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Design, synthesis and pharmacological evaluation of spirocyclic σ_1 receptor ligands with exocyclic amino moiety (increased distance 1)

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ABSTRACT

Various pharmacophore models for potent σ_1 ligands specify a basic amino group flanked by two different hydrophobic regions in defined distances to the basic amine (distance 1 and distance 2, respectively). According to these models distance 1 of the potent spirocyclic σ_1 ligand **1** is too short. In order to find a new class of more potent σ_1 ligands and to verify the distance hypothesis of the pharmacophore models spirocyclic compounds **2** with an exocyclic amino group were designed and synthesized. The secondary amines **8** and **9** with N-benzyl residues are >100-fold less potent than the spirocyclic piperidine **1**. However, the tertiary methylamines *trans*-**11** and *cis*-**11** represent potent σ_1 ligands with K_i-values of 43 and 24 nM, respectively. Whereas one large benzyl moiety is required for high σ_1 receptor binding, a second large N-substituent is not tolerated by the σ_1 receptor protein. As a rule, *cis*-configured diastereomers with a longer distance 1 (predominantly 7.16–7.23 Å) show higher σ_1 affinities than their *trans*-configured counterparts (distance 1 is predominantly 5.88–6.26 Å).

1. Introduction

Initially the σ receptor was classified as an opioid receptor subtype.[1] The fact that classical opioid receptor antagonists like naloxone and naltrexone did not inhibit the effects of σ ligands led to classification of σ receptors as an independent receptor class.[2,3] Differentiation via ligand binding experiments and biochemical analysis defined two σ receptor subtypes, which were termed σ_1 and σ_2 receptors.[4]

The σ_1 receptor is a 25.3 kDa membrane bound protein, which has been cloned.[5–7] The amino acid sequence does not show any homology or even similarity with any other known mammalian protein. On the contrary a 30% homology with the yeast enzyme ergosterol-Δ^8/Δ^7-isomerase was detected.[6] σ_1 receptors are located with high density in the central nervous system (CNS)—especially in regions, which are associated with motoric, cognitive and sensoric functions.[8,9] Moreover, large amounts of σ_1 receptors were discovered in some organs and tissues in the periphery including the heart,[10] liver,[11] kidney[12] and the eye[13] as well as in some human tumor cell lines.[14]

The exact σ_1 mediated signal transduction pathway is not known yet. But it has been clearly shown that σ_1 receptors are neither ion channel receptors, soluble receptors, tyrosine kinase receptors nor classical G-protein coupled receptors.[15,16] They basically show modulatory properties, for example, modulation of Ca^{2+}-, K^+-, Na^+-, Cl^--channels as well as NMDA and IP_3 receptors.[17] Several σ_1 mediated effects occur only after preactivation of another biological system.[18] Some neurosteroids interact with σ_1 receptors and are therefore assumed to be the endogenous σ_1 ligands.[19] In contrast to most known σ_1 receptor ligands they do not contain a basic element. Dehydroepiandrosterone and pregnenolone sulfate show σ_1 agonistic and progesterone σ_1 antagonistic activity.[20] The steroid levels in the brain are modified especially in diseases associated with σ_1 receptors.[21,22] Very recently *N*,*N*-dimethyltryptamine with a K_i-value of 15 μM has been postulated as endogenous σ_1 receptor ligand.[23]

Since many known centrally active drugs also display high affinity to σ_1 receptors, σ_1 ligands are considered as useful drugs for the treatment of several psychiatric disorders.[24,25] Pharmacological analyses lead to potential applications as antidepressants[26,27] and anxiolytic agents.[28] There is particular interest in σ_1 ligands for the treatment of memory disorders,[22,29] Alzheimer disease as well as cocaine abuse.[22,28] Moreover, σ_1 ligands can be used as neuroprotectants at the eye.[13,30] Chen and Pasternak describe the σ system as an endogenous anti-opioid system. (+)-Pentazocine, a known σ agonist, antagonizes μ, κ, and δ receptor mediated analgesia, whilst σ_1 antagonists enhance opioid induced antinociception.[31] Down regulation of σ_1 receptors potentiates opioid mediated analgesia.[17] Therefore, σ_1 antagonists show a high potential as new analgesics with reduced side effects. Furthermore the high density of σ_1 receptors in some human tumor cell lines

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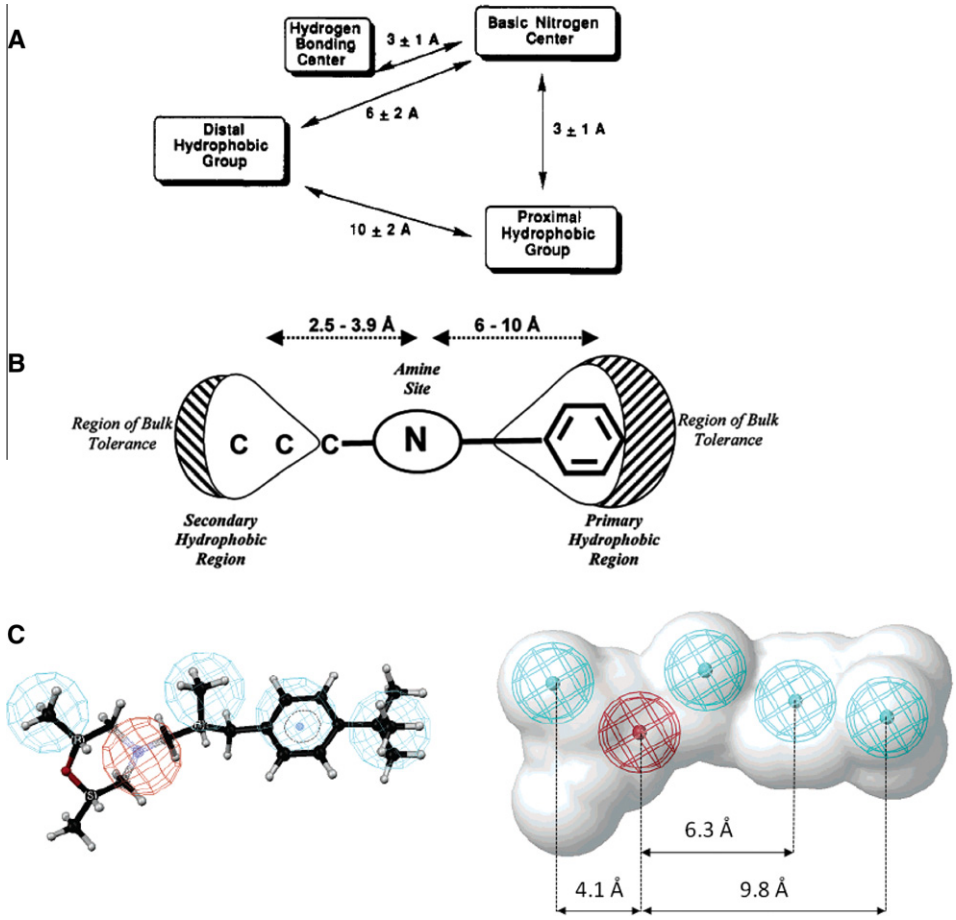


Figure 1. Different pharmacophore models for σ_1 ligands. (A) σ Pharmacophore model according to Gilligan et al.[46] (B) σ_1 pharmacophore model according to Glennon et al.[33,47,48] (C) 3D-σ_1 pharmacophore model according to Langer et al. including calculations of distances and surface of the highly potent σ_1 receptor model ligand fenpropimorph.[49]

will be exploited for the development of novel tumor diagnostics and antitumor drugs.[25,32]

In this article we report on the synthesis and pharmacological evaluation of spirocyclic compounds bearing an exocyclic amino moiety. Theoretical considerations taking various pharmacophore models and the corresponding distances of pharmacophoric elements into account provided the rationale for the development of these compounds.

2. Pharmacophore models of σ_1 ligands

Several structural diverse σ_1 ligands with high affinity are known today, including benzomorphans, conformationally constrained and flexible arylalkylamines,[33] spirocyclic compounds,[34–37] bicyclic ligands,[38,39] guanidines and steroids[20] as well as clinically used drugs like fluvoxamine[40] and haloperidol.[41]

For the development of novel σ_1 ligands the structural properties, which are crucial for high σ_1 receptor affinity were investigated. In 1986 Lloyd and Andrews postulated general requirements for CNS active compounds—a phenyl ring and a basic amino group.[42] Based on this hypothesis Manallack et al. and Gund et al. superimposed independently different σ ligands and defined a distance between the phenyl ring and the basic amino moiety of 5.06 Å (Manallack) and 5.3–5.7 Å (Gund), respectively.[43–45] In 1992 Gilligan et al. published a σ pharmacophore model, which defines the orientation and distances of two 'hydrophobic groups' and one 'basic nitrogen center' to each other for potent σ_1 ligands.[46] (Fig. 1A)

The differentiation of the σ receptor into σ_1 and σ_2 subtypes required some modifications of the Gilligan model. In 1994 Glennon et al. described the first 2D σ_1 pharmacophore model. (Fig. 1B) Based on deconstruction–reconstruction–elobaration analyses of different flexible σ_1 ligands a pharmacophore comprising one basic amine flanked by two 'hydrophobic regions' was defined. The distances between the basic amino group and the 'primary' and 'secondary hydrophobic regions' should be in the range of 6–10 Å and 2.5–3.9 Å, respectively. The basic group can be a secondary or tertiary amine, but in the case of a tertiary amine the third N-substituent should be rather small (e.g., a CH_3 moiety).[47] The term hydrophobic region is slightly confusing here, because in pharmacophore models ligand properties are usually described, but the expression 'region' refers better to the properties of the receptor surface. According to Glennon's model the binding

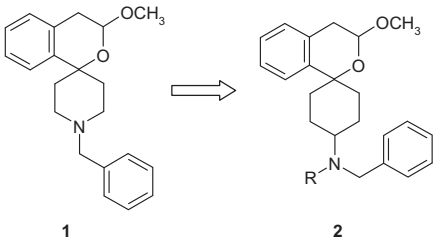


Figure 2. Novel spirocyclic σ_1 ligands **2** are derived from the potent spirocyclic piperidine **1**.

pocket of the receptor tolerates sterically demanding hydrophobic substituents at both ends.[33,48]

The first 3D computer-based σ_1 pharmacophore model was developed by Langer et al. and consists of four hydrophobic groups and one positive ionizable group. (Fig. 1C)The postulated distances between the basic amino moiety and the hydrophobic groups are 4.1, 6.3 and 9.8 Å, respectively. These distances correspond pretty well with the distances defined in the Glennon model. Compared with the Glennon model the Langer model defines two additional hydrophobic groups and the three dimensional orientation to each other.[49]

Recently Zampieri et al. developed a computer based model consisting of five pharmacophoric features—one basic amine, two hydrophobic aromatic groups, one hydrophobic group and one H-bond acceptor. The distances of 7.01 and. 8.50 Å, respectively as well as 3.58 Å are in good accordance with the Glennon and Langer models. However in this model an additional H-bond acceptor was postulated, which has been previously postulated in the Gilligan model.[50]

Very recently, a pseudoreceptor model for spirocyclic σ_1 ligands has been reported, which simulates the binding characteristics of σ_1 receptor ligands and visualizes potential structural features of the receptor surface. In this model an H-bond acceptor group was postulated on the receptor surface, which interacts with the protonated amino group of the σ_1 ligand. The receptor surface consists of many hydrophobic particles leading to many hydrophobic interactions during ligand binding. The large amount of hydrophobic interactions supports the hypothesis that σ_1 ligands interact with the steroid binding domain like region 1 (SBDL1) of the σ_1 receptor protein.[51]

3. Theoretical considerations and design of novel σ_1 ligands

The majority of reported potent σ_1 receptor ligands are conformationally rather flexible. In our group several spirocyclic compounds with high σ_1 affinity have been synthesized.[34–37,52–54] The rigid spirocyclic framework of these compounds reduces the conformational flexibility and defines the relative orientation of the pharmacophoric elements to each other. The spirocyclic 2-benzopyran **1**, which represents a highly potent (K_i = 1.29 nM) and subtype selective (K_i (σ_2) >1000 nM) σ_1 ligand,[52] was selected as lead compound for this project. (Fig. 2)

The high σ_1 affinity of **1** can be explained with the described pharmacophore models. (see Fig. 1) For the determination of the distances of the pharmacophoric elements a stochastic conformational search (MOE—Molecular Operating Environment) was performed with energy cut off of 7 kcal/mol. Since it is assumed that the basic amino moiety is protonated in physiological milieu, the corresponding protonated compound **1H$^+$** was included into the study. Moreover, both enantiomers were included in the calculations, respectively. The distances between the basic amino moiety and the two hydrophobic regions were calculated for all energetically favored conformations using the center of the phenyl rings. The definitions of distance 1 and distance 2 are given in Figure 3.

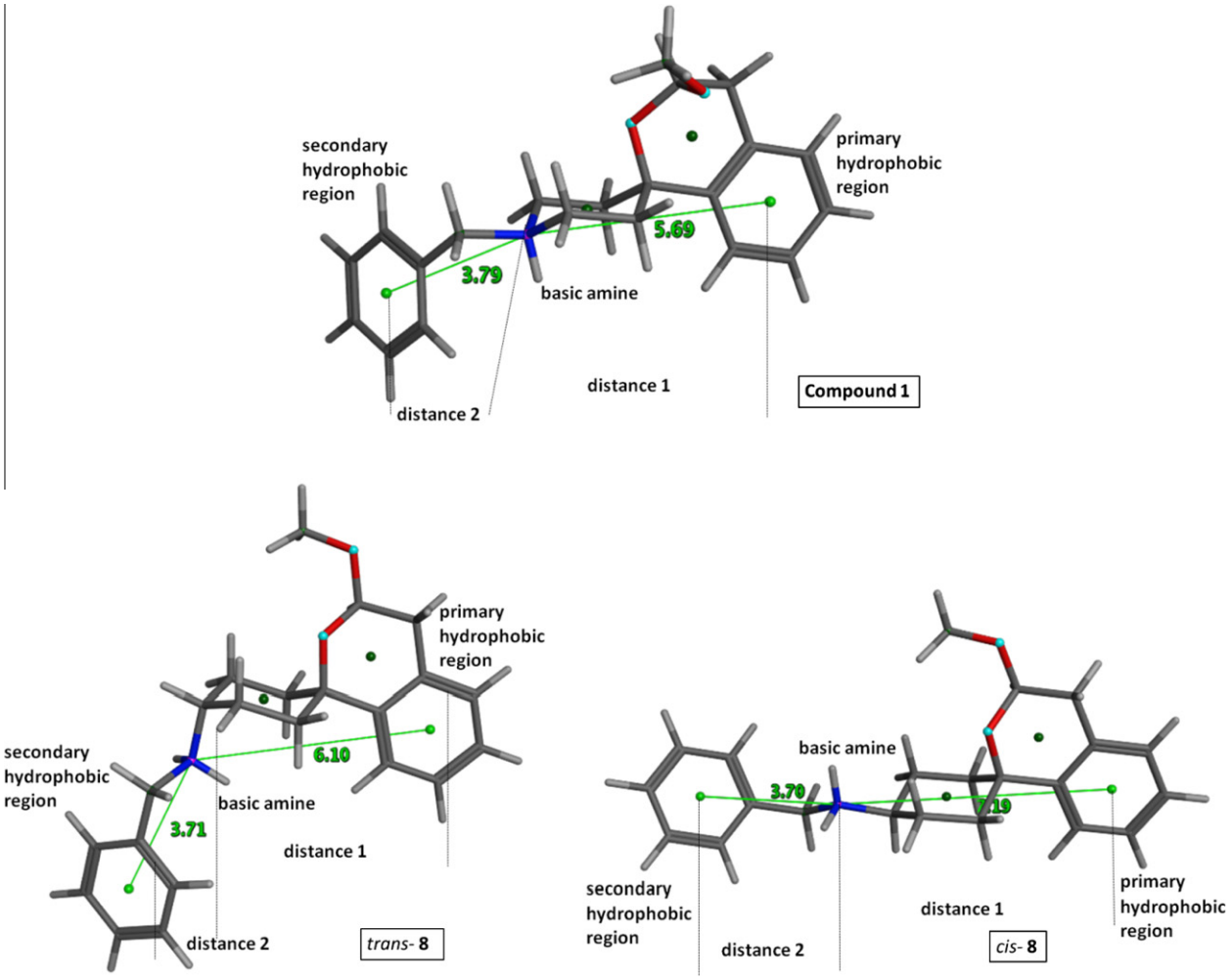


Figure 3. Visualization of distances 1 and 2 for the lead compound 1 and the designed ligands *trans*-**8** and *cis*-**8**.

Generally the spirocyclic compound **1** can adopt two types of conformations: in the first type of conformations **1-1** the oxygen atom of the benzopyran ring adopts the axial position at the piperidine chair (Fig. 4, left), whereas in the second type of conformations **1-2** the oxygen atom adopts the equatorial position (Fig. 4, right). Within the energy frame of 7 kcal/mol both types of conformations were found for the free piperidine **1**. Nevertheless, the number of energetically favored conformations of type **1-1** with the oxygen atom in the axial orientation is much higher. For the protonated compound **1H$^+$** only conformations of type **1-1** were observed. (Table 1)

In Table 1 the distances between the N-atom and the centers of the phenyl moieties are summarized. The distance 2 between the N-atom and the center of the *N*-benzyl residue is exactly within the range, which has been postulated by Gilligan (3 ± 1 Å), Glennon (2.5–3.9 Å) and Langer (around 4.1 Å). However, distance 1 between the N-atom and the phenyl moiety of the benzopyran system is not matching to the models. The analysis led to two types of conformers. Whereas distance 1 in the less populated conformation type **1-2** is 5.09–5.18 Å the conformations of type **1-1** have a distance 1 of 5.65–5.75 Å. These distances are considerably shorter than the postulated distances of 6–10 Å (Glennon) and 6.3 Å and 9.5 Å (Langer).

These results stimulated the idea to increase the distance 1 between the N-atom and the phenyl moiety of the benzopyran system of the spirocyclic σ_1 ligands in order to improve the σ_1 affinity and selectivity over other receptor systems. One possibility to increase distance 1 is the removal of the N-atom from the piperidine ring and attach it at the ring system. This modification results in the spirocyclic compounds **2** with an exocyclic amino moiety attached to the spirocyclic connected cyclohexane ring. (Fig. 2) Attachment of the amino moiety at the cyclohexane ring provides two diastereomeric compounds *trans*-**2** and *cis*-**2** reflecting the relative orientation of N- and O-substituents at the cyclohexane ring. The central cyclohexane ring of both diastereomers can adopt two chair conformations, which are termed *trans*/*cis*-**2-1** and *trans*/*cis*-**2-2**. (Fig. 4)

In order to compare the distances 1 and 2 of the designed spirocyclic compounds **2** with those of the spirocyclic piperidine **1** a stochastic conformational search was performed with both benzylamines *trans*-**8** and *cis*-**8**. Again an energy cut off of 7 kcal/mol was defined and both types of conformers **8-1** (O-atom of the benzopyran ring axially oriented at the cyclohexane ring) and **8-2** (O-atom in equatorial position) as well as both enantiomers were considered, respectively.

In case of *trans*-**8** energetically favored conformations of type *trans*-**8-1** and *trans*-**8-2** were found. However, distances 1 of both conformation types are very similar 5.88–6.26 Å. In addition to clear chair conformations of the cyclohexane ring two energetically allowed (E <7 kcal/mol) distorted chair conformations were found for the protonated compound *trans*-**8H$^+$** with an increased distance 1 of 6.52–6.56 Å.

The *cis*-configured compound *cis*-**8** can also adopt both types of conformations, although the conformations of type *cis*-**8-1** with axial orientation of the benzopyran-O-atom are slightly preferred. Whereas distances 1 of type *cis*-**8-2** conformations are in the range of 6.25–6.30 Å, these distances are considerably longer (7.16–7.23 Å) for *cis*-**8-1** type conformations.

Distance 1 of the spirocyclic ligands with exocyclic amino moiety is increased in the following order: *tans*-**8-1** = *trans*-**8-2** < *cis*-**8-2** < *cis*-**8-1**. These distances are longer than the distances 1 in the lead compound **1** and fulfill exactly the requirements defined in the pharmacophore models of Glennon (6–10 Å), Langer (6.3 and 9.5 Å) and Zampieri (7.01 and 8.56 Å). (Fig. 1)

Since in this study only benzylamines were considered, distances 2 of the lead compound **1** and the cyclohexane derivatives **8** are very similar (3.69–3.85 Å), independently on the position of the N-atom in or at the ring system, the configuration or the protonation state. Initially the *N*-benzyl substituent was not modified, since the corresponding distances fit well into the described models of Glennon (2.5–3.9 Å), Laggner (4.1 Å) and Zampieri (3.58 Å).

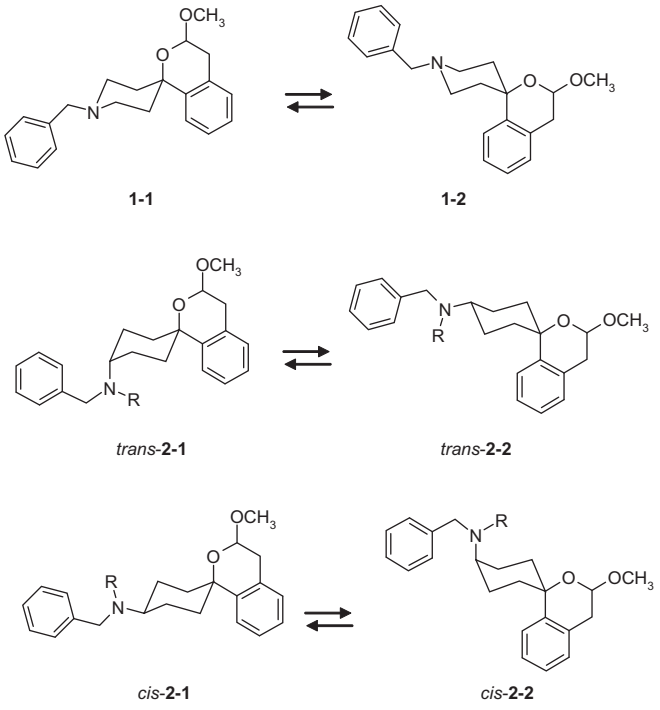


Figure 4. Conformational analysis of the lead compound **1** and the designed spirocyclic compounds **2** with an exocyclic amino group.

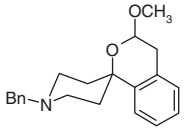
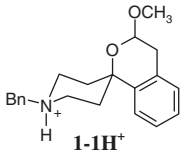
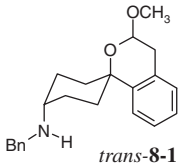
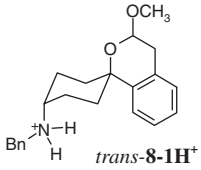
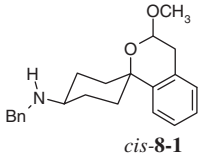
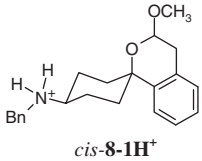
4. Synthesis

The synthesis of the designed spirocyclic σ_1 ligands of type **2** with an exocyclic amino moiety started with 2-bromobenzaldehyde (**3**). (Scheme 1) Homologation of the aldehyde was performed via a Wittig reaction with (methoxymethyl)triphenylphosphonium chloride and KO^{t}Bu and subsequent addition of methanol to the resulting enol ether **4**, which gave the dimethyl acetal **5**.[55]

Aryl bromide **5** was treated with *n*-BuLi at −78 °C and the formed aryllithium intermediate was trapped with an excess of cyclohexane-1,4-dione to afford the hydroxy acetal **6** in 50–60% yields. During NMR-spectroscopy of the hydroxy acetal **6** in $CDCl_3$ cyclization was observed, which was due to the presence of small amounts of HCl in $CDCl_3$. Therefore, the intramolecular transacetalization of the hydroxy acetal **6** to the 2-benzopyran **7** was conducted in $CHCl_3$ after addition of catalytic amounts of HCl. After stirring the reaction mixture for 90 min at room temperature, the racemic spirocyclic benzopyran **7** was isolated in 81% yield. Prolongation of the reaction time led to reduced yields, since elimination of methanol occurred as side reaction.

Reductive amination of the spirocyclic ketone **7** with benzylamine, $NaBH(OAc)_3$[56] and one equivalent of acetic acid led to the diastereomeric benzylamines *trans*-**8** and *cis*-**8**, which were separated by flash chromatography and isolated in 35% and 52% yields, respectively. The slight preference of *cis*-**8** with an equatorially oriented amino moiety is explained by stereoelectronic effects, that is, the intermediate iminium ion is preferably attacked from the axial side, which is favored by binding overlap between the occupied σ-molecular orbital of the newly formed C–H bond and

Table 1
Calculated distances 1 and 2 for the energetically most favored conformers of spirocyclic piperidine **1** and the benzylmaines *trans*-**8** and *cis*-**8**

Compd	Structure	Config.	Conform.	Number conform.	Distance 1 arom 1–N [Å]	Distance 2 arom 2–N [Å]
1	 **1-1**	(*R*)	**1-1**	22	5.65–5.74	3.75–3.84
			2-2	6	5.09–5.18	
		(*S*)	**1-1**	22	5.67–5.74	3.75–3.84
			1-2	3	5.10–5.18	
1H$^+$	 **1-1H$^+$**	(*R*)	**1-1**	17	5.67–5.75	3.78–3.85
			1-2	0	—	—
		(*S*)	**1-1**	15	5.67–5.75	3.78–3.85
			1-2	0	—	—
trans-**8**	 *trans*-**8-1**	(*R*)	*trans*-**8-1**	39	5.98–6.15	3.69–3.77
			trans-**8-2**			
		(*S*)	*trans*-**8-1**	49	5.88–6.17	3.69–3.77
			trans-**8-2**			
trans-**8H$^+$**[a]	 *trans*-**8-1H$^+$**	(*R*)	*trans*-**8-1**	42	5.99–6.26	3.70–3.78
			trans-**8-2**			
		(*S*)	*trans*-**8-1**	42	5.99–6.26	3.70–3.77
			trans-**8-2**			
cis-**8**	 *cis*-**8-1**	(*R*)	*cis*-**8-1**	36	7.16–7.22	3.69–3.80
			cis-**8-2**	9	6.25–6.29	
		(*S*)	*cis*-**8-1**	43	7.16–7.22	3.69–3.81
			cis-**8-2**	11	6.25–6.30	
cis-**8H$^+$**	 *cis*-**8-1H$^+$**	(*R*)	*cis*-**8-1**	32	7.18–7.23	3.70–3.81
			cis-**8-2**	9	6.26–6.28	
		(*S*)	*cis*-**8-1**	39	7.18–7.23	3.70–3.80
			cis-**8-2**	11	6.26–6.29	

The conformations are grouped into two classes **8-1** and **8-2** according to Figure 4. The calculations were performed for the free bases and the corresponding protonated species, respectively. Both enantiomers were taken into account.

[a] For the protonated isomer *trans*-**8H$^+$** two additional conformations with a distorted geometry were found. Distance 1 of these conformations was 6.52–6.56 Å.

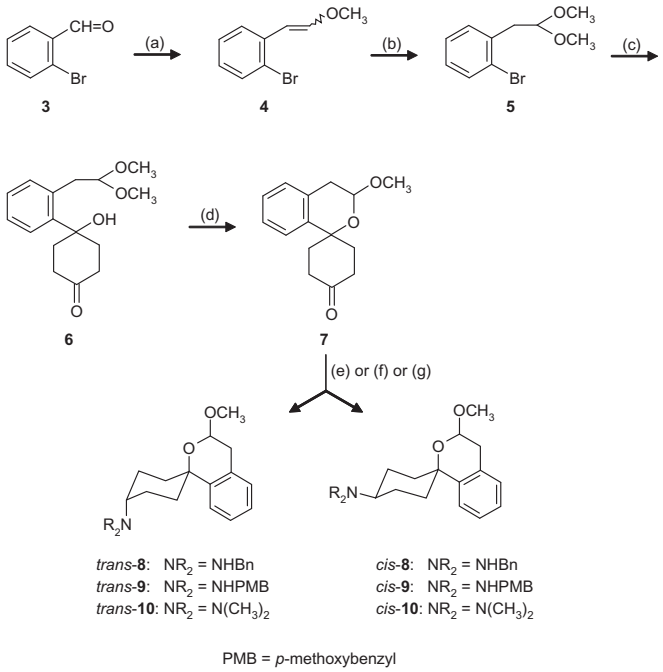
the non-occupied, parallel oriented, antibonding σ^*-molecular orbitals of the adjacent axially oriented C–H bonds in 3′- and 5′-position of the cyclohexane ring.[57]

Reaction of the spirocyclic ketone **7** with *p*-methoxybenzylamine (PMB-NH_2) and $NaBH(OAc)_3$ provided *trans*-**9** and *cis*-**9** in 23% and 35% yield, respectively. Again stereoelectronic effects are responsible for the production of the *cis*-configured diastereomer *cis*-**9** in higher amounts than *trans*-**9**. The diastereoselectivity was increased considerably, when performing the reductive amination with a secondary amine. After reductive amination of ketone **7** with dimethylamine and $NaBH(OAc)_3$ the diastereomeric tertiary amines *trans*-**10** and *cis*-**10** were isolated in 18% and 66% yields, respectively.

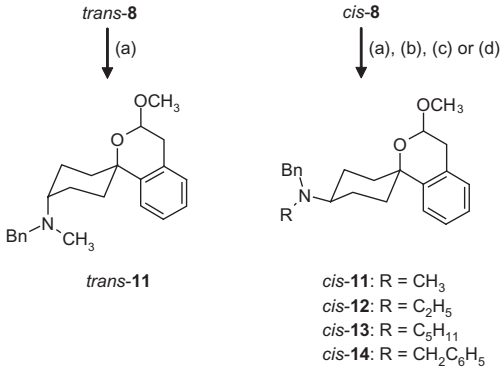
In order to investigate the influence of a second substituent at the amino moiety, benzylamines **8** were converted into various tertiary amines. (Scheme 2) Reductive methylation of *trans*-**8** and *cis*-**8** with formalin and $NaBH(OAc)_3$ led to the tertiary amines *trans*-**11** and *cis*-**11** in 88% and 84% yields, respectively. Since the *cis*-configured diastereomers *cis*-**8** and *cis*-**11** revealed higher σ_1 receptor affinity than their *trans*-configured analogues *trans*-**8** and *trans*-**11** and, moreover, *cis*-**8** was obtained in higher yields, further alkyl residues were introduced into the *cis*-configured benzylamine *cis*-**8**. The ethyl derivative *cis*-**12** was obtained by alkylation of *cis*-**8** with 1-iodoethane using microwave irradiation. Alkylation of benzylamine *cis*-**8** with valeraldehyde or benzaldehyde using the reducing agent $NaBH(OAc)_3$ provided the pentyl and benzyl derivatives *cis*-**13** and *cis*-**14**, respectively.

The axial orientation of the amino group of *trans*-**8** was shown by the quintet-type signal at 3.03 ppm (J = 2.7 Hz) for the equatorially oriented proton in 4′-position of the cyclohexane ring. The triplet of triplets at 2.66 ppm with two large (J = 10.5 Hz) and two small coupling constants (J = 3.8 Hz) is caused by the axially oriented proton at 4′-position of the cyclohexane ring of *cis*-**8**. Similar signals are also found for *trans*-**9**-**11** and *cis*-**9**-**14**. However the arrangement of the amino moiety relative to the benzopyran ring could not be determined unequivocally, since both diastereomers *trans*-**8** and *cis*-**8** can adopt conformations with axially (*trans*-**8-1**, *cis*-**8-2**) and equatorially oriented (*trans*-**8-2**, *cis*-**8-1**) amino substituent.

In order to prove the relative configuration unequivocally the *trans*-configured diastereomer *trans*-**9** was recrystallized from ethyl acetate, which led to crystals suitable for X-ray crystal structure analysis. The X-ray crystal structure shows the amino moiety and the O-atom of the benzopyran ring on opposite sides of the



Scheme 1. Synthesis of spirocyclic σ_1 receptor ligands with exocyclic amino moiety. Reagents and conditions: (a) methoxymethyltriphenylphosphonium chloride, KO^{t}Bu, THF, start at −10 °C, then 16 h rt, 71%;[55] (b) *p*TosOH·H_2O, MeOH, 72 h, reflux, 92%;[55] (c) *n*-BuLi, THF, −78 °C, 20 min, then cyclohexane-1,4-dione, 2 h, −78 °C, 1 h, rt, 56%; (d) $CHCl_3$, HCl, 1.5 h, rt, 81%; (e) benzylamine, THF, HOAc, $NaBH(OAc)_3$, 2 h, rt, 35% (*trans*-**8**), 52% (*cis*-**8**); (f) 4-methoxybenzylamine, THF, HOAc, $NaBH(OAc)_3$, 2.5 h, rt, 23% (*trans*-**9**), 35% (*cis*-**9**); (g) dimethylamine in EtOH, THF, HOAc, $NaBH(OAc)_3$, 2.5 h, rt, 18% (*trans*-**10**), 66% (*cis*-**10**).



Scheme 2. Alkylation of secondary amines to obtain tertiary amines. Reagents and conditions: (a) CH_2=O, CH_2Cl_2, $NaBH(OAc)_3$, 23 h or 3 h, rt, 88% (*trans*-**11**), 84% (*cis*-**11**); (b) C_2H_5I, K_2CO_3, CH_3CN, microwave, 45%; (c) valeraldehyde, CH_2Cl_2, $NaBH(OAc)_3$, 23 h, rt, 90%; (d) benzaldehyde, CH_2Cl_2, $NaBH(OAc)_3$, 23 h, rt, 41%.

cyclohexane ring plain indicating *trans*-configuration. (Fig. 5) In the crystals the cyclohexane ring adopts the conformation *trans*-**8-1** (see Fig. 4) with the amino moiety in axial orientation. Apparently, *trans*-**8-1** represents an energetically favored conformation in the solid state (X-ray crystal structure) as well as in solution (compare ^{1}H NMR data).

5. Receptor affinity

The σ receptor affinities of the spirocyclic compounds **8**–**14** were determined in competition experiments with radioligands. In the σ_1 assay homogenates of guinea pig brains were used as receptor material and the σ_1 selective ligand [^{3}H]-(+)-pentazocine

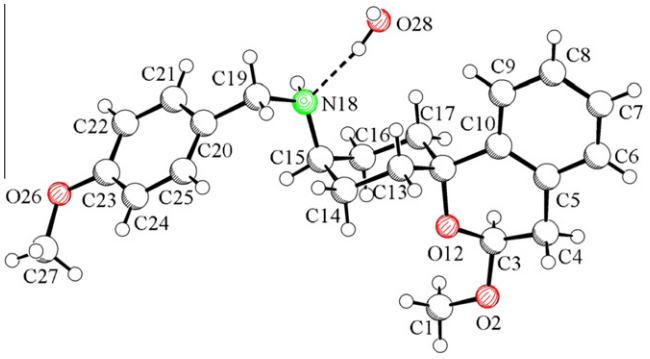


Figure 5. X-ray crystal structure of the *trans*-configured *p*-methoxybenzylamine *trans*-**9**.

was employed as radioligand. Homogenates of rat liver served as source for σ_2 receptors in the σ_2 assay. Since a σ_2 selective radioligand is not commercially available, the non-selective radioligand [^{3}H]-1,3-di(*o*-tolyl)guanidine was employed in the presence of an excess of non-tritiated (+)-pentazocine, which selectively occupies σ_1 receptors.[52–54]

In Table 2 the σ_1 and σ_2 receptor affinities of the exocyclic amines **8**–**14** are compared with the σ affinities of the spirocyclic piperidine **1** and the reference compounds (+)-pentazocine and haloperidol. The σ_1 receptor affinities of the secondary amines *trans*-**8**, *cis*-**8**, *trans*-**9**, and *cis*-**9** are >100-fold lower than the σ_1 receptor affinity of the lead piperidine **1**. However, transformation of the secondary amines **8** into methylated tertiary amines *trans*-**11** and *cis*-**11** led to a considerable increase of the σ_1 affinity: the K_i-value of *trans*-**11** is 43 nM the K_i-value of the *cis*-configured diastereomer *cis*-**11** is 24 nM. The low σ_1 affinities of the diastereomeric dimethylamines *trans*-**10** and *cis*-**10** demonstrate that at least one large substituent (e.g., a benzyl moiety) is necessary for high σ_1 affinity. However, the size of the second alkyl substituent is rather limited, since the σ_1 affinity was decreased with increasing size of this substituent: *cis*-**11** (Me) > *cis*-**12** (Et) > *cis*-**13** (Pent) ≈ *cis*-**14** (Bn). This observation is in good accordance with the reported pharmacophore models, which postulate a small third substituent (H, CH_3) at the basic N-atom.

A considerable decrease of σ_1 affinity was observed after shifting the amino group at the cyclohexane ring as demonstrated with the secondary amines **8** and **9**. Only tertiary amines *trans*-**11** and *cis*-**11** show σ_1 receptor affinities in the range of reference

Table 2
σ_1 and σ_2 receptor affinities of spirocyclic ligands with an exocyclic amino group compared with reference compounds.

Compd	NR_2	K_i ± SEM [nM]	
		σ_1	σ_2
1	–	1.29 ± 0.18	>1000
trans-**8**	NHBn	538 ± 56	>1000
cis-**8**	NHBn	158 ± 5	>1000[#]
trans-**9**	NHPMB	169 ± 19	664 ± 178
cis-**9**	NHPMB	174 ± 12	142 ± 33
trans-**10**	$N(CH_3)_2$	>1000	>1000
cis-**10**	$N(CH_3)_2$	>1000	>1000
trans-**11**	NCH_3Bn	43 ± 18	>1000
cis-**11**	NCH_3Bn	24 ± 4.7	329
cis-**12**	$N(C_2H_5)Bn$	107 ± 25	666 ± 106
cis-**13**	$N(C_5H_{11})Bn$	>1000	719
cis-**14**	$N(Bn)_2$	>1000	>1000
(+)-Pentazocine	–	5.7 ± 2.2	–
Haloperidol	–	6.3 ± 1.6	78 ± 2.3

[#] No correlation between dose and receptor affinity.

compounds and the lead compound **1**. But despite the enlargement of distance 1 in *cis*-**11** the σ_1 receptor affinity is still about 15-fold lower than the σ_1 affinity of **1**. The reduced σ_1 affinity of *cis*-**11** may be attributed to entropic factors, since attachment of the benzylamino moiety at the ring leads to increased flexibility.

With exception of the equipotent diastereomeric *p*-methoxybenzyl derivatives **9**, generally, the *cis*-configured diastereomers show higher σ_1 receptor affinities than the *trans*-configured diastereomers. The higher σ_1 receptor affinity correlates well with the increased distance 1. For the more populated conformations of *cis*-**8** distance 1 (7.16–7.23 Å) is about 1 Å longer than distance 1 of the conformations of *trans*-**8** (5.88–6.26 Å).

Almost all compounds show negligible affinity towards σ_2 receptors indicating high selectivity against the σ_2 subtype, at least for the most potent tertiary σ_1 ligands *trans*-**11** and *cis*-**11**. Only the *p*-methoxybenzylamine *cis*-**9** and the tertiary amine *cis*-**13** with an *N*-pentyl residue are equipotent at both σ receptor subtypes.

6. Conclusion

Despite an increased distance 1, which fits exactly into various σ_1 pharmacophore models, the σ_1 receptor affinity of the secondary amines **8** and **9** is >100-fold reduced compared with the σ_1 affinity of the spirocyclic piperidine **1** with a shorter distance 1. In contrast to the established models secondary amines **8** and **9** are less tolerated by the σ_1 receptor protein than the corresponding tertiary methylamines **11**. The affinities of the tertiary amines **10**–**14** demonstrate that one large N-substituent (e.g., a benzyl group) is required, but two large substituents (e.g., two benzyl groups) are not accepted by the receptor protein. As a rule, *cis*-configured diastereomers show higher σ_1 receptor affinities than their *trans*-configured counterparts, which leads to *cis*-**11** as the most potent σ_1 ligand of this series (K_i = 24 nM). The high σ_1 affinity of *cis*-**11** represents a promising starting point for the development of novel potent σ_1 ligands, since the exocyclic amino moiety allows fine tuning of the pharmacological properties of this compound class by modifying *two* N-substituents.

7. Experimental

7.1. Conformational analysis

3D-Structures were generated with MOE (Molecular Operating Environment), Version 2009.10, Chemical computing group AG (CCG, Montreal, Canada). Structures were drawn with modul Molecule Builder. Stochastic conformational search was carried out at standard conditions. Method: Stochastic, Rejection Limit: 100, Iteration Limit 10,000, RMS Gradient: 0.005, MM Iteration Limit: 500, RMSD Limit: 0.25, Strain cutoff: 7 kcal/mol, Conformation Limit: 10,000).

7.2. Experimental, chemistry

7.2.1. General

Unless otherwise noted, moisture sensitive reactions were conducted under dry nitrogen. THF was dried with sodium/benzophenone and was freshly distilled before use. Thin layer chromatography (TLC): Silica Gel 60 F_{254} plates (Merck). Flash chromatography (FC): Silica Gel 60, 40–64 μm (Merck); parentheses include: diameter of the column, length of the column, eluent, fraction size, R_f value. Melting point: Melting point apparatus SMP 3 (Stuart Scientific), uncorrected. MS: MAT GCQ (Thermo-Finnigan); EI = electron impact; Thermo Finnigan LCQ® ion trap mass spectrometer with an ESI = electrospray ionization interface. IR: IR spectrophotometer 480Plus FT-ATR-IR (Jasco). ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz): Mercury-400BB spectrometer (Varian); δ in ppm related to tetramethylsilane; coupling constants are given with 0.5 Hz resolution. HPLC: Merck Hitachi Equipment; UV detector: L-7400; autosampler: L-7200; pump: L-7100; degasser: L-7614; Method A: column: LiChrospher® 60 RP-select B (5 μm), 250–4 mm; flow rate: 1.00 mL/min; injection volume: 5.0 μL; detection at λ = 210 nm; solvents: A: water with 0.05% (v/v) trifluoroacetic acid; B: acetonitrile with 0.05% (v/v) trifluoroacetic acid: gradient elution: (A%): 0–4 min: 90% , 4–29 min: gradient from 90% to 0%, 29–31 min: 0%, 31–31.5 min: gradient from 0% to 90%, 31.5–40 min: 90%. The purity of all test compounds was greater than 95%.

7.2.2. 2-[2-(1-Hydroxy-4-oxocyclohexyl)phenyl]acetaldehyde dimethyl acetal (6)

Under N_2 2-(2-bromophenyl)acetaldehyde dimethyl acetal[55] (**5**, 1.03 g, 4.20 mmol) was dissolved in THF abs (32 mL) and cooled down to −78 °C. Subsequently, *n*-BuLi (1.48 M in *n*-hexane, 3.12 mL, 4.62 mmol) was added slowly. After 20 min a solution of cyclohexane-1,4-dione (0.946 g, 8.4 mmol in THF, 12 mL) was added rapidly and the mixture was stirred for 2 h at −78 °C and 1 h at rt. Then H_2O was added (30 mL), after addition of CH_2Cl_2 the layers were separated and the aqueous layer was extracted with CH_2Cl_2 (2x), the combined organic layers were dried (Na_2SO_4), concentrated in vacuo and the residue was purified by FC (5 cm, cyclohexane/ethyl acetate 2:1, 20 cm, 30 mL, R_f = 0.20). Colorless solid, mp 45 °C, yield 652 mg (56%). $C_{16}H_{22}O_4$ (278.4). MS (EI): *m*/*z* (%) = 278 [M, 1], 246 [M−MeOH, 2], 189 [M−MeOH, −CH_3CH_2C=O, 50], 157 [M−2 MeOH, −CH_3CH_2C=O, 100]. MS (ESI$^-$): *m*/*z* (%) = 555 [(2M−H)$^-$,15], 277[(M−H)$^-$, 100]. IR: $\tilde{\nu}$ (cm^{-1}) = 3424 (m, broad, v, OH), 3057 (w, v, C–H, arom), 2938 (s,v, C–H, alkyl), 2833 (m, v, OCH_3), 1710 (s, v, C=O), 1443 (m, δ, C–H, alkyl), 760 (s, δ, C–H, o-disubst. arom). ^{1}H NMR (DMSO-d_6): δ (ppm) = 2.14–2.28 (m, 6H, (CH_2CH_2)$_2$C=O), 2.84 ('dt', *J* = 14.4/9.5 Hz, 2H, (CH_2CH_2)$_2$C=O), 3.30 (s, 6H, Ar-$CH_2CH(OCH_3)_2$), 3.33 (d, *J* = 5.3 Hz, 2H, Ar-$CH_2CH(OCH_3)_2$), 4.67 (t, *J* = 5.4 Hz, 1H, Ar-$CH_2CH(OCH_3)_2$), 5.33 (s, 1H, O*H*), 7.16–7.24 (m, 2H, Ar-H), 7.30–7.34 (m, 1H, Ar-H), 7.38–7.42 (m, 1H, Ar-H).

7.2.3. 3-Methoxy-3,4-dihydrospiro[[2]benzopyran-1,1′-cyclohexan]-4′-one (7)

A solution of 2 M HCl (0.06 mL) and hydroxy acetal **6** (300 mg, 1.08 mmol) in $CHCl_3$ (24 mL) was stirred vigorously at rt for 90 min. Afterwards CH_2Cl_2 (24 mL) was added, and the organic layer was washed with 0.2 M NaOH (12 mL) and H_2O (12 mL). The organic layer was dried (Na_2SO_4), concentrated in vacuo and the residue was purified by FC (4 cm, cyclohexane/ethyl acetate 4:1, 20 cm, 30 mL, R_f = 0.23). Colorless solid, mp 126.4 °C, yield 214 mg (81%). $C_{15}H_{18}O_3$ (246.3). MS (EI): *m*/*z* (%) = 246 [M, 4], 214 [M–MeOH, 4], 189 [M–CH_3CH_2C*=O, 66], 157 [M–MeOH, –$CH_3CH_2C^*$=O, 100]. MS (ESI): *m*/*z* (%) = 515 [2M+Na, 33], 269 [M+Na, 62], 264 [M+NH_4, 100]. IR: $\tilde{\nu}$ (cm^{-1}) = 2948 (s, v, C–H, alkyl), 2905 (m, v, C–H, alkyl), 2872 (m, v, OCH_3), 1717 (s, v, C=O), 773 (s, δ, C–H, o-disubst. arom). ^{1}H NMR ($CDCl_3$): δ (ppm) = 2.12 ('td', *J* = 14.0/4.4 Hz, 1H, (CH_2CH_2)$_2$C=O, 2.22–2.42 (m, 5H, (CH_2CH_2)$_2$C=O), 2.82–2.92 (m, 1H, (CH_2CH_2)$_2$C=O), 2.92–3.06 (m, 3H, (CH_2CH_2)$_2$C=O (1H), ArCH_2CHOCH_3 (2H)), 3.58 (s, 3H, OCH_3), 4.99 (dd, *J* = 6.8/3.5 Hz, 1H, ArCH_2CHOCH_3), 7.08–7.11 (m, 1H, Ar-H), 7.12–7.16 (m, 1H, Ar-H), 7.19 - 7.22 (m, 2H, Ar-H). ^{13}C NMR ($CDCl_3$): δ (ppm) = 35.5 (1C, CH_2CHOCH_3), 37.4 (1C, (CH_2CH_2)$_2$O), 37.7 (1C, (CH_2CH_2)$_2$C=O), 37.8 (1C, (CH_2CH_2)$_2$C=O), 39.3 (1C, (CH_2CH_2)$_2$C=O), 56.6 (1C, OCH_3), 75.6 (1C, spiro-C), 97.4 (1C, CH_2CHOCH_3), 124.5 (1C, arom), 127.1 (1C, arom), 127.5 (1C, aroma), 129.7 (1C, arom), 131.6 (1C, arom), 139.9 (1C, arom), 211.8 (1C, C=O). Purity (HPLC): 99.6%, t_R = 18.87 min.

7.2.4. *trans*-*N*-Benzyl-3-methoxy-3,4-dihydrospiro-[[2]benzopyran-1,1′-cyclohexan]-4′-amine (*trans*-8) and *cis*-*N*-Benzyl-3-methoxy-3,4-dihydrospiro[[2]benzopyran-1,1′-cyclohexan]-4′-amine (*cis*-8)

Under N_2 ketone **7** (145 mg, 0.588 mmol) was dissolved in THF (7 mL). Benzylamine (64 μL, 0.588 mmol), acetic acid (34 μL, 0.588 mmol) and $NaBH(OAc)_3$ (95%, 175 mg, 0.784 mmol) were added and the mixture was stirred for 2 h at rt. Subsequently 1 M NaOH (8 mL) was added and the mixture was extracted with CH_2Cl_2 (4 × 8 mL), the combined organic layers were dried (K_2CO_3), concentrated in vacuo and the residue was purified by FC (3 cm, cyclohexane : ethyl acetate 9:1 + 2% *N*,*N*-dimethylethanamine, 20 cm, 10 mL).

trans-**8** (R_f = 0.31): Colorless solid, mp 96 °C, yield 70 mg (35%). $C_{22}H_{27}NO_2$ (337.5). MS (ESI): *m*/*z* (%) = 338 [MH,100]. IR: $\tilde{\nu}$ (cm^{-1}) = 3043 (m, v, C–H, arom), 2928, 2870 (s, v, C–H, alkyl), 1604, 1486 (w, C=C, arom), 1441 (m, δ, C–H, alkyl), 757 (s, δ, C–H, o-disubst. arom), 737, 699 (s, δ, C–H, monosubst. arom). ^{1}H NMR ($CDCl_3$): 1.61–1.73 (m, 3H, 2′-H_e, 3′-H_e, 5′-H_e), 1.80 (ddd, *J* = 13.1/5.6/3.0 Hz, 1H, 6′-H_e) ,1.99 ('tt', *J* = 13.3/ 3.1 Hz, 1H, 5′-H_a), 2.04–2.16 (m, 2H, 3′-H_a, 6′-H_a), 2.34 ('td', *J* = 13.4/4.0 Hz, 1H, 2′-H_a), 2.89 (dd, *J* = 15.6/6.8 Hz, 1H, ArC*H*$_2$CHOCH$_3$), 2.94 (dd, *J* = 15.6/4.2 Hz, 1H, ArC*H*$_2$CHOCH$_3$), 3.03 ('quint', *J* = 2.7 Hz, 1H, CH$_2$C*H*-NH), 3.57 (s, 3H, OC*H*$_3$), 3.83 (s, 2H, Ar-C*H*$_2$-NH), 4.87 (dd, *J* = 6.7/4.2 Hz, 1H, ArCH$_2$C*H*OCH$_3$), 7.07–7.11 (m, 1H, Ar-H), 7.14–7.20 (m, 1H, Ar-H), 7.21–7.25 (m, 2H, Ar-H), 7.26–7.31 (m, 1H, Ar-H), 7.34–7.43 (m, 4H, Ar-H), a signal for the NH-proton is not seen in the spectrum. ^{13}C NMR ($CDCl_3$): δ (ppm) = 26.2 (1C, 3′-C or 5′-C), 26.5 (1C; 3′-C or 5′-C), 30.7 (1C, 6′-C), 33.5 (1C, 2′-C), 35.7 (1C, *C*H$_2$CHOCH$_3$), 50.6 (1C, 4′-C), 52.0 (1C, NH*C*H$_2$Ar), 56.4 (1C, OCH$_3$), 77.5 (1C, spiro-C), 96.6 (1C, CH$_2$*C*HOCH$_3$), 125.4 (1C, arom), 126.7 (1C, arom), 126.8 (1C, arom), 127.2 (1C, arom), 128.4 (2C, arom), 128.7 (2C, arom), 129.4 (1C, arom), 131.6 (1C, arom), 141.6 (1C, arom), 142.7 (1C, arom). Purity (HPLC): 99.4%, t_R = 18.81 min.

cis-**8** (R_f = 0.12): Colorless oil, yield 102 mg (52%). $C_{22}H_{27}NO_2$ (337.5). MS (ESI): *m*/*z* (%) = 338 [MH,100]. IR: $\tilde{\nu}$ (cm^{-1}) = 3027 (m, v, C–H, arom), 2927, 2855 (s, v, C–H, arom), 1603, 1491 (w, C=C, arom), 1451 (m, δ, C–H, alkyl), 754 (s, δ, C–H, *o*-disubst. arom), 730, 698 (s, δ, C–H, mono-subst. arom). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.58–1.74 (m, 2H, (C*H*$_2$C*H*$_2$)$_2$CHN), 1.74–1.99 (m, 5H, (C*H*$_2$C*H*$_2$)$_2$CHN), 2.09 (ddd, *J* = 13.2/5.2/2.6 Hz, 1H, 2′-H_e), 2.66 ('tt', *J* = 10.5/3.8 Hz, 1H, 4′-H_a), 2.89 (dd, *J* = 15.3/6.2 Hz, 1H, ArC*H*$_2$CHOCH$_3$), 2.94 (dd, *J* = 15.4/3.7 Hz, 1H, ArC*H*$_2$CHOCH$_3$), 3.57 (s, 3H, OC*H*$_3$), 3.89 (s, 2H, Ar-C*H*$_2$-NH), 4.86 (dd, *J* = 6.7/3.9 Hz, 1H, ArCH$_2$C*H*OCH$_3$), 7.06–7.10 (m, 2H, Ar-H), 7.13–7.20 (m, 2H, Ar-H), 7.25–7.30 (m, 2H, Ar-H), 7.33–7.38 (m, 3H, Ar-H), a signal for the NH-proton is not seen in the spectrum. ^{13}C NMR ($CDCl_3$): δ (ppm) = 29.3 (1C, 3′-C or 5′-C or 6′-C), 29.4 (1C, 3′-C or 5′-C or 6′-C), 36.6 (1C, ArCH$_2$CHOCH$_3$), 36.1 (1C, 2′-C), 38.5 (1C, 3′-C or 5′-C or 6′-C), 51.4 (1C, NHCH$_2$Ar), 55.9 (1C, 4′-C), 56.5 (1C, OCH$_3$), 76.6 (1C, spiro-C), 96.7 (1C, ArCH$_2$*C*HOCH$_3$), 124.9 (1C, arom), 126.7 (1C, arom), 126.9 (1C, arom), 127.2 (1C, arom), 128.4 (2C, arom), 128.8 (2C, arom), 129.5 (1C, arom), 131.9 (1C, arom), 141.1 (1C, arom), 141.9 (1C, arom). Purity (HPLC): 99.9%, t_R = 18.64 min.

7.2.5. *trans*-*N*-(*p*-Methoxybenzyl)-3-methoxy-3,4-dihydrospiro-[[2]benzopyran-1,1′-cyclohexan]-4′-amine (*trans*-9) and *cis*-*N*-(*p*-Methoxybenzyl)-3-methoxy-3,4-dihydrospiro-[[2]benzopyran-1,1′-cyclohexan]-4′-amine (*cis*-9)

Under N_2 ketone **7** (79 mg, 0.32 mmol) was dissolved in THF (4 mL), 4-methoxybenzylamine (46 μL, 0.35 mmol), acetic acid (18 μL, 0.32 mmol) and $NaBH(OAc)_3$ (95%, 95 mg, 0.43 mmol) were added and the mixture was stirred for 2.5 h at rt. Subsequently 1 M NaOH (5 mL) was added and the mixture was extracted with CH_2Cl_2 (4 × 5 mL), the combined organic layers were dried (K_2CO_3), concentrated in vacuo and the residue was purified by FC (2 cm, CH_2Cl_2/cyclohexane 1:1 + 2% *N*,*N*-dimethylethanamine, 23 cm, 10 mL). After concentration in vacuo NaOH (1 M, 4 mL) was added to the separated and purified compounds and the mixture was extracted with CH_2Cl_2 (4×). The organic layers were dried (K_2CO_3) and concentrated in vacuo.

trans-**9** (R_f = 0.23): Colorless solid, mp 84 °C, yield 26.3 mg (23%). $C_{23}H_{29}NO_3$ (367.5). MS (ESI): *m*/*z* (%) = 368 [MH,100]. FT-IR: $\tilde{\nu}$ (cm^{-1}) = 3061 (m, v, C–H, arom), 2993, 2926 (s, v, C–H, alkyl), 2833 (s, v, OCH$_3$), 1611,1510 (w, C=C, arom), 1442 (m, δ, C–H, alkyl), 823 (s, δ, C–H, p-disubst. arom), 754 (s, δ, C–H, o-disubst. arom). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.61–1.72 (m, 3H, 2′-H_e, 3′-H_e, 5′-H_e), 1.80 (ddd, *J* = 13.0/5.3/2.7 Hz, 1H, 6′-H_e), 1.98 ('tt', *J* = 13.4/3.2 Hz, 1H, 5′-H_a), 2.02–2.14 (m, 2H, 3′-H_a, 6′-H_a), 2.32 ('td', *J* = 13.7/4.2 Hz, 1H, 2′-H_a), 2.89 (ddd, *J* = 15.6/6.8/0.9 Hz, 1H, ArC*H*$_2$CHOCH$_3$), 2.94 (ddd, *J* = 15.7/4.1/0.7 Hz, 1H, ArC*H*$_2$CHOCH$_3$), 3.02 ('quint', *J* = 2.8 Hz, 1H, 4′-H_e), 3.57 (s, 3H, OC*H*$_3$), 3.75 (s, 2H, ArC*H*$_2$NH), 3.82 (s, 3H, ArOC*H*$_3$), 4.86 (dd, *J* = 6.7/4.2 Hz,1H, ArCH$_2$C*H*OCH$_3$), 5.30 (s, 1H, N*H*), 6.88–6.92 (m, 2H, Ar-H), 7.06–7.10 (m, 1H, Ar-H), 7.14–7.19 (m, 1H, Ar-H), 7.20–7.23 (m, 2H, Ar-H), 7.29–7.33 (m, 2H, Ar-H). Purity (HPLC): 99.4%, t_R = 19.20 min. A sample was recrystallized from ethyl acetate to obtain crystals, which were suitable for X-ray crystal structure analysis.

X-ray crystal structure analysis for *trans*-**9**: formula $C_{23}H_{29}NO_3 \cdot H_2O$, *M* = 385.49, colorless crystal 0.35 × 0.30 × 0.20 mm, *a* = 12.0010(4), *b* = 13.0086(5), *c* = 13.8753(1) Å, β = 105.890 (2)°, *V* = 2083.39(14) Å^3, ρ_{calcd} = 1.229 g cm^{-3}, μ = 0.668 mm^{-1}, empirical absorption correction (0.800 ⩽ *T* ⩽ 0.878), *Z* = 4, monclinic, space group $P2_1/c$ (No. 14), λ = 1.54178 Å, *T* = 223 K, ω and φ scans, 15594 reflections collected (±*h*, ±*k*, ±*l*), [(sin θ)/λ] = 0.60 Å^{-1}, 3672 independent (R_{int} = 0.039) and 3419 observed reflections [$I \geqslant 2\sigma(I)$], 267 refined parameters, *R* = 0.043, wR^2 = 0.116, max. residual electron density 0.27 (−0.17) e Å^{-3}, hydrogen atoms at N and water from difference fourier calculations, others calculated and refined as riding atoms.

Data set was collected with a Nonius KappaCCD diffractometer. Programs used: data collection COLLECT (Nonius B.V., 1998), data reduction Denzo-SMN,[58] absorption correction Denzo,[59] structure solution SHELXS-97,[60] structure refinement SHELXL-97,[61] graphics SCHAKAL.[62]

CCDC 804299 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html [or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (internat.) +44 (1223)336 033, E-mail: deposit@ccdc.cam.ac.uk].

cis-**9** (R_f = 0.16): Colorless solid, mp 91 °C, yield 41 mg (35%). $C_{23}H_{29}NO_3$ (367.5). MS (ESI): *m*/*z* (%) = 368 [MH, 100]. FT-IR: $\tilde{\nu}$ (cm^{-1}) = 3067, 3029 (m, v, C–H, arom), 2993, 2930 (s, v, C–H, arom), 2833 (s, v, OCH$_3$), 1610, 1510 (w, C=C, arom), 1442 (m, δ, C–H, alkyl), 811 (s, δ, C–H, p-disubst. arom), 755 (s, δ, C–H, o-disubst. arom). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.58–1.72 (m, 2H, (C*H*$_2$C*H*$_2$)$_2$CHN), 1.76–1.98 (m, 5H, (C*H*$_2$C*H*$_2$)$_2$CHN), 2.05–2.12 (m, 1H, 2′-H_e), 2.64 ('tt', *J* = 10.5/3.9 Hz, 1H, 4′-H_a), 2.89 (dd, *J* = 15.3/6.5 Hz, 1H, ArC*H*$_2$CHOCH$_3$), 2.94 (dd, *J* = 15.5/3.7 Hz, 1H, ArC*H*$_2$CHOCH$_3$), 3.57 (s, 3H, OC*H*$_3$), 3.81 (s, 3H, ArOC*H*$_3$), 3.82 (s, 2H, ArC*H*$_2$NH), 4.86 (dd, *J* = 6.7/3.9 Hz, 1H, ArCH$_2$C*H*OCH$_3$), 5.80 (s, 1H, N*H*), 6.86–6.91 (m,2H, Ar-H), 7.06–7.11 (m,2H, Ar-H), 7.13–7.21 (m,2H, Ar-H), 7.24–7.29 (m,2H, Ar-H). Purity (HPLC): 98.7%, t_R = 19.01 min.

7.2.6. *trans*-3-Methoxy-*N*,*N*-dimethyl-3,4-dihydrospiro-[[2]benzopyran-1,1′-cyclohexan]-4′-amine (*trans*-10) and *cis*-3-Methoxy-*N*,*N*-dimethyl-3,4-dihydrospiro-[[2]benzopyran-1,1′-cyclohexan]-4′-amine (*cis*-10)

Under N_2 ketone **7** (70.6 mg, 0.287 mmol) was dissolved in THF (4 mL). A solution of dimethylamine in ethanol (2 M, 158 μL,

0.32 mmol), acetic acid (16 μL, 0.287 mmol) and $NaBH(OAc)_3$ (95%, 85 mg, 381 mmol) were added and the mixture was stirred for 2.5 h at rt. Then, 1 M NaOH (5 mL) was added and the mixture was extracted with CH_2Cl_2 (4 × 5 mL), the combined organic layers were dried (K_2CO_3), concentrated in vacuo and the residue was purified by FC (2 cm, cyclohexane/ethyl acetate 9/1 + 2% *N*,*N*-dimethylethanamine, 20 cm, 5 mL).

trans-**10** (R_f = 0.38): Colorless solid, mp 67 °C, yield 13.8 mg (18%). $C_{17}H_{25}NO_2$ (275.4). MS (ESI): *m*/*z* (%) = 276 [MH, 100]. IR: $\tilde{\nu}$ (cm^{-1}) = 2959, 2926 (s, v, C–H, alkyl), 2854 (s, v, OCH_3), 2805, 2760 (m, v, N-CH_3), 1449 (s, δ, C–H, alkyl), 759 (s, δ, C–H, o-disubst. arom).^{1}H NMR ($CDCl_3$): δ (ppm) = 1.57–1.65 (m, 1H, 2′-H_e), 1.76 (ddd, *J* = 13.4/5.8/2.9 Hz, 1H, 6′-H_e), 1.84–1.92 (m, 3H, 3′-H_e, 5′-H_a, 5′-H_e), 1.92–2.05 (m, 2H, 3′-H_a, 6′-H_a), 2.10 ('quint', *J* = 2.8 Hz, 1H, 4′-H_e), 2.15 ('td', *J* = 13.3/3.7 Hz, 1H, 2′-H_a), 2.28 (s, 6H, N(CH_3)$_2$), 2.89 (dd, *J* = 15.6/6.9 Hz, 1H, ArCH_2CHOCH_3), 2.94 (dd, *J* = 15.6/3.9 Hz, 1H, ArCH_2CHOCH_3), 3.57 (s, 3H, OCH_3), 4.86 (dd, *J* = 6.8/4.1 Hz, 1H, ArCH_2C*H*OCH_3), 7.05–7.09 (m, 1H, Ar-H), 7.15 ('td', *J* = 7.3/1.6 Hz, 1H, Ar-H), 7.20 ('td', *J* = 7.4/1.6 Hz, 1H, Ar-H), 7.26–7.29 (m, 1H, Ar-H). Purity (HPLC): 99.6%, t_R = 15.09 min.

cis-**10** (R_f = 0.07): Colorless solid, mp 83 °C, yield 51.9 mg (66%). $C_{17}H_{25}NO_2$ (275.4). MS (ESI): *m*/*z* (%) = 276 [MH, 100]. IR: $\tilde{\nu}$ (cm^{-1}) = 2979, 2946, 2921, 2857 (s, v, C–H, alkyl), 2824, (m, v, OCH_3), 2774 (m, v, N-CH_3), 1446 (s, δ, C–H, alkyl), 767 (s, δ, C–H, o-disubst. arom). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.61–1.67 (m, 1H, (CH_2CH_2)$_2$CHN), 1.74–1.96 (m, 5H, (CH_2CH_2)$_2$CHN), 1.96–2.02 (m, 1H, (CH_2CH_2)$_2$CHN), 2.14 (ddd, *J* = 13.9/6.2/3.1 Hz, 1H, (CH_2CH_2)$_2$CHN), 2.35 (s, 6H, N(CH_3)$_2$), 2.32–2.40 (m, 1H, 4′-H_a), 2.90 (dd, *J* = 15.3/6.5 Hz, 1H, ArCH_2CHOCH_3), 2.94 (dd, *J* = 15.7/3.5 Hz, 1H, ArCH_2CHOCH_3), 3.58 (s, 3H, OCH_3), 4.85 (dd, *J* = 6.9/3.9 Hz, 1H, ArCH_2C*H*OCH_3), 7.07–7.12 (m, 2H, Ar-H), 7.14–7.22 (m, 2H, Ar-H). Purity (HPLC): 95.4%, t_R = 15.35 min.

7.2.7. *trans*-*N*-Benzyl-3-methoxy-*N*-methyl-3,4-dihydrospiro-[[2]benzopyran-1,1′-cyclohexan]-4′-amine (trans-11)

Under N_2 benzylamine *trans*-**8** (56.6 mg, 0.168 mmol) was dissolved in CH_2Cl_2 (2.5 mL) and formalin 37% (250 μL, 3.36 mmol) and $NaBH(OAc)_3$ (95%, 57 mg, 0.256 mmol) were added. The reaction mixture was stirred for 23 h at rt. Subsequently H_2O (10 mL) was added and the mixture was extracted with CH_2Cl_2 (4x20 mL). The combined organic layers were dried (K_2CO_3), concentrated in vacuo and the residue was purified by FC (2 cm, cyclohexane + 1% *N*,*N*-dimethylethanamine, 20 cm, 10 mL, R_f = 0.25). Colorless solid, mp 62 °C, yield 51.8 mg (88%). $C_{23}H_{29}NO_2$ (351.5). MS (ESI): *m*/*z* (%) = 352 [MH, 100]. FT-IR: $\tilde{\nu}$ (cm^{-1}) = 3024 (m, v, C–H, arom), 2952 (s, v, C–H, alkyl), 2834 (s, v, OCH_3), 2784 (m, v, N–CH_3), 1604, 1494 (w, v, C=C, arom), 1494 (s, δ, C–H, alkyl), 752 (s, δ, C–H, o-disubst. arom), 732, 697 (s, δ, C–H, mono-subst. arom). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.66 (ddd, *J* = 13.5/5.8/3.0 Hz, 1H, 2′-H_e), 1.81 (ddd, *J* = 13.6/5.8/3.0 Hz, 1H, 6′-H_e), 1.94–2.22 (m, 5H, 3′-H_e, 3′-H_a, 5′-H_a, 5′-H_e, 6′-H_a), 2.17 (s, 3H, NCH_3), 2.39 ('td', *J* = 13.2/4.1 Hz, 1H, 2′-H_a), 2.46 ('quint', *J* = 3.0 Hz, 1H, 4′-H_e), 2.91 (dd, *J* = 15.6/6.9 Hz, 1H, ArCH_2CHOCH_3), 2.96 (dd, *J* = 15.7/4.1 Hz, 1H, ArCH_2CHOCH_3), 3.59 (s, 3H, OCH_3), 3.60 (s, 2H,NHCH_2Ar), 4.89 (dd, *J* = 6.7/4.2 Hz, 1H, Ar-CH_2-C*H*-OCH_3), 7.07–7.11 (m, 1H, Ar-H), 7.16–7.20 (m, 1H, Ar-H), 7.22–7.30 (m, 3H, Ar-H), 7.33–7.42 (m, 4H, Ar-H). Purity (HPLC) 98.6%, t_R = 18.88 min.

7.2.8. *cis*-*N*-Benzyl-3-methoxy-*N*-methyl-3,4-dihydrospiro-[[2]benzopyran-1,1′-cyclohexan]-4′-amine (cis-11)

Under N_2 benzylamine *cis*-**8** (54 mg, 0.16 mmol) was dissolved in CH_2Cl_2 (2.5 mL) and formalin 37% (238 μL, 3.2 mmol) and $NaBH(OAc)_3$ (95%, 57 mg, 0.256 mmol) were added. The reaction mixture was stirred for 3 h at rt. Subsequently H_2O (10 mL) was added and the mixture was extracted with CH_2Cl_2 (4 × 20 mL). The combined organic layers were dried (K_2CO_3), concentrated in vacuo and the residue was purified by FC (2 cm, cyclohexane + 1% *N*,*N*-dimethylethanamine, 20 cm, 5 mL, R_f = 0.10). Colorless solid, mp 91 °C, yield 47 mg (84%). $C_{23}H_{29}NO_2$ (351.5). MS (ESI): *m*/*z* (%) = 352 [MH, 100]. FT-IR: $\tilde{\nu}$ (cm^{-1}) = 3024 (m, v, C–H, arom), 2928 (s, v, C–H, alkyl), 2839 (s, v, OCH_3), 2788 (m, v, N-CH_3) 1603, 1492 (w, v, C=C, arom), 754 (s, δ, C–H, *o*-disubst. arom), 732, 698 (s, δ, C–H, mono-subst. arom). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.64 ('td', *J* = 14.0/3.9 Hz, 1H, 2′-H_a), 1.75–1.84 (m, 2H, (CH_2CH_2)$_2$CHN), 1.84–1.95 (m, 2H, (CH_2CH_2)$_2$CHN), 1.96 - 2.09 (m, 2H, (CH_2CH_2)$_2$CHN), 2.11 (ddd, *J* = 14.1/6.4/3.2 Hz, 1H, 2′-H_e), 2.28 (s, 3H, NCH_3), 2.67 ('tt', *J* = 11.6/3.6 Hz, 1H, 4′-H_a), 2.90 (dd, *J* = 15.3/6.7 Hz, 1H, ArCH_2CHOCH_3), 2.95 (dd, *J* = 15.4/3.5 Hz, 1H, ArCH_2CHOCH_3), 3.59 (s, 3H, OCH_3), 3.65 (s, 2H, NHCH_2Ar), 4.88 (dd, *J* = 6.9/3.9 Hz, 1H, Ar-CH_2-C*H*–OCH_3), 7.07–7.12 (m, 2H, Ar-H), 7.13–7.22 (m, 2H, Ar-H), 7.22–7.29 (m, 1H, Ar-H), 7.30–7.39 (m, 4H, Ar-H). Purity (HPLC): 99.8%, t_R = 17.68 min.

7.2.9. *cis*-*N*-Benzyl-*N*-ethyl-3-methoxy-3,4-dihydrospiro-[[2]benzopyran-1,1′-cyclohexan]-4′-amine (cis-12)

Benzylamine *cis*-**8** (54 mg, 0.16 mmol), 1-iodoethane (18 μL, 0.226 mmol) and K_2CO_3 were dissolved and suspended in acetonitrile (3 mL) in a microwave tube (10 mL) and irradiated with microwaves. Reaction parameter: Program: Standard, power: max. 220 W, pressure: max. 4 bar, temperature: 100 °C, reaction time: 5 min ramp time, 40 min hold time, 5 min cool off time. The reaction mixture was filtered and concentrated in vacuo. H_2O (4 mL) was added and the mixture was extracted with CH_2Cl_2. The organic layers were dried (K_2CO_3) concentrated in vacuo and the residue was purified by FC (2 cm, cyclohexane/ethyl acetate 9:1 + 2% *N*,*N*-dimethylethanamine, 15 cm, 5 mL, R_f = 0.55). Colorless solid, mp 74 °C, yield 26.1 mg (45%). $C_{24}H_{31}NO_2$ (365.6). MS (ESI): *m*/*z* (%) = 366 [MH,100]. FT-IR: $\tilde{\nu}$ (cm^{-1}) = 3021 (m, v, C–H, arom), 2946, 2919 2860 (s, v, C–H, alkyl), 2807 (–H_2C–N–), 1602, 1491 (w, v, C=C, arom), 768 (s, δ, C–H, o-disubst. arom), 732, 696 (s, δ, C–H, mono-subst. arom). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.05 (t, *J* = 7.1 Hz, 3H, NCH_2CH_3), 1.60 ('td', *J* = 13.7/4.0 Hz, 1H, 2′-H_a), 1.69–1.79 (m, 2H, (CH_2CH_2)$_2$CHN), 1.81–1.89 (m, 2H, (CH_2CH_2)$_2$CHN), 1.91–2.05 (m, 2H, (CH_2CH_2)$_2$CHN), 2.13 (ddd, *J* = 14.1/6.1/3.1 Hz, 1H, 2′H_e), 2.57–2.66 (m, 2H, N-CH_2CH$_3$), 2.74 ('tt', *J* = 11.7/3.7 Hz, 1H, 4′-H_a), 2.88 (dd, *J* = 15.5/6.5 Hz, 1H, Ar-CH_2CHOCH_3), 2.94 (dd, *J* = 15.5/3.6 Hz, 1H, Ar-CH_2CHOCH_3), 3.58 (s, 3H, OCH_3), 3.68 (d, *J* = 14.6 Hz, 1H, N–CH_2–Ar), 3.72 (d, *J* = 14.7 Hz, 1H, N-CH_2-Ar), 4.86 (dd, *J* = 6.9/3.9 Hz, 1H, ArCH_2-C*H*OCH_3), 7.06–7.11 (m, 2H, Ar-H), 7.13–7.19 (m, 2H, Ar-H), 7.19–7.25 (m, 1H, Ar-H), 7.29–7.34 (m, 2H, Ar-H), 7.37–7.43 (m, 2H, Ar-H). Purity (HPLC): 97.2%, t_R = 19.43 min.

7.2.10. *cis*-*N*-Benzyl-3-methoxy-*N*-pentyl-3,4-dihydrospiro-[[2]benzopyran-1,1′-cyclohexan]-4′-amine (cis-13)

Under N_2 benzylamine *cis*-**8** (46.5 mg, 0.138 mmol) was dissolved in CH_2Cl_2 (5 mL). Valeraldehyde (18 μL, 0.169 mmol) and $NaBH(OAc)_3$ (95%, 45 mg, 0.213 mmol) were added and the reaction mixture was stirred for 23 h at rt. Then H_2O (10 mL) was added and the mixture was extracted with CH_2Cl_2 (4 × 20 mL) the combined organic layers were dried (K_2CO_3), concentrated in vacuo and the residue was purified by FC (2 cm, cyclohexane + 2% *N*,*N*-dimethylethanamine, 20 cm, 10 mL, R_f = 0.37). Colorless oil, yield 50.3 mg (90%). $C_{27}H_{37}NO_2$ (407.6). MS (ESI): *m*/*z* (%) = 408 [MH,100]. IR: $\tilde{\nu}$ (cm^{-1}) = 3062, 3022 (m, v, C–H, arom), 2926, 2858 (s, v, C–H, alkyl), 1603, 1492 (w, v, C=C, arom), 1451 (m, δ, C–H, alkyl), 754 (s, δ, C–H, o-disubst. arom), 731, 697 (s, δ, C–H, monosubst. arom). ^{1}H NMR ($CDCl_3$): δ (ppm) = 0.86 (t, *J* = 7.0 Hz, 3H, NCH_2CH_2CH_2CH_2CH_3), 1.24–1.27 (m, 4H, NCH_2CH_2CH_2CH_2CH$_3$), 1.39–1.46 (m, 2H, NCH_2CH_2CH_2CH_2CH$_3$), 1.57 ('td', *J* = 13.7/3.9 Hz, 1H, 2′-H_a), 1.67–1.77 (m, 2H, (CH_2CH_2)$_2$CHN), 1.78–1.91 (m, 2H, (CH_2CH_2)$_2$CHN), 1.92–2.04 (m, 2H, (CH_2CH_2)$_2$CHN), 2.09 (ddd,

J = 14.0/6.1/3.0 Hz, 1H, 2′-H_e), 2.53 (m, 2H, NCH_2CH$_2$CH$_2$CH$_2$CH$_3$), 2.65–2.72 ('tt', J = 11.7/3.7 Hz, 1H, 4′-H_a), 2.88 (dd, J = 15.6/6.6 Hz, 1H, ArCH_2CHOCH$_3$), 2.93 (dd, J = 15.4/3.8 Hz, 1H, ArCH_2CHOCH$_3$), 3.58 (s, 3H, OCH_3), 3.69 (s, 2H, NHCH_2Ar), 4.85 (dd, J = 6.9/4.0 Hz, 1H, Ar-CH$_2$–CH–OCH$_3$), 7.06–7.09 (m, 2H, Ar-H), 7012–7.19 (m, 2H, Ar-H), 7.20–7.25 (m, 1H, Ar-H), 7.28–7.34 (m, 2H, Ar-H), 7.37–7.41 (m, 2H, Ar-H). Purity (HPLC): 98.3%, t_R = 22.33 min.

7.2.11. *cis*-*N*,*N*-Dibenzyl-3-methoxy-3,4-dihydrospiro-[[2]benzopyran-1,1′-cyclohexan]-4′-amine (cis-14)

Under N_2 benzylamine *cis*-**8** (65 mg, 0.193 mmol) was dissolved in CH_2Cl_2 (5 mL). Benzaldehyd (23.5 μL, 0.232 mmol) and NaBH(OAc)$_3$ (95%, 65 mg, 0.291 mmol) were added and the reaction mixture was stirred for 23 h at rt. Then H_2O (10 mL) was added and the mixture was extracted with CH_2Cl_2 (4 × 20 mL), the combined organic layers were dried (K_2CO_3), concentrated in vacuo and the residue was purified by FC (2 cm, cyclohexane + 2% *N*,*N*-dimethylethanamine, 20 cm, 10 mL, R_f = 0.39). Colorless solid, mp 136 °C, yield 34 mg (41%). $C_{29}H_{33}NO_2$ (427.6). MS (ESI): m/z (%) = 428 [MH,100]. IR: $\tilde{\nu}$ (cm^{-1}) = 3061, 3028 (m, v, C–H, arom), 2942, 2921, 2857 (s, v, C–H, alkyl), 1601, 1491 (w, v, C=C, arom), 1451 (m, δ, C–H, alkyl), 761 (s, δ, C–H, o-disubst. arom), 745, 697 (s, δ, C–H, monosubst. arom). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.54 ('td', J = 13.6/4.1 Hz, 1H, 2′-H_a), 1.74–1.87 (m, 3H, (CH_2CH_2)$_2$CHN), 1.90–2.03 (m, 2H, (CH_2CH_2)$_2$CHN), 2.03–2.15 (m, 2H, (CH_2CH_2)$_2$CHN), 2.71 ('tt', J = 11.9/3.6 Hz, 1H, CH$_2$-CH-NH, 4′-H_a), 2.88 (dd, J = 15.4/6.4 Hz, 1H, ArCH_2CHOCH$_3$), 2.93 (dd, J = 15.4/3.6 Hz, 1H, ArCH_2CHOCH$_3$), 3.57 (s, 3H, OCH_3), 3.70 (d, J = 14.7 Hz, 2H, N(CH_2-Ph)$_2$), 3.73 (d, J = 14.7 Hz, 2H, N(CH_2-Ph)$_2$), 4.86 (dd, J = 6.9/3.9 Hz, 1H, ArCH$_2$CHOCH$_3$), 7.00–7.09 (m, 2H, Ar-H), 7.12–7.17 (m, 2H, Ar-H), 7.20–7.25 (m, 2H, Ar-H), 7.28–7.34 (m, 4H, Ar-H), 7.40–7.44 (m, 4H, Ar-H). Purity (HPLC): 97.7%, t_R = 21.79 min.

7.3. Receptor binding studies

7.3.1. Materials and general procedures

Guinea pig brains and rat livers were commercially available (Harlan-Winkelmann, Germany). Homogenizer: Elvehjem Potter (B. Braun Biotech International). Centrifuge: High-speed cooling centrifuge model Sorvall RC-5C plus (Thermo Finnigan). Filter: Printed Filtermat Type A (Perkin Elmer), presoaked in 0.5% aqueous polyethylenimine for 2 h at rt before use. The filtration was carried out with a MicroBeta FilterMate-96 Harvester (Perkin Elmer). The scintillation analysis was performed using Meltilex (Type A) solid scintillator (Perkin Elmer). The radioactivity bound to the filter was measured using a MicroBeta Trilux scintillation analyzer (Perkin Elmer). The overall counting efficiency was 20%.

7.3.2. Membrane preparation for the σ_1 assay[52,54]

Five guinea pig brains were homogenized with the potter (500–800 rpm, 10 up-and-down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1200*g* for 10 min at 4 °C. The supernatant was separated and centrifuged at 23,500*g* for 20 min at 4 °C. The pellet was resuspended in 5–6 volumes of buffer (50 mM TRIS, pH 7.4) and centrifuged again at 23,500*g* (20 min, 4 °C). This procedure was repeated twice. The final pellet was resuspended in 5–6 volumes of buffer, the protein concentration was determined according to the method of Bradford[63] using bovine serum albumin as standard, and subsequently the preparation was frozen (−80 °C) in 1.5 mL portions containing about 1.5 mg protein/mL.

7.3.3. Protocol of the σ_1 assay[52,54]

The test was performed with the radioligand [^{3}H]-(+)-pentazocine (42.5 Ci/mmol; Perkin Elmer). The thawed membrane preparation (about 75 μg of the protein) was incubated with various concentrations of test compounds, 2 nM [^{3}H]-(+)-pentazocine, and buffer (50 mM TRIS, pH 7.4) in a total volume of 200 μL for 180 min at 37 °C. The incubation was terminated by rapid filtration through the presoaked filtermats by using the cell harvester. After washing each well five times with 300 μL of water, the filtermats were dried at 95 °C. Subsequently, the solid scintillator was put on the filtermat and melted at 95 °C. After 5 min, the solid scintillator was allowed to solidify at rt. The bound radioactivity trapped on the filters was counted in the scintillation analyzer. The non-specific binding was determined with 10 μM unlabeled (+)-pentazocine. The K_d-value of the radioligand [^{3}H]-(+)-pentazocine is 2.9 nM.[64]

7.3.4. Membrane preparation for the σ_2 assay[52,54]

Two rat livers were cut into small pieces and homogenized with a potter (500–800 rpm, 10 up-and-down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1200*g* for 10 min at 4 °C. The supernatant was separated and centrifuged at 31,000*g* for 20 min at 4 °C. The pellet was resuspended in buffer (50 mM TRIS, pH 8.0) and incubated at rt for 30 min. After the incubation, the suspension was centrifuged again at 31,000*g* for 20 min at 4 °C. The final pellet was resuspended in buffer, the protein concentration was determined according to the method of Bradford[63] using bovine serum albumin as standard, and subsequently the preparation was frozen (−80 °C) in 1.5 mL portions containing about 2 mg protein/mL.

7.3.5. Protocol of the σ_2 assay[52,54]

The test was performed with the radioligand [^{3}H]-di-*o*-tolylguanidine (50 Ci/mmol; ARC). The thawed membrane preparation (about 100 μg of the protein) was incubated with various concentrations of test compounds, 3 nM [^{3}H]-di-*o*-tolylguanidine, 500 nM (+)-pentazocine and buffer (50 mM TRIS, pH 8.0) in a total volume of 200 μL for 180 min at rt. The incubation was terminated by rapid filtration through the presoaked filtermats using a cell harvester. After washing each well five times with 300 μL of water, the filtermats were dried at 95 °C. Subsequently, the solid scintillator was put on the filtermat and melted at 95 °C. After 5 min, the solid scintillator was allowed to solidify at rt. The bound radioactivity trapped on the filters was counted in the scintillation analyzer. The non-specific binding was determined with 10 μM unlabeled ditolylguanidine. The K_d-value of the radioligand [^{3}H]-ditolylguanidine is 17.9 nM.[65]

7.3.6. Data analysis

Usually, all experiments were carried out in triplicates using standard 96-well-multiplates (Diagonal). The IC_{50}-values were determined in competition experiments with six concentrations of the test compounds and were calculated with the program GraphPad Prism® 3.0 (GraphPad Software) by non-linear regression analysis. The K_i-values were calculated according to Cheng and Prusoff.[66] The K_i-values of potent compounds are given as mean values + SEM from three independent experiments.

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