *J* = 14.5, 7.0 Hz), 3.57–3.63 (m, 2H), 6.86 (br t, 1H), 7.08–7.37 (m, 9H), 7.62 (d, 1H, *J* = 7.7 Hz), 8.04 (br s, 1H). ESI-MS *m*/*z* 404 $(M+H)^+$, ESI-MS/MS *m*/*z* 283 (100), 273 (87), 263 (63), 144 (23).

*4.1.10.4. (S)-2-[Benzylmethylamino-3-(1H-indol-3-yl)-N-(cyclohexylmethyl)]propanamide ((S)-**9f**).* 30% Yield. ^{1}H NMR ($CDCl_3$): δ 0.77–0.90 (m, 2H), 1.06–1.19 (m, 3H), 1.23–1.39 (m, 1H), 1.57–1.68 (m, 5H), 2.39 (s, 3H), 3.05 (t, 2H, *J* = 6.5 Hz), 3.16 (dd, 1H, *J* = 14.5, 6.0 Hz), 3.38–3.67 (m, 4H), 6.86 (br t, 1H), 7.06–7.37 (m, 9H), 7.58–7.63 (m, 1H), 8.10 (br s, 1H). ESI-MS *m*/*z* 404 $(M+H)^+$, ESI-MS/MS *m*/*z* 283 (100), 273 (84), 263 (69), 144 (31).

*4.1.11. General procedure for the preparation of compounds (S)-**10a**,**d**, (R)- and (S)-**10f***

A solution of *N*-benzyl derivatives (*S*)-**9a**,**d**, (*R*)- or (*S*)-**9f** in ethanol (20 mL) was saturated with H_2 (5 atm) in presence of a catalytic amount of 10% Pd/C. The reaction mixture was stirred at room temperature overnight. Then, the mixture was filtered through a Celite pad and the filtrate was evaporated to dryness. The crude residue was chromatographed ($CHCl_3$/CH_3OH, 19:1, as eluent) to afford the pure compound as pale yellow oil.

*4.1.11.1. (S)-2-[Methylamino-3-(1H-indol-3-yl)-N-[1-(5-methoxypyridin-2-yl)cyclohexyl methyl]propanamide ((S)-**10a**).* 46% Yield. ^{1}H NMR ($CDCl_3$): δ 1.25–1.44 (m, 4H), 1.53–1.61 (m, 4H), 1.91 (br s, 1H), 2.08–2.14 (m, 2H), 2.14 (s, 3H), 2.73–2.82 (m, 1H), 3.20–3.28 (m, 2H), 3.43 (dq, 2H, *J* = 13.2, 6.1 Hz), 3.84 (s, 3H), 7.01 (d, 1H, *J* = 2.2 Hz), 7.09–7.22 (m, 4H), 7.31 (br s, 1H), 7.35 (d, 1H, *J* = 8.0 Hz), 7.64 (d, 1H, *J* = 8.0 Hz), 8.10 (br s, 1H), 8.25 (d, 1H, *J* = 3.0 Hz). ESI-MS *m*/*z* 421 $(M+H)^+$, ESI-MS/MS *m*/*z* 390 (51), 290 (100), 221 (23), 204 (14).

*4.1.11.2. (S)-2-[Methylamino-3-(1H-indol-3-yl)-N-[1-(pyridin-2-yl)-cyclohexylmethyl]propanamide ((S)-**10d**).* 60% Yield. ^{1}H NMR ($CDCl_3$): δ 1.25–1.49 (m, 5H), 1.62–1.64 (m, 4H), 2.04–2.16 (m, 2H), 2.10 (s, 3H), 2.73 (dt, 1H, *J* = 11.0, 4.0 Hz), 3.17–3.26 (m, 2H), 3.41–3.56 (m, 2H), 7.01 (d, 1H, *J* = 2.2 Hz), 7.07–7.14 (m, 2H), 7.19 (dt, 1H, *J* = 7.2, 1.1 Hz), 7.26–7.39 (m, 2H), 7.58–7.66 (m, 2H), 8.14 (br s, 1H), 8.55–8.58 (m, 1H). ESI-MS *m*/*z* 389 $(M-H)^-$, ESI-MS/MS *m*/*z* 358 (3), 260 (21), 228 (100), 157 (61), 130 (25).

*4.1.11.3. (R)-2-[Methylamino-3-(1H-indol-3-yl)-N-(cyclohexylmethyl)propanamide ((R)-**10f**).* 70% Yield. ^{1}H NMR ($CDCl_3$): δ 0.83–0.97 (m, 2H), 1.14–1.28 (m, 3H), 1.36–1.48 (m, 1H), 1.63–1.68 (m, 6H), 2.27 (s, 3H), 2.85–2.93 (m, 1H), 3.02–3.18 (m, 2H), 3.28–3.49 (m, 2H), 7.06 (d, 1H, *J* = 2.2 Hz), 7.13 (dt, 1H, *J* = 7.0, 1.0 Hz) 7.20 (dt, 1H, *J* = 7.0, 1.0 Hz), 7.24 (br s, 1H), 7.37 (d, 1H, *J* = 8.0 Hz), 7.68 (d, 1H, *J* = 8.0 Hz), 8.20 (br s, 1H). ESI-MS *m*/*z* 312 $(M-H)^-$, ESI-MS/MS *m*/*z* 312 (69), 181 (51), 130 (100).

*4.1.11.4. (S)-2-[Methylamino-3-(1H-indol-3-yl)-N-(cyclohexylmethyl)]propanamide ((S)-**10f**).* 90% Yield. ^{1}H NMR ($CDCl_3$): δ 0.78–0.87 (m, 2H), 1.09–1.28 (m, 3H), 1.30–1.38 (m, 1H), 1.62–1.66 (m, 6H), 2.38 (s, 3H), 2.94–3.10 (m, 3H), 3.18 (dd, 1H, *J* = 14.5, 8.0 Hz), 3.39 (dd, 1H, *J* = 14.5, 6.0 Hz), 7.04–7.18 (m, 3H), 7.34–7.40 (m, 2H), 7.63 (d, 1H, *J* = 7.7 Hz), 8.57 (br s, 1H). ESI-MS *m*/*z* 314 $(M+H)^+$, ESI-MS/MS *m*/*z* 283 (69), 183 (100), 173 (22), 132 (15).

*4.1.12. General procedure for the preparation of compounds (S)-**11a**,**d**, (R)- and (S)-**11f***

To solution of 4-nitroaniline (1.0 mmol) in anhydrous THF (10 mL) was added *N*,*N*′-carbonyldiimidazole (1.0 mmol), under N_2, and the reaction mixture was stirred at room temperature overnight. A solution of amine (*S*)-**10a**,**d**, (*R*)- and (*S*)-**10f** (1.0 mmol) in anhydrous THF was added and the resulting mixture was stirred at room temperature for 24 h. Then the solvent was removed in vacuo and methanol was added to the residue. The solid was filtered off and the filtrate was evaporated under reduced pressure. The crude residue was purified on a silica gel column by eluting first with $CHCl_3$, then with $CHCl_3$/MeOH, 49:1. The obtained solid was crystallized (EtOAc) to give a yellow solid.

*4.1.12.1. (S)-N-[1-(5-Methoxypyridin-2-yl)-cyclohexylmethyl]-2-[3-(4-nitrophenyl)-1-methyl-ureido]-3-(1H-indol-3-yl)propanamide ((S)-**11a**).* 12% Yield. ^{1}H NMR ($CDCl_3$): δ 1.25–1.63 (m, 8H), 2.01–2.03 (m, 2H), 2.93 (s, 3H), 3.25 (dd, 1H, *J* = 15.0, 9.5 Hz), 3.42 (dt, 2H, *J* = 12.6, 5.8 Hz), 3.75 (dd, 1H, *J* = 13.0, 6.0 Hz), 3.78 (s, 3H), 4.98 (br s, 1H), 6.87 (br s, 1H), 6.95 (br s, 2H), 6.99 (br s, 1H), 7.07–7.24 (m, 6H), 7.34–7.37 (m, 1H), 7.49 (br s, 2H), 8.11 (d, 1H, *J* = 3.0 Hz), 8.25 (br s, 1H). ESI-MS *m*/*z* 583 $(M-H)^-$, ESI-MS/MS *m*/*z* 445 (100), 316 (92), 301 (14). Anal. ($C_{32}H_{36}N_6O_5$) C, H, N.

*4.1.12.2. (S)-N-[1-(Pyridin-2-yl)-cyclohexylmethyl]-2-[3-(4-nitrophenyl)-1-methylureido]-3-(1H-indol-3-yl)propanamide ((S)-**11d**).* 6% Yield. ^{1}H NMR ($CDCl_3$): δ 1.24–1.64 (m, 8H), 2.00–2.07 (m, 2H), 1.37–1.39 (m, 1H), 2.92 (s, 3H), 3.22–3.30 (m, 1H), 3.42–3.49 (m, 2H), 3.64 (dd, 1H, *J* = 13.5, 6.0 Hz), 4.98 (br s, 1H), 6.84 (br s, 1H), 6.95 (br s, 1H), 7.00 (d, 1H, *J* = 1.2 Hz), 7.07–7.15 (m, 3H), 7.17–7.29 (m, 4H), 7.36 (d, 1H, *J* = 8.0 Hz), 7.48 (br s, 1H), 7.61 (dt, 1H, *J* = 7.7, 1.9 Hz), 8.08–8.11 (m, 1H), 8.15 (br s, 1H), 8.42–8.44 (m, 1H). ESI-MS *m*/*z* 553 $(M-H)^-$, ESI-MS/MS *m*/*z* 415 (100), 286 (57). Anal. ($C_{31}H_{34}N_6O_4$) C, H, N.

*4.1.12.3. (R)-N-Cyclohexylmethyl-2-[3-(4-nitrophenyl)-1-methylureido]-3-(1H-indol-3-yl)propanamide ((R)-**11f**).* 13% Yield. ^{1}H NMR (DMSO-d_6): δ 0.78–0.82 (m, 2H), 1.11–1.18 (m, 3H), 1.37–1.39 (m, 1H) 1.60–1.76 (m, 5H), 2.92 (s, 3H), 2.94–3.15 (m, 4H), 5.02–5.07 (m, 1H), 6.92–7.07 (m, 3H), 7.27 (d, 1H, J = 8.0 Hz), 7.59–7.64 (m, 3H), 7.99 (br s, 1H), 8.09 (d, 2H, J = 9.4 Hz), 9.07 (br s, 1H), 10.78 (s, 1H). ESI-MS m/z 476 $(M-H)^-$, ESI-MS/MS m/z 338 (100), 209 (24), 137 (17). Anal. ($C_{26}H_{31}N_5O_4$) C, H, N.

*4.1.12.4. (S)-N-Cyclohexylmethyl-2-[3-(4-nitrophenyl)-1-methylureido]-3-(1H-indol-3-yl)propanamide ((S)-**11f**).* 16% Yield. ^{1}H NMR (DMSO-d_6): δ 0.75–0.86 (m, 2H), 1.04–1.21 (m, 3H), 1.32–1.38 (m, 1H) 1.57–1.60 (m, 5H), 2.92 (s, 3H), 2.94–3.15 (m, 4H), 4.91–5.02 (q, 1H, J = 7.8 Hz), 6.91–7.06 (m, 3H), 7.27 (d, 1H, J = 8.0 Hz), 7.59–7.64 (m, 3H), 7.97 (br s, 1H), 8.10 (d, 2H, J = 9.3 Hz), 9.09 (br s, 1H), 10.77 (s, 1H). ESI-MS m/z 476 $(M-H)^-$, ESI-MS/MS m/z 338 (100), 209 (24), 137 (17). Anal. ($C_{26}H_{31}N_5O_4$) C, H, N.

*4.1.13. General procedure for the preparation of compounds **13a**,**g**, **15a**, (S)-**13h**,**J**–**l***

To a solution of amino acid **12**, **14**, or (*S*)-**12**, (0.75 mmol) in THF (50 mL) were added PyBOP (1.11 mmol), *N*-methylmorpholine (3.00 mmol) and the amine **2a**,**g**,**h**,**j**–**l** (0.90 mmol). The reaction mixture was stirred at room temperature overnight. The mixture was taken up with EtOAc (30 mL) and washed with H_2O (30 mL). The organic layer was dried (Na_2SO_4) and concentrated in vacuo. The crude residue was chromatographed ($CHCl_3$/MeOH, 19:1) to afford the pure target compound as colorless oil in 80–90% yield.

*4.1.13.1. 2-Amino-3-(1H-indol-3-yl)-N-[1-(5-methoxypyridin-2-yl)-cyclohexylmethyl]-2-methylpropionamide (**13a**).* ^{1}H NMR ($CDCl_3$): δ 1.31 (s, 3H), 1.37–1.58 (m, 12H), 1.92–2.11 (m, 2H), 3.32–3.39 (m, 2H), 3.79 (s, 3H), 6.97 (d, 1H, J = 6.5 Hz), 7.01 (d, 1H, J = 2.5 Hz), 7.05–7.19 (m, 3H), 7.32–7.36 (m, 1H), 7.56–7.61 (m, 2H), 8.19–8.20 (m, 1H), 8.33 (br s, 1H). ESI-MS m/z 421 $(M+H)^+$, ESI-MS/MS m/z 404 (88), 376 (20), 290 (95), 247 (100), 221 (39).

*4.1.13.2. 2-Amino-3-(1H-indol-3-yl)-N-[4-(4-methoxyphenyl)tetrahydropyran-4-yl-methyl]-2-methylpropionamide (**13g**).* ^{1}H NMR ($CDCl_3$): δ 1.33 (s, 3H), 1.56–1.98 (m, 8H), 3.40–3.65 (m, 4H), 3.69–3.79 (m, 2H), 3.75 (m, 3H), 6.70–6.75 (m, 2H), 6.90–6.93 (m, 2H), 6.96 (d, 1H, J = 2.2 Hz). 7.08–7.24 (m, 3H), 7.37 (d, 1H, J = 6.0 Hz), 7.60 (d, 1H, J = 9.0 Hz), 8.53 (br s, 1H). ESI-MS m/z 422 $(M+H)^+$, ESI-MS/MS m/z 405 (100), 291 (56), 248 (47), 158 (84).

*4.1.13.3. 2-Amino-N-[1-(5-methoxypyridin-2-yl)-cyclohexylmethyl]-2-methyl-3-phenylpropionamide (**15a**).* ^{1}H NMR ($CDCl_3$): δ 1.27 (s, 3H), 1.29–1.58 (m, 12H), 1.92–2.11 (m, 2H), 3.27–3.36 (m, 2H), 3.82 (s, 3H), 7.09–7.17 (m, 4H), 7.19–7.24 (m, 3H), 7.59 (br s, 1H), 8.22 (t, 1H, J = 1.8 Hz). ESI-MS m/z 382 $(M+H)^+$, ESI-MS/MS m/z 337 (17), 247 (34), 221 (100), 204 (72).

*4.1.13.4. (S)-2-Amino-3-(1H-indol-3-yl)-N-[1-(5-hydroxypyridin-2-yl)cyclohexylmethyl]-2-methylpropionamide ((S)-**13h**).* ^{1}H NMR (DMSO-d_6): δ 1.16–1.41 (m + s, 13H), 1.91–1.95 (m, 1H), 2.04–2.07 (m, 1H), 3.03–3.25 (m, 4H), 6.90–7.04 (m, 5H), 7.29 (d, 1H, J = 7.8 Hz), 7.51 (d, 1H, J = 7.8 Hz), 7.60 (br t, 1H, D_2O exchanged), 8.04 (s, 1H), 9.55 (s, 1H, D_2O exchanged), 10.88 (s, 1H, D_2O exchanged). ESI-MS m/z 405 $(M-H)^-$, ESI-MS/MS m/z 405 (11), 274 (100), 216 (50), 205 (41), 188 (22).

*4.1.13.5. (S)-2-Amino-3-(1H-indol-3-yl)-N-[4-(2-fluoroethoxy) phenyl)cyclohexylmethyl]-2-methylpropionamide ((S)-**13j**).* ^{1}H NMR (DMSO-d_6): δ 1.10–1.32 (m + s, 7H), 1.37–1.41 (m, 4H), 1.64–1.69 (m, 1H), 1.72 (br s, 2H), 1.87–1.89 (m, 1H), 2.73 (d, 1H, J = 14.0 Hz), 2.99–3.02 (m, 2H), 3.10–3.17 (m, 1H), 4.14 (dt, 2H, J_{H-F} = 30.3 Hz, J_{H-H} = 3.6 Hz), 4.70 (2H, J_{H-F} = 48.0 Hz, J_{H-H} = 3.6 Hz), 6.76 (d, 2H, J = 8.8 Hz), 6.92–7.06 (m, 4H), 7.26 (br t, 1H), 7.31 (d, 1H, J = 8.0 Hz), 7.53 (d, 1H, J = 7.7 Hz), 8.31 (s, 1H), 10.94 (s, 1H). ESI-MS m/z 450 $(M-H)^-$, ESI-MS/MS m/z 430 (100).

*4.1.13.6. (S)-2-Amino-3-(1H-indol-3-yl)-N-[1-(2-fluoropyridin-4-yl)-cyclohexylmethyl]-2-methylpropionamide ((S)-**13k**).* ^{1}H NMR ($CDCl_3$): δ 1.10–1.32 (m + s, 7H), 1.37–1.41 (m, 4H), 1.64–1.69 (m, 1H), 1.72 (br s, 2H), 1.87–1.89 (m, 1H), 2.72 (d, 1H, J = 14.3 Hz), 2.99–3.02 (m, 2H), 3.43 (d, 1H, J = 14.3 Hz), 6.65 (d, 1H, J = 0.8 Hz), 6.88–6.91 (m, 2H), 7.01–7.19 (m, 2H), 7.25–7.27 (m, 2H), 7.36–7.39 (m, 1H), 8.01 (d, 1H, J = 5.2 Hz), 8.30 (br s, 1H). ESI-MS m/z 407 $(M-H)^-$, ESI-MS/MS m/z 228 (100).

*4.1.13.7. (S)-2-Amino-3-(1H-indol-3-yl)-N-[1-(2-fluoropyridin-5-yl)-cyclohexylmethyl]-2-methylpropionamide ((S)-**13l**).* ^{1}H NMR ($CDCl_3$): δ 1.23–1.60 (m + s, 13H), 1.76–1.88 (m, 2H), 2.72 (d, 1H, J = 14.0 Hz), 3.21–3.27 (m, 2H), 3.41 (d, 1H, J = 14.0 Hz), 6.73 (dd, 1H, J = 8.8, 3.3 Hz), 6.90 (d, 1H, J = 2.2 Hz), 7.07–7.19 (m, 2H), 7.35–7.40 (m, 1H), 7.40 (br t, 1H), 7.48 (dt, 1H, J = 7.7, 2.8 Hz), 7.59 (d, 1H, J = 8.0 Hz), 7.96 (br d, 1H), 8.46 (br s, 1H). ESI-MS m/z 407 $(M-H)^-$, ESI-MS/MS m/z 279 (11), 278 (100), 276 (60).

*4.1.14. General procedure for the preparation of compounds **1a**,**g**, **16a**, **17a**, and (S)-**1j**–**l***

A mixture of the appropriate amine (1.0 mmol) and substituted phenylisocyanate (1.2 mmol) in anhydrous THF (20 mL) was stirred at room temperature for 24 h. The solvent was evaporated and the residue was treated with boiling MeOH. The mixture was filtered and the filtrate was concentrated under reduced pressure. The crude residue was chromatographed ($CHCl_3$/EtOAc, 1:1, as eluent) to afford the pure compound as a pale yellow solid.

*4.1.14.1. 3-(1H-Indol-3-yl)-N-[1-(5-methoxypyridin-2-yl)cyclohexylmethyl]-2-methyl-2-[3-(4-nitrophenyl)ureido]propionamide (**1a**).* 33% Yield. ^{1}H NMR (DMSO-d_6): δ 1.10–1.23 (m, 4H), 1.34–1.48 (m + s, 7H), 2.10 (br d, 2H), 2.99–3.07 (m, 2H), 3.23–3.28 (m, 2H), 3.66 (s, 3H), 6.55 (s, 1H), 6.80 (t, 1H, J = 7.2 Hz), 6.92–7.04 (m, 3H), 7.16 (d, 1H, J = 8.8 Hz), 7.26 (d, 2H, J = 8.0 Hz), 7.40 (d, 2H, J = 7.7 Hz), 7.60 (d, 2H, J = 9.1 Hz), 8.13–8.17 (m, 2H), 9.50 (s, 1H), 10.80 (s, 1H). ESI-MS m/z 583 $(M-H)^-$, ESI-MS/MS m/z 445 (100), 316 (8). Anal. ($C_{32}H_{36}N_6O_5$) C, H, N.

*4.1.14.2. 3-(1H-Indol-3-yl)-N-[4-(4-methoxyphenyl)tetrahydropyran-4-ylmethyl]-2-methyl-2-[3-(4-nitrophenyl)ureido]propionamide (**1g**).* 44% Yield. ^{1}H NMR ($CDCl_3$): δ 1.22 (s, 3H), 1.61–1.92 (m, 4H), 2.96 (dd, 1H, J = 8.5, 13.8 Hz), 3.03 (d, 1H, J = 14.6 Hz), 3.29–3.48 (m + s, 7H), 3.56–3.63 (m, 2H), 5.89 (br s, 2H), 6.38 (d, 2H, J = 9.0 Hz), 6.71–6.90 (m, 4H), 6.92 (d, 1H, J = 9.0 Hz), 7.13 (d, 1H, J = 8.0 Hz), 7.32 (d, 1H, J = 8.0 Hz), 7.44 (d, 2H, J = 9.0 Hz), 7.99 (d, 2H, J = 9.0 Hz), 8.73 (s, 1H), 9.73 (s, 1H). ESI-MS m/z 584 $(M-H)^-$, ESI-MS/MS m/z 446 (100), 317 (8). Anal. ($C_{32}H_{35}N_5O_6$) C, H, N.

*4.1.14.3. N-[1-(5-Methoxypyridin-2-yl)cyclohexylmethyl]-2-methyl-2-[3-(4-nitrophenyl)ureido]-3-phenylpropionamide (**16a**).* 38% Yield. ^{1}H NMR ($CDCl_3$): δ 1.15–1.20 (m, 2H), 1.25–1.37 (m, 2H), 1.41 (s, 3H), 1.51–1.51 (m, 4H), 1.96–2.11 (m, 2H), 3.18–3.30 (m, 3H), 3.44 (dd, 1H, J = 5.8, 12.9 Hz), 3.62 (s, 3H), 6.18 (br d, 1H), 6.92–7.00 (m, 3H), 7.02–7.12 (m, 3H), 7.13–7.18 (m, 2H), 7.43–7.47 (m, 2H), 7.99–8.09 (m, 3H), 8.95 (s, 1H). ESI-MS m/z 544 $(M-H)^-$, ESI-MS/MS m/z 406 (100). Anal. ($C_{30}H_{35}N_5O_5$) C, H, N.

*4.1.14.4. 3-(1H-Indol-3-yl)-2-[3-(4-methoxyphenyl)ureido]-N-[1-(5-methoxypyridin-2-yl)cycloexylmethyl]-2-methylpropionamide (**17a**).* 52% Yield. ^{1}H NMR (DMSO-d_6): δ 1.13–1.23 (m, 4H), 1.31 (s, 3H), 1.37–1.49 (m, 4H), 2.13 (br d, 2H), 3.05 (dd, 1H, J = 12.9,

5.8 Hz), 3.18–3.26 (m, 1H), 3.34–3.38 (m, 2H), 3.65 (s, 3H), 3.69 (s, 3H), 6.04 (s, 1H), 6.81–6.86 (m, 3H), 6.94–7.00 (m, 3H), 7.18–7.30 (m, 5H), 7.42 (d, 1H, *J* = 8.0 Hz), 8.17 (d, 1H, *J* = 3.0 Hz), 8.42 (s, 1H), 10.82 (s, 1H). ESI-MS *m/z* 568 $(M-H)^-$, ESI-MS/MS *m/z* 445 (100), 419 (90), 316 (16). Anal. ($C_{33}H_{39}N_5O_4$) C, H, N.

*4.1.14.5. (S)-3-(1H-Indol-3-yl)-N-[1-(5-hydroxypyridin-2-yl)cyclohexylmethyl]-2-methyl-2-[3-(4-nitrophenyl)ureido]propionamide ((S)-**1h**).* 19% Yield: ^{1}H NMR (DMSO-d_6): δ 1.10–1.23 (m, 4H), 1.34–1.48 (m + s, 7H), 2.10 (br d, 2H), 2.99–3.07 (m, 2H), 3.23–3.28 (m, 2H), 6.55 (s, 1H), 6.80 (t, 1H, *J* = 7.2 Hz), 6.92–7.04 (m, 3H), 7.16 (d, 1H, *J* = 8.8 Hz), 7.26 (d, 2H, *J* = 8.0 Hz), 7.40 (d, 2H, *J* = 7.7 Hz), 7.60 (d, 2H, *J* = 9.1 Hz), 8.13–8.17 (m, 2H), 9.50 (s, 1H), 10.80 (s, 1H), 10.99 (s, 1H). ESI-MS *m/z* 569 $(M-H)^-$, ESI-MS/MS *m/z* 430 (100), 242 (8).

*4.1.14.6. (S)-3-(1H-Indol-3-yl)-N-[1-[4-(2-fluoroethoxy)phenyl]cyclohexylmethyl]-2-methyl-2-[3-(4-nitrophenyl)ureido]propionamide ((S)-**1j**).* Yield: 33%. ^{1}H NMR (DMSO-d_6): δ 1.13–1.18 (m, 4H), 1.36–1.47 (m + s, 7H), 2.01 (br s, 2H), 2.92 (dd, 1H, *J* = 12.9 Hz) 3.21 (dd, 1H, *J* = 12.9 Hz), 3.38 (br s, 2H), 3.97–4.10 (m, 2H), 4.57–4.75 (m, 2H), 6.50 (s, 1H), 6.70 (d, 2H, *J* = 8.0 Hz), 6.77–6.82 (m, 1H), 6.92–6.99 (m, 2H), 7.15–7.27 (m, 4H), 7.42 (d, 1H, *J* = 8.0 Hz), 7.63–7.66 (m, 2H), 8.15–8.19 (m, 2H), 9.49 (br s, 1H), 10.82 (br s, 1H). ^{13}C NMR (DMSO-d_6): 176.8; 158.9; 156.9; 152.1; 150.9; 146.7; 145.7; 141.3; 132.6; 130.4; 128.8; 126.1; 123.7; 123.4; 123.1; 121.9; 116.4; 114.8; 84.4; 72.9; 58.7; 53.8; 50.2; 45.3; 37.5; 37.4; 33.8; 30.8; 27.1; 22.9. ESI-MS *m/z* 614 $(M-H)^-$, ESI-MS/MS *m/z* 476 (100), 347 (7). Anal. ($C_{34}H_{38}FN_5O_5$) C, H, N.

*4.1.14.7. (S)-3-(1H-Indol-3-yl)-N-[1-(2-fluoropyridin-4-yl)cyclohexylmethyl]-2-methyl-2-[3-(4-nitrophenyl)ureido]propionamide ((S)-**1k**).* ^{1}H NMR (DMSO-d_6): δ 1.06–1.09 (m, 2H), 1.21–1.26 (m, 2H), 1.39 (s, 3H), 1.51–1.54 (m, 4H), 2.03 (br d, 2H), 3.00 (dd, 1H, *J* = 13.2, 5.2 Hz), 3.26–3.44 (m, 3H), 6.50 (br s, 1H), 6.77 (t, 1H, *J* = 7.4 Hz), 6.94–7.02 (m, 3H), 7.15 (br d, 1H), 7.25 (d, 1H, *J* = 8.3 Hz), 7.40 (d, 1H, *J* = 8.3 Hz), 7.53 (br t, 1H), 7.63 (d, 2H, *J* = 9.1 Hz), 7.90 (d, 1H, *J* = 5.2 Hz), 8.15 (d, 2H, *J* = 9.1 Hz), 9.53 (br s, 1H), 10.81 (br s, 1H). ^{13}C NMR (DMSO-d_6): 171.9; 166.0; 164.1; 157.4; 147.4; 143.5; 136.5; 136.3; 128.3; 127.8; 125.7; 123.9; 121.3; 118.9; 118.7; 117.1; 113.9; 111.7; 110.1; 55.1; 53.7; 49.0; 42.2; 33.5; 33.1; 29.0; 25.3; 22.9; 21.3. ESI-MS *m/z* 571 $(M-H)^-$, ESI-MS/MS *m/z* 433 (100), 304 (9). Anal. ($C_{31}H_{33}FN_6O_4$) C, H, N.

*4.1.14.8. (S)-3-(1H-Indol-3-yl)-N-[1-(2-fluoropyrid-5-yl)cyclohexylmethyl]-2-methyl-2-[3-(4-nitrophenyl)ureido]propionamide ((S)-**1l**).* 10% Yield. ^{1}H NMR (DMSO-d_6): δ 1.12–1.28 (m, 4H), 1.40 (s, 3H), 1.50–1.54 (m, 4H), 1.97–2.04 (m, 2H), 3.00 (dd, 1H, *J* = 13.2, 5.2 Hz), 3.26–3.44 (m, 3H), 6.48 (br s, 1H), 6.77–6.89 (m, 3H), 6.96 (t, 1H, *J* = 7.1 Hz), 7.25 (d, 1H, *J* = 8.0 Hz), 7.41 (d, 1H, *J* = 8.0 Hz), 7.60–7.63 (m, 3H), 7.75 (dt, 1H, *J* = 8.0, 2.5 Hz), 8.12–8.17 (m, 3H), 9.52 (br s, 1H), 10.81 (br s, 1H). ^{13}C NMR (DMSO-d_6): 171.9; 163.1; 157.4; 154.2; 147.4; 140.9; 136.5; 136.3; 128.3; 127.8; 125.7; 123.9; 121.3; 118.9; 118.7; 117.1; 113.9; 111.7; 110.1; 55.1; 53.7; 49.0; 42.2; 33.5; 33.1; 29.0; 25.3; 22.9; 22.3. ESI-MS *m/z* 571 $(M-H)^-$, ESI-MS/MS *m/z* 433 (100), 304 (6). Anal. ($C_{31}H_{33}FN_6O_54$ C, H, N.

*4.1.14.9. (S)-3-(1H-Indol-3-yl)-N-[1-[5-(2-fluoroethoxy)pyridin-2-yl]-cyclohexylmethyl]-2-methyl-2-[3-(4-nitrophenyl)ureido]propionamide ((S)-**1m**).* 2-Fluoroethyltosylate (0.039 g, 0.18 mmol) was added to a mixture of (*S*)-**1h** (0.050 g, 0.88 mmol) and Cs_2CO_3 (0.118 g, 0.36 mmol) in acetone. The reaction mixture was refluxed for 8 h (TLC monitoring), then the solvent was removed in vacuo. The residue was taken up with EtOAc, washed with H_2O, dried over Na_2SO_4, and concentrated under reduced pressure. The crude residue was chromatographed ($CHCl_3$/EtOAc, 7:3 as eluent) to afford pure (*S*)-**1m** (0.015 g, 28% yield). ^{1}H NMR (DMSO-d_6): δ 1.10–1.32 (m, 4H), 1.43 (s, 3H), 1.47–1.56 (m, 4H), 2.00–2.13 (m, 2H), 3.03 (dd, 1H, *J* = 12.9, 5.8 Hz), 3.10–3.17 (m, 3H), 4.14 (dt, 2H, J_{H-F} = 30.3 Hz, J_{H-H} = 3.8 Hz), 4.53 (2H, J_{H-F} = 40.4 Hz, J_{H-H} = 3.8 Hz), 6.52 (s, 1H), 6.80 (t, 1H, *J* = 7.2 Hz), 6.92–7.04 (m, 3H), 7.16 (d, 1H, *J* = 8.8 Hz), 7.26 (d, 2H, *J* = 8.0 Hz), 7.40 (d, 2H, *J* = 7.7 Hz), 7.60 (d, 2H, *J* = 9.1 Hz), 8.13–8.17 (m, 2H), 9.50 (s, 1H), 10.80 (s, 1H). ^{13}C NMR (DMSO-d_6): 171.9; 157.4; 154.2; 147.4; 140.9; 136.5; 136.3; 128.3; 127.8; 125.7; 123.9; 121.3; 118.9; 118.7; 117.1; 113.9; 111.7; 110.1; 84.4; 72.9; 55.1; 53.9; 49.0; 42.2; 33.5; 33.1; 29.0; 26.3; 22.9; 22.1. ESI-MS *m/z* 615 $(M-H)^-$, ESI-MS/MS *m/z* 477 (100), 348 (5). Anal. ($C_{33}H_{37}FN_6O_5$) C, H, N.

4.2. Semi-preparative HPLC enantioseparation on chiral stationary phase

4.2.1. Instrumentation

The chromatographic apparatus consisted of a Shimadzu LC-10AD Pump (Shimadzu Italia, Milan), a Rheodyne 7125 manual injector equipped with a 200 μl sample loop (Jasco Europe, Italy, Milan). A Merck Hitachi L-7400UV (Merck KGaA, Darmstadt, Germany) was used as detector. Chromatograms were recorded with a HSM Hitachi chromatography software. The column used was a Chiralcel OD (cellulose tris(3,5-dimethylphenylcarbamate), 250 × 10 mm, 10 μm particle size)) purchased from Chiral Technologies Europe (Illkirch, France). HPLC-grade *n*-hexane and 2-propanol (IPA) were obtained from Sigma-Aldrich (Milan, Italy); triethylamine (TEA) was obtained from Fluka (Milan, Italy).

4.2.2. Chromatographic parameters

The separation factor (α) was calculated as k'_2/k'_1 and retention factors (k'_1 and k'_2) as $k'_1 = (t_1 - t_0)/t_0$ where t_1 and t_2 refer to retention times of the first and second eluted enantiomers. The resolution factor (R_s) was calculated by the formula $R_s = 2(t_2 - t_1)/(w_1 + w_2)$ where w_1 and w_2 are the peak widths at base for the first and second eluted enantiomers. The dead time of the column (t_0) was determined by injection of 1,3,5-tri-*tert*-butylbenzene.

*4.2.3. Semipreparative enantioseparation of compounds **1a**,**g**, **17a***

Details on the separation of the enantiomerically enriched samples **1aE1**, **1aE2**, **1gE1**, **1gE2**, **17aE1**, **17aE2** are given in Table 4.

4.3. Radiochemistry

*4.3.1. (S)-3-(1H-Indol-3-yl)-N-[1-[5-(2-[^{18}F]fluoroethoxy)pyridin-2-yl]cyclohexylmethyl]-2-methyl-2-[3-(4-nitrophenyl)ureido]propionamide ([^{18}**F**](S)-**1m**)*

Aqueous [^{18}F]fluoride (2–5 GBq) was produced by irradiation of ^{18}O-water with an IBA 18/18 cyclotron via the ^{18}O(p,n)^{18}F nuclear reaction. The [^{18}F]-fluoride solution was passed through a SepPak Light Accell plus QMA anion exchange cartridge (Waters, Milford, MA, USA) to recover the ^{18}O-enriched water. [^{18}F]fluoride was eluted with a solution of K_2CO_3 (1 mg/mL) into a V-vial containing 15 mg of Kryptofix 2.2.2. [^{18}F]-Fluoride was dried by three consecutive azeptropical evaporations with dry acetonitrile. 2-[^{18}F]-Fluoroethyl tosylate was prepared by fluorination at 125 °C for 5 min of the ditosylate precursor (5 mg in 0.5 mL of dry acetonitrile) followed by Sep-Pak Silica plus purification using 14 mL of hexane/diethyl ether (3:1, v/v). After evaporation to dryness, 2-[^{18}F]fluoroethyl tosylate was dissolved in DMF (0.8 mL) and added to a solution of **1h** (2 mg, 0.0035 mmol) and NaH (2 mg) in DMF (0.2 mL). The reaction mixture was heated for 5 min at 125 °C. Hereafter water (2 mL) was added to the cooled mixture. The reactor was rinsed with an additional 1 mL of water. The reaction mixture was purified by HPLC using a microBondapak C18

Table 4
Experimental details for the separation of compounds **1aE1**, **1aE2**, **1gE1**, **1gE2**, **17aE1**, **17aE2**.

Compd	Mobile Phase	Flow rate	k′	α	ee%
1aE1[a]	*n*-Hexane/IPA (8:2, v/v)	4.5 mL/min	4.11	1.30	93
1aE2[b]			5.32		90
1gE1	*n*-Hexane/IPA (8:2, v/v)	2.5 mL/min	9.37	1.18	92
1gE2			11.03		89
17aE1	*n*-Hexane/IPA/TEA(8:2:0.01, v/v)	2.5 mL/min	15.14	1.20	92
17aE2			18.24		90

[a] E1: faster eluting enantiomer.
[b] E2: later eluting enantiomer.

(300 × 7.8 mm) with 10 mM sodium acetate/MeCN (4:6, v/v) as mobile phase with a flow rate of 3 mL/min. The retention time was 16 min. The collected HPLC-fraction was diluted with 20 mL of water and passed through an C18-Sep-Pak. [^{18}F](*S*)-**1m** was eluted with ethanol (0.8 mL) and formulated with saline (5 mL). Quality control was performed by UPLC, using a HSS T3 column (1.8 μm, 3.0 × 50 mm) with the following gradient: t = 0–5 min: 90% 10 mM ammonium bicarbonate (pH 9.5) and 10% MeCN; t = 5–6 min: 40% 10 mM ammonium bicarbonate (pH 9.5) and 60% MeCN; t = 6–7 min 70% 10 mM ammonium bicarbonate (pH 9.5) and 30% MeCN at a flow rate of 0.8 mL/min (retention time: [^{18}F]fluoride = 0.5 min, [^{18}F](*S*)-**1m** = 4.75 min). Thus, [^{18}F](*S*)-**1m** was obtained within 120 min in 3.37 ± 0.26% radiochemical yield (decay-corrected). At the end of synthesis (EOS), the specific activity was 9.64 ± 5.29 GBq/mmol and the radiochemical purity was always higher than 95%.

4.4. Receptor binding studies

4.4.1. General

PC3 human prostate cell line was obtained from Interlab Cell Line Collection (ICLC, Genoa, Italy). Cell culture reagents were purchased from EuroClone (Milan, Italy). Human cloned BB2 receptor expressed in HEK-293 cells, [^{125}I]-(Tyr4)-bombesin (2200 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). hGRP and bombesin were purchased from Tocris Bioscience (Bristol, UK).

4.4.2. Cells culture

PC3 cell lines was routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin in a humidified incubator at 37 °C with a 5% CO_2 atmosphere.

4.4.3. Saturation binding assay

The saturation experiments were carried out as described by Benya et al. with minor modifications [24]. Membranes of human PC3 prostate cells were prepared according to Colabufo et al. [42] BB2 receptor were radiolabeled using [^{125}I]-(Tyr4)-bombesin concentrations of 0.01–0.7 nM. Samples contained 100 μg of PC3 membranes, radioligand and hGRP (1 μM) to determine non-specific binding were incubated in a final volume of 500 μL (Hepes 20 mM pH 7.4, 3 mM $MgCl_2$, 1 mM EDTA, 0.3% BSA) for 60 min at 27 °C. The suspension was filtered through Whatman GF/C glass microfiber filters presoaked in 0.5% polyethylenimine for at least 30 min prior to use. The filters were washed 3 × 1 mL ice-cold buffer (50 mM Tris-HCl, pH 7.4). Scatchard parameters were determined by nonlinear curve fitting, using the Prism, version 3.0, GraphPad software [43] (K_d = 0.66 nM; B_{max} = 358 fmol/mg of protein).

4.4.4. Competition binding assays

BB2 receptor binding was carried out according to Benya et al. with minor modifications [24]. In 0.5 mL of incubation buffer (20 mM Hepes pH 7.4, 3 mM $MgCl_2$, 1 mM EDTA, 0.3% BSA) were suspended 100 μg of PC3 cells membranes or 0.32 μg of human cloned BB2 receptor, 0.02 nM [^{125}I]-(Tyr4)-bombesin, test or reference compounds. The samples were incubated for 60 min at 27 °C. The incubation was stopped by rapid filtration on Whatman GF/C glass microfiber filters (presoaked in 0.5% polyethylenimine for 30 min). The filters were washed with 3 × 1 mL of ice-cold buffer (50 mM Tris-HCl, pH 7.4). Nonspecific binding was determined in the presence of 1 μM hGRP. Approximately 90% of specific binding was determined under these conditions. Concentration required to inhibit 50% of radioligand specific binding (IC_{50}) was determined by using six to nine different concentrations of the drug studied in two or three experiments with samples in duplicate. Apparent inhibition constant (K_i) values were determined by nonlinear curve fitting, using the Prism, version 3.0, GraphPad software [43].

4.5. Intracellular calcium mobilization

Changes in intracellular Ca^{2+} was measured in human PC3 cells with a Infinite® Tecan multimode reader using the Fluo-4 Direct™ Calcium Assay Kit (Molecular Probes, Inc.) as previously described [9]. Cells (25,000 cells per well) were suspended in culture medium, seeded in clear bottom black 96-well plates and cultured for 1 day at 37 °C and 5% CO_2. After incubation time, the culture medium was removed and replaced with 50 μL/well of culture medium and 50 μL/well of Fluo-4 Direct™ dye dissolved in assay buffer containing 5 mM probenecid. The plate was incubated for 90 min at 37 °C and 5% CO_2. After addition of test compound, changes in fluorescence were recorded (λ_{ex} = 494 nm, λ_{em} = 520 nm) every 5 s for 120 s. Maximum change in fluorescence, expressed in arbitrary units over baseline, was used to determine agonist response. Maximum fluorescence was measured after addition of ionomycin and baseline measurements were taken for untreated cells. The dose-response curve for bombesin (Tocris Bioscience, UK) was obtained incubating cells with 5–6 concentrations of agonist (10^{-11}–10^{-6} M). The dose-response curves for the antagonists were obtained incubating cells with 1 nM bombesin after 10 min pretreatment with different concentrations of test compounds (10^{-10}–10^{-5} M). All experiments were performed 3 times in triplicate. Curve fitting (5–6 points) and calculation of median effective concentration values (EC_{50}) were performed by nonlinear regression analysis of the dose-response curves generated using Prism 3 [43].

4.6. Serum stability

The rat serum was diluted in PBS (1:1, v/v) and pre-incubated at 37 °C for 5 min. The reaction was initiated by adding the appropriate amount of the stock solution of the tested compounds in DMSO. The final concentration of the tested compound was 10 μM (the amount of DMSO did not exceed 1.25%, v/v). Time samples aliquots (100 μl) were removed and immediately mixed with cold acetonitrile (400 μl) containing the internal standard. Quenched samples

were centrifugated at 3500 rpm for 5 min and the supernatants were directly injected for quantification analysis. Samples (100 μl) were analyzed by using an Agilent 1260 Infinity Binary LC System equipped with a diode array detector (Open Lab software was used to analyze the chromatographic data) and a Phenomenex Gemini C-18 column (250 × 4.6 mm, 5 μm particle size). The samples were eluted using MeOH/water (70:30, v/v) as eluent (1 mL/min). The *in vitro* serum half life ($t_{1/2}$) was calculated using the expression $t_{1/2} = 0.693/b$, where b is the slope found in the linear fit of the natural logarithm of the fraction remaining of the parent compound vs incubation time.

4.7. Stability assays in rat liver microsomes

Test compounds were pre-incubated at 37 °C with rat liver microsomes (Tebu-Bio, Milan, Italy) (1.0 mg/mL microsomal protein) at 10 μM final concentration in 100 mM potassium phosphate buffer (pH 7.4) for 10 min. Metabolic reactions were initiated by the addition of the NADPH regenerating system (containing 10 mM NADP, 50 mM glucose-6-phosphate, and 10 unit/mL glucose-6-phosphate dehydrogenase, final glucose-6-phosphate dehydrogenase concentration, 1 unit/mL). Aliquots were removed at specific time endpoints and immediately mixed with an equal volume of cold acetonitrile containing the internal standard. Test compound incubated with microsomes without NADPH regenerating system was included. Quenched samples were centrifugated at 4500 rpm for 15 min and the supernatants were injected for quantification analysis. Samples (100 μL) were analyzed by using an Agilent 1260 Infinity Binary LC System equipped with a diode array detector (Open Lab software was used to analyze the chromatographic data) and a Phenomenex Gemini C-18 column (250 × 4.6 mm, 5 μm particle size). The samples were eluted using CH_3CN/20 mM ammonium formate pH 5.5 (70:30, v/v) as eluent (1 mL/min). Concentrations were quantified by measuring the area under the peak.

The *in vitro* half life ($t_{1/2}$) was calculated using the expression $t_{1/2} = 0.693/b$, where b is the slope found in the linear fit of the natural logarithm of the fraction remaining of the parent compound vs incubation time [44]. *In vitro* half-life was then used to calculate the intrinsic plasma clearance (CL_{int}) according to the following equation:

$$CL_{int} : \frac{0.693}{\text{In vitro } t_{1/2}} \times \frac{1}{\text{mg/ml microsomial protein}}$$

4.8. [^{18}F](*S*)-***1m*** stability

The formulated tracer (ethanol/water) was incubated at room temperature and analyzed by UPLC at 1 and 2 h post incubation.

4.9. Uptake study

For uptake experiments, PC-3 cells were plated in 24-well plates at a density of 0.1 million cells per well. After 24 h, the medium was discarded, the cells were washed twice with warm and new medium was added. Then, [^{18}F](*S*)-**1m** (ca 100 kBq/well) was incubated with cells at 37 °C for 0, 15, 30, 60 and 120 min in triplicate to allow for cellular uptake. Glu[Aca-BN(7–14)]$_2$ (20 μg) was co-incubated with [^{18}F](*S*)-**1m** in blocking groups at the same time points. To remove unbound radioactivity, the cells were washed twice with cold PBS and the cells were harvested with trypsin (100 μL/well) and resuspended in 900 μL of RPMI 1640 (Vt = 1 mL). The cell suspensions were collected for each well and the radioactivity was measured using a gamma counter. A 50 μl sample of each suspension was mixed with 50 μl of Trypan blue and used for cell counting. Cell numbers were manually determined, using a phase-contrast microscope, a Bulker bright-line chamber (depth, 0.1 mm:0.0025 mm^2) and a hand-tally counter. Cellular uptake of the radioactivity was corrected for the number of viable cells. Results are expressed as percentage of incubated radioactivity per million cells (mean ± SD).

4.10. Dissociation kinetic study

For the dissociation study, 0.1 million PC-3 cells were plated per well in 24-well plates one day before the experiments and kept at 37 °C in a humidified 5% CO_2 atmosphere. On the day of the experiments, the cells were washed twice with warm PBS and new RPMI 1640 was added to the wells. The cells were then incubated with 50 μl of [^{18}F](*S*)-**1m** (ca 100 kBq/well) for 1 h at 37 °C to allow for maximum internalization. To remove unbound radioactivity, the cells were washed twice afterwards with cold PBS and were then incubated with warm RPMI 1640 medium at 37 °C for 0, 15, 30, 60 and 120 min in triplicate to allow for externalization. At the end of each time point, the medium was discarded, the cells washed with cold PBS, harvested with trypsin (100 μL/well) and resuspended in 900 μL of RPMI 1640 (Vt = 1 mL). The cell suspensions were collected for each well and the radioactivity was measured using a gamma counter. A 50 μL sample of each suspension was mixed with 50 μL of Trypan blue and used for cell counting. Cell numbers were manually determined in the same manner as mentioned above. Cellular uptake of the radioactivity was corrected for the number of viable cells. Results are expressed as the percentage of maximum intracellular radioactivity (remaining activity at specific time-point/activity at time point 0, mean ± SD).

4.11. Competition study with [^{18}F](*S*)-***1m***

PC-3 cells were plated in duplicate in a 24-well plate at a density of 0.1 million cells per well. After one day, the medium was discarded and the cells were washed twice with PBS and 900 μL of warm RPMI 1640 medium was added per well. Next, 50 μL of [^{18}F](*S*)-**1m** (ca 100 kBq/well) solution was added in the absence or presence of different concentrations of unlabeled Glu[Aca-BN(7–14)]$_2$. After the cells were incubated for 60 min, the medium was discarded. The cells were washed with cold PBS (3 × 0.5 mL/well), harvested with trypsin (100 μL) and resuspended in 900 μL of RPMI. The cell suspensions were collected separately for each well and the radioactivity was measured using a gamma counter. A 50 μL sample of the suspension was mixed with 50 μL of trypan blue and used for cell counting. Cell numbers were manually determined in the same manner as mentioned above. Cellular uptake of the radioactivity was corrected for the number of viable cells. Results are plotted as sigmoid curves for the displacement of [^{18}F](*S*)-**1m** as a function of increasing concentrations of Glu[Aca-BN(7–14)]$_2$ (mean ± SD).

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2016.10.031.

References

[1] Jensen RT, Battey JF, Spindel ER, Benya RV. *Pharmacol. Rev*. 2008;60:1–42.
[2] Markwalder R, Reubi JC. *Cancer Res*. 1999;59:1152–1159.
[3] Fleischmann A, Waser B, Reubi JC. *Cell. Oncol*. 2007;29:421–433.
[4] Gugger M, Reubi JC. *Am. J. Pathol*. 1999;155:2067–2076.
[5] Sancho V, Di Florio A, Moody TW, Jensen RT. *Curr. Drug Deliv*. 2011;8:79–134.
[6] Yu Z, Ananias HJ, Carlucci G, Hoving HD, Helfrich W, Dierckx RA, Wang F, de Jong IJ, Elsinga PH. *Curr. Pharm. Des*. 2013;19:3329–3341.
[7] Maina T, Nock B, Mather S. *Cancer Imaging*. 2006;6:153–157.
[8] Mansi R, Wang X, Forrer F, Kneifel S, Tamma ML, Waser B, Cescato R, Reubi JC, Maecke HR. *Clin. Cancer Res*. 2009;15:5240–5249.
[9] Cescato R, Maina T, Nock B, Nikolopoulou A, Charalambidis D, Piccand V, Reubi JC. *J. Nucl. Med*. 2008;49:318–326.
[10] Kwekkeboom DJ, Bakker WH, Kooij PPM, Erion J, Srinivasan A, de Jong M, Reubi JC, Krenning EP. *Eur. J. Nucl. Med*. 2000;27:1312–1317.
[11] Cai H, Conti PS. *J. Labelled Comp. Radiopharm*. 2013;56:264–279.
[12] Ma Y, Yang M, Gao H, Niu G, Yan Y, Lang L, Kiesewetter DO, Chen X. *Amino Acids*. 2012;43:1625–1632.
[13] Marsouvanidis PJ, Maina T, Sallegger W, Krenning EP, de Jong M, Nock BA. *J. Nucl. Med*. 2013;54:1797–1803.
[14] Zhang X, Cai W, Cao F, Schreibmann E, Wu Y, Wu JC, Xing L, Chen X. *J. Nucl. Med*. 2006;47:492–501.
[15] Zhang H, Abiraj K, Thorek DL, Waser B, Smith-Jones PM, Honer M, Reubi JC, Maecke HR. *PLoS One*. 2012;7:e44046.
[16] Mason JM. Future. *Med. Chem*. 2010;2:1813–1822.
[17] Ametamey SM, Honer M, Schubiger PA. *Chem. Rev*. 2008;108:1501–1516.
[18] Okarvi SM. *Eur. J. Nucl. Med*. 2001;28:929–938.
[19] Moody TW, Leyton J, Unsworth E, John C, Lang LX, Eckelman WC. *Peptides*. 1998;19:585–592.
[20] Wester HJ, Hamacher K, Stocklin G. *Nucl. Med. Biol*. 1996;23:365–372.
[21] Ting R, Harwig C, auf dem Keller U, McCormick S, Austin P, Overall CM, Adam TJ, Ruth TJ, Perrin DM. *J. Am. Chem. Soc*. 2008;130:12045–12055.
[22] Campbell-Verduyn LS, Mirfeizi L, Schoonen AK, Dierckx RA, Elsinga PH, Feringa BL. *Angew. Chem. Int. Ed. Engl*. 2011;50:11117–11120.
[23] Ashwood V, Brownhill V, Higginbottom M, Horwell DC, Hughes J, Lewthwaite AT, McKnight AT, Pinnock RD, Pritchard MC, Suman-Chauhan N, Webb C, Williams SC. *Bioorg. Med. Chem. Lett*. 1998;8:2589–2594.
[24] Benya RV, Kusui T, Pradhan TK, Battey JF, Jensen RT. *Mol. Pharm*. 1995;47:10–20.
[25] Gonzalez N, Mantey SA, Pradhan TK, Sancho V, Moody TW, Coy DH, Jensen RT. *Peptides*. 2009;30:1473–1486.
[26] D. C. Horwell, M. C. Pritchard, PCT Int. Appl. (1998), WO 9,807,718 A1.
[27] Lacivita E, Schepetkin IA, Stama ML, Kirpotina LN, Colabufo NA, Perrone R, Khlebnikov AI, Quinn MT, Leopoldo M. *Bioorg. Med. Chem*. 2015;23:3913–3924.
[28] Zhang L, Finn JM. *J. Org. Chem*. 1995;60:5719–5720.
[29] Nájera C, Abellán T, Sansano JM. *Eur. J. Org. Chem*. 2000;2000:2809–2820.
[30] Eden JM, Hall MD, Higginbottom M, Horwell DC, Howson W, Hughes J, Jordan RA, Lewthwaite RA, Martin K, McKnight AT, O'Toole JC, Pinnock RD, Pritchard N, Suman-Chauhan N, Williams SC. *Bioorg. Med. Chem. Lett*. 1996;6:2589–2594.
[31] Tannert R, Milroy LG, Ellinger B, Hu TS, Arndt HD, Waldmann H. *J. Am. Chem. Soc*. 2010;132:3063–3077.
[32] Carrieri A, Lacivita E, Belviso BD, Caliandro R, Mastrorilli P, Gallo V, Niso M, Leopoldo M. *Curr. Top. Med. Chem*. 2017. In press.
[33] K. Hasumi, S. Ohta, T. Saito, S. Sato, J.-Y. Kato, J. Sato, H. Suzuki, H. Asano, M. Okada, Y. Matsumoto, S.K. Shirota, PTC Int. Appl. (2008) WO 2,008,001,930, A1.
[34] J. A. McCauley, C. R. Theberge, N. J. Liverton, D. A. Claremon, C. F. Claiborne, PCT Int. Appl. (2001), WO 2,001,032,174, A1.
[35] Lacivita E, De Giorgio P, Patarnello D, Niso M, Colabufo NA, Berardi F, Perrone G, Satala G, Duszynska B, Bojarski AJ, Leopoldo M. *Exp. Brain Res*. 2013;230:569–582.
[36] Carlucci G, Kuipers A, Ananias HJ, de Paula Faria D, Dierckx RA, Helfrich W, Rink R, Moll GN, de Jong IJ, Elsinga PH. *Peptides*. 2015;67:45–54.
[37] Elsinga PH, Kawamura K, Kobayashi T, Tsukada H, Senda M, Vaalburg W, Ishiwata K. *Synapse*. 2002;43:259–267.
[38] Carlucci G, Ananias HJ, Yu Z, Hoving HD, Helfrich W, Dierckx RA, Liu S, de Jong PH, Elsinga PH. *Mol. Pharm*. 2013;10:1716–1724.
[39] S.G. Mueller, C.J. Brown, A. Heifetz, B. Nosse, J. Prestle, N. Prevost, K. Rudolf, S. Scheuerer, M. Schindler, D. Stenkamp, L. Thomas, H. Tye, PCT Int. Appl. (2010), WO 2,010,060,952, A1.
[40] A. Marinier, C. Quesnelle, M. Dodier, S. Roy, P. Gill, M. Wittman, D. R. Langley, PTC Int. Appl. (2008) WO 2,008,079,873, A2.
[41] Shuttleworth SJ, Lizarzaburu ME, Chai A, Coward P. *Bioorg. Med. Chem. Lett.*. 2004;14:3037–3042.
[42] Colabufo NA, Berardi F, Contino M, Niso M, Abate C, Perrone R, Tortorella V. *Naunyn-Schmiedeberg's Arch. Pharmacol*. 2004;370:106–113.
[43] *Prism Software, version 3.0 for Windows*. San Diego, CA: GraphPad Software Inc; 1998.
[44] Obach RS, Baxter JG, Liston TE, Silber BM, Jones BC, MacIntyre F, Rance DJ, Wastall P. *J. Pharmacol. Exp. Ther*. 1997;283:46–58.