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Biotin Ergopeptide Probes for Dopamine Receptors

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The incorporation of chemical modifications into the structure of bioactive compounds is often difficult because the biological properties of the new molecules must be retained with respect to the native ligand. Ergopeptides, with their high affinities at D_1 and D_2 dopamine receptors, are particularly complex examples. Here, we report the systematic derivatization of two ergopeptides with different peptide-based spacers and their evaluation by radioligand binding assays. Selected spacer-containing ergopeptides with minimal biological alteration and a proper anchoring point were further derivatized with a biotin reporter. Detailed characterization studies identified **13** as a biotin ergopeptide maintaining high affinity and agonist behavior at dopamine receptors, being a useful tool for the study of heteromers involving D_1R, D_2R, or D_3R.

Introduction

Ergopeptides, with their high affinity at D_1 and D_2 dopamine receptors (D_1Rs and D_2Rs[a]), are valuable molecules to study dopamine receptors.[1] The therapeutic significance of dopamine receptors containing heteromers has been extensively reported.[2−4] Dual ligands have been successfully applied to the study of G-protein coupled receptors (GPCRs) oligomerization.[5,6] While ergopeptides may provide insights into the D_1 or D_2 receptors containing heteromers, they require a proper reporter tag prior to their use in protein localization and profiling studies.[7−10] The modification of hit compounds is a critical step as the biological properties of the labeled molecules can be significantly altered with respect to the native ligand.[11] This step is particularly important when applied to small molecule ligands, such as ergopeptides, because the ligand affinity and efficacy of the pharmacophore is likely to be compromised.[12] In the present work, we report a systematic study to optimize the length and chemical nature of different spacer moieties attached to two ergopeptides with high affinity at D_1R and D_2R (**1** and **2**, Chart 1) and identify those linkers that retain their binding profile. Trifunctional amino acids, such as lysine and glutamic acid, are excellent scaffolds for the synthesis of peptide-based spacers. In addition to their low toxicity, they can be easily adapted to a solid-phase synthesis approach to allow the incorporation of a range of functional groups (amines, anilides, and carboxamides) within the spacer structure. A number of peptide-based spacers were incorporated to **1** and **2** to render a 40-member library of new ergopeptides, and the evaluation of their affinities at D_1R and D_2R identified two linker moieties with minimal biological interference. The subsequent incorporation of a biotin reporter led to identification of **13** as a biotin ergopeptide for dopamine receptors with nanomolar binding affinities and agonist behavior.

Results

Design of the Library. The incorporation of a spacer moiety into a bioactive molecule requires the selection of a suitable attachment point, which must be well separated

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[a] Abbreviations: $A_{2A}R$, adenosine A_{2A} receptor; A_1R, adenosine A_1 receptor; Ac_2O, acetic anhydride; ACN, acetonitrile; Alloc, allyloxycarbonyl; All, allyl ester; Ahx, aminohexanoic acid; Akt, protein kinase B; Boc, *t*-butoxycarbonyl; CDI, 1,1′-carbonyldiimidazole; CHO, Chinese hamster ovary; DCM, dichloromethane; cDNA, cDNA; [^{3}H]-CP55940, tritium labeled 2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxypropyl)-cyclohexyl]-5-(2-methyloctan-2-yl)phenol; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; D_1R, dopamine D_1 receptor; D_2R, dopamine D_2 receptor; D_3R, dopamine D_3 receptor; ERK, extracellular-signal-regulated kinases; Fmoc, fluorenylmethyloxycarbonyl; GPCR, G-protein coupled receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOBt, hydroxybenzotriazole; HOAt, 1-hydroxy-7-azabenzotriazole; HRMS, high resolution mass spectrometry; MAPK, mitogen-activated protein kinases; PEG, polyethylene glycol; PEI, polyethylenimine; Rink-MBHA-PS, 4-(2′,4′dimethoxyphenyl-Fmoc-aminomethyl)-pheoxyacetamido *p*-methylbenhidrylamine resin; RP-HPLC, reversed phase-high performance liquid chromatography; RP-HPLC-MS, reversed phase-high performance liquid chromatography−mass spectroscopy; SKF81297, (±)-6-chloro-2,3,4,5-tetrahydro-1-phenyl-1*H*-3-benzazepine hydrobromide; TFA, trifluoroacetic acid; TMUCl Cl, *N*-[chloro(dimethylamino)methylene]-*N*-methylmethanaminium chloride; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloric acid; [^{3}H]-RAMH, tritium labeled *R*-methyl histamine; [^{3}H]-*R*-PIA, tritium labeled *R*-phenylisopropyladenosine; [^{3}H]-SCH23390, tritium labeled [*R*-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol); [^{3}H]-YM09151-2, tritium labeled nemonapride (*N*-(1-benzyl-2-methylpyrrolidin-3-yl)-5-chloro-2-methoxy-4-(methylamino)benzamide); [^{3}H]-ZM241358, tritium labeled 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol. Abbreviations used for amino acids follow the IUPAC-IUB Commission of Biochemical Nomenclature in Jones, J. H. *J. Pept. Sci.* **2003**, *9*, 1−8.

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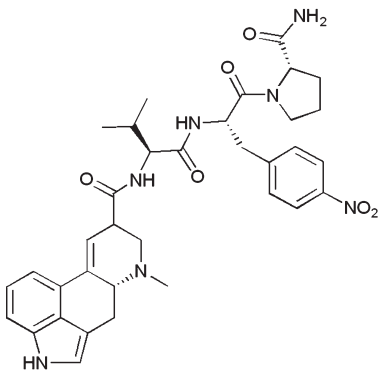
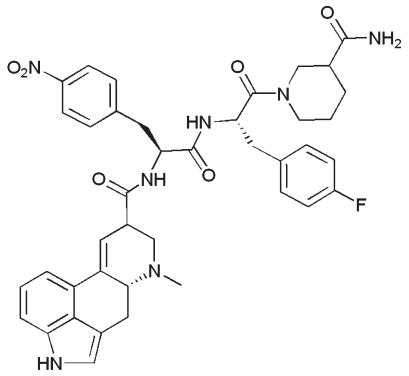
Chart 1. Ergopeptides with High Affinity at D_1 and D_2 Dopamine Receptors



1



2

from the pharmacophore in order to prevent any interference on the binding between biomolecule and receptor. The binding mode analysis of **1** and **2** revealed the interaction of the ergolene scaffold at the transmembrane binding sites while the peptide moieties interacted with adjacent amino acids.[1] Bearing this in mind, the attachment of distinct spacer moieties into **1** and **2** was designed by means of the C-terminal carboxamide group modification, which would presumably produce a minor change to the biological properties of the final compounds.

Several spacer moieties were designed on the basis of two trifunctional amino acids, lysine and glutamic acid, which are key scaffolds for the derivatization of biomolecules when a suitable protecting group scheme is used.[13] The introduction of a number of building blocks onto the lysine and glutamic acid side chains afforded 19 spacer moieties with various length and chemical functionalities (Table 1), including aromatic and saturated rings, primary amines, carboxamides, anilides, and poliethylenglycol (PEG) units. Moreover, a common N^{α}-group at the C-terminus was acetylated to mimic the further incorporation of a reporter molecule.

Synthesis of the Spacer-Containing Ergopeptides. The synthesis of the peptide-based spacers was entirely performed on solid-phase, using Rink-MBHA-PS as a polymeric support. Coupling Fmoc-Lys(Alloc)-OH or Fmoc-Glu(OAll)-OH onto the resin, Fmoc elimination, acetylation of the N^{α}-group, and removal of the side chain protecting groups led to resins **3** and **4**, which were used for the construction of the 19 peptide-based spacers (Scheme S1 in Supporting Information (SI)).

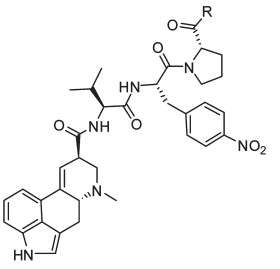
The introduction of distinct units (amino acids, diamines, anhydrides, and dicarboxylic acids) onto resins **3** and **4** was carried out using standard solid-phase peptide synthesis (SPPS) protocols and 1,1′-carbonyldiimidazole (CDI) for the activation of supported carboxylic acids. The poor nucleophilicity of some anilines required the use of a previously described procedure, based on *N*-[chloro(dimethylamino)-methylene]-*N*-methylmethanaminium chloride (TMUCl Cl) as the activating reagent, for the solid-phase synthesis of anilides[14] (compounds **1p**, **2p**, **1r**, and **2r**). The incorporation of the 19 spacer moieties into the ergopeptides structure was carried out in two steps: (1) N^{α}-Boc-tripeptides **5** and **6** were attached to the supported spacers in a convergent protocol instead of a stepwise procedure to favor the parallelization synthetic process; (2) the crude mixtures released after cleavage (TFA-H_2O, 95:5) were further coupled to the D-lysergic acid in solution using DIPCDI and HOAt (Scheme 1). Although this method involved an extra step compared to the direct coupling of ergopeptides onto the spacer moieties, the amount of the costly D-lysergic acid could be minimized. The purification of the whole library by semipreparative RP-HPLC afforded the 38 final products with excellent purities (**1a**−**s** and **2a**−**s**, Table S1 in SI).

Biological Assays: Binding Properties of Spacer-Containing Ergopeptides. The binding properties of **1a**−**s** and **2a**−**s** were assayed by displacement experiments of D_1R or D_2R radiolabeled ligands (concentration indicated in the legends of Figures 1 and 2) by 25 μM **1a**−**s** or **2a**−**s**. The binding screening at D_1R and D_2R (Figures 1 and 2) indicated a significant decrease of binding affinity when aromatic rings were included within the spacer moieties (**1p** (mainly at D_2R), **2i**, **2j**, **2p**, and **2r**). In contrast, the incorporation of linear aliphatic spacers resulted in compounds showing a more favorable binding, with slightly stronger interactions in the presence of medium-length spacers (**1c**, **1f**, **1n** at D_1R; **1l**, **1n**, **2f**, and **2k** at D_2R) and some shorter ones (**1a**, **1m** at D_1R; **1a**, **1m**, **2a** at D_2R). Regarding the inclusion of primary amines within the spacer structure, the results were both ergopeptide and receptor-dependent; **1d** did not show a significantly different binding at D_1R, but **1d** and **1e** binding affinities were remarkably improved at D_2R. Whereas determining the exact nature of this enhancement would require further studies, the incorporation of medium-sized aliphatic spacers (**c** and **f**, Table 1) at the C-terminus of ergopeptides **1** and **2** proved to be successful in preserving their binding affinities at both D_1R and D_2R.

We further investigated the behavior of the modified ergopeptides (**1c**, **1f**, **2c**, and **2f**) at A_1 and A_{2A} adenosine receptors because heteromers containing adenosine-dopamine receptors have been very well described.[15−17] The binding affinities of **1c**, **1f**, **2c**, and **2f** were assayed by displacement experiments of A_1R or $A_{2A}R$ radiolabeled ligands (concentration indicated in the Figure S1 and S2 legends in SI) by 25 μM ergopeptides. The binding of **1c**, **1f**, **2c**, and **2f** at adenosine receptors (Figures S1 and S2 in SI) proved to be much lower than at D_1R and D_2R and confirmed their specificity for dopamine receptors.

Synthesis and Pharmacological Characterization of Biotin Ergopeptides (9−16). Biotin has been extensively used for protein profiling studies because its tight interaction with avidin can facilitate the isolation of protein complexes.[18−20] Moreover, the availability of numerous biotin/streptavidin-labeled antibodies and reagents makes biotin one of the most versatile reporters for protein characterization. To construct the corresponding biotin ergopeptide probes, a biotin molecule

Table 1. Chemical Structures of Ergopeptides after the Incorporation of Peptide-Based Spacers



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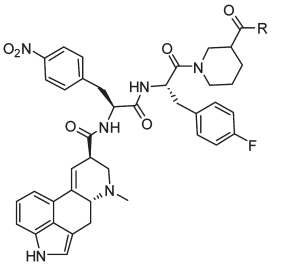
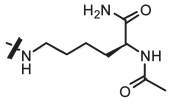
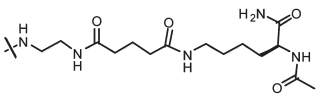
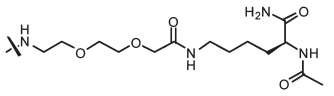
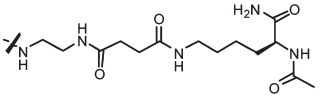
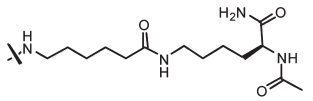
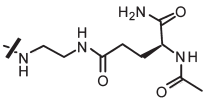
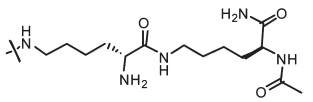
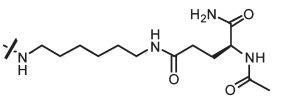
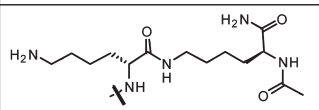
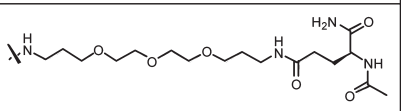
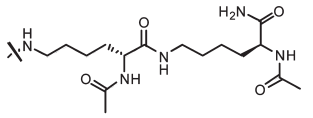
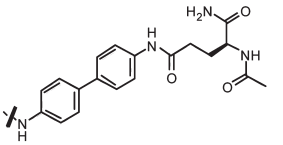
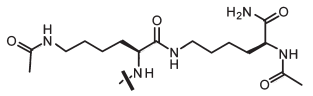
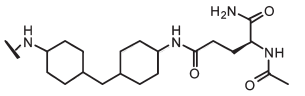
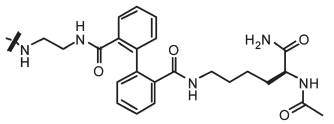
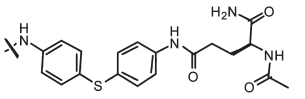
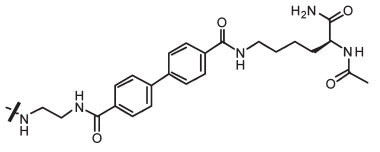
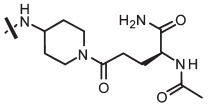
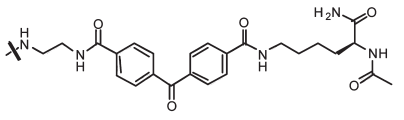


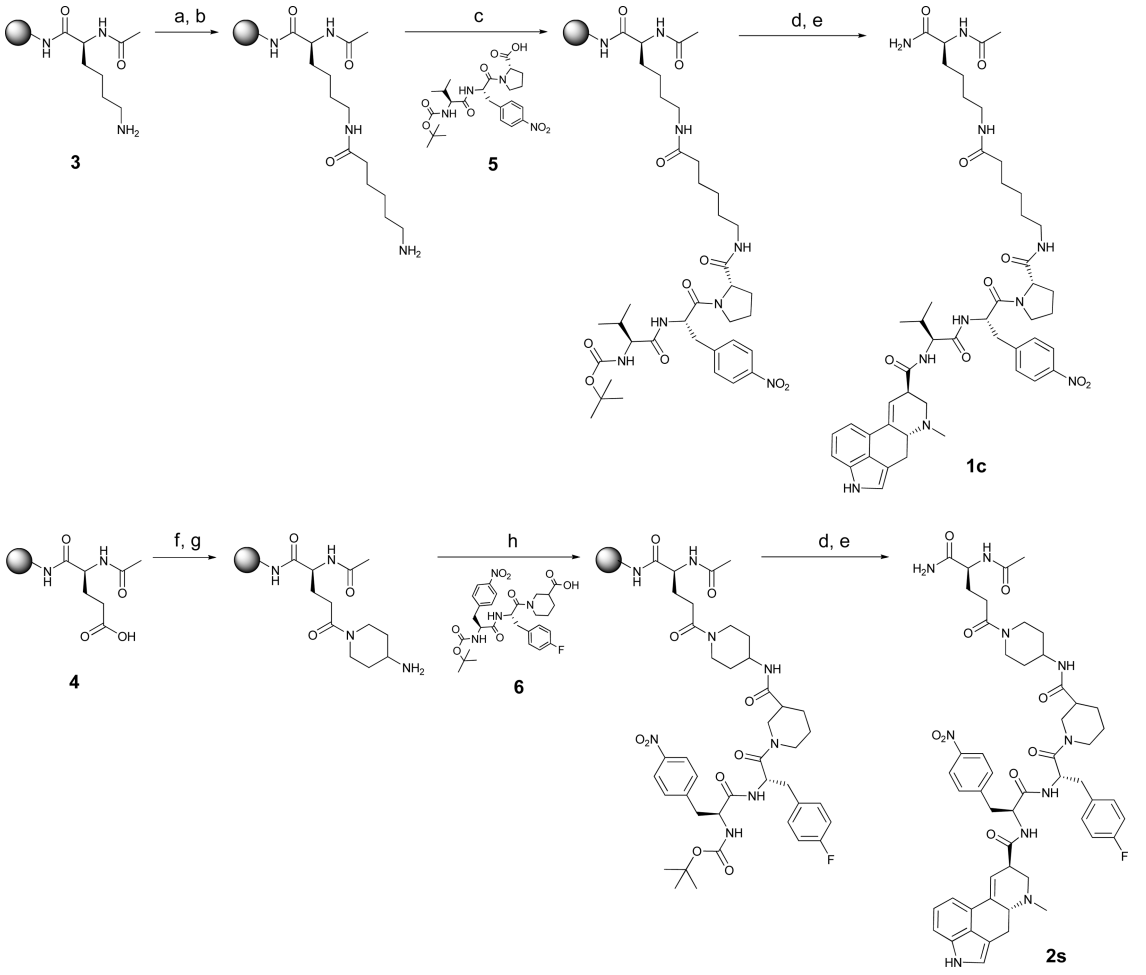
2x

compd	R	compd	R
1a, 2a		**1k, 2k**	
1b, 2b		**1l, 2l**	
1c, 2c		**1m, 2m**	
1d, 2d		**1n, 2n**	
1e, 2e		**1o, 2o**	
1f, 2f		**1p, 2p**	
1g, 2g		**1q, 2q**	
1h, 2h		**1r, 2r**	
1i, 2i		**1s, 2s**	
1j, 2j			

was incorporated to the C-terminus of **1c**, **2c**, **1f**, and **2f**. Biotin ergopeptides were synthesized using a slightly modified procedure from the previously described (Scheme 2), and the biotin reporter was coupled to the N^{α}-group of the C-terminus of ergopeptides using TBTU and HOBt.

The larger size of the biotin ergopeptides facilitated the isolation of the two ergolene diastereomers derived from the epimerization of D-lysergic acid under the coupling conditions,[21] and eight biotin ergopeptides (**9**−**16**, Chart 2) were subjected to primary radioligand binding assays at D_1R and

Scheme 1. Synthesis of Representative Compounds **1c** and **2s**



[a] Conditions: (a) Fmoc-Ahx-OH, DIPCDI/HOBt; (b) piperidine-DMF (2:8); (c) **5**, DIPCDI/HOBt; (d) TFA-H_2O (95:5); (e) D-lysergic acid, DIPCDI/HOAt, DIEA; (f) (Fmoc-4-amino) piperidine·HCl, DIEA, CDI; (g) piperidine-DMF (2:8); (h) **6**, DIPCDI/HOBt.

D_2R. The binding properties of **9**−**16** were initially evaluated using displacement experiments of a fixed concentration of D_1R, D_2R, A_1R or $A_{2A}R$ radiolabeled ligands (indicated in the legends of Figures S3−S6 in SI) by 25 μM **9**−**16** (Figures S3−S6 in SI). The incorporation of the biotin molecule retained the binding properties of the parent compounds **1** and **2** and confirmed that both selected spacers (**c** and **f**) separate well the pharmacophore from the reporter tag. We selected compounds **9**, **12**, **13**, and **14** for their pharmacological characterization, and their affinity constants at D_1R and D_2R were calculated by competition experiments: brain membranes (0.5 mg/mL) were incubated with a fixed concentration of D_1R or D_2R radiolabeled antagonists in the absence or in the presence of increasing concentrations of compounds **9**, **12**, **13**, or **14**, as described in the Experimental Section. From the resulting competition curves (Figure 3 for compound **13**, and Figures S7−S9 in SI for compounds **9**, **12**, and **14**), their corresponding K_D values were determined (Table 2). The biotin ergopeptide **13** proved to be the compound that best maintained the nanomolar range affinities at both dopamine receptor subtypes.

To test whether the compound **13** behaved as an agonist, we examined two different signal transduction pathways (e.g., MAPK and Akt (PKB)) in cells that were separately transfected with D_1R and D_2R. As shown in Figure 4a,b, **13** increased the ERK1/2 phosphorylation in cells expressing D_1R or D_2R in a dose-dependent manner and to a similar extent than a D_1R full agonist (e.g., SKF 81297)[22] or a D_2R agonist (e.g., quinpirole).[23] The **13**-mediated effect (at 0.1 or 1 μM) was also reverted when cells were preincubated with 5 or 50 μM of D_1R (e.g., SCH 23390) or D_2R antagonists (e.g., raclopride), further demonstrating the agonist behavior of **13** at both dopamine receptor subtypes. Regarding the Akt (PKB) pathway, both SKF 81297 and quinpirole (D_1R and D_2R agonists, respectively) induced a decrease in the Akt Ser^{473} phosphorylation, a signaling that has been also observed in mouse brain.[24,25] Similarly, **13** decreased the Akt phosphorylation in a dose-dependent manner in cells expressing D_1R or D_2R, and its effect was also reverted upon preincubation with D_1R (e.g., SCH 23390) or D_2R antagonists (e.g., raclopride), confirming the agonist behavior of **13** at both D_1R and D_2R (Figure 4c,d).

Since it has been described that D_1Rs can also heteromerize with dopamine D_3Rs,[26] we analyzed the binding affinity of compound **13** at D_3R. Because of the low expression level of D_3R in the striatum when compared to D_2R, we transiently transfected CHO cells with D_3R to study the binding affinity of compound **13** to this receptor. Membranes (0.5 mg/mL) from transfected cells were incubated with 4 nM [^{3}H]-raclopride, a D_3R antagonist, in the absence or in the presence of increasing concentrations of compound **13**. The competition curve was used to determine the corresponding

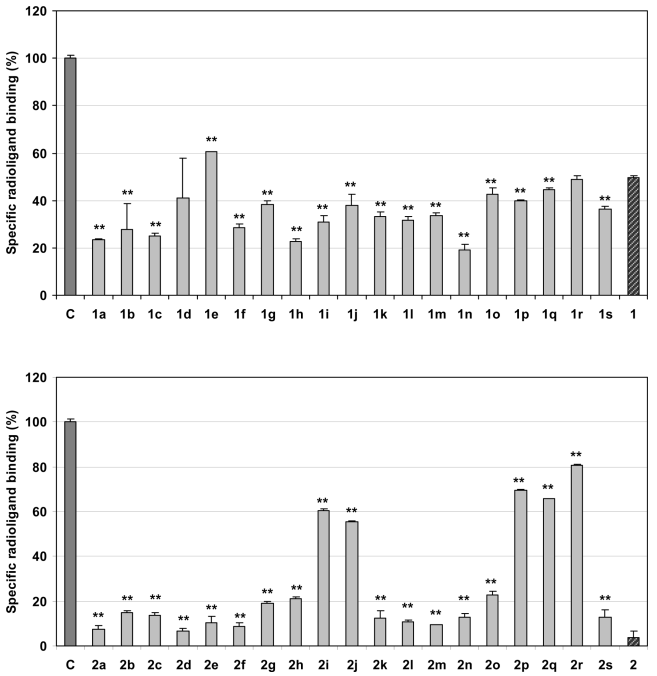


Figure 1. Displacement experiments at D_1R. Specific binding of 0.9 nM D_1R antagonist [^{3}H]-SCH23390 in the absence or in the presence of competing ligands was measured as indicated in the Experimental Section. C, control of radioligand binding without competing ligand; **1a**−**2s**, radioligand specific binding in the presence of 25 μM ergopeptides; **1**−**2**, radioligand specific binding in the presence of 25 μM ergopeptides **1** and **2**, respectively. Values are represented as means ± SD (n = 3). Student's t-test for unpaired samples showed significant differences (**$p < 0.01$) compared to the ergopeptides **1** or **2**.

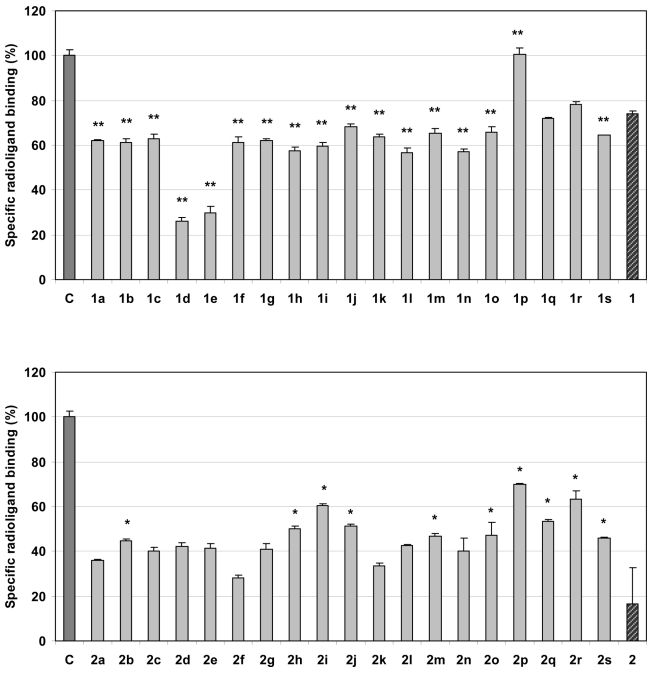


Figure 2. Displacement experiments at D_2R. Specific binding of 0.7 nM D_2R antagonist [^{3}H]-YM 09151-2 in the absence or in the presence of competing ligands was measured as indicated in the Experimental Section. C, control of radioligand binding without competing ligand; **1a**−**2s**, radioligand specific binding in the presence of 25 μM ergopeptides; **1**−**2**, radioligand specific binding in the presence of 25 μM ergopeptides **1** and **2**, respectively. Values are represented means ± SD (n = 3). Student's t-test for unpaired samples showed significant differences (*$p < 0.05$; **$p < 0.01$) compared to the ergopeptides **1** or **2**.

K_D values (K_{DB1} = 23 ± 9 nM and K_{DB2} = 93 ± 36 nM), proving that **13** can be a useful tool for the study of heteromers involving D_1, D_2, or D_3 dopamine receptors (Figure 5). The binding properties of **13** were also examined at other GPCRs that can form heteromers with D_1Rs or D_2Rs (e.g., histamine H_3, metabotropic glutamate 5, somatostatin SST5, and cannabinoids CB_1 receptors).[27−31] Displacement experiments (Figure S10 in SI) showed that high concentrations of **13** did not significantly decrease the radioligand binding at the studied receptors and proved that the binding of **13** at other GPCRs was very low when compared to dopamine receptors.

Conclusions

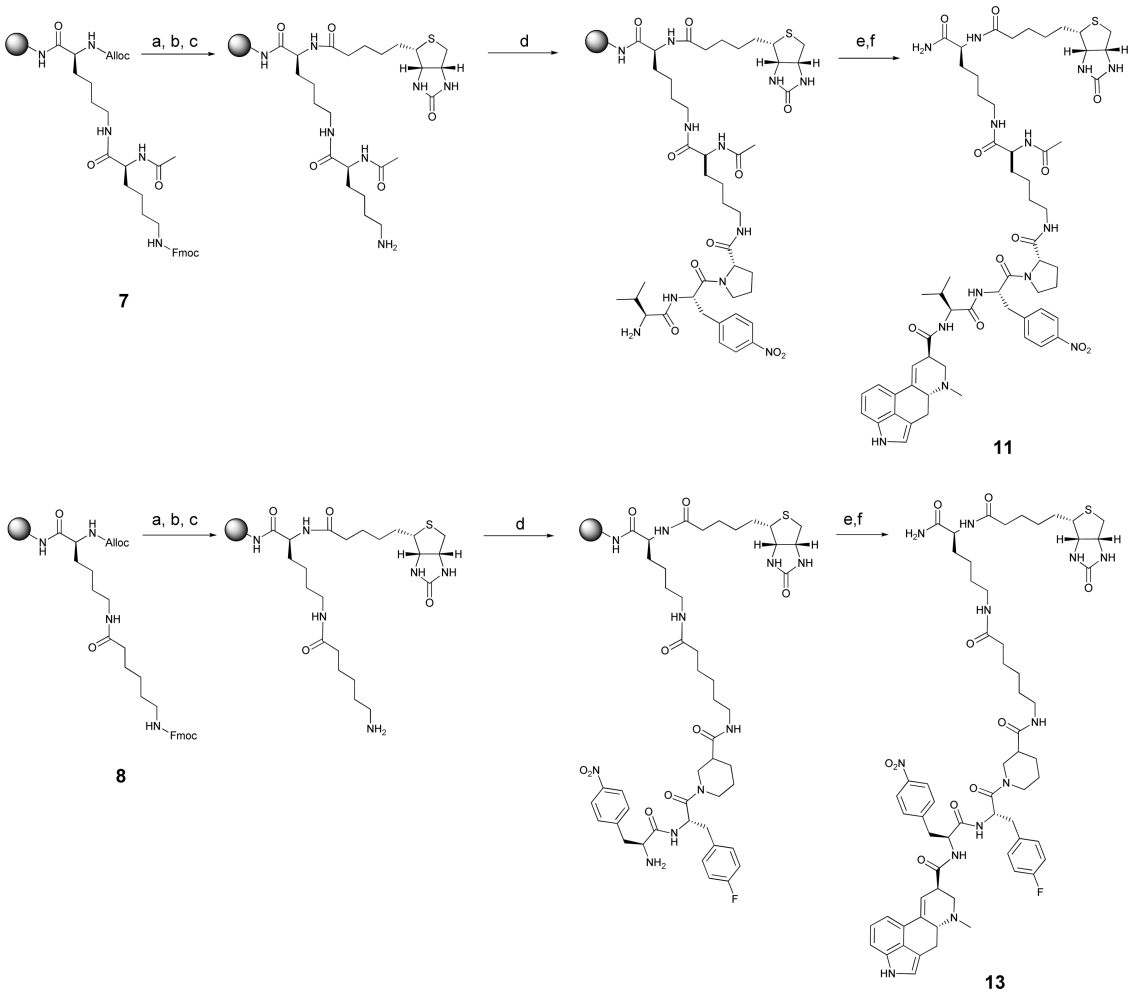
The derivatization of two ergopeptides showing high affinity at dopamine receptors has been optimized using a combinatorial chemistry approach to develop of a novel biotin ergopeptide that maintained both nanomolar binding affinities and an agonist behavior at dopamine receptors. The systematic modification of the two parent ergopeptides using a solid-phase synthesis approach afforded a 40-member library including different peptide-based spacers at the C-terminus of the ergopeptides. The binding analysis of the library identified two modified ergopeptides incorporating medium-length aliphatic spacers as the compounds that best retained the affinity profile at D_1R and D_2R. Subsequent derivatization of the spacer-containing ergopeptides with a biotin molecule rendered a set of biotin ergopeptides that bound at D_1R and D_2R with K_D values in the nanomolar range. Further characterization studies identified **13** as a biotin ergopeptide with agonist behavior and significantly higher binding affinities at D_1R, D_2R, and D_3R when compared to adenosine (A_1/A_{2A}), histamine H_3, metabotropic glutamate 1/5, somatostatin SST, and cannabinoid CB_1 receptors. These results attest the potential application of **13** to study heteromer complexes involving dopamine receptors. Experiments with biotin ergopeptides to study dopamine receptors heteromers are currently ongoing and will be reported in due course.

Experimental Section

Materials and Equipment. All Fmoc-amino acids were purchased from Neosystem (Strasbourg, France), and Fmoc-Rink-PS and 2-chlorotrityl resins were supplied by Calbiochem-Novabiochem AG. DIPCDI was obtained from Fluka Chemika (Buchs, Switzerland) and HOBt from Albatross Chem, Inc. (Montreal, Canada). Solvents for peptide synthesis and RP-HPLC equipment were obtained from Scharlau (Barcelona, Spain). Trifluoroacetic acid was supplied by KaliChemie (Bad Wimpfen, Germany). Other chemicals of the highest commercially available purity were purchased from Aldrich (Milwaukee, WI). All commercial reagents and solvents were used as received.

Adenosine deaminase (EC 3.5.4.4) was purchased from Roche (Basel, Switzerland), and [^{3}H]-*R*-PIA was supplied by Amersham Biosciences (Buckinghamshire, UK). Raclopride, polyethylenimine (PEI), $MgCl_2$, DPCPX, mouse antiphospho-ERK1/2 antibody, rabbit anti-ERK1/2 antibody, IRDye 800 antimouse antibody, and IRDye 680 antirabbit antibodies were purchased from Sigma (St Louis, MO). Rabbit anti-P-Ser^{473}Akt antibody was purchased from SAB Signalway (Pearland, USA). ZM241385, SCH23390, RAMH, quisqualic acid, somatostatin, and CP55940 were supplied by Tocris Biosciences (Avonmouth, UK). [^{3}H]-SCH23390, [^{3}H]-YM09151−2, [^{3}H]-quisqualic acid,

Scheme 2. Synthesis of Representative Biotin Ergopeptides **11** and **13**. [a]



[a] Conditions: (a) $Pd(PPh_3)_4$-$PhSiH_3$ in DCM_{anh}; (b) biotin, TBTU/DIEA, HOBt; (c) piperidine-DMF (2:8); (d) sequential peptide synthesis: couplings in DIPCDI/HOBt, Fmoc removal in piperidine-DMF (2:8); (e) TFA-H_2O (95:5); (f) D-lysergic acid, DIPCDI/HOAt.

[^{3}H]-CP55940, [^{125}I]-Tyr^{11}-somatostatin 14, and [^{3}H]-ZM241385 were supplied by Perkin-Elmer (Boston, MA). [^{3}H]-RAMH was purchased from GE Healthcare (Buckinghamshire, U.K). Ecoscint H scintillation cocktail was purchased from National Diagnostics (Atlanta, GA). Bradford assay kit was purchased from Bio-Rad (Munich, Germany). All other supplements were purchased from Invitrogen (Paisley, UK).

Analytical RP-HPLC-MS was performed using 2795 Waters (Milford, MA) Alliance with a Micromass ZQ mass spectrometer and a 996 PDA detector. Semipreparative RP-HPLC was performed on a 2767 Waters chromatography system with a Micromass ZQ mass spectrometer. Multiple sample evaporation was carried out in a Discovery SpeedVac ThermoSavant (Waltham, MA). Radioligand binding experiments were performed using a Brandel (Gaithersburg, MD) cell harvester and a Packard 1600 TRI-CARB scintillation counter. Fitting data binding program GRAFIT was obtained from Erithacus Software (Surrey, UK). For ERK1/2 or P-Ser^{473}Akt phosphorylation determination, the Odyssey infrared scanner (LI-COR Biosciences, Lincoln, Nebraska, USA) was used. Band densities were quantified using the scanner software and exported to Excel (Microsoft, Redmond, WA, USA).

Synthesis. Solid-Phase General Procedure. Peptide syntheses were performed manually in a polypropylene syringes, each fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by filtration. Washings between deprotection, coupling, and subsequent deprotection steps were carried out with DMF (5 × 1 min) and DCM (5 × 1 min) using 10 mL of solvent/g of resin each time.

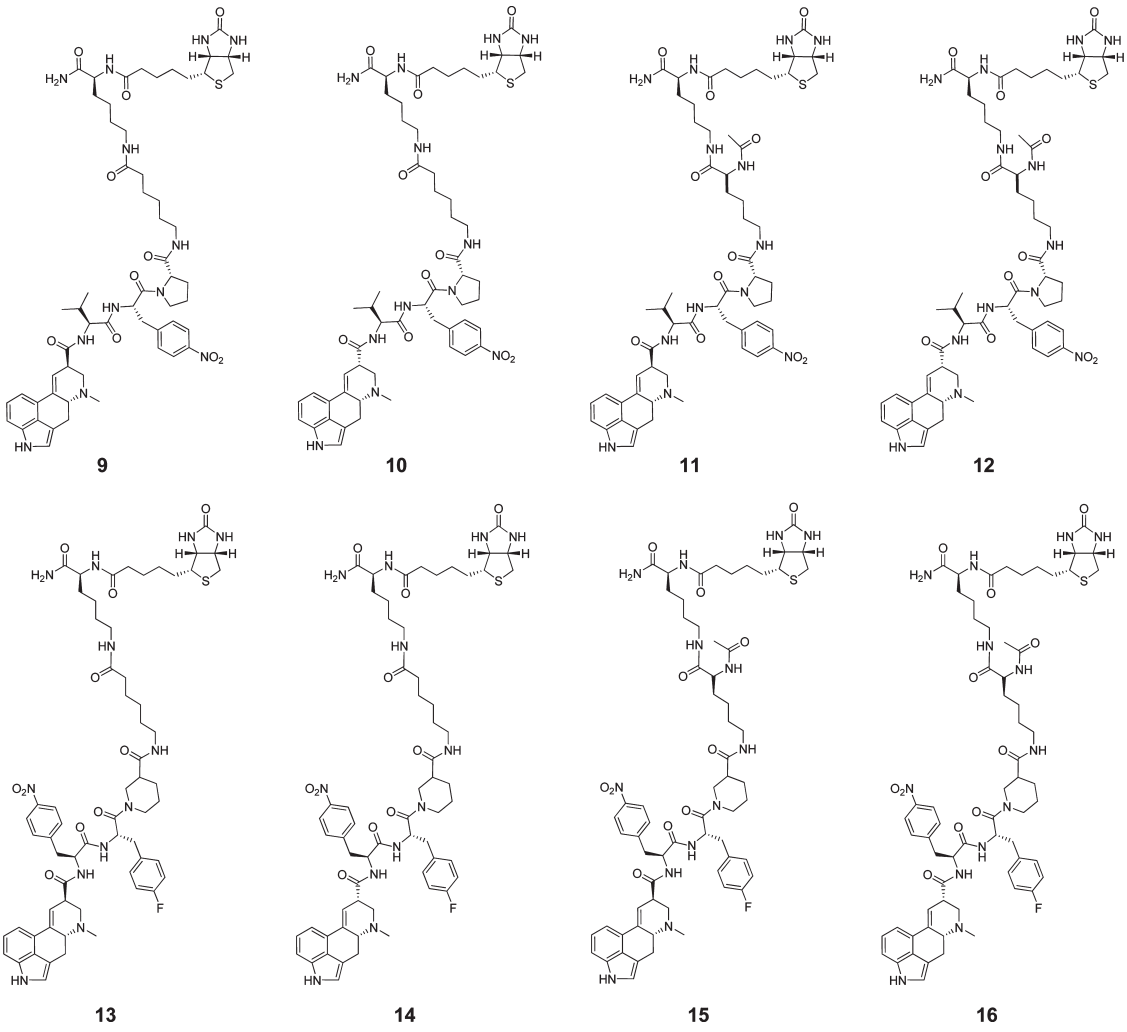
Coupling using DIPCDI and HOBt-HOAt. Fmoc-AA-OH, carboxylic acids or Boc-peptides (3 equiv) were coupled using DIPCDI (3 equiv) as coupling reagent and HOBt (3 equiv) or HOAt (3 equiv) as additives in DCM-DMF (1:1) for 2−4 h at rt. After each coupling, the resin was washed with DMF (5 × 1 min) and DCM (5 × 1 min). Reaction completion was checked by means of the Kaiser or chloranil tests.[32]

Anhydrides Coupling. Anhydrides (10 equiv) were coupled using DIEA (10 equiv) in DCM for 2−4 h at rt. After each coupling, the resin was washed with DMF (5 × 1 min) and DCM (5 × 1 min). Reaction completion was checked by means of the Kaiser or chloranil tests.

Coupling using CDI.[33] Solid-supported carboxylic acids were washed with DCM (5 × 1 min) and DMF (5 × 1 min) and treated with CDI (25 equiv) in DMF (30 min). After filtering the resin and washing with DMF, amines (5 equiv) were added in DCM-DMF (1:1) and kept under orbital agitation for 2−4 h at rt. After each coupling, the resin was washed with DMF (5 × 1 min) and DCM (5 × 1 min). Reaction completion was checked by means of the malachite green test.[34]

Coupling using TMUCl Cl.[14] Solid-supported carboxylic acids were washed with DCM (5 × 1 min) and treated with TMUCl Cl (10 equiv) and DIEA (10 equiv) in DCM (10 min). After filtering the resin and washing with DCM and DMF, anilines (5 equiv) were added in DCM-DMF (1:1) and left

Chart 2. Structures of Biotin Ergopeptides **9**−**16**



under orbital agitation for 2 h at rt. After each coupling, the resin was washed with DMF (5 × 1 min) and DCM (5 × 1 min).

Fmoc Group Removal involved the following sequence: (i) DMF (5 × 1 min); (ii) piperidine-DMF (2:8) (1 × 1 min + 2 × 15 min); (iii) DMF (5 × 1 min).

Alloc/All Group Removal involved the following sequence: (i) DCM (5 × 1 min); (ii) $Pd(PPh_3)_4$ (0.1 equiv) and $PhSiH_3$ (10 equiv) in anhydrous DCM (3 × 15 min); (iii) anhydrous DCM (5 × 1 min); (iv) DCM (5 × 1 min); (v) DMF (5 × 1 min); (vi) 0.02 M solution of sodium diethyldithiocarbamate in DMF (3 × 15 min), DMF (5 × 1 min), DCM (5 × 1 min), and DMF (5 × 1 min).

N^{α}-Terminus Acetylation. The resins were treated with Ac_2O (10 equiv) and DIEA (10 equiv) in DCM (2 × 15 min). Reaction completion was checked by the Kaiser test.

Cleavage Conditions. A. 2-Chlorotrityl-Based Resins. The resins were treated with a solution of TFA-DCM (5:95) (5 × 1 min). Filtrates were collected, washed with DCM (3 × 1 min), and evaporated under vacuum.

B. Rink-Based Resins. The resins were treated with a solution of TFA-H_2O (95:5) and orbitally shaken for 2 h at rt. Filtrates were collected, washed with TFA (2 × 1 min) and DCM (3 × 1 min), and evaporated under vacuum.

Synthesis of Boc-Val-pNO$_2$Phe-Pro-OH (5). Starting from 2-chlorotrityl resin (6.0 g, loading: 1.3 mmol/g), the peptide was synthesized as described above. Cleavage and lyophilization in H_2O−ACN (2:1) rendered a white solid powder; 1.75 g (yield: 39%); M_{exp} 507.1 (M_{calc} 506.3), 96%.

Synthesis of Boc-pNO$_2$Phe-pFPhe-Nip-OH (6). Starting from 2-chlorotrityl resin (6.0 g, loading: 1.3 mmol/g), the peptide was synthesized as described above. Cleavage and lyophilization in H_2O−ACN (2:1) yielded a white solid powder; 3.58 g (yield: 68%); M_{exp} 587.0 (M_{calc} 586.2), 95%.

Coupling of D-Lysergic Acid. Evaporated crude cleavages for the different peptide moieties (23.7−79.0 mg, 0.03−0.09 mmol) were dissolved in individual vials using 0.5 mL of DMF, treated with DIEA (1 equiv, 5−15 μL, 0.03−0.09 mmol), and stirred for 5 min at rt. Second, D-lysergic acid (1.2 equiv, 11−29 mg, 0.04−0.11 mmol) and HOAt (1.5 equiv, 6−18 mg, 0.05−0.14 mmol) were dissolved in 1 mL of DMF (each reaction) and added to every vial. Finally, DIPCDI was added (2 equiv, 9−27 μL, 0.06−0.18 mmol) and the set of reactions was stirred in a parallel synthesizer for 16 h. Multiple sample evaporation rendered the crude mixtures further purified by semipreparative RP-HPLC.

Library Characterization. The purified compounds **1a**−**1s** and **2a**−**2s** were characterized by analytical RP-HPLC-MS, using a reverse-phase Symmetry C_{18} (5 μm, 3.9 mm × 150 mm) column and 1 mL/min flow. Elution system A: H_2O−TFA, 99.9:0.1; B: ACN−TFA, 99.9:0.1; gradient 0% B to 100% B in 25 min. The purities of all library compounds (average ≥95%) were determined by UV absorption at 220 nm (HPLC-MS data, quantities, and purities of all library compounds are included in Table S1 in SI).

Synthesis of Biotin Ergopeptides (9−16). Rink-MBHA PS (1 g, loading: 0.56 mmol/g) was swollen with DCM (1 × 1 min, 2 × 10 min) and DMF (5 × 1 min, 1 × 15 min) before

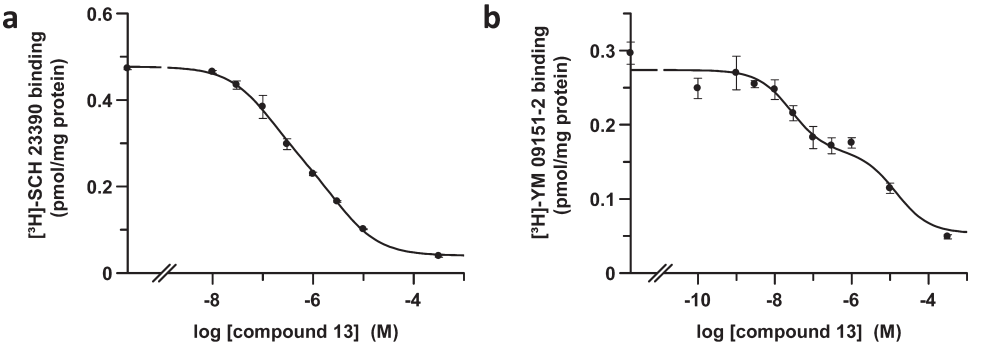


Figure 3. Competition curves of dopamine receptors antagonists binding versus increasing concentrations of compound **13**. Competition experiments of 0.9 nM D_1R antagonist [^{3}H]-SCH23390 (a) or 0.7 nM D_2R antagonist [^{3}H]-YM 09151−2 (b) versus increasing concentrations of compound **13** were performed with brain striatal membranes (0.5 mg prot/mL) as indicated in the Experimental Section. Data are represented as means ± SD from a representative experiment (n = 3) performed in triplicate.

Table 2. K_D Values of Selected Biotin Ergopeptides at D_1R and D_2R

	D_1R		D_2R	
	K_{DB1} (nM)	K_{DB2} (μM)	K_{DB1} (nM)	K_{DB2} (μM)
9	107 ± 9	3.2 ± 0.3	260 ± 60	7 ± 2
12	2100 ± 300	> 50	2000 ± 400	23 ± 8
13	56 ± 6	1.5 ± 0.2	17 ± 5	9 ± 3
14	490 ± 80	> 50	160 ± 50	10 ± 4

[a] K_{DB1} and K_{DB2} are respectively the equilibrium dissociation constants of the high and low binding affinities of ergopeptides to the dimeric D_1R and D_2R.

use. After washing, Alloc-L-Lys(Fmoc)-OH (3 equiv) was coupled to the resin, using DIPCI (3 equiv) and HOBt (3 equiv) as a coupling system in DMF. The resin was washed and the Fmoc group removed, yielding Alloc-L-Lys(NH_2)-AM-MBHA. At this point, the resin was split into two equal aliquots. Ac-Lys(Fmoc)-OH (3 equiv) and the Fmoc-Ahx-OH (3 equiv) were respectively coupled on each one as previously described to render the resins **7** and **8**. After that, Alloc group was eliminated in both resins and biotin (3 equiv) was introduced using TBTU (3 equiv), DIEA (6 equiv), and HOBt (3 equiv) in DMF for 1 h at rt. After the Fmoc group removal, the resins were again divided in two parts, obtaining at that point four different resins. From this point onward, the attachment of the three different amino acids (corresponding to the tripeptides of **1** and **2**) was performed according to the procedure described previously. The biotin peptide moieties were cleaved following the B cleavage conditions, and the coupling of D-lysergic acid was carried out as described above. Evaporated crude cleavages for the different biotin peptide moieties (95−127 mg, 0.10−0.13 mmol) were dissolved in individual vials using 0.5 mL of DMF, treated with DIEA (1 equiv), and stirred for 5 min at rt. Second, D-lysergic acid (1.2 equiv) and HOAt (1.5 equiv) were dissolved in 1 mL of DMF (each reaction) and added to every vial. Finally, DIPCDI was added (2 equiv) and the set of reactions were stirred for 16 h at rt. Evaporation rendered the crude mixtures further purified by semipreparative RP-HPLC-MS under basic conditions (A: 20 mM NH_4COOCH_3, pH 9; B: ACN) using a reverse-phase X-Bridge C_{18} column (5 μm, 19 mm × 100 mm^2) to yield the biotin ergopeptides **9**−**16**.

Biotin Ergopeptides Characterization (9−16). Biotin ergopeptides **9**−**16** were characterized by analytical RP-HPLC-MS and RP-HPLC using a reverse-phase XBridge C_{18} column (3.5 μm, 4.6 mm × 50 mm^2) at 2 mL/min (two separate elution solvent systems: (a) A, H_2O−HCOOH (99.9:0.1); B, ACN-HCOOH (99.93:0.07). (b) A, H_2O−TFA (99.9:0.1); B, ACN−TFA (99.9:0.1). The purities of **9**−**16** were determined by RP-HPLC at 220 nm UV absorption using as elution system b and a gradient 5% B to 50% B in 4.5 min. All purities were confirmed to be ≥95%. HRMS spectra for **9**−**16** were recorded confirming the identity of each biotin ergopeptides (Table 3).

Identity of compound **13** was confirmed by ^{1}H NMR (400 MHz, DMSO-d_6): 10.7 (s, 1H), 8.44 (s, 1H), 8.37 (s, 1H), 8.29 (s, 2H), 8.17 (d, 2H), 7.90 (s,1H), 7.54 (d, 2H), 7.3−6.9 (m, 7H), 7,30 (s,1H), 7.09 (s, 1H), 7.02 (s, 1H), 6.42 and 6.36 (s, 2H), 6.26 (s, 1H), 4.98 (m, 1H), 4.64 (m, 1H), 4.4−3.8 (m, 7H), 3.00 (dd, 1H), 2.78 (m, 1H), 2.59 (m, 2H), 2.51 (s, 3H), 2.3−1.24 (m, 41H).

Biological Assays. Radioligand Binding Experiments. General Procedure. Membrane suspensions from lamb brain striatum or D_3R transiently transfected CHO cells were obtained by following the method previously described.[35] Radioligand binding assays using membrane suspensions (0.5 mg prot/mL determined with bicinchoninic acid kits) were carried out at 22 °C in 50 mM Tris-HCl buffer, pH 7.4 (see conditions below used for each receptor). After radioligand incubation, free and membrane-bound ligand were separated by rapid filtration of 500 μL aliquots in a cell harvester through Whatman GF/C filters embedded in 0.3% polyethylenimine (PEI) that were subsequently washed for 5 s with 5 mL of ice-cold Tris-HCl buffer. The filters were incubated with 10 mL of Ecoscint H scintillation cocktail overnight at room temperature, and radioactivity counts were determined using a scintillation counter with an efficiency of 62% for tritium labeled compounds and 99% for the ^{125}I-labeled compound.

Screening of the Library. Binding experiments of the whole library were performed at a concentration of 25 μM for compounds **1**, **2**, **1a**−**1s**, **2a**−**2s** and **9**−**16.**

Dopamine D_1, D_2, and D_3, Histamine H_3, and Metabotropic Glutamate 1/5 Receptors. Membranes were incubated with 0.9 nM [^{3}H]-SCH23390 (85 Ci/mmol), 0.7 nM [^{3}H]-YM09151-2 (85.5 Ci/mmol), 4 nM [^{3}H]-raclopride (82.8 Ci/mmol), 2.5 nM [^{3}H]-RAMH (34 Ci/mmol), or 27 nM [^{3}H]-quisqualic acid (30.9 Ci/mmol) in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$ for 2 h in the absence or in the presence of tested compounds. Nonspecific binding was measured in the presence of 10 μM SCH23390, raclopride, RAMH, or quisqualic acid, respectively.

Somatostatin Receptors. Membranes were incubated with 0.1 nM [^{125}I]-Tyr^{11}-somatostatin 14 (2,200 Ci/mmol) in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$ for 3 h in the absence or in the presence of tested compound. Nonspecific binding was measured in the presence of 100 nM somatostatin.

Adenosine A_1 and A_{2A} Receptors. Membranes were incubated with 1.0 nM [^{3}H]-*R*-PIA (30.5 Ci/mmol) or 1.6 nM [^{3}H]-ZM241385 (27.4 Ci/mmol) in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$ and 0.2 U/mL ADA for 2 h in the absence or in the presence of tested compounds. Nonspecific binding was measured in the presence of 10 μM DPCPX or ZM 241385, respectively.

Cannabinoid CB_1 Receptor. Membranes were incubated in siliconated tubes with 0.5 nM [^{3}H]-CP 55940 (144 Ci/mmol) in

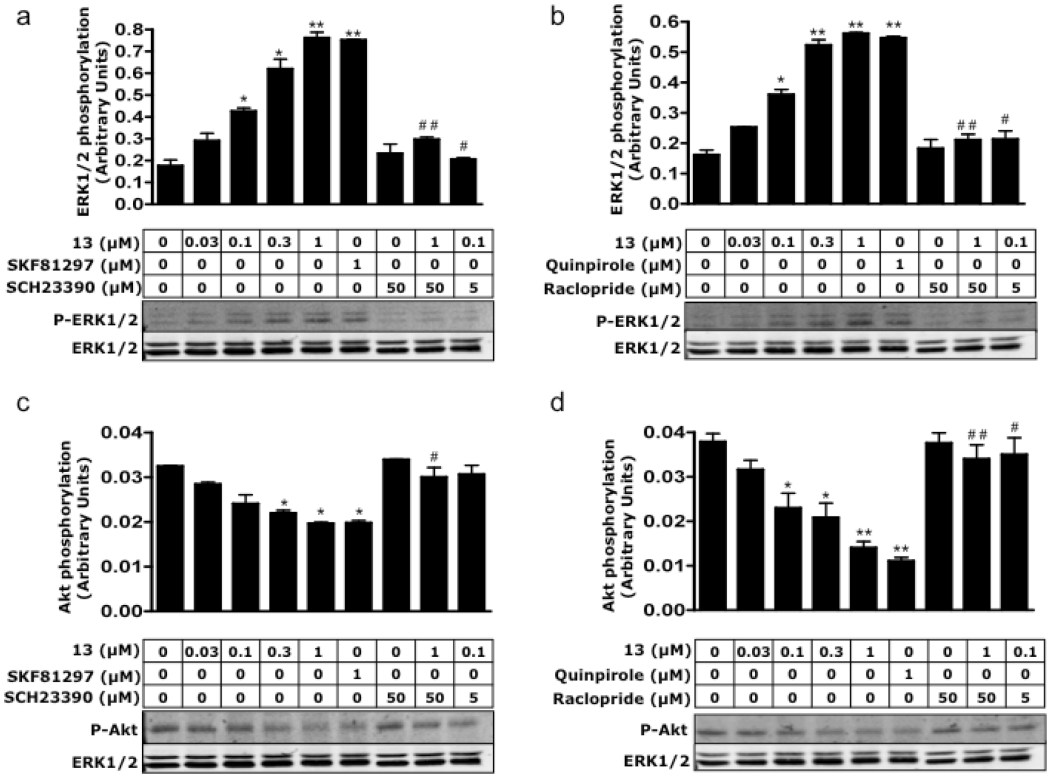


Figure 4. Functional characterization of the biotin ergopeptide **13**. CHO cells expressing D_1R (a and c) or D_2R (b and d) were cultured in serum-free medium for 16 h prior to the addition of any ligand. Cells were treated (or not) with 50 μM of the D_1R antagonist SCH23390 (a and c) or the D_2R antagonist raclopride (b and d). After 5 min, increasing concentrations of compound **13**, D_1R agonist SKF 81297, or the D_2R agonist quinpirole were added for further 5 min of incubation, and ERK1/2 phosphorylation (a and b) or P-Ser^{473}Akt (c and d) were determined as indicated in the Experimental Section. Results are expressed as means ± SEM of four independent experiments. Student's *t*-test for unpaired samples showed significant increases (a and b) or decreases (c and d) over basal (not treated cells, $*p < 0.05$, $**p < 0.01$) or significant decreases (a and b) or increases (c and d) respect to cells stimulated with the same concentration of compound **13** in the absence of antagonist ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$).

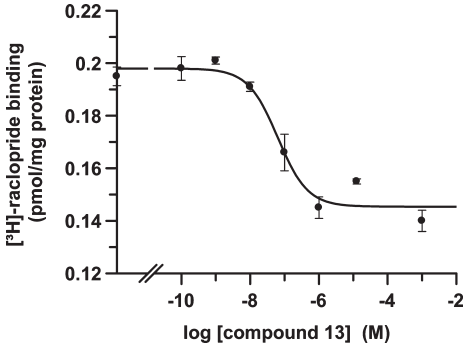


Figure 5. Competition curve of a D_3R antagonist binding versus increasing concentrations of compound **13**. Competition experiments of 4 nM D_3R antagonist [^{3}H]-raclopride versus increasing concentrations of compound **13** were performed with membranes (0.5 mg prot/mL) from CHO cells transiently transfected with 2 μg of the cDNA corresponding to D_3R, as indicated in the Experimental Section. Data are represented as means ± SD from a representative experiment ($n = 3$) performed in triplicate.

50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$ and 1 mg/mL fatty acid-free bovine serum albumin (BSA) for 2 h in the absence or in the presence of tested compounds. In this case, filters were presoaked with buffer containing BSA, and BSA was maintained in the washing medium. Nonspecific binding was measured in the presence of 10 μM CP55940.

***K_D* Determination.** Competition experiments of 0.9 nM [^{3}H]-SCH23390, 0.7 nM [^{3}H]-YM09151-2, or 4 nM [^{3}H]-raclopride binding versus increasing concentrations of compounds **9**, **12**, **13**, or **14** were performed by incubating membranes under the same conditions as described above for D_1R, D_2R, or D_3R binding. Nonspecific binding was determined as previously outlined. Radioligand displacement curves were analyzed by nonlinear regression using the commercial program GRAFIT (Erithacus Software, Surrey, UK) by fitting the specific binding data to the mechanistic two-state dimer receptor model.[35,36] To calculate the macroscopic equilibrium dissociation constants, the equations used for a competition binding experiment were deduced by Casadó.[35] Goodness-of-fit was tested following the reduced χ^2 value given by the nonlinear regression program GRAFIT. A modified *F* test was used to analyze whether the fit to cooperativity model significantly improved upon the fit to noncooperative model, and $p < 0.05$ was taken as a criterion of significance; when no significant improvement over the non-cooperative model was detected, the *p* values were > 0.30.

Cell Culture, Transient Transfection, and Protein Determination. Chinese hamster ovary (CHO) cells were cultured in MEM α medium without nucleosides supplemented with 100 U/mL penicillin/streptomycin and 10% (v/v) heat inactivated fetal bovine serum (FBS). CHO cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and were passaged when they were 80−90% confluent, i.e., approximately twice a week. CHO cells were transiently transfected with 2 μg cDNA corresponding to human D_1R, D_2R, or D_3R by the ramified PEI method. Cells were incubated for 4 h with the corresponding cDNA together with ramified PEI (5 mL/mg cDNA of 10 mM PEI) and 150 mM NaCl in a serum-starved medium. After 4 h, the medium was changed to a fresh complete culture medium. Forty-eight h after transfection, cells were washed twice in quick succession in HBSS (Hanks' balanced salt solution: 137 mM

Table 3

compd	t_R (min)	molecular formula	M_{calc}	M_{exp} (HRMS)	purity (220 nm) (%)	mg
9	3.33	$C_{57}H_{78}N_{12}O_{10}S$	1122.5685	1123.5747	95	12.6
10	3.68	$C_{57}H_{78}N_{12}O_{10}S$	1122.5685	1123.5747	99	14.3
11	3.20	$C_{59}H_{81}N_{13}O_{11}S$	1179.5899	1180.5961	99	14.5
12	3.58	$C_{59}H_{81}N_{13}O_{11}S$	1179.5899	1180.5963	98	13.6
13	3.88	$C_{62}H_{79}FN_{12}O_{10}S$	1202.5747	1203.5815	99	14.7
14	3.85	$C_{62}H_{79}FN_{12}O_{10}S$	1202.5747	1203.5804	94	15.5
15	3.81	$C_{64}H_{82}FN_{13}O_{11}S$	1259.5961	1260.6015	95	12.9
16	3.77	$C_{64}H_{82}FN_{13}O_{11}S$	1259.5961	1260.6016	96	25.1

NaCl, 5 mM KCl, 0.34 mM $Na_2HPO_4 \cdot 12H_2O$, 0.44 mM KH_2PO_4, 1.26 mM $CaCl_2 \cdot 2H_2O$, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM $MgCl_2$, 10 mM HEPES, pH 7.4) supplemented with 0.1% glucose (w/v), detached by gently pipetting and resuspended in the same buffer. To control the cell number, sample protein concentration was determined using a Bradford assay kit using BSA dilutions as standards.

ERK and Akt Phosphorylation Assay. Transfected CHO cells were cultured in serum-free medium for 16 h before the addition of any agent. Cells were treated or not with 5 or 50 μM of the D_1R antagonist SCH 23390 or the D_2R antagonist raclopride. After 5 min, increasing concentrations of compound **13**, 1 μM D_1R agonist SKF 81297, or 1 μM D_2R agonist quinpirole were added for further 5 min incubation. Cells were rinsed with ice-cold phosphate-buffered saline and lysed by the addition of 500 μL of ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 50 mM NaF, 150 mM NaCl, 45 mM β-glycerophosphate, 1% Triton X-100, 20 μM phenyl-arsine oxide, 0.4 mM $NaVO_4$, and protease inhibitor cocktail). The cellular debris was removed by centrifugation at 13000*g* for 5 min at 4 °C, and the protein was quantified by the bicinchoninic acid method using bovine serum albumin dilutions as standard. To determine the level of ERK1/2 or Akt-phosphorylation, equivalent amounts of protein (10 μg) were separated by electrophoresis on a denaturing 7.5% SDS-polyacrylamide gel and transferred onto PVDF-FL membranes. Odyssey blocking buffer was then added, and the membrane was rocked for 90 min. The membranes were then probed with a mixture of antiphospho-ERK1/2 antibody (1:2500) or anti-P-Ser^{473}Akt antibody (1:2500) and anti-ERK1/2 antibody that recognizes both phosphorylated and nonphosphorylated ERK1/2 (1:40000) for 2−3 h. Bands were visualized by the addition of a mixture of IRDye 800 (antimouse) antibody (1:10000) and IRDye 680 (antirabbit) antibody (1:10000) for 1 h and scanned by the Odyssey infrared scanner. Bands densities were quantified using the scanner software, exported to Excel. The level of phosphorylated ERK1/2 isoforms or P-Ser^{473}Akt was normalized for differences in loading using the total ERK protein band intensities.

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Supporting Information Available: Additional synthetic schemes, tables with chemical structures, and complete characterization data for the ergopeptide library, radioligand binding assays of **1c**, **1f**, **2c**, and **2f** (A_1R and $A_{2A}R$) and **9**−**16** (D_1R, D_2R, A_1R, and $A_{2A}R$), competition curves of **9**, **12**, and **14** at D_1R and D_2R and binding experiments of **13** at different GPCRs. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Vendrell, M.; Angulo, E.; Casadó, V.; Lluis, C.; Franco, R.; Albericio, F.; Royo, M. Novel ergopeptides as dual ligands for adenosine and dopamine receptors. *J. Med. Chem.* **2007**, *50*, 3062–3069.
(2) Maggio, R.; Aloisi, G.; Silvano, E.; Rossi, M.; Millan, M. J. Heterodimerization of dopamine receptors: new insights into functional and therapeutic significance. *Parkinsonism Relat. Disord.* **2009**, *15* (S4), S2–S7.
(3) So, C. H.; Verma, V.; O'Dowd, B. F.; George, S. R. Desensitization of the dopamine D1 and D2 receptor hetero-oligomer mediated calcium signal by agonist occupancy of either receptor. *Mol. Pharmacol.* **2007**, *72*, 450–462.
(4) So, C. H.; Varghese, G.; Curley, K. J.; Kong, M. M.; Alijaniaram, M.; Ji, X.; Nguyen, T.; O'Dowd, B. F.; George, S. R. D_1 and D_2 dopamine receptors form heterooligomers and cointernalize after selective activation of either receptor. *Mol. Pharmacol.* **2005**, *68*, 568–578.
(5) Bhushan, R. G.; Sharma, S. K.; Xie, Z.; Daniels, D. J.; Portoghese, P. S. A bivalent ligand (KDN-21) reveals spinal delta and kappa opioid receptors are organized as heterodimers that give rise to delta(1) and kappa(2) phenotypes. Selective targeting of delta−kappa heterodimers. *J. Med. Chem.* **2004**, *47*, 2969–2972.
(6) Waldhoer, M.; Fong, J.; Jones, R. M.; Lunzer, M. M.; Sharma, S. K.; Kostenis, E.; Portoghese, P. S.; Whistler, J. L. A heterodimer-selective agonist shows in vivo relevance of G protein-coupled receptor dimers. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 9050–9055.
(7) Adam, G. C.; Sorensen, E. J.; Cravatt, B. F. Proteomic profiling of mechanistically distinct enzyme classes using a common chemotype. *Nature Biotechnol.* **2002**, *20*, 805–809.
(8) Speers, A. E.; Adam, G. C.; Cravatt, B. F. Activity-based protein profiling in vivo using a copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. *J. Am. Chem. Soc.* **2003**, *125*, 4686–4687.
(9) Walsh, D. P.; Chang, Y. T. Chemical genetics. *Chem. Rev.* **2006**, *106*, 2476–2530.
(10) Middleton, R. J.; Briddon, S. J.; Cordeaux, Y.; Yates, A. S.; Dale, C. L.; George, M. W.; Baker, J. G.; Hill, S. J.; Kellam, B. New fluorescent adenosine A_1-receptor agonists that allow quantification of ligand−receptor interactions in microdomains of single living cells. *J. Med. Chem.* **2007**, *50*, 782–793.
(11) Lokey, R. S. Forward chemical genetics: progress and obstacles on the path to a new pharmacopoeia. *Curr. Opin. Chem. Biol.* **2003**, *7*, 91–96.
(12) Middleton, R. J.; Kellam, B. Fluorophore-tagged GPCR ligands. *Curr. Opin. Chem. Biol.* **2005**, *9*, 517–525.
(13) Gellerman, G.; Elgavi, A.; Salitra, A.; Kramer, M. Novel Gly building units for backbone cyclization: synthesis and incorporation into model peptides. *J. Pept. Res.* **2001**, *57*, 277–291.
(14) Vendrell, M.; Ventura, R.; Ewenson, A.; Royo, M.; Albericio, F. *N*-[Chloro(dimethylamino)methylene]-*N*-methylmethanaminium chloride (TMUCl Cl), the reagent of choice for the solid-phase synthesis of anilides. *Tetrahedron Lett.* **2005**, *46*, 5383–5386.
(15) Baraldi, P. G.; Tabrizi, M. A.; Gessi, S.; Borea, P. A. Adenosine receptor antagonists: translating medicinal chemistry and pharmacology into clinical utility. *Chem. Rev.* **2008**, *108*, 238–263.
(16) Agnati, L. F.; Ferré, S.; Lluis, C.; Franco, R.; Fuxe, K. Molecular mechanisms and therapeutical implications of intramembrane receptor/receptor interactions among heptahelical receptors with

examples from the striatopallidal GABA neurons. *Pharmacol. Rev.* **2003**, *55*, 509–550.
(17) Franco, R.; Casadó, V.; Mallol, J.; Ferré, S.; Fuxe, K.; Cortés, A.; Ciruela, F.; Lluis, C.; Canela, E. I. Dimer-based model for heptaspanning membrane receptors. *Trends Biochem. Sci.* **2005**, *300*, 360–366.
(18) Bayer, E. A.; Wilchek, M. Application of avidin−biotin technology to affinity-based separations. *J. Chromatogr.* **1990**, *510*, 3–11.
(19) Elia, G. Biotinylation reagents for the study of cell surface proteins. *Proteomics* **2008**, *8*, 4012–4024.
(20) Bratthauer, G. L. The avidin−biotin complex (ABC) and other avidin-biotin binding methods. *Methods Mol. Biol.* **1999**, *115*, 203–214.
(21) Smith, D. J.; Shappell, N. W. Technical note: epimerization of ergopeptine alkaloids in organic and aqueous solvents. *J. Anim. Sci.* **2002**, *80*, 1616–1622.
(22) Chausmer, A. L.; Katz, J. L. Comparison of interactions of D1-like agonists, SKF 81297, SKF 82958, and A-77636, with cocaine: locomotor activity and drug discrimination studies in rodents. *Psychopharmacology* **2002**, *159*, 145–153.
(23) Levant, B.; Grigoriadis, D. E.; De Souza, E. B. Characterization of [3H]-quinpirole binding to D_2-like dopamine receptors in rat brain. *J. Pharmacol. Exp. Ther.* **1992**, *262*, 929–935.
(24) Beaulieu, J.; Gainetdinov, R. R.; Caron, M. G. The Akt/GSK-3 signaling cascade in the actions of dopamine. *Trends Pharmacol. Sci.* **2007**, *28*, 166–172.
(25) Beaulieu, J.; Gainetdinov, R. R.; Caron, M. G. Akt/GSK3 signaling in the action of psychotropic drugs. *Annu. Rev. Pharmacol. Toxicol.* **2009**, *49*, 327–347.
(26) Marcellino, D.; Ferré, S.; Casadó, V.; Cortés, A.; Le Foll, B.; Mazzola, C.; Drago, F.; Saur, O.; Stark, H.; Soriano, A.; Barnes, C.; Goldberg, S. R.; Lluis, C.; Fuxe, K; Franco, R. Identification of dopamine D_1−D_3 receptor heteromers. Indications for a role of synergistic D_1−D_3 receptor interactions in the striatum. *J. Biol. Chem.* **2008**, *283*, 26016–26025.
(27) Ferrada, C.; Moreno, E.; Casadó, V.; Bongers, G.; Cortés, A.; Mallol, J.; Canela, E. I.; Leurs, R.; Ferré, S.; Lluís, C.; Franco, R. Marked changes in signal transduction upon heteromerization of dopamine D_1 and histamine H_3 receptors. *Br. J. Pharmacol.* **2009**, *157*, 64–75.
(28) Ferrada, C.; Ferré, S.; Casadó, V.; Cortés, A.; Justinova, Z.; Barnes, C.; Canela, E. I.; Goldberg, S. R.; Leurs, R.; Lluis, C.; Franco, R. Interactions between histamine H_3 and dopamine D_2 receptors and the implications for striatal function. *Neuropharmacology* **2008**, *55*, 190–197.
(29) Cabello, N.; Gandía, J.; Bertarelli, D. C.; Watanabe, M.; Lluís, C.; Franco, R.; Ferré, S.; Luján, R.; Ciruela, F. Metabotropic glutamate type 5, dopamine D_2 and adenosine A_{2A} receptors form higher-order oligomers in living cells. *J. Neurochem.* **2009**, *109*, 1497–1507.
(30) Kearn, C. S.; Blake-Palmer, K.; Daniel, E.; Mackie, K.; Glass, M. Concurrent stimulation of cannabinoid CB_1 and dopamine D_2 receptors enhances heterodimer formation: a mechanism for receptor cross-talk? *Mol. Pharmacol.* **2005**, *67*, 1697–1704.
(31) Rocheville, M.; Lange, D. C.; Kumar, U.; Patel, S. C.; Patel, R. C.; Patel, Y. C. Receptors for dopamine and somatostatin: formation of hetero-oligomers with enhanced functional activity. *Science* **2000**, *288*, 154–157.
(32) Vazquez, J.; Qushair, G.; Albericio, F. Qualitative colorimetric tests for solid phase synthesis. *Methods Enzymol.* **2003**, *369*, 21–35.
(33) Strohmeier, G. A.; Haas, W.; Kappe, C. O. Synthesis of functionalized 1,3-thiazine libraries combining solid-phase synthesis and post-cleavage modification methods. *Chem.—Eur. J.* **2004**, *10*, 2919–2926.
(34) Attardi, M. E.; Porcu, G.; Taddei, M. Malachite green, a valuable reagent to monitor the presence of free COOH on the solid-phase. *Tetrahedron. Lett.* **2000**, *41*, 7391–7394.
(35) Casadó, V.; Ferrada, C.; Bonaventura, J.; Gracia, E.; Mallol, J.; Canela, E. I.; Lluís, C.; Cortés, A.; Franco, R. Useful pharmacological parameters for G-protein-coupled receptor homodimers obtained from competition experiments. Agonist−antagonist binding modulation. *Biochem. Pharmacol.* **2009**, *78*, 1456–1463.
(36) Casadó, V.; Cortés, A.; Ciruela, F.; Mallol, J.; Ferré, S.; Lluís, C.; Canela, E. I.; Franco, R. Old and new ways to calculate the affinity of agonists and antagonists interacting with G-protein-coupled monomeric and dimeric receptors: the receptor−dimer cooperativity index. *Pharmacol. Ther.* **2007**, *116*, 343–354.