Bioorganic & Medicinal Chemistry 23 (2015) 3938–3947

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

# Fluoro-substituted phenylazocarboxamides: Dopaminergic behavior and N-arylating properties for irreversible binding

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ARTICLE INFO

*Article history:*
Received 24 October 2014
Revised 8 December 2014
Accepted 10 December 2014
Available online 17 December 2014

*Keywords:*
Covalent ligands
Bioisosteres
Azocarboxamide
Dopamine
$D_3$ receptor
GPCR

ABSTRACT

Phenylazocarboxamides can serve as bioisosteres for cinnamides, which are widely occurring substructures in medicinal chemistry. Starting from our lead compound **2**, the introduction of additional fluoro substituents and the exchange of the methoxyphenylpiperazine head group by an aminoindane moiety was investigated resulting in dopamine $D_3$ receptor antagonists and agonists with $K_i$ values in the sub- and low-nanomolar range. As a potentially irreversible ligand, the 3,4,5-trifluoro-substituted phenylazocarboxamide **7** was investigated for its N-arylating properties by incubation with the protected lysine analog **18** and with the L89K mutant of the dopamine $D_3$ receptor. Whereas covalent bond formation with the lysine unit in TM2 of $D_3$ could not be detected, substantial N-arylation of the side chain of the model compound **18** has been observed.



## 1. Introduction

G protein-coupled receptors (GPCRs) of the dopamine $D_2$ family including the subtypes $D_2$, $D_3$ and $D_4$ are generally known as important targets for the treatment of CNS disorders including Parkinson's disease, schizophrenia, depression and drug addiction.[1] In recent years, innovative studies have focused on the development of ligands for the dopamine $D_3$ receptor[2] exhibiting an inhibitory effect on adenylyl cyclase upon activation.[3] The particular regional distribution of the $D_3$ subtype in different brain areas points to a special role of these receptors in disorders related to drug abuse,[4] and $D_3$-subtype selective antagonists are considered to be antipsychotics with a favorable pharmacological profile and less side effects.[5]

Among dopamine $D_3$ subtype-selective ligands that have been synthesized as candidates for therapeutics,[6] compounds with phenylpiperazine head groups, such as BP 897[3a] and FAUC 346[7] have become of particular importance as lead structures (Fig. 1). In recent studies, the favorable phenylpiperazine unit, mimicking the dihydroxyphenethylamine structure of dopamine, has been combined with cinnamic acid amides,[8] which led to $D_3$ receptor ligands of type **1**.[9]

In parallel to the studies aiming at highly potent and subtype-selective dopamine $D_3$ receptor ligands, several attempts have been made to develop $^{18}$F-fluorine-labeled derivatives, which could correctly display the regional distribution of the $D_3$ receptor subtype in brain.[10–12] We found that the cinnamide moiety present in ligand **1** can be replaced by a *para*-fluorophenyl substituted azocarboxamide unit as it occurs as a substructure of ligand **2**.[13] Albeit this replacement led to a decrease in binding affinity to the dopamine $D_3$ subtype by almost one order of magnitude and also to a lower subtype selectivity over $D_2$, the general suitability of the azocarboxamide substructure, which was moreover shown to be reasonably stable regarding metabolism,[13] suggests further investigations on structural modifications.

Moreover, the aromatic core of structurally related phenylazocarboxylic esters is highly activated towards nucleophilic aromatic substitutions.[14] Depending on whether a nitro group, a phenoxy group or a fluorine atom is located in *para*-position relative to the azo unit to act as a leaving group, selective substitutions with phenols, aliphatic or aromatic amines could be achieved under mild conditions.[15] Due to the particularly high activation of the aromatic core, whereby the azocarbonyl unit exerts an even stronger effect than a nitro group,[16] we reasoned that compounds of type **2** might be valuable prototypes of irreversible ligands, given that a suitable nucleophile-containing amino acid side chain would be present in vicinity of the respective binding region of the receptor.

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http://dx.doi.org/10.1016/j.bmc.2014.12.012
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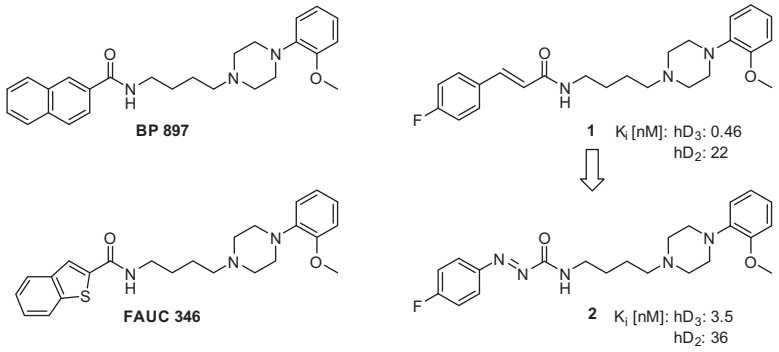



**Figure 1.** Lead compounds BP 897 and FAUC 346, and bioisosteric replacement of a cinnamide by a phenylazocarboxamide substructure.

Covalent binding of ligands to receptors[17] has mainly been accomplished by exploiting the reactivity of carbenes or nitrenes, generated under irradiation from diazirines[18] or azides,[19] oxygen-centered radicals photochemically generated from suitable ketones,[20] as well as by employing disulfides,[21] alkylating groups[22] or Michael acceptors,[23] which are able to undergo covalent bond formation with nucleophilic amino acid side chains. Irreversible ligand binding based on nucleophilic aromatic substitution, as it is the well-known reaction principle of Sanger's reagent,[24] has so far only rarely been exploited.[25]

In this article, we report the synthesis and biological studies on novel fluoro-substituted phenylazocarboxamides as well as model investigations on the 3,4,5-trifluoro-substituted test compound **7** regarding its N-arylating properties towards a protected lysine equivalent and a mutated dopamine $D_3$ receptor.

## 2. Results and discussion

### 2.1. Chemistry

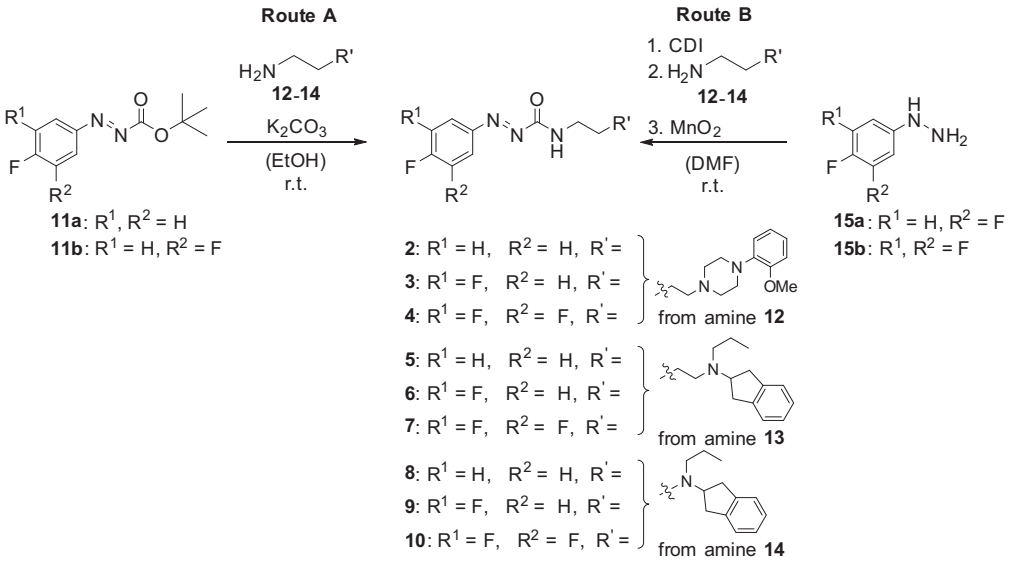
All azocarboxamide ligands **2**–**10** included in this study were prepared following the two routes A and B depicted in Scheme 1.

On route A, the mono- and difluorinated phenylazocarboxylic *tert*-butyl esters **11a** and **11b**, which were readily accessible by established procedures,[14] served as starting materials. Nucleophilic substitutions with amines **12**–**14** at the carbonyl unit provided the desired products **2**, **3**, **5**, **6**, **8** and **9**. Since the reactions of the difluorinated azoester **11b** thereby turned out not to be fully selective for the ester functionality, and substitution at the aromatic core was also observed, an alternative route B to the azocarboxamide ligands was investigated. Following this strategy, hydrazines **15a** and **15b** were first treated with carbonyldiimidazole (CDI) to give azocarbonyl imidazolides, which were subsequently reacted with the respective amines **12**–**14** to provide phenyl semicarbazides. Final oxidation with manganese dioxide gave the ligands **4**, **7** and **10**, as well as again **3**, **6** and **9**. Within the synthesis of the ligands **2**–**10** slight variations were made to further optimize the conditions. A generally applicable, preferred procedure could however not yet be established, which might partially be due to the different properties of the amines **12**–**14**.

Regarding the results and observations from both routes, it turned out that the monofluorinated (**2**, **5**, **8**) and difluorinated ligands (**3**, **6**, **9**) are accessible via both pathways, whereas the highly activated trifluorinated compounds (**4**, **7**, **10**) can only be prepared via route B. Attempts to synthesize ligands with a nitro group on the aromatic core (e.g., $R^1 = NO_2$, $R^2 = H$) failed on both routes A and B, which was due to an overly increased reactivity of the benzene core towards nucleophiles.

### 2.2. Receptor binding affinity

The amines **12**–**14** were chosen to investigate their receptor binding properties. Amine **12** contains a *N*-(2-methoxyphenyl)piperazine substructure, which represents one of the




**Scheme 1.** Synthesis of novel phenylazocarboxamide-type dopamine $D_3$ ligands.

well-established head groups for dopamine receptor antagonists.[1a,7] The aminoindane-based amine **13** was included in the study due to the known effect that related ligands show agonistic activity at dopamine receptors.[26] Inspired by computational studies, we also considered ligands derived from amine **14** with a shortened side chain. Competition binding experiments were done using the human dopamine receptors $D_{2long}$, $D_{2short}$,[27] $D_3$[28] and $D_{4.4}$[29] stably expressed in CHO cells. Binding affinities to human $D_1$, the serotonin receptors 5-$HT_{1A}$ and 5-$HT_{2A}$ and the $\alpha_1$ adrenoreceptor were also determined.[30] Biological data obtained are summarized in Table 1.

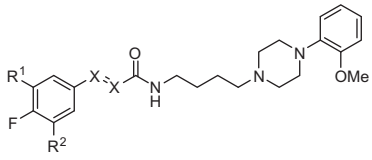
$D_3$ binding affinity of the phenylpiperazine derivative **2** was significantly reduced by the introduction of the N=N-unit (as compared to cinnamide **1**), but was largely regained when the degree of fluorination was increased, with a maximum affinity for the difluorinated azocarboxamide ligand **3** ($K_i$ = 0.72 nM). Within the aminobutyl substituted ligands **16** and **5**–**7**, no significant loss of $D_3$ binding affinity occured through the replacement of the cinnamide **16** by the azocarboxamide **5**. However, the introduction of further fluorine atoms on the aromatic core, as in compounds **6** and **7**, led to a remarkable loss in affinity towards $D_3$. Comparison of the $D_3$ binding affinity of the aminoethyl substituted carboxamides (**17**, **8**–**10**)[7] displayed an increase of affinity through the introduction of the N=N-unit. Whereas the related difluoro derivative **9** showed almost unchanged properties towards all dopamine receptor subtypes compared to **8**, the trifluorinated compound **10** displayed much lower binding affinities. Replacement of the C=C- for the N=N-unit led in most cases to a decrease of selectivity for $D_3$ over $D_{2long}$, but an increase in selectivity for $D_3$ over $D_{2short}$. In general, the trends observed for $D_3/D_2$ subtype selectivity follow known rules for dopamine ligands of this type.[4d] Whereas a variation of aliphatic chain length from aminobutyl to aminoethyl leads to a recognizable effect on subtype selectivity (groups A and B compared to group C), variations on the lipophilic azophenyl moiety, such as fluorination, as well as on the aminergic head group (group A vs B) have only minor influence.

### 2.3. Studies on irreversible binding and functional assays

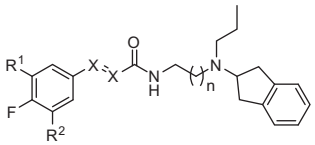
For initial experiments on the reactivity of the ligands towards nucleophilic aromatic substitution under assay conditions, ligand **7** was chosen as a representative structure (Scheme 2).[32] The reaction of **7** with *N*-acetyl lysine methyl ester **18** in DMF provided **19** as reference compound for further investigations in high yield. Under the conditions of a biological assay in PBS buffer at nearly neutral pH values, the same reaction proceeded much slower, but the desired conjugate **19** could still be identified unambiguously by HPLC-MS analysis (see Supporting information).[33] Increased reaction rates were determined upon addition of DMSO (1% or 1:1 v/v mixture) to the buffered solution, whereby the latter conditions gave **19** in 10% yield.

To incorporate a nucleophilic position into the binding pocket, the amino acid leucine at position 89 in the extracellular region of transmembrane helix 2 of the dopamine $D_3$ receptor was exchanged for lysine. The amino acid side chain in this position points to the binding pocket and may allow a covalent bond

**Table 1**
Receptor binding data at the dopamine $D_1$–$D_5$, the serotonin 5-$HT_{1A}$ and 5-$HT_{2A}$ and the adrenergic $\alpha_1$ receptors for the phenylazocarboxamides **2**–**10** and the cinnamides **1**, **16** and **17**[31]




group A: **1**: X = CH, $R^1 = R^2$ = H
**2**: X = N, $R^1 = R^2$ = H
**3**: X = N, $R^1$ = H, $R^2$ = F
**4**: X = N, $R^1 = R^2$ = F

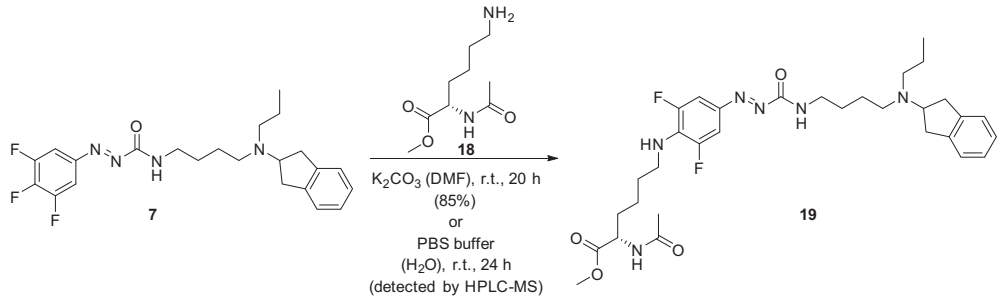



group B: **16**: X = CH, n = 3, $R^1 = R^2$ = H
**5**: X = N, n = 3, $R^1 = R^2$ = H
**6**: X = N, n = 3, $R^1$ = H, $R^2$ = F
**7**: X = N, n = 3, $R^1 = R^2$ = F

group C: **17**: X = CH, n = 1, $R^1 = R^2$ = H
**8**: X = N, n = 1, $R^1 = R^2$ = H
**9**: X = N, n = 1, $R^1$ = H, $R^2$ = F
**10**: X = N, n = 1, $R^1 = R^2$ = F

| Compd | $K_i$ values[a] (nM ± SEM) | | | | | | | | | Ratio $D_2/D_3$[b] |
|---|---|---|---|---|---|---|---|---|---|---|
| | $hD_1$ | $hD_5$ | $hD_{2long}$ | $hD_{2short}$ | $hD_3$ | $hD_{4.4}$ | p5-$HT_{1A}$ | h5-$HT_{2A}$ | p$\alpha_1$ | |
| | [$^3$H]SCH23390 | | [$^3$H]spiperone | | | | [$^3$H]WAY 600135 | [$^3$H]ketanserin | [$^3$H]prazosin | |
| **1**[9] | 760 ± 120[c] | nd | 31 ± 4.1 | 22 ± 1.1 | 0.46 ± 0.077 | 75 ± 23 | 28 ± 1.4[c] | 330 ± 160[c] | 5.2 ± 1.4[c] | 67 (48) |
| **2**[13] | 1300 ± 320 | 2800 ± 550 | 38 ± 4.6 | 34 ± 3.0 | 3.0 ± 0.41 | 94 ± 13 | 3.3 ± 1.1 | 540 ± 75 | 5.2 ± 0.38 | 13 (11) |
| **3** | nd | nd | 7.5 ± 0.92[c] | 12 ± 4.4[c] | 0.72 ± 0.18[c] | 36 ± 5.7[c] | nd | nd | nd | 10 (16) |
| **4** | nd | nd | 6.6 ± 3.1[c] | 9.5 ± 3.5[c] | 1.4 ± 0.074[c] | 47 ± 24[c] | nd | nd | nd | 4.7 (6.8) |
| **16** | 1300 ± 70[c] | 2900 ± 71[c] | 19 ± 5.0[c] | 2.9 ± 0.21[c] | 0.31 ± 0.12[c] | 16 ± 5.0[c] | 5.0 ± 2.1[c] | 89 ± 7.8[c] | 120 ± 29[c] | 61 (9.4) |
| **5** | 3300 ± 750 | 7000 ± 2200 | 23 ± 3.4 | 36 ± 10 | 0.85 ± 0.22 | 34 ± 5.4 | 5.8 ± 0.60 | 550 ± 75 | 170 ± 13 | 27 (42) |
| **6** | nd | nd | 25 ± 7.1[c] | 13 ± 4.2[c] | 2.0 ± 0.28[c] | 31 ± 5.7[c] | nd | nd | nd | 13 (6.5) |
| **7** | nd | nd | 40 ± 14[c] | 12 ± 3.0[c] | 4.9 ± 1.6[c] | 40 ± 2.8[c] | nd | nd | nd | 8.2 (2.4) |
| **17** | 2200 ± 370 | 11000 ± 35000 | 43 ± 2.8 | 13 ± 4.0 | 14 ± 4.6 | 1.4 ± 0.10 | 4.3 ± 1.0 | 210 ± 23 | 58 ± 4.1 | 3.1 (0.93) |
| **8** | nd | nd | 5.0 ± 2.0[c] | 4.0 ± 0.071[c] | 1.4 ± 0.21[c] | 0.99 ± 0.041[c] | nd | nd | nd | 3.6 (2.9) |
| **9** | nd | nd | 3.9 ± 1.2[c] | 8.5 ± 5.0[c] | 2.8 ± 1.3[c] | 2.0 ± 0.21[c] | nd | nd | nd | 1.4 (3.0) |
| **10** | 2600 ± 540 | 3800 ± 88 | 110 ± 31 | 73 ± 9.6 | 87 ± 14 | 10 ± 3.0 | 3.3 ± 1.5 | 510 ± 54 | 900 ± 61 | 1.3 (0.84) |

nd = not determined.
[a] $K_i$-values in nM ± SEM are mean values of 3–7 independent experiments each done in triplicate.
[b] Selectivity of binding for $D_3$ over $D_2$ displayed as ration of $K_i(D_{2long})/K_i(D_3)$ and $K_i(D_{2short})/K_i(D_3)$ in parentheses.
[c] $K_i$-values in nM ± SD are mean values of two independent experiments each done in triplicate.




**Scheme 2.** Nucleophilic aromatic substitution of **7** with protected lysine **18** in dimethylformamide and under conditions of a biological assay.

formation with an appropriately positioned ligand. Covalent linkage at the analogous position $His^{89}$ in the $\beta_2$AR has been reported.[34]

HEK293T cells transiently transfected with the $D_3^{wt}$ receptor and the $D_3^{L89K}$ receptor mutant, respectively, were stimulated with the reference agonist quinpirole in an IP accumulation assay[21a,35] to determine the ability of the mutant to be stimulated similarly to the wild type. The mutant thereby showed no loss in receptor function compared to the wild-type, even a slightly improved activation profile could be observed (see Supporting information).

The azocarboxamides **5**, **6** and **7** were then investigated for their efficacy and potency to activate the $D_3$ wild-type receptor and the $D_3^{L89K}$ receptor mutant. In general, the activation pattern of all three compounds did not notably differ. At the wild-type receptor, potencies in a low nanomolar range (2.6–11 nM) and nearly full agonist properties were observed, which is similar to the potency of the reference agent quinpirole with an $EC_{50}$ of 2.4 nM (Fig. 2, left). A slightly different pattern of receptor activation could be observed at the $D_3^{L89K}$ mutant, whereby the mutation caused a rightward shift to the dose–response curves. The activity of quinpirole was not significantly influenced by that mutation. The potencies of the aminoindanes **5**, **6** and **7** at the wild-type receptor decreased 3–6-fold compared to the reference but indicated still nearly full agonist properties (Fig. 2, right). At the $D_3^{L89K}$ mutant, the test compounds **5** and **6** showed strong partial agonism whereas the trifluoro-substituted azocarboxamide **7** was able to fully activate the receptor. (Fig. 2, see also Supporting information)

The study on the irreversibility of binding was performed by an IP accumulation assay which allows the detection of continued [$^3$H]IP production in the presence of an antagonist in cells expressing the receptor of interest.[21a,35] HEK cells transiently expressing the $D_3^{L89K}$ mutant were stimulated with 3 μM of each of the fluorinated phenylazocarboxamides **5**, **6** and **7** for 60 min or 240 min, respectively. In parallel after preincubation for 60 min, the $D_3$ antagonist haloperidol (10 μM) was added and incubation was continued for further 180 min. Accumulated radioactivity was determined and the resulting IP-response was expressed as fold over basal.

In these experiments the reference agent quinpirole caused an activation level of 3.2-fold over basal after 60 min further increasing to 6.8-fold over basal after 4 h of incubation time. Adding haloperidol after 60 min, the IP-response of the reference remained at 3.7-fold over basal representing approximately the level after 60 min incubation without antagonist. For the test compounds **5**, **6** and **7**, a similar behavior could be observed (see Supporting information). Any irreversible blocking of the receptor by an agonist should result in an increased formation of IP also after addition of an antagonist. As we could not observe any further increase of IP accumulation for **5**–**7**, this result lead us to the conclusion that these compounds were not able to conjugate within the receptor under the investigated conditions.

## 3. Summary

Based on the lead structure of phenylazocarboxamide **2**, the introduction of additional fluoro substituents on the aromatic core combined with a replacement of the methoxyphenylpiperazine head group by an aminoindane moiety led to new dopamine $D_3$ receptor antagonists and agonists, of which compound **3** and **5** even showed $K_i$ values in the subnanomolar range. The present study therefore strongly supports the assumption that phenylazocarboxamides represent a so far unknown group of bioisosteres for cinnamides. The remarkable potential of phenylazocarboxamides for future studies was further underlined by functional assays with the aminoindane derivatives **5**–**7**. Attempts to exploit the particularly high reactivity at the aromatic core of the 3,4,5-trifluoro-substituted phenylazocarboxamide **7** were successful with the protected lysine analog **18**, since irreversible conjugation

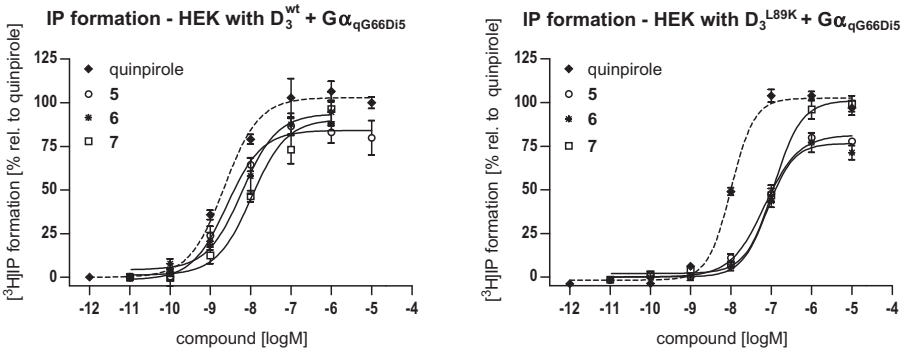



**Figure 2.** Dose–response curves of the fluorinated phenylazocarboxamides **5**–**7** and the reference quinpirole at $D_3^{wt}$ or $D_3^{L89K}$ expressing cells. In an IP accumulation assay the functional properties of the test compounds were measured at cells transiently expressing $D_3^{wt}$ and the promiscuous G-protein $G\alpha_{qG66Di5}$ (left) or the mutant $D_3^{L89K}$ and $G\alpha_{qG66Di5}$ (right). Curves represent pooled and normalized means ± SEM of one to three independent experiments each done in triplicate.

through N-arylation could be observed under the conditions of a biological cell assay. Initial experiments with ligand **7** and the L89K mutant of the dopamine $D_3$ receptor did not lead to covalent binding, which might however be due to the protonation of the newly incorporated lysine unit by unfavorably placed glutamic acid residues at positions 90 (TM2) and 363 (TM7). Having shown that the reactivity of the aromatic core of phenylazocarboxamides is generally sufficient to achieve N-arylation, our current efforts focus on the design and the synthesis of ligands for receptor subtypes naturally presenting lysine at their binding sites. Moreover, experiments are underway to explore an extension of the principle towards a selective labeling of tyrosine residues with 4-nitrophenylazocarboxamides.[14,15]

## 4. Experimental

### 4.1. Chemistry

Solvents and reagents were obtained from commercial sources and used as received. NMR spectra were recorded on Bruker Avance 600 ($^1$H: 600 MHz, $^{13}$C: 151 MHz) and Bruker Avance 360 ($^1$H: 360 MHz, $^{13}$C: 91 MHz). For $^1$H NMR $CDCl_3$, $CD_3OD$, $D_2O$ and $CD_3CN$ were used as solvents referenced to TMS (0 ppm), $CHCl_3$ (7.26 ppm) or $CHD_2CN$ (1.94 ppm). For $^{13}$C NMR $CDCl_3$, $CD_3OD$ and $CD_3CN$ were used as solvents with $CDCl_3$ (77.0 ppm) and $CD_3CN$ (1.24 ppm) as standard. $^{19}$F NMR spectra were recorded at 339 MHz using $C_6F_6$ (−164.9 ppm) as standard. Chemical shifts were reported in parts per million (ppm). Coupling constants are reported in Hertz (*J* Hz). The following abbreviations are used for the description of signals: s (singlet), d (doublet), t (triplet), q (quadruplet), quin (quintet), m (multiplet), br s (broad signal). Mass spectra were recorded on Jeol GC mate II GC–MS-system using electron impact (EI) or electron spray ionization (ESI) and a sector field mass analyzer for MS and HRMS measurements. High-performance liquid chromatography (HPLC) is performed on a Varian 940-LC with a XRs C8 column (21.2 × 150 mm × 5 µm) and PDA detection. (Solvent A: 0.1% aqueous TFA solution, solvent B: acetonitrile, flow rate 20 mL/min, Method **A**: Gradient A/B: min 0–7, 90/10% → 80/20%, min 7–15, 80/20% → 0/100%, min 15–22, 0/100%; Method **B**: Gradient A/B: min 0–10, 100/0%→ 70/30%, min 10–18, 70/30% → 20/80%, min 18–25, 20/80%, min 25–26, 20/80% → 0/100%, min 26–30 0/100%). HPLC–MS is performed on an Agilent 1100 series with a Zorbax Eclipse XDB-C8 column (4.6 × 150 mm × 5 µm) and VWL detection coupled with a Bruker-Esquire 2000 mass spectrometer with EI-ionisation. (Solvent A: 0.1% aqueous HCOOH solution, solvent B: methanol, flow rate 0.5 mL/min. Gradient A/B: 0–3, 90/10%, min 3–18, 90/10% → 100/0%, min 18–24, 100/0%, min 24–26, 100/0% → 10/90%, 26–30 10/90%.) Analytical TLC was carried out on Merck silica gel plates using short wave (254 nm) UV light, $KMnO_4$ [3.0 g $KMnO_4$, 20 g potassium carbonate, 5.0 mL aqueous sodium hydroxide (5% w/w) in 300 mL $H_2O$] and ninhydrin [200 mg ninhydrin in 100 mL ethanol] to visualise components. Silica gel (Kieselgel 60, 40–63 µm, Merck) was used for flash column chromatography. The phosphate buffered saline (PBS) was adjusted to a pH value of 7.3 [137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \times 7$ $H_2O$, 1.3 mM $KH_2PO_4$ in 200 mL $H_2O$]. Compounds **1**,[9] **2**,[13] **11a**,[14] **12**[9] and **13**[26] were prepared according to literature procedures.

#### 4.1.1. General procedure for the synthesis of phenylhydrazines[36]

A solution of the respective aniline (8.01–10.0 mmol) in glacial acetic acid (5.0 mL) was treated with concentrated hydrochloric acid (20–50 mL, 36%) at room temperature and cooled to 0 °C. Subsequently a pre-cooled solution of sodium nitrite (11.0–12.0 mmol) in water (3.0 mL) was added slowly and the mixture was stirred for 1 h at 0 °C. The chilled solution was filtered and then a solution of tin(II)–chloride dihydrate (20.0–22.0 mmol) in concentrated hydrochloric acid (10 mL) was added dropwise at 0 °C. The precipitate was filtered off and washed with a saturated aqueous solution of sodium chloride (30 mL). Due to stability reasons hydrazines which were not used immediately, were dried and stored as hydrochlorides. They were later extracted under basic conditions directly before using them in a reaction. Therefore the solid hydrochloride (1.00 mmol) was dissolved in a saturated aqueous solution of sodium carbonate (40.0 mL) and then extracted with diethyl ether (4 × 50 mL). The combined organic phases were washed with a saturated aqueous solution of sodium chloride (20 mL) and dried over sodium sulfate. After filtration the solvent was removed under reduced pressure. The phenylhydrazine could be used without further purification.

**4.1.1.1. 3,4-Difluorophenylhydrazine hydrochloride (15a).** Compound **15a** was prepared from 3,4-difluoroaniline (1.03 g, 8.01 mmol), sodium nitrite (0.83 g 12.0 mmol) and tin(II)–chloride dihydrate (4.51 g, 20.0 mmol) in hydrochloric acid (20 mL). Hydrochloride **15a** (1.15 g, 6.36 mmol, 79%) was obtained as a white solid. $^1$H NMR (600 MHz, $CDCl_3$ as free base) $\delta$ 3.57 (br s, 2H), 5.16 (br s, 1H), 6.48 (dtd, *J* = 1.6 Hz, *J* = 3.2 Hz, *J* = 7.9 Hz, 1H), 6.70 (ddd, *J* = 2.8 Hz, *J* = 6.7 Hz, *J* = 12.0 Hz, 1H), 7.00 (td, *J* = 8.6 Hz, *J* = 10.0 Hz, 1H); $^{19}$F NMR (339 MHz, HCl salt, $D_2O$) $\delta$ −138.0 (m, 1F), −146.8 (m, 1F), (the analytical data obtained is in agreement with those reported in literature).[37]

**4.1.1.2. 3,4,5-Trifluorophenylhydrazine hydrochloride (15b).** Compound **15b** was prepared from 3,4,5-trifluoroaniline (1.47 g, 10.0 mmol), sodium nitrite (0.76 g, 11.0 mmol) and tin(II)–chloride dihydrate (5.00 g, 22.0 mmol) in hydrochloric acid (50 mL). Hydrochloride **15b** (576 mg, 2.91 mmol, 29%) was obtained as a white solid. $^1$H NMR (360 MHz, $CDCl_3$ + 1% $CD_3OD$) $\delta$ 6.67–6.76 (m, 2H); $^{13}$C NMR (151 MHz, $CDCl_3$ + 1% $CD_3OD$) $\delta$ 99.4 (dd, $J_{CF}$ = 6.1 Hz, $J_{CF}$ = 19.9 Hz, 2× CH), 135.3 (td, $J_{CF}$ = 15.3 Hz, $J_{CF}$ = 246.0 Hz, $C_q$), 140.5 (dt, $J_{CF}$ = 3.4 Hz, $J_{CF}$ = 10.5 Hz, $C_q$), 151.3 (ddd, $J_{CF}$ = 5.2 Hz, $J_{CF}$ = 10.5 Hz, $J_{CF}$ = 248.9 Hz, 2× $C_q$).

#### 4.1.2. *tert*-Butyl (*E*)-2-(3,4-difluorophenyl)diazene-1-carboxylate (11b)

To a solution of hydrazine **15a** (440 mg, 3.05 mmol) in dry acetonitrile (30 mL) was added di-*tert*-butyldicarbonate (800 mg, 3.66 mmol) under argon atmosphere at room temperature. The mixture was stirred at room temperature and after complete consumption of the reactants, as monitored by TLC, the solvent was removed under reduced pressure. The crude product was purified by column chromatography (silica gel, hexane/ethyl acetate = 6:1) to give the difluorophenylhydrazine carboxylate (542 mg, 2.22 mmol, 73%) as a light orange solid. $R_f$ = 0.2 (hexane/ethyl acetate = 4:1) [UV, $KMnO_4$]; $^1$H NMR (600 MHz, $CDCl_3$) $\delta$ 1.47 (s, 9H), 6.37 (br s, 1H), 6.49–6.53 (m, 1H), 6.66 (ddd, *J* = 2.7 Hz, *J* = 6.7 Hz, *J* = 11.9 Hz, 1H), 7.01 (td, *J* = 2.7 Hz, *J* = 8.7 Hz, 1H); $^{13}$C NMR (151 MHz, $CDCl_3$) $\delta$ 28.2 (3× $CH_3$), 81.7 ($C_q$), 102.4 (d, $J_{CF}$ = 21.4 Hz, CH), 108.2–108.3 (m, $C_q$), 117.5 (dd, $J_{CF}$ = 1.3 Hz, $J_{CF}$ = 18.3 Hz, CH), 145.0 (dd, $J_{CF}$ = 12.9 Hz, $J_{CF}$ = 239.6 Hz, $C_q$), 145.4 (d, $J_{CF}$ = 7.5 Hz, CH), 150.8 (dd, $J_{CF}$ = 13.6 Hz, $J_{CF}$ = 246.4 Hz, $C_q$), 156.1 ($C_q$); $^{19}$F NMR (339 MHz, $D_2O$) $\delta$ −139.8 (ddd, *J* = 9.0 Hz, *J* = 11.8 Hz, *J* = 21.0 Hz, 1F), −151.9 (dddd, *J* = 3.4 Hz, *J* = 6.8 Hz, *J* = 10.0 Hz, *J* = 13.1 Hz, 1F). In the second step manganese dioxide (535 mg, 6.15 mmol) was added to a solution of the difluorophenylhydrazine carboxylate (300 mg, 1.23 mmol) in dry dichloromethane (10 mL) under argon atmosphere at room temperature. After complete consumption of the reactants, as monitored by TLC, the mixture was filtered over Celite® and washed with dichloromethane. The solvent was removed under reduced pressure and the crude

product was purified by column chromatography (silica gel, hexane/ethyl acetate = 19:1) to give **11b** (296 mg, 1.22 mmol, 99%) as a light orange solid. $R_f$ = 0.5 (hexane/ethyl acetate = 19:1) [UV, $KMnO_4$]; $^1$H NMR (600 MHz, $CDCl_3$) $\delta$ 1.66 (s, 9H), 7.33 (ddd, $J$ = 7.9 Hz, $J$ = 8.8 Hz, $J$ = 9.5 Hz, 1H), 7.71 (ddd, $J$ = 2.4 Hz, $J$ = 7.4 Hz, $J$ = 10.5 Hz, 1H), 7.78–7.81 (m, 1H); $^{13}$C NMR (151 MHz, $CDCl_3$) $\delta$ 27.8 (3× $CH_3$), 85.5 ($C_q$), 110.3 (dd, $J_{CF}$ = 1.7 Hz, $J_{CF}$ = 18.6 - Hz, CH), 117.7 (d, $J_{CF}$ = 18.7 Hz, CH), 123.0 (dd, $J_{CF}$ = 3.4 Hz, $J_{CF}$ = 7.2 Hz, CH), 148.1 (dd, $J_{CF}$ = 3.5 Hz, $J_{CF}$ = 4.6 Hz, $C_q$), 150.9 (dd, $J_{CF}$ = 14.1 Hz, $J_{CF}$ = 252.6 Hz, $C_q$), 153.6 (dd, $J_{CF}$ = 13.5 Hz, $J_{CF}$ = 257.9 Hz, $C_q$), 160.7 ($C_q$); $^{19}$F NMR (339 MHz, $CDCl_3$) $\delta$ −132.2 (m, 1F), −137.8 (m, 1F).

### 4.1.3. General procedures for the synthesis of phenylazocarboxamides 3–10

*From phenylazocarboxylic acid tert-butyl esters (Route A, Scheme 1)*: To a stirred solution of the phenylazocarboxylic acid *tert*-butyl ester (**11a** or **11b**, 0.3–0.4 mmol) and $K_2CO_3$ (4 equiv) or triethyl amine (2.5 equiv) in dry ethyl acetate (5.0 mL) or dry ethanol (2.0 mL) was added the amine (**12**, **13** or **14**) (0.5 equiv) and the resulting mixture was stirred at room temperature. The reaction course was monitored by TLC. The reaction mixture was either concentrated under reduced pressure or diluted with water (30 mL) and extracted with ethyl acetate (3× 30 mL). The combined organic phases were then washed with a saturated aqueous solution of sodium chloride (20 mL) and dried over sodium sulfate. After filtration the solvent was removed under reduced pressure. The crude product was purified by column chromatography, and for the biological testing additionally via preparative HPLC (Method **A** or **B**) to give the required product.

*From phenylhydrazines (Route B, Scheme 1)*: A solution of phenylhydrazine hydrochloride (**15a** or **15b**, 0.3–0.7 mmol) in dry *N*,*N*-dimethylformamide (2.0–3.0 mL) was added dropwise to a solution of 1,1′-carbonyldiimidazole (1 equiv) in dry *N*,*N*-dimethylformamide (3.0–9.0 mL) over a period of 15–30 min at room temperature under nitrogen atmosphere and stirring was continued for 2 h. To the resulting mixture a solution of the respective amine (0.6–1.3 equiv) in dry *N*,*N*-dimethylformamide (2.0–3.0 mL) was added over a period of 30 min and stirring was continued for 2.5 h to overnight. Subsequently manganese dioxide (4.0–8.0 equiv) was added and the mixture was stirred for 2 h. The solid was filtered off and washed with dichloromethane or chloroform. The combined organic phases were concentrated under reduced pressure and the crude product was purified via column chromatography as described for every product. For the biological testing an additional purification via preparative HPLC (Method **A** or **B**), to give the required product, was made. Variations from this procedure are described with every product.

#### 4.1.3.1. (*E*)-2-(3,4-Difluorophenyl)-*N*-(4-(4-(2-methoxyphenyl) piperazin-1-yl)-butyl)diazene-1-carboxamide (3).

According to the general procedure for Route B, a solution of 3,4-difluorophenylhydrazine hydrochloride (**15a**) (42.3 mg, 293 μmol) in dry *N*,*N*-dimethylformamide (3.0 mL) was added dropwise to a solution of 1,1′-carbonyldiimidazole (47.5 mg, 293 μmol) in dry *N*,*N*-dimethylformamide (3.0 mL) over a period of 15 min at room temperature under nitrogen atmosphere and stirred for further 2 h. To this mixture a solution of 4-(4-(2-methoxyphenyl) piperazin-1-yl)butan-1-amine (**12**) (47.5 mg, 293 μmol) in dry *N*,*N*-dimethylformamide (2.0 mL) was added over a period of 15 min and the resulting mixture was stirred overnight. Subsequently manganese dioxide (209 mg, 2.40 mmol) was added and stirring was continued for 1 h. The solid was filtered off and was washed with dichloromethane. The combined organic solutions were concentrated under reduced pressure and the crude product was purified via column chromatography (silica gel, desact. with $NEt_3$, hexane/ethyl acetate = 1:3 → 100% ethyl acetate). Azocarboxamide **3** (52.2 mg, 121 μmol, 41%) was obtained as red-brown viscous oil. For biological testing it was additionally purified using HPLC method A. $R_f$ = 0.4 (100% ethyl acetate desact. $NEt_3$) [UV]; $^1$H NMR (360 MHz, TFA salt, $CDCl_3$) $\delta$ 1.70–1.85 (m, 4H), 2.54 (t, $J$ = 6.4 Hz, 2H), 2.72 (br s, 4H), 3.10 (br s, 4H), 3.54 (q, $J$ = 6.0 Hz, 2H), 3.85 (s, 3H), 6.79 (dd, $J$ = 2.0 Hz, $J$ = 7.6 Hz, 1H), 6.83–6.90 (m, 2H), 6.96–7.03 (m, 1H), 7.27 (ddd, $J$ = 7.9 Hz, $J$ = 8.8 Hz, $J$ = 9.5 Hz, 1H), 7.71 (ddd, $J$ = 2.4 Hz, $J$ = 7.4 Hz, $J$ = 10.5 Hz, 1H), 7.80 (dddd, $J$ = 1.5 Hz, $J$ = 2.4 Hz, $J$ = 4.1 Hz, $J$ = 8.7 Hz, 1H), 8.06 (br s, 1H); $^{13}$C NMR (151 MHz, TFA salt, $CDCl_3$) $\delta$ 24.2 ($CH_2$), 27.4 ($CH_2$), 40.7 ($CH_2$), 50.1 (2× $CH_2$), 53.2 (2× $CH_2$), 55.3 ($CH_2$), 57.8 ($CH_3$), 110.5 (dd, $J_{CF}$ = 1.6 Hz, $J_{CF}$ = 18.6 Hz, CH), 111.2 (CH), 117.7 (d, $J_{CF}$ = 18.7 Hz, CH), 118.0 (CH), 120.9 (CH), 123.1 (CH), 123.2 (dd, $J_{CF}$ = 3.3 Hz, $J_{CF}$ = 7.1 Hz, CH), 140.8 ($C_q$), 147.8 (dd, $J_{CF}$ = 3.3 Hz, $J_{CF}$ = 4.8 Hz, $C_q$), 150.8 (dd, $J_{CF}$ = 13.9 Hz, $J_{CF}$ = 252.5 Hz, $C_q$), 152.1 ($C_q$), 153.6 (dd, $J_{CF}$ = 13.3 Hz, $J_{CF}$ = 257.9 Hz, $C_q$), 160.6 ($C_q$); $^{19}$F NMR (339 MHz, TFA salt, $CDCl_3$) $\delta$ −78.8 (TFA), −131.8 (dd, $J$ = 9.4 Hz, $J$ = 21.7 Hz, 1F), −137.7 (m, 1F); MS (EI) $m/z$ (%): 431 (22) [$M^+$], 290 (100), 205 (34), 193 (24), 150 (27), 120 (21), 70 (67); HRMS (ESI) $m/z$ calcd for $C_{22}H_{28}F_2N_5O_2$ [$MH^+$] 432.2206, found 432.2199.

#### 4.1.3.2. (*E*)-*N*-(4-(4-(2-Methoxyphenyl)piperazin-1-yl)butyl)-2-(3,4,5-trifluoro-phenyl)diazene-1-carboxamide (4).

According to the general procedure for Route B, a solution of 3,4,5-trifluorophenylhydrazine hydrochloride (**15b**) (85.2 mg, 0.53 mmol) in dry *N*,*N*-dimethylformamide (2.5 mL) was added dropwise to a solution of 1,1′-carbonyldiimidazole (84.9 mg, 0.52 mmol) in dry *N*,*N*-dimethylformamide (4.0 mL) over a period of 15 min at room temperature under nitrogen atmosphere and stirring was continued for 2 h. To the resulting mixture a solution of 4-(4-(2-methoxyphenyl)piperazin-1-yl)butan-1-amine (**12**) (140 mg, 0.53 mmol) in dry *N*,*N*-dimethylformamide (2.0 mL) was added over a period of 15 min and stirring was continued overnight. In this particular case no $MnO_2$ was added. The organic solution was concentrated under reduced pressure and the crude product was purified via column chromatography (silica gel, desact. with $NEt_3$, hexane/ethyl acetate = 1:2 → 100% ethyl acetate). Azocarboxamide **4** (24.0 mg, 5.3 μmol, 10%) was obtained as brown viscous oil. For biological testing it was additionally purified using HPLC method A. $R_f$ = 0.4 (100% ethyl acetate desact. $NEt_3$) [UV, $KMnO_4$], $^1$H NMR (360 MHz, $CDCl_3$) $\delta$ 1.68–1.86 (m, 4H), 2.53 (t, $J$ = 6.4 Hz, 2H), 2.71 (br s, 4H), 3.08 (br s, 4H), 3.54 (dd, $J$ = 5.6 Hz, $J$ = 11.3 Hz, 2H), 3.85 (s, 3H), 6.77 (dd, $J$ = 1.7 Hz, $J$ = 8.2 Hz, 1H), 6.82–6.88 (m, 2H), 6.96–7.02 (m, 1H), 7.60 (dd, $J$ = 6.5 Hz, $J$ = 7.8 Hz, 2H), 8.26 (br s, 1H); $^{13}$C NMR (91 MHz, $CDCl_3$) $\delta$ 24.4 ($CH_2$), 27.4 ($CH_2$), 40.1 ($CH_2$), 50.1 (2× $CH_2$), 53.2 (2× $CH_2$), 55.3 ($CH_2$), 57.8 ($CH_3$), 108.4 (d, $J_{CF}$ = 22.7 - Hz, 2× CH), 108.4 ($J_{CF}$ = 9.7 Hz, $C_q$), 111.3 (CH), 117.9 (CH), 120.9 (CH), 123.1 (CH), 140.8 ($C_q$), 152.1 ($C_q$), 160.3 ($C_q$), (three $C_q$ signals missing); $^{19}$F NMR (339 MHz, $CDCl_3$) $\delta$ −134.7 (dd, $J$ = 7.7 Hz, $J$ = 20.1 Hz, 2F), −154.9 (tt, $J$ = 6.3 Hz, $J$ = 20.1 Hz, 1F).

#### 4.1.3.3. (*E*)-*N*-(4-((2,3-Dihydro-1*H*-inden-2-yl)(prop-1-yl)amino)-butyl)-2-(4-fluoro-phenyl)diazene-1-carboxamide (5).

According to the general procedure for Route A, azocarboxamide **5** was prepared from *tert*-butyl 2-(4-fluorophenyl)azocarboxylate (**11a**) (91.9 mg, 0.41 mmol), $K_2CO_3$ (150 mg, 1.02 mmol) and $N^1$-(2,3-dihydro-1*H*-inden-2-yl)-$N^1$-(prop-1-yl)butane-1,4-diamine (**13**) (50.0 mg, 0.20 mmol) in dry ethyl acetate (5.0 mL). The reaction mixture was diluted with water (30 mL) and extracted with ethyl acetate (3 × 30 mL). The combined organic phases were washed with a saturated aqueous solution of sodium chloride (20 mL) and dried over sodium sulfate. After filtration the solvent was removed under reduced pressure. The crude product was purified via column chromatography (silica gel desact. with $NEt_3$, hexane/ethyl

acetate = 2:1 → 100% ethyl acetate) to give azocarboxamide **5** (41.4 mg, 107 μmol, 53%) as brown viscous oil. For biological testing it was additionally purified using HPLC method B. $R_f$ = 0.5 (ethyl acetate/methanol = 9:1) [UV, $KMnO_4$]; $^1$H NMR (360 MHz, $CDCl_3$) $\delta$ 0.87 (t, $J$ = 7.4 Hz, 3H), 1.45–1.58 (m, 2H), 1.69 (dd, $J$ = 6.1 Hz, $J$ = 12.6 Hz, 2H), 1.72–1.79 (m, 2H), 2.51–2.58 (m, 2H), 2.61 (t, $J$ = 6.5 Hz, 2H), 2.91 (dd, $J$ = 8.8 Hz, $J$ = 15.3 Hz, 2H), 3.02 (dd, $J$ = 7.7 Hz, $J$ = 15.2 Hz, 2H), 3.52 (dd, $J$ = 6.2 Hz, $J$ = 12.2 Hz, 2H), 3.62–6.72 (m, 1H), 7.08–7.15 (m, 6H), 7.87 (dd, $J$ = 5.2 Hz, $J$ = 9.2 Hz, 2H), 8.03 (br s, 1H); $^{13}$C NMR (91 MHz, $CDCl_3$): $\delta$ 11.9 ($CH_3$), 18.9 ($CH_2$), 25.2 ($CH_2$), 28.0 ($CH_2$), 36.3 (2× $CH_2$), 40.9 ($CH_2$), 51.1 ($CH_2$), 52.9 ($CH_2$), 63.0 (CH), 116.4 (d, $J_{CF}$ = 23.1 Hz, 2× CH), 124.4 (2× CH), 126.1 (d, $J_{CF}$ = 9.5 Hz, 2× CH), 126.4 (2× CH), 141.5 (2× $C_q$), 147.79 (d, $J_{CF}$ = 3.0 Hz, $C_q$), 160.8 ($C_q$), 165.7 (d, $J_{CF}$ = 255.7 Hz, $C_q$); $^{19}$F NMR (339 MHz, $CDCl_3$) $\delta$ −108.4 (m, 1F); HRMS (ESI) $m/z$ calcd for $C_{23}H_{30}FN_4O$ [$MH^+$] 397.2398, found 397.2398.

#### 4.1.3.4. (*E*)-2-(3,4-Difluorophenyl)-*N*-(4-((2,3-dihydro-1*H*-inden-2-yl)(prop-1-yl)-amino)butyl)diazene-1-carboxamide (6).

According to the general procedure for Route B, a solution of 3,4-difluorophenylhydrazine hydrochloride (**15a**) (130 mg, 0.72 mmol) in dry *N*,*N*-dimethylformamide (2.0 mL) was added dropwise to a solution of 1,1′-carbonyldiimidazole (113 mg, 0.72 mmol) in dry *N*,*N*-dimethylformamide (3.0 mL) over a period of 20 min at room temperature under nitrogen atmosphere and stirring was continued for 2 h. To the resulting mixture a solution of $N^1$-(2,3-dihydro-1*H*-inden-2-yl)-$N^1$-(prop-1-yl)butane-1,4-diamine (**13**) (230 mg, 0.93 mmol) in dry *N*,*N*-dimethylformamide (2.0 mL) was added over a period of 20 min and stirring was continued for 2 h. Subsequently manganese dioxide (250 mg, 2.88 mmol) was added and the mixture was stirred overnight. The solid was filtered off and was washed with chloroform. The combined organic solutions were concentrated under reduced pressure and the crude product was purified via column chromatography (silica gel, desact. with $NEt_3$, hexane/ethyl acetate = 1:1) to give azocarboxamide **6** (55.0 mg, 0.13 mmol, 18%) as a brown viscous oil. For biological testing it was additionally purified using HPLC method B. $R_f$ = 0.4 (ethyl acetate/methanol = 9:1) [UV, $KMnO_4$]; $^1$H NMR (600 MHz, TFA salt, $CD_3CN$) $\delta$ 0.86 (t, $J$ = 7.4 Hz, 3H), 1.48 (qd, $J$ = 7.4 Hz, $J$ = 14.8 Hz, 2H), 1.56–1.61 (m, 2H), 1.63–1.68 (m, 2H), 2.49 (t, $J$ = 7.7 Hz, 2H), 2.56 (t, $J$ = 6.9 Hz, 2H), 2.81 (dd, $J$ = 8.6 Hz, $J$ = 15.4 Hz, 2H), 2.99 (dd, $J$ = 7.7 Hz, $J$ = 15.2 Hz, 2H), 3.37 (dd, $J$ = 6.6 Hz, $J$ = 12.4 Hz, 2H), 3.60–3.66 (m, 1H), 7.08–7.11 (m, 2H), 7.12–7.15 (m, 2H), 7.43–7.48 (m, 1H), 7.71 (ddd, $J$ = 2.4 Hz, $J$ = 7.5 Hz, $J$ = 11.0 Hz, 1H), 7.75–7.78 (m, 1H); $^{13}$C NMR (151 MHz, TFA salt, $CD_3CN$) $\delta$ 12.2 ($CH_3$), 20.6 ($CH_2$), 25.7 ($CH_2$), 28.4 ($CH_2$), 37.2 (2× $CH_2$), 41.4 ($CH_2$), 51.7 ($CH_2$), 53.7 ($CH_2$), 63.9 (CH), 111.2 (dd, $J_{CF}$ = 1.4 Hz, $J_{CF}$ = 18.8 Hz, CH), 119.1 (d, $J_{CF}$ = 18.9 Hz, CH), 123.7 (dd, $J_{CF}$ = 3.4 Hz, $J_{CF}$ = 7.7 Hz, CH), 125.4 (2× CH), 127.2 (2× CH), 143.1 (2× $C_q$), 149.2 (dd, $J_{CF}$ = 3.4 Hz, $J_{CF}$ = 5.0 Hz, $C_q$), 151.8 (dd, $J_{CF}$ = 14.3 Hz, $J_{CF}$ = 249.8 Hz, $C_q$), 154.2 (dd, $J_{CF}$ = 13.3 Hz, $J_{CF}$ = 254.3 Hz, $C_q$), 162.9 ($C_q$); $^{19}$F NMR (339 MHz, $CDCl_3$) $\delta$ −137.4 (m, 1F), −133.0 (dddd, $J$ = 4.5 Hz, $J$ = 7.3 Hz, $J$ = 9.9 Hz, $J$ = 20.3 Hz, 1F); HRMS (ESI) $m/z$ calcd for $C_{23}H_{29}F_2N_4O$ [$MH^+$] 415.2304, found 415.2307.

#### 4.1.3.5. (*E*)-*N*-(4-((2,3-Dihydro-1*H*-inden-2-yl)(prop-1-yl)amino)butyl)-2-(3,4,5-tri-fluorophenyl)diazene-1-carboxamide (7).

According to the general procedure for Route B, a solution of 3,4,5-trifluorophenylhydrazine hydrochloride (65.9 mg, 0.33 mmol) in dry *N*,*N*-dimethylformamide (3.0 mL) was added dropwise to a solution of 1,1′-carbonyldiimidazole (53.8 mg, 0.32 mmol) and diisopropylethylamine (58 μL, 64.4 mg, 0.50 mmol) in dry *N*,*N*-dimethylformamide (9.0 mL) over a period of 20 min at room temperature under nitrogen atmosphere and stirring was continued for 1.5 h. To the resulting mixture a solution of $N^1$-(2,3-dihydro-1*H*-inden-2-yl)-$N^1$-(prop-1-yl)butane-1,4-diamine (**13**) (78.8 mg, 0.32 mmol) in dry *N*,*N*-dimethylformamide (3.0 mL) was added over a period of 30 min and stirring was continued for 2.5 h. Subsequently manganese dioxide (116 mg, 1.33 mmol) was added and the mixture was stirred for 2 h. The solid was filtered off and washed with chloroform. The combined organic solutions were concentrated under reduced pressure and the crude product was purified via column chromatography (silica gel, desact. with $NEt_3$, hexane/ethyl acetate = 1:1). Purification using HPLC method B gave the TFA salt of **7** (26.8 mg, 49.1 μmol, 15%) as light brown solid. $R_f$ = 0.5 (ethyl acetate/methanol = 9:1) [UV, $KMnO_4$]; HPLC-MS $t_R$ = 17.6 min, $m/z$ 433.8; $^1$H NMR (600 MHz, $CDCl_3$) $\delta$ 0.88 (t, $J$ = 7.4 Hz, 3H), 1.52 (dt, $J$ = 7.4 Hz, $J$ = 14.8 Hz, 2H), 1.67–1.74 (m, 2H), 1.78 (td, $J$ = 7.4 Hz, $J$ = 14.8 Hz, 2H), 2.51–2.60 (m, 2H), 2.63 (t, $J$ = 6.3 Hz, 2H), 2.88 (dd, $J$ = 8.7 Hz, $J$ = 15.5 Hz, 2H), 3.02 (dd, $J$ = 7.8 Hz, $J$ = 15.4 Hz, 2H), 3.52 (dd, $J$ = 5.8 Hz, $J$ = 12.1 Hz, 2H), 3.67 (td, $J$ = 8.3 Hz, $J$ = 16.9 Hz, 1H), 7.04–7.21 (m, 4H), 7.53 (dd, $J$ = 6.5 Hz, $J$ = 7.9 Hz, 2H), 8.35 (br s, 1H); $^{13}$C NMR (151 MHz, TFA salt, $CD_3CN$) $\delta$ 11.3 ($CH_3$), 17.5 ($CH_2$), 21.5 ($CH_2$), 27.0 ($CH_2$), 35.4 ($CH_2$), 35.5 ($CH_2$), 40.3 ($CH_2$), 51.3 ($CH_2$), 53.1 ($CH_2$), 64.3 (CH), 109.1 (dd, $J_{CF}$ = 4.7 Hz, $J_{CF}$ = 18.3 Hz, 2× CH), 125.5 (2× CH), 128.3 (2× CH), 140.2 (2× $C_q$), 162.6 ($C_q$), (four $C_q$-signals missing); $^{19}$F NMR (339 MHz, $CDCl_3$) $\delta$ −76.3 (TFA), −134.7 (dd, $J$ = 8.2 Hz, $J$ = 19.5 Hz, 2F), −155.6 (m, 1F); HRMS (ESI) $m/z$ calcd for $C_{23}H_{28}F_3N_4O$ [$MH^+$] 433.2210, found 433.2214.

#### 4.1.3.6. (*E*)-*N*-(2-((2,3-Dihydro-1*H*-inden-2-yl)(prop-1-yl)amino)ethyl)-2-(4-fluoro-phenyl)diazene-1-carboxamide (8).

According to the general procedure for Route A, azocarboxamide **8** was synthesized from *tert*-butyl 4-fluorophenylazocarboxylate (**11a**) (86.7 mg, 0.36 mmol), amine **14** (39.0 mg, 0.18 mmol) and triethyl amine (1.12 mL, 0.90 mg, 0.89 mmol) in dry ethanol (2.0 mL). After concentration of the reaction mixture under reduced pressure the crude product was purified by column chromatography (silica gel desact. with $NEt_3$, hexane/ethyl acetate = 10:1 → 100% ethyl acetate) to give azocarboxamide **8** (20.4 mg, 53.3 μmol, 30%) as a brown oil. For the biological testing it was additionally purified using preparative HPLC method B. $R_f$ = 0.5 (hexane/ethyl acetate = 1:1) [UV, $KMnO_4$]; $^1$H NMR (600 MHz, TFA salt, $CDCl_3$) $\delta$ 1.01 (t, $J$ = 7.3 Hz, 3H), 1.75–1.92 (m, 2H), 3.00–3.20 (m, 2H), 3.27–3.39 (m, 5H), 3.48 (br s, 1H), 3.86 (br s, 1H), 3.94 (br s, 1H), 4.23 (quin, $J$ = 7.6 Hz, 1H), 7.18–7.24 (m, 6H), 7.99 (dd, $J$ = 5.2 Hz, $J$ = 9.0 Hz, 2H), 8.88 (br s, 1H), 12.51 (br s, 1H); $^{13}$C NMR (91 MHz, TFA salt, $CDCl_3$) $\delta$ 11.2 ($CH_3$), 16.9 ($CH_2$), 34.2 ($CH_2$), 34.6 ($CH_2$), 36.7 ($CH_2$), 51.2 ($CH_2$), 54.4 ($CH_2$), 64.2 (CH), 116.4 (d, $J_{CF}$ = 23.2 Hz, 2× CH), 124.6 (2× CH), 126.6 (d, $J_{CF}$ = 9.7 Hz, 2× CH), 127.9 (2× CH), 138.2 (2× $C_q$), 147.8 ($C_q$), 162.0 ($C_q$), 166.2 (d, $J_{CF}$ = 256.4 Hz, $C_q$); $^{19}$F NMR (339 MHz, TFA salt, $CDCl_3$) $\delta$ −78.8 (TFA), −107.9 (s, 1F); HRMS (ESI) $m/z$ calcd for $C_{21}H_{26}FN_4O$ [$MH^+$] 369.2085, found 369.2080.

#### 4.1.3.7. (*E*)-2-(3,4-difluorophenyl)-*N*-(2-((2,3-dihydro-1*H*-inden-2-yl)(prop-1-yl)-amino)ethyl)diazene-1-carboxamide (9).

According to the general procedure for Route A, azocarboxamide **9** was synthesized from *tert*-butyl 3,4-difluorophenylazocarboxylate (**11b**) (78.3 mg, 0.32 mmol), amine **14** (35.3 mg, 0.16 mmol) and triethyl amine (1.11 mL, 0.82 mg, 0.81 mmol) in dry ethanol (2.0 mL). After concentration of the reaction mixture under reduced pressure the crude product was purified by column chromatography (silica gel desact. with $NEt_3$, hexane/ethyl acetate = 10:1 → 100% ethyl acetate) to give azocarboxamide **9** (30.5 mg, 78.9 μmol, 48%) as a brown oil. For the biological testing it was additionally purified using preparative HPLC method B. $R_f$ = 0.5 (hexane/ethyl acetate = 1:1) [UV, $KMnO_4$], $^1$H NMR (360 MHz, TFA salt, $CDCl_3$) $\delta$ 1.01 (t, $J$ = 7.3 Hz, 3H), 1.78–1.91 (m, 2H), 3.05 (br s, 1H), 3.14 (br s, 1H), 3.35 (br s, 5H), 3.48 (br s, 1H), 3.86 (br s, 1H), 3.93 (br s,

1H), 4.23 (quin, $J$ = 7.6 Hz, 1H), 7.20–7.24 (m, 4H), 7.33 (dd, $J$ = 8.8 Hz, $J$ = 17.3 Hz, 1H), 7.77 (t, $J$ = 8.1 Hz, 1H), 7.83–7.86 (m, 1H), 9.08 (br s, 1H); $^{13}$C NMR (151 MHz, $CDCl_3$) $\delta$ 11.2 ($CH_3$), 16.9 ($CH_2$), 34.2 ($CH_2$), 34.6 ($CH_2$), 36.7 ($CH_2$), 51.2 ($CH_2$), 54.4 ($CH_2$), 64.2 (CH), 110.9 (dd, $J_{CF}$ = 1.7 Hz, $J_{CF}$ = 18.6 Hz, CH), 117.7 (d, $J_{CF}$ = 18.7 Hz, CH), 123.6 (dd, $J_{CF}$ = 3.2 Hz, $J_{CF}$ = 7.4 Hz, CH), 124.6 (2× CH), 127.9 (2× CH), 138.1 (2× $C_q$), 147.8 ($C_q$), 150.9 (dd, $J_{CF}$ = 14.1 Hz, $J_{CF}$ = 252.9 Hz, $C_q$), 153.9 (dd, $J_{CF}$ = 13.6 Hz, $J_{CF}$ = 258.4 Hz, $C_q$), 161.8 ($C_q$); $^{19}$F NMR (339 MHz, $CDCl_3$) $\delta$ −78.8 (TFA), −131.6 (m, 1F), −137.7 (m, 1F); MS (EI) $m/z$ (%): 386 [$M^+$] (5), 245 (19), 189 (13), 188 (100), 146 (11), 117 (60), 116 (17), 115 (19), 113 (12), 85 (10), 83 (12), 72 (12), 32 (14), 28 (25); HRMS (ESI) $m/z$ calcd for $C_{21}H_{25}F_2N_4O$ [$MH^+$] 387.1991, found 387.1990.

**4.1.3.8. (*E*)-*N*-(2-((2,3-Dihydro-1*H*-inden-2-yl)(prop-1-yl)amino) ethyl)-2-(3,4,5-tri-fluorophenyl)diazene-1-carboxamide (10).** According to the general procedure for Route A, a solution of 3,4,5-trifluorophenylhydrazine (**15b**) (50.0 mg, 0.26 mmol) in dry *N*,*N*-dimethylformamide (3.0 mL) was added dropwise to a solution of 1,1′-carbonyldiimidazole (40.2 mg, 0.25 mmol) in dry *N*,*N*-dimethylformamide (5.0 mL) over a period of 30 min at room temperature under nitrogen atmosphere and stirring was continued for 3 h. To the resulting mixture a solution of $N^1$-(2,3-dihydro-1*H*-inden-2-yl)-$N^1$-(prop-1-yl)ethane-1,4-diamine (**14**) (36.0 mg, 0.17 μmol) in dry *N*,*N*-dimethylformamide (3.0 mL) was added over a period of 30 min and stirring was continued overnight. Subsequently manganese dioxide (120 mg, 1.40 mmol) was added and the mixture was stirred for 4 h. The solid was filtered off and washed with chloroform. The combined organic solutions were concentrated under reduced pressure and the crude product was purified via column chromatography (silica gel, desact. with $NEt_3$, hexane/ethyl acetate = 1:1). After purification using HPLC method B the TFA salt of azocarboxamide **10** (3.8 mg, 9.40 μmol, 6%) was obtained as light brown solid. $R_f$ = 0.5 (hexane/ethyl acetate = 1:1) [UV, $KMnO_4$]; $^1$H NMR (600 MHz, TFA salt, $CDCl_3$) $\delta$ 1.01 (t, $J$ = 7.3 Hz, 3H), 1.75–1.92 (m, 2H), 3.00–3.20 (m, 2H), 3.27–3.39 (m, 5H), 3.45–3.52 (m, 1H), 3.80 (br s, 2H), 4.23 (quin, $J$ = 7.6 Hz, 1H), 7.18–7.24 (m, 6H), 7.99 (dd, $J_{HF}$ = 5.2 Hz, $J$ = 9.0 Hz, 2H), 8.88 (br s, 1H), 12.51 (br s, 1H); $^{13}$C NMR (151 MHz, TFA salt, $CD_3CN$) $\delta$ 11.2 ($CH_3$), 14.5 ($CH_2$), 32.3 (2× $CH_2$), 37.1 ($CH_2$), 52.1 ($CH_2$), 54.3 ($CH_2$), 64.9 (CH), 125.5 (2× CH), 128.4 (2× CH), (nine signals missing); $^{19}$F NMR (339 MHz, TFA salt, $CDCl_3$) $\delta$ −76.4 (TFA), −134.7 (dd, $J$ = 8.0 Hz, $J$ = 19.5 Hz, 2F), −155.2 (m, 1F); HRMS (ESI) $m/z$ calcd for $C_{21}H_{24}F_3N_4O$ [$MH^+$] 405.1897, found 405.1881.

### 4.1.4. Synthesis of $N^1$-(2,3-dihydro-1*H*-inden-2-yl)-$N^1$-prop-1-ylethane-1,2-diamine (14)

*N*-(2,3-Dihydro-1*H*-inden-2-yl)-N-(prop-1-yl)amine (416 mg, 2.37 mmol) was added to a solution of N-Boc-2-aminoacetaldehyde (754 mg, 4.74 mmol) in dry dichloromethane (20 mL) under argon atmosphere at room temperature and the resulting mixture was stirred for 20 min. Subsequently sodium triacetoxyborohydride (1.00 g, 4.74 mmol) was added and the mixture was stirred overnight. Water (30 mL) and a saturated aqueous solution of sodium carbonate (30 mL) were added. After extraction with dichloromethane (3 × 80 mL) the combined organic phases were washed with a saturated solution of sodium chloride (30 mL) and dried over sodium sulfate. After filtration the solvent was removed under reduced pressure and the crude product was purified via column chromatography (silica gel, desact. with NEt3, hexane/ethyl acetate = 4:1) to give tert-butyl (2-((2,3-dihydro-1*H*-inden-2-yl)(prop-1-yl)amino)ethyl)carbamate (733 mg, 2.30 mmol, 97%) as a colorless oil. $R_f$ = 0.1 (ethyl acetate/hexane = 4:1) [UV, $KMnO_4$]; $^1$H NMR (600 MHz, $CDCl_3$) $\delta$ 0.89 (t, $J$ = 7.3 Hz, 3H), 1.45 (s, 9H), 1.51 (br s, 2H), 2.49 (br s, 2H), 2.61 (br s, 2H), 2.87 (br s, 2H), 3.02 (br s, 2H), 3.19 (br s, 2H), 3.71 (br s, 1H), 4.96 (br s, 1H), 7.12–7.18 (m, 4H); $^{13}$C NMR (151 MHz, $CDCl_3$) $\delta$ 11.8 ($CH_3$), 20.3 ($CH_2$), 28.5 (3× $CH_3$), 36.2 (2× $CH_2$), 38.6 ($CH_2$), 50.5 ($CH_2$), 53.7 ($CH_2$), 62.8 (CH), 79.0 ($C_q$), 124.4 (2× CH), 126.4 (2× CH), 141.6 (2× $C_q$), 156.1 ($C_q$); HRMS (ESI) $m/z$ calcd for $C_{19}H_{31}N_2O_2$ [$MH^+$] 319.2380, found 319.2378. In the second step, a solution of the above prepared *N*-Boc-protected amine (238 mg, 0.75 mmol) in dichloromethane (10 mL) was treated with trifluoroacetic acid (0.58 mL, 854 mg, 7.49 mmol) and the resulting mixture was stirred at room temperature. The reaction course was monitored by TLC after micro-workup with an aqueous solution of sodium carbonate and ethyl acetate. Upon completion of the reaction a saturated aqueous solution of sodium carbonate (30 mL) was added. After extraction with chloroform (5 × 30 mL) the combined organic phases were washed with a saturated solution of sodium chloride (30 mL) and dried over sodium sulfate. After filtration the solvent was removed under reduced pressure and amine **14** (164 mg, quant.) was obtained as a clear, light brown oil and was used without further purification. $^1$H NMR (360 MHz, $CDCl_3$) $\delta$ 0.89 (t, $J$ = 7.4 Hz, 3H), 1.45–1.55 (m, 2H), 2.48–2.52 (m, 2H), 2.61 (t, $J$ = 6.1 Hz, 2H), 2.80 (t, $J$ = 6.0 Hz, 2H), 2.89 (dd, $J$ = 8.0 Hz, $J$ = 15.9 Hz, 2H), 3.03 (dd, $J$ = 7.9 Hz, $J$ = 15.5 Hz, 2H), 3.69–3.77 (m, 1H), 7.12–7.18 (m, 4H).

### 4.1.5. General procedure for the synthesis of cinnamides 16 and 17

1-Chloro-*N*,*N*,2-trimethylpropenylamine (79.0 μL, 0.60 mmol) was added to a solution of (E)-3-(4-fluorophenyl)acrylic acid (100 mg, 0.60 mmol) in dry dichloromethane (1.5 mL) and the resulting mixture was stirred for 2 h at room temperature under nitrogen atmosphere. Subsequently a solution of the amine 13 or 14 (0.60 mmol) in dry dichloromethane (1.0 mL) was added to the in situ generated acid chloride and the mixture was stirred for 24 h at room temperature. A saturated aqueous solution of ammonium chloride (20 mL) was added and the aqueous layer was extracted with dichloromethane (8 × 20 mL). The combined organic phases were washed with a saturated aqueous solution of sodium chloride (20 mL) and dried over sodium sulfate. After filtration the solvent was removed under reduced pressure and the crude product was purified via column chromatography as described for each product.

**4.1.5.1. (*E*)-*N*-(4-((2,3-Dihydro-1*H*-inden-2-yl)(prop-1-yl)amino)butyl)-3-(4-fluoro-phenyl)acrylamide (16).** Cinnamide **16** was synthesized from $N^1$-(2,3-dihydro-1*H*-inden-2-yl)-$N^1$-(prop-1-yl)butane-1,4-diamine (**13**) (177 mg, 0.72 mmol) according to the general procedure described above. The crude product was purified via column chromatography (silica gel, desact. with NEt3, ethyl acetate/methanol = 9:1) to give **16** (23.6 mg, 59.8 μmol, 10%) as a beige solid. For biological testing it was additionally purified using HPLC method B. $R_f$ = 0.2 (ethyl acetate/methanol = 9:1) [UV, ninhydrin]; $^1$H NMR (600 MHz, TFA salt, $CDCl_3$) $\delta$ 0.99 (t, $J$ = 7.3 Hz, 3H), 1.60–1.67 (m, 1H), 1.73–1.92 (m, 4H), 1.95–2.03 (m, 1H), 2.97–3.04 (m, 2H), 3.08 (dt, $J$ = 4.8 Hz, $J$ = 12.5 Hz, 1H), 3.23–3.41 (m, 6H), 3.53 (td, $J$ = 6.4 Hz, $J$ = 12.0 Hz, 1H), 3.59 (br s, 1H), 4.09–4.15 (m, 1H), 6.51 (d, $J$ = 15.7 Hz, 1H), 7.04 (t, $J$ = 8.7 Hz, 2H), 7.19–7.25 (m, 4H), 7.38 (br s, 1H), 7.49 (dd, $J$ = 5.4 Hz, $J$ = 8.8 Hz, 2H), 7.58 (d, $J$ = 15.7 Hz, 1H); $^{13}$C NMR (151 MHz, TFA salt, $CDCl_3$) $\delta$ 11.1 ($CH_3$), 16.4 ($CH_2$), 21.2 ($CH_2$), 25.9 ($CH_2$), 34.3 ($CH_2$), 34.5 ($CH_2$), 37.6 ($CH_2$), 50.6 ($CH_2$), 52.0 ($CH_2$), 63.3 (CH), 115.8 (d, $J_{CF}$ = 21.9 Hz, 2× CH), 120.4 (d, $J_{CF}$ = 2.3 Hz, CH), 124.5 (2× CH), 127.7 (2× CH), 129.6 (d, $J_{CF}$ = 8.4 Hz, 2× CH), 131.1 (d, $J_{CF}$ = 3.4 Hz, $C_q$), 138.2 (2× $C_q$), 139.6 (CH), 163.4 (d, $J_{CF}$ = 250.0 Hz, $C_q$), 166.7 ($C_q$); $^{19}$F NMR (339 MHz, TFA salt, $CDCl_3$) $\delta$ −78.7 (TFA), −114.2 (m, 1F); MS (EI) $m/z$ (%): 394 [$M^+$] (3), 366 (14), 365 (55), 278 (13), 189 (18), 188 (100), 149 (63), 121 (18), 117 (70), 116 (17), 115 (22), 101 (13), 72 (22), 70 (11),

43 (12); HRMS (EI) *m*/*z* calcd for $C_{25}H_{31}FN_2O$ [$M^+$] 394.2420, found 394.2422.

**4.1.5.2. (*E*)-*N*-(2-((2,3-Dihydro-1*H*-inden-2-yl)(prop-1-yl)amino)ethyl)-3-(4-fluoro-phenyl)acrylamide (17).** Cinnamide **17** was prepared from $N^1$-(2,3-dihydro-1*H*-inden-2-yl)-$N^1$-(prop-1-yl)ethane-1,4-diamine (**14**) (70 mg, 0.32 mmol) according to the general procedure described above. The crude product was purified via column chromatography (silica gel, desact. with NEt3, hexane/ethyl acetate = 1:1 → ethyl acetate) to give **17** (23.6 mg, 51.8 μmol, 16%) as a beige solid. For biological testing it was additionally purified using HPLC method B. $R_f$ = 0.4 (ethyl acetate = 9:1) [UV, ninhydrin]; $^1$H NMR (600 MHz, TFA salt, $CDCl_3$) $\delta$ 1.01 (t, $J$ = 6.4 Hz, 3H), 1.75–1.89 (m, 2H), 3.00–3.07 (m, 1H), 3.08–3.15 (m, 1H), 3.25–3.40 (m, 5H), 3.42–3.52 (m, 1H), 3.72–3.83 (br s, 1H), 3.84–3.94 (m, 1H), 4.17–4.25 (m, 1H), 5.04 (br s, 2H), 6.52 (d, $J$ = 15.0 Hz, 1H), 7.04 (t, $J$ = 8.4 Hz, 2H), 7.20–7.23 (m, 4H), 7.51 (dd, $J_{HF}$ = 5.4 Hz, $J$ = 8.2 Hz, 2H), 7.60 (d, $J$ = 15.0 Hz, 1H); $^{13}$C NMR (151 MHz, TFA salt, $CDCl_3$) $\delta$ 11.1 ($CH_3$), 17.0 ($CH_2$), 34.4 (2× $CH_2$), 36.1 ($CH_2$), 52.4 ($CH_2$), 54.3 ($CH_2$), 64.2 (CH), 115.8 (d, $J_{CF}$ = 22.6 Hz, 2× CH), 119.3 (CH), 124.6 (2× CH), 127.9 (2× CH), 129.9 (d, $J_{CF}$ = 8.1 Hz, 2× CH), 130.9 (d, $J_{CF}$ = 3.6 Hz, $C_q$), 138.0 (2× $C_q$), 140.9 (CH), 165.7 (d, $J_{CF}$ = 250.8 Hz, $C_q$), 168.1 ($C_q$); $^{19}$F NMR (339 MHz, $CDCl_3$) $\delta$ −78.7 (TFA), −113.4 (s, 1 F); HRMS (ESI) *m*/*z* calcd for $C_{23}H_{28}FN_2O$ [$MH^+$] 367.2180, found 367.2182.

**4.1.6. Methyl (*E*)-$N^2$-acetyl-$N^6$-(4-(((4-((2,3-dihydro-1*H*-inden-2-yl)(prop-1-yl)amino) butyl)carbamoyl)diazenyl)-2,6-difluoro-phenyl)-D-lysinate (19)**

*Conditions A*: A solution of azocarboxamide **7** (3.26 mg, 5.97 μmol) and $N^{\alpha}$-acetyl-L-lysine methyl ester hydrochloride **18** (2.91 mg, 12.2 μmol) in dry *N*,*N*-dimethylformamide (0.5 mL) was treated with potassium carbonate (5.00 mg, 36.2 μmol) under argon atmosphere at room temperature. The mixture was stirred for 12 h at room temperature, stored for two days at −18 °C and then stirred for additional 7 h at room temperature. Subsequently the solvent was removed under reduced pressure and the residue was purified using HPLC method B to give **19** (3.70 mg, 5.08 μmol, 85%) as a deep red solid. *Conditions B*: A mixture of **7** (4.04 mg, 7.39 μmol) was dissolved in dimethylsulfoxide (0.25 mL), and PBS buffer (pH = 7.4, 0.25 mL) and subsequently Nα-acetyl-L-lysine methyl ester hydrochloride **18** (22.3 mg, 93.4 μmol) were added. The mixture was stirred at room temperature. The reaction course and product formation were monitored via LCMS. Compound **19** could be detected by mass analysis and retention time. $R_f$ = 0.1 (ethyl acetate/methanol = 9:1) [UV, $KMnO_4$]; HPLC-MS $t_R$ = 16.8 -min, *m*/*z* 616.2; $^1$H NMR (600 MHz, TFA salt, $CDCl_3$) $\delta$ 0.96 (br s, 3H), 1.40–1.45 (m, 2H), 1.62–1.68 (m, 3H), 1.71–1.83 (m, 5H), 1.87 (s, 3H), 1.95–2.03 (m, 2H), 3.26–3.40 (br s, 6H), 3.45 (t, $J$ = 6.0 Hz, 2H), 3.65 (s, 3H), 4.20 (br s, 1H), 4.34 (dd, $J$ = 7.9 Hz, $J$ = 13.1 Hz, 1H), 5.34 (br s, 1H), 6.71 (br s, 1H), 7.18–7.27 (m, 6H), 8.02 (br s, 1H), 10.70 (br s, 1H), $^{19}$F NMR (339 MHz, TFA salt, $CDCl_3$) $\delta$ −133.7 (2 F); HRMS (ESI) *m*/*z* calcd for $C_{32}H_{45}F_2N_6O_4$ [$MH^+$] 615.3465, found 615.3474.

### 4.2. Binding assays

Receptor binding studies were carried out as described.[30] In brief, radioligand displacement experiments with human $D_{2long}$, $D_{2short}$,[27] $D_3$[5,28] and $D_{4.4}$[29] receptors were carried out with preparations of membranes from CHO cells stably expressing the corresponding receptor and [$^3$H]spiperone (specific activity 73 Ci/mmol; PerkinElmer, Rodgau, Germany) at final concentrations of 0.1–0.2 nM, 0.2 nM, 0.2–0.4 nM and 0.15–0.35 nM for $D_{2long}$, $D_{2short}$, $D_3$ and $D_{4.4}$, respectively according to the particular $K_D$ value. The assays were run at protein concentrations of 4–6 μg/well, 1–4 μg/well, 1–6 μg/well and 5–6 μg/well with $K_D$ values of 0.058–0.10 nM, 0.062–0.095 nM, 0.098–0.32 nM, and 0.15–0.35 nM and $B_{max}$ values of 610–140 fmol/mg, 1500–4400 fmol/mg, 1400–8000 fmol/mg and 650–2000 fmol/mg protein for the $D_{2long}$, $D_{2short}$, $D_3$ and $D_{4.4}$ receptors, respectively. $D_1$, $D_5$ and 5-$HT_{2A}$ binding was achieved using homogenates of membranes from HEK 293 cells, which were transiently transfected with the pcDNA3.1 vector containing the appropriate human gene (all purchased from Missouri S&T cDNA Resource Center (UMR), Rolla, MO) by the calcium phosphate method.[38] Membranes were incubated at final concentrations of 3–4 μg/well, 5–10 μg/well or 4–10 μg/well with receptor densities ($B_{max}$ values) of 2200–5000 fmol/mg, 350–450 fmol/mg and 880–3200 fmol/mg protein and specific affinities ($K_D$ values) of 0.22–0.75 nM, 0.35–0.37 nM and 0.19–0.45 nM for $D_1$, $D_5$ and 5-$HT_{2A}$, respectively. The radioligands [$^3$H]SCH23390 (specific activity 80 Ci/mmol; Biotrend, Cologne, Germany) and [$^3$H]ketanserin (specific activity 53 Ci/mmol; PerkinElmer, Rodgau, Germany) were used at concentrations of 0.3–0.7 nM for $D_1$, 0.4–0.5 nM for $D_5$ and 0.3–0.5 nM for 5-$HT_{2A}$, respectively. Receptor binding experiments at 5-$HT_{1A}$ and $\alpha_1$ were performed with homogenates prepared from porcine cerebral cortex.[30] Assays were run with membranes at a protein concentration of 60–80 μg/well or 20–40 μg/well, $B_{max}$ values of 50–130 fmol/mg and 160–210 fmol/mg, $K_D$ values of 0.060–0.30 nM and 0.060–0.17 nM and radioligand concentrations of 0.2–0.33 nM for [$^3$H]WAY600135 (specific activity 80 Ci/mmol; Biotrend, Cologne, Germany) and 0.2 nM for [$^3$H]prazosin (specific activity 85 Ci/mmol; PerkinElmer, Rodgau, Germany) for 5-$HT_{1A}$ and $\alpha_1$ receptor, respectively. Unspecific binding was determined in the presence of haloperidol (10 μM), WAY600135 (10 μM), ketanserin (10 μM) or prazosin (10 μM) for $D_1$–$D_5$, 5-$HT_{1A}$, 5-$HT_{2A}$ or $\alpha_1$, respectively. Protein concentration was established by the method of Lowry using bovine serum albumin as standard.[39]

Data analysis of the competition curves from the radioligand binding experiments was accomplished by non-linear regression analysis using the algorithms in PRISM5.0 (GraphPad Software, San Diego, CA). $EC_{50}$ values derived from the resulting dose response curves were transformed into the corresponding $K_i$ values utilizing the equation of Cheng and Prusoff.[40]

### 4.3. Preparation of receptor mutants by site directed mutagenesis

The $hDRD_3^{wt}$ cDNA subcloned into a pcDNA3.1(+) expression vector was used as described previously.[41] Oligonucleotidic primers were purchased from Biomers.net. Point mutations were introduced by polymerase chain reaction with oligonucleotidic primers bearing the desired mutation and the $hDRD_3^{wt}$ receptor cDNA as a template. All mutations were verified by DNA sequence analysis using the service of LGC Genomics, Berlin.

### 4.4. Determination of $D_3$ activation via inositol phosphate assays

Agonist-induced activation of the human Dopamine $D_3$ wild-type receptor and receptor mutants was studied in inositol phosphate (IP) accumulation assays as described previously.[35] In brief, HEK293T cells were transiently co-transfected with cDNAs encoding the human Dopamine $D_3$ wild-type receptor and receptor mutants, respectively and the G protein $G\alpha_{qG66Di5}$. (The $G\alpha_{qG66Di5}$-protein was a kind gift from Prof. Dr. Evi Kostenis[42] of the University of Bonn.) Twenty-four h after transfection, cells were seeded into 24-well plates and incubated with *myo*-[$^3$H]inositol. On the next day, test compounds (diluted in binding buffer containing 50 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, 0.1 mM dithiothreitol,

100 μg/ml Bacitracin, 5 μg/ml Soybean Trypsin Inhibitor, pH 7.4, supplemented with10 mM LiCl) were added at 37 °C for 120 min. After lysis, cell extract was separated by anion-exchange chromatography and total IP was eluted. Radioactivity was measured by scintillation counting. Data were analyzed by normalizing disintegrations per minute (dpm) values with 0% for the unstimulated receptor and 100% for the full effect of the reference quinpirole. Concentration–response curves were fitted by nonlinear regression using the GraphPad Prism 5 software.

Irreversible activation of the $D_3$ receptor mutants was tested at 3 μM test compounds in comparison to the reversible reference ligand quinpirole (3 μM). After incubation for 60 min, the antagonist haloperidol (10 μM) was added to one-half of the sample (buffer was added to the other half) and incubations were continued for an additional 180 min. Total IP accumulation was determined as described above. Data were analyzed by defining dpm values for the unstimulated control as 1. IP-response of the test compounds was expressed as *x*-fold over basal.

## Acknowledgements

The authors would like to thank the Deutsche Forschungsgemeinschaft DFG for financial support within the projects HE5413/3-1 and GRK1910/B3. The experimental assistance by Karina Wicht, Markus Lang, Sarah Höfling and Stefanie Fehler is gratefully acknowledged. The authors are further grateful for computational support by Ralf Kling.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.12.012.

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