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Combination of cyclohexane and piperazine based κ-opioid receptor agonists: Synthesis and pharmacological evaluation of *trans,trans*-configured perhydroquinoxalines

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ABSTRACT

Desymmetrization of the pseudochiral (2*r*)-configured cyclohexane-1,2,3-triamines **8** with dimethyl oxalate led to racemic aminoquinoxaline-2,3-diones **9**. Selective introduction of the κ pharmacophoric structural elements pyrrolidine and 3,4-dichlorophenylacetamide with a two-carbon distance afforded conformationally restricted κ agonists **13**–**15** based on the quinoxaline ring system. In competitive radioligand receptor binding studies the benzylamine **13b**, the secondary amine **14b**, and the carbamate **15** displayed high κ receptor affinity. The K_i value of the lead compound derived methoxycarbonyl derivative **15** is 9.7 nM. However, the κ affinity of **15** is exceeded by **13b** and **14b** with a basic functional group instead of the methoxycarbonyl group in 1-position of the quinoxaline system. The chlorine atoms of the dichlorophenylacetyl residue are essential, since the corresponding phenylacetyl analogs show considerably reduced κ affinity. The potent κ ligands **13b**, **14b** and **15** are selective over the related μ- and δ-opioid receptors, σ_1, σ_2 and NMDA receptors. In the [^{35}S]GTPγS-binding assay **13b** behaved as partial agonist with lower activity than U-69,593.

1. Introduction

κ receptors belonging to the class of opioid receptors are widely distributed throughout the central nervous system and the periphery. Activation of centrally localized κ opioid receptors leads to strong analgesia.[1] Compared to clinically used μ receptor agonists (e.g. morphine, fentanyl, pethidine, levomethadone), κ receptor agonists show a quite different side effect profile. In particular dangerous μ receptor mediated side effects like respiratory depression and addiction are not caused by activation of κ opioid receptors. However κ receptor agonists are not devoid of side effects: dysphoria, sedation and strong diuresis are the most common side effects associated with activation of κ receptors.[2,3] Since κ receptors are also found in the periphery, κ agonists which are restricted to the periphery can be used for the treatment of visceral pain and inflammatory and itching skin diseases.[4–6]

The known κ agonists can be classified into four compound classes: peptides including the physiological agonist dynorphin A,[2,7] morphinoids with the prototypical ligand ketocyclazocine giving name to this opioid receptor subtype,[2,7] the natural product salvinorin A without a basic functional group[8,9] and ethylenediamines (arylacetamides) with the first synthetic κ agonist U-50,488 (**1**)[10] (Fig. 1). The pharmacophore of the fourth class of κ agonists is represented by an ethylenediamine, whose terminal N-atoms are incorporated into a pyrrolidine ring and an arylacetamide moiety, respectively. In U-50,488 (**1**, K_i = 0.34 nM) this ethylenediamine structural element is part of a *trans*-configured cyclohexane ring. In the potent κ agonist **2** (K_i = 0.31 nM) the ethylenediamine pharmacophore is found as part of a piperazinylmethylamine system.[11]

Since we are interested in conformationally restricted κ agonists with novel scaffolds, it was planned to combine the structural features of the potent κ agonists **1** and **2** containing the ethylenediamine substructure (Fig. 1). A superposition of the ethylenediamine substructures of **1** and **2** is only possible by annulation of the cyclohexane and piperazine rings to afford the perhydroquinoxalines **3**, which also result from addition of an ethylene moiety between the cyclohexane ring and the *N*-methyl moiety of **1** or between the piperazine ring and the methyl group of **2** (see arrows at compounds **1** and **2**). Whereas the configuration of the centers of chirality in 8- and 8a-position are defined by the lead compounds **1** and **2**, the configuration of the third center of chirality (C-4a) is not defined by the lead compounds **1** and **2**.

In this communication we report on the synthesis of perhydroquinoxalines **3** with the relative *trans,trans*-configuration of the three N-functionalities attached to the cyclohexane ring (Fig. 1). The additional N-4-atom of the perhydroquinoxaline ring, which

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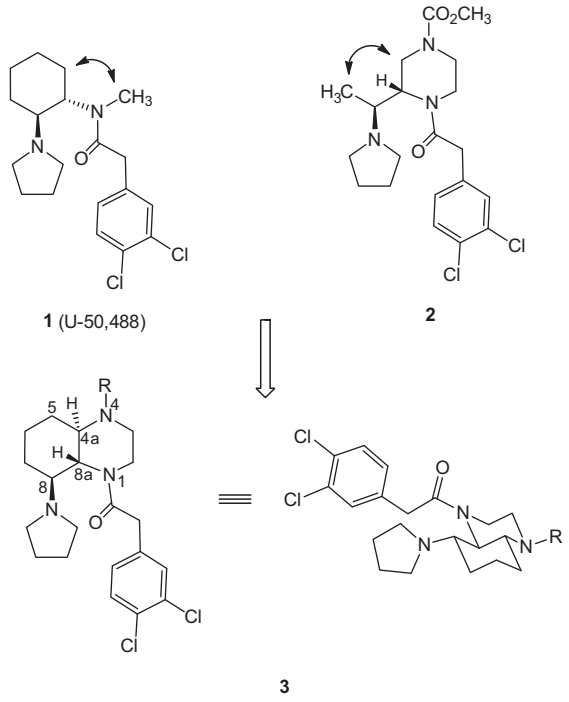


Figure 1. Development of the novel κ agonists **3** by combining the cyclohexane and piperazine rings of the lead compounds **1** and **2** to give a perhydroquinoxaline ring system.

is located outside of the κ pharmacophore, will allow fine tuning of the pharmacodynamic and more importantly the pharmacokinetic properties including the passage of the blood brain barrier of the new compounds by introducing various substituents.

2. Synthesis

According to literature,[12,13] the one-pot reaction of glutaraldehyde (**4**), nitromethane (**5**) and an excess of benzylamine led to the nitrocyclohexanediamine **7a** in 60% yield (Scheme 1). However, due to the formation of several side products and subsequent purification problems the stepwise synthesis was preferred. Thus, a double *Henry* reaction (nitroaldol reaction) of nitromethane (**5**) with glutaraldehyde (**4**) in the presence of NaOH[13,14] afforded the nitrocyclohexanediol **6** in 80% yield. Subsequent treatment of **6** with benzylamine in an aqueous solution provided the nitrodiamine **7a**.[13] Reduction of the amount of benzylamine and increasing the amount of H_2O resulted in an optimized yield of 92%, exceeding the previously reported yield of 75%.[13] The nitrodiamines **7b**, **7d**, and **7e** were obtained under the same conditions in 86–93% yields. Due to purification problems the *p*-chlorobenzylamine **7c** could only be isolated in 49% yield.

The exchange of the OH moieties of the nitrodiol **6** with amino groups is explained either by a base induced β-elimination of water followed by conjugated addition of amine or by a retro-nitroaldol reaction followed by imine formation and subsequent addition of the nitroalkane to the intermediate imine. The formation of the nitrodiol **6** and its transformation into nitrodiamines **7** occurred with high diastereoselectivity giving only the thermodynamically most stable *trans*,*trans*-configured diastereomers with all substituents in the favorable equatorial orientation. The *trans*,*trans*-configuration of **6** and **7** is confirmed by the triplet for the proton adjacent to the nitro moiety (CH-NO_2) with large coupling constants of 10.3–10.6 Hz indicating the axial orientation of three adjacent protons.

Although the reduction of the nitrodiamine **7a** to form the triamine **8a** has been reported in literature using H_2 and Raney Ni,[12,13] this reaction step required special attention and careful selection of the reaction conditions. Reproducible yields of **8a** could only be achieved by a certain combination of reaction conditions (H_2 pressure, temperature) and amount and quality of Raney Ni. Alternative reduction methods (Zn/HCl, Sn/HCl, SmI_2, $LiAlH_4$) did not improve the reproducibility and yields. Finally, the triamines **8a–c** and **8e** were prepared by reduction of the nitrodiamines **7a–c** and **7e** with H_2 (1 bar) in the presence of Raney Ni as catalyst.

For the establishment of the quinoxaline ring two amino moieties of the triamines **8** had to be connected by a C_2-building block. For this purpose the benzyl derivative **8a** was reacted with oxalyl chloride at −78 °C. According to tlc several products were formed, but the desired quinoxalinedione **9a** could not be isolated. Therefore the triamines **8a–c** were treated with the less reactive dimethyl oxalate in boiling methanol for 48 h (Scheme 1). After complete conversion the quinoxalinediones **9a–c** were isolated by recrystallization in yields of 33–64%. The corresponding 3,4-dichlorobenzyl derivative **9d** was not obtained due to side reactions during the reduction of the nitro moiety of nitrodiamine **7d**.

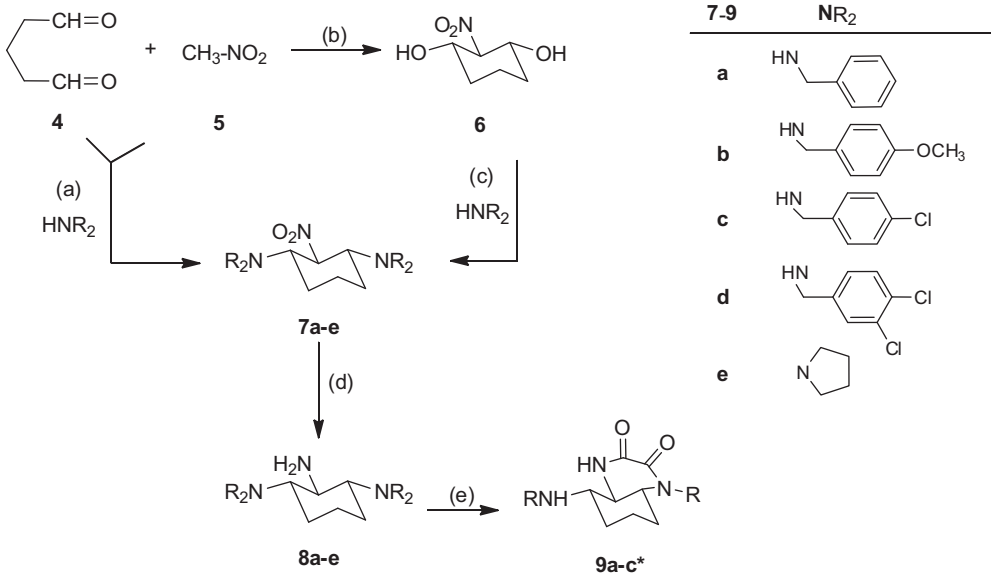
The nitrodiol **6**, the nitrodiamines **7** and the triamines **8** represent achiral compounds with a pseudochiral center in 2-position having (2*r*)-configuration. Treatment of the triamines **8** with dimethyl oxalate led to desymmetrization since both enantiotopic benzylamino moieties reacted with the same probability with dimethyl oxalate. However the original *trans*,*trans*-configuration of the triamines **8** is retained in the quinoxalinediones **9**. Although the quinoxalinediones **9** contain three centers of chirality, this synthetic strategy led to only one out of four possible diastereomers. In this manuscript only one enantiomer of the racemic mixtures is shown in the Schemes.

The construction of the quinoxalinedione **9a** led to a differentiation of the benzylamino moieties; one benzylamine is part of an amine, whereas the other one is part of an amide allowing their chemoselective conversion. Treatment of **9a** with ammonium formate in the presence of Pd/C as catalyst[15] cleaved chemoselectively the benzyl moiety of the benzylamine affording the primary amine **10** in 97% yield (Scheme 2). Reaction of the primary amine **10** with 1,4-diiodobutane provided the pyrrolidine **11** in 76% yield. Several reducing agents were tried for the reduction of the quinoxalinedione **11**. A 3:1 mixture of $LiAlH_4$ and $AlCl_3$ forming AlH_3 in situ[16] turned out to give the highest yield (96%) of the quinoxaline **12**. The reduced quinoxaline **12** contains a secondary (N-4) and a tertiary amine (N-1) within the heterocycle. Acylation of the secondary amino moiety (N-4) with phenylacetyl chloride and 3,4-dichlorophenylacetyl chloride led to the phenylacetamides **13a** and **13b**, respectively.

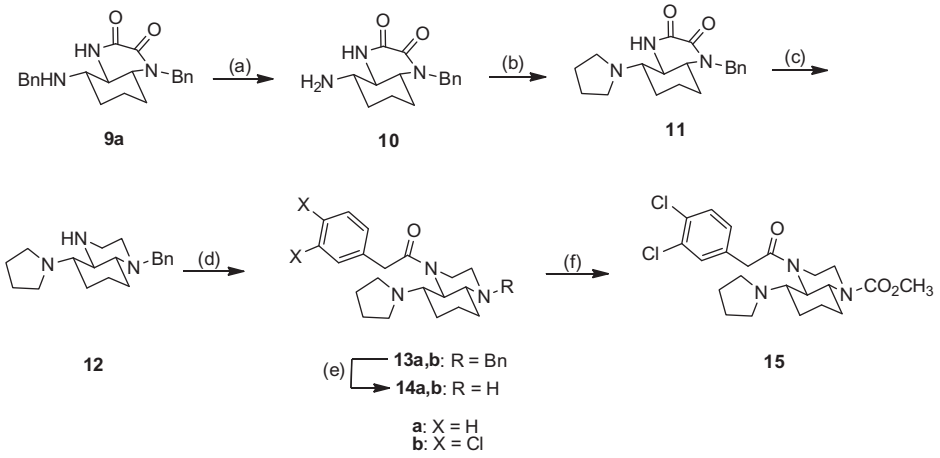
In order to introduce the methoxycarbonyl moiety of the lead compound **2** the remaining benzyl moiety should be removed by conventional hydrogenolysis. However, treatment of the benzylamines **13** with H_2 and Pd/C in a THF/H_2O mixture led to incomplete transformation and provided considerable amounts (approx. 30%) of dechlorinated products from **13b**. Therefore concd HCl (10%) was added to the solvent, which should increase the hydrogenolysis rate. After limitation of the reaction time to 30 min, the secondary amines **14a** and **14b** were isolated in 90% and 94% yield, respectively. The amount of dechlorinated products found after hydrogenolysis of **13b** was less than 1%. Finally, acylation of the secondary amine **14b** with methyl chloroformate afforded the carbamate **15** in 59% yield.

3. Pharmacological evaluation

The κ receptor affinity of the quinoxalines **9–15** was determined in competition receptor binding studies. Guinea pig brain



Scheme 1. Synthesis of racemic quinoxalinediones **9**. Reagents and reaction conditions: (a) $BnNH_2$, 60%;[12,13] (b) CH_3OH, NaOH, rt, 4 h, 80%;[13,14] (c) HNR_2, H_2O, rt, 16–96 h; (d) H_2, 1 bar, Raney Ni, CH_3OH, rt, 3–20 h; (e) dimethyl oxalate ($MeO_2C–CO_2Me$), CH_3OH, 65 °C, 24–48 h. *Residues R in **9** refer to benzyl (**9a**), *p*-methoxybenzyl (**9b**) and *p*-chlorobenzyl (**9c**). Only one enantiomer of the racemic mixture **9** is shown.



Scheme 2. Synthesis of the quinoxaline based κ receptor agonist **15**. Reagents and reaction conditions: (a) $NH_4{\cdot}HCO_2$, Pd/C, CH_3OH, 65 °C, 2 h, 97%; (b) 1,4-diiodobutane, CH_3CN, $NaHCO_3$, 80 °C, 18 h, 76%; (c) $AlCl_3/LiAlH_4$ 1:3, THF, 0 °C, 45 min, then rt, 20 min, 96%; (d) phenylacetyl chloride or (3,4-dichlorophenyl)acetyl chloride, CH_2Cl_2, NaOH, rt, 2–18 h, 91% (**13a**), 86% (**13b**); (e) H_2, 1 bar, Pd/C, THF, H_2O, HCl, rt, 30 min, 90% (**14a**), 94% (**14b**); (f) $ClCO_2CH_3$, CH_2Cl_2, rt, 2 h, 59%. Only one enantiomer of the racemic mixtures is shown.

membrane preparations were used as receptor material and tritium labeled [^{3}H]-U-69,593 served as radioligand. The non-specific binding of the radioligand was determined after addition of a large excess of non-tritiated U-69,593 (10 μM).[17,18]

In Table 1 the κ receptor affinities of the quinoxaline derivatives are summarized. It is obvious that the quinoxalinediones **9**–**11** with lactam moieties within the piperazine ring do not interact significantly with the κ receptor. Additionally the quinoxaline **12** without a substituent at N-4 shows very low κ affinity. However, introduction of the dichlorophenylacetyl moiety as second κ pharmacophoric element resulted in the potent κ receptor agonist **13b** with a K_i value of 9.4 nM. Removal of the benzyl moiety led to a fourfold increase of the κ affinity (**14b**: K_i = 2.1 nM), whereas the introduction of the methoxycarbonyl moiety of the lead compound **2** did not change the κ affinity (**15**: K_i = 9.7 nM).

In the κ agonists **13**–**15** the ethylenediamine κ pharmacophore is embedded in the quinoxaline ring, which contains an additional N-atom for modifications. The κ receptor tolerates various substituents at that position including a proton (**14b**), a benzyl moiety (**13b**) and a methoxycarbonyl group (**15**). The tolerance of a second basic structural element within the κ agonists **13b** and **14b** is of particular interest. This observation correlates nicely with the results obtained with κ agonists based on the 6,8-diazabicyclo[3.2.2]nonane scaffold.[18]

The relevance of the two chlorine atoms of the dichlorophenylacetyl moiety is documented by the data in Table 1. The ligands **13a** and **14a** without chlorine atoms at the phenyl moiety show considerably reduced κ affinity when compared with their chlorinated analogs **13b** and **14b**.

In order to test the opioid receptor selectivity, the μ- and δ-opioid receptor affinities of the κ agonists **13**–**15** were recorded in radioligand receptor binding studies.[18] At a concentration of 1 μM the compounds did not interact significantly with μ- and δ-opioid receptors (IC_{50} >1 μM) indicating high selectivity for the κ receptor over these related opioid receptors.

Table 1
κ-Opioid receptor affinity of quinoxaline derivatives **9**–**15** and reference compounds

Compd	$K_i \pm SEM^{\#}$ (nM)
	κ ([^{3}H]-U-69,593)
9a	0%
9b	5%
9c	10%
10	0%
11	3%
12	2%
13a	129 ± 46
13b (WMS-0610)	9.4 ± 1.6
14a	435 ± 91
14b (WMS-0611)	2.1 ± 0.4
15 (WMS-0622)	9.7 ± 1.8
U-50, 488 (**1**)	0.34 ± 0.07
2	0.31 ± 0.04
U-69,593	0.88 ± 0.10
Naloxone	6.9 ± 0.50

[#] Generally the K_i values were determined in triplicates ($n = 3$). For compounds showing very low affinity in the first experiment repetitions were not performed ($n = 1$). For low affinity compounds the residual binding of the radioligand (in %) at a test compound concentration of 1 μM is given.

Additionally the affinity towards σ_1, σ_2,[19,20] and NMDA (PCP binding site)[21,22] receptors was recorded. The κ agonists **13**–**15** did not compete with the radioligands even at the high concentration of 1 μM. It can be concluded that among the new κ agonists the potent dichlorophenylacetamides **13b**, **14b**, and **15** are very selective over σ_1, σ_2, and NMDA receptors.

The synthetic intermediates **9**–**12** were also included into the μ, δ, σ_1, σ_2, and NMDA (PCP binding site) assays. With exception of **9b** and **9c**, these test compounds did not show affinity towards these receptor systems. Unexpectedly, submicromolar σ_1 affinity was found for the quinoxalinediones **9b** ($K_i(\sigma_1) = 368$ nM) and **9c** ($K_i(\sigma_1) = 543$ nM), which did not interact with the κ receptor. Although the σ_1 affinity is rather low, **9b** and **9c** can be regarded as lead compounds for optimizations.

In order to demonstrate the agonistic activity at κ receptors, the benzyl derivative **13b** was tested exemplarily in the [^{35}S]GTPγS-binding assay employing human κ-opioid receptors.[18,23] In this assay **13b** demonstrated the properties of a partial κ agonist with 70% relative efficacy compared with the full agonist U-69,593 (Fig. 2). The recorded EC_{50} value of 490 nM for **13b** indicates reduced κ agonistic activity compared to the full agonist U-69,593 ($EC_{50} = 80$ nM). This result documents that an additional basic moiety in κ-opioid receptor ligands does not inhibit activation of κ receptors.

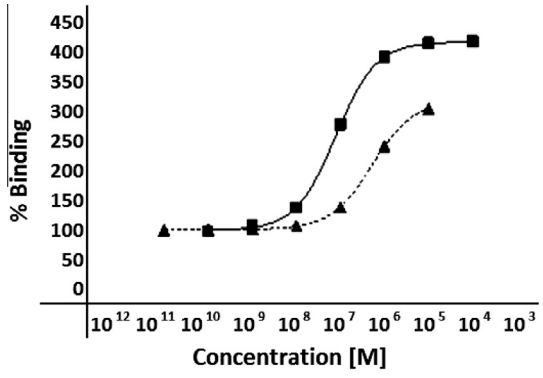


Figure 2. [^{35}S]GTPγS-binding assay of **13b** (▲) in comparison with U-69,593 (■) employing human κ-opioid receptors.

4. Conclusion

The conformational flexibility of κ agonists was restricted by introduction of the ethylenediamine κ pharmacophore into a quinoxaline ring system. The quinoxalines **13b**, **14b**, and **15** represent potent κ agonists with low nanomolar κ affinity. Unexpectedly, the κ affinity of the quinoxalines **14b** with a basic functional group in 4-position is higher than the κ affinity of the methoxycarbonyl derivative **15**. The impact of the aromatic chlorine atoms on the κ affinity is shown by comparison of the κ affinity of the dichlorophenylacetyl derivatives **13b** and **14b** with those of unsubstituted phenylacetyl analogs **13a** and **14a.** The high κ affinity and receptor selectivity of **13b**, **14b**, and **15** together with the agonistic activity of **13b** render these compounds a new compound class with promising κ agonistic properties. The additional N-atom outside the κ pharmacophore allows further fine tuning of the pharmacokinetic and pharmacodynamics properties.

5. Experimental

5.1. Experimental, chemistry

5.1.1. General

Unless otherwise noted, moisture sensitive reactions were conducted under dry nitrogen. THF was dried with sodium/benzophenone and was distilled freshly 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. Elemental analysis: CHN-Rapid Analysator (Foss-Heraeus). 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 1: column: LiChrospher® 60 RP-select B (5 μm), 250–4 mm; flow rate: 1.00 mL/min; injection volume: 5.0 μL; detection at $\lambda = 210$ 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%; method 2: column: phenomenex® Gemini C6-Phenyl 110A (5 μm), 250–4.6 mm; flow rate: 1.00 mL/min; injection volume: 5.0 μL; detection at $\lambda = 220$ nm; solvents: A: water with 0.05% (v/v) trifluoroacetic acid; B: acetonitrile with 0.05% (v/v) trifluoroacetic acid: gradient elution: (A%): 0–3 min: 90%, 3–28 min: gradient from 90% to 0%, 28–31 min: 0%, 31–31.5 min: gradient from 0% to 90%, 31.5–40 min: 90%. According to two different HPLC methods the purity of all test compounds was greater than 95%.

5.1.2. Synthetic procedures

5.1.2.1. (*2r*)-2-Nitrocyclohexane-1,3-diol (6)[13,14]. An aqueous solution of glutaraldehyde (**4**, 25 %, 182 mL, 460 mmol), nitromethane (**5**, 38 mL, 0.71 mol) and CH_3OH (600 mL) was cooled to 0–5 °C. Then 2 M NaOH (12 mL) was added dropwise and the mixture was stirred at rt for 4 h. An acid cation exchange resin (16.8 g) was added and the mixture was stirred for 20 min. Then it was filtered and the filtrate was concentrated in vacuo. The residue was treated with EtOH (100 mL) and toluene (250 mL) and the solvents were removed in vacuo to obtain a solid, which was dissolved in EtOH (a00 mL). Crystallization was induced by addition of toluene

(250 mL). Colorless solid, mp 154 °C, yield 58.7 g (80%). $C_6H_{11}NO_4$ (161.2). R_f = 0.69 (CH_3OH). ^1H NMR (DMSO-d_6): δ (ppm) = 1.13–1.32 (m, 3H, 4-H, 5-H, 6-H), 1.48–1.66 (m, 1H, 5-H), 1.74–1.86 (m, 2H, 4-H, 6-H), 3.28–3.39 (m, 2H, 1-H, 3-H), 4.17 (t, ^3J = 9.9 Hz, 1H, 2-H), 5.45 (d, ^3J = 6.1 Hz, 2H, OH). IR: $\tilde{\nu}$ (cm^{-1}) = 3.500–3.100 (s, ν (OH)), 1550 (s, ν (NO_2)), 1339 (m, ν (NO_2)).

5.1.2.2. (2*r*)-N^1,N^3-Dibenzyl-2-nitrocyclohexane-1,3-diamine (7a)[13]. The nitrodiol **6** (19.3 g, 0.12 mol) was added to a solution of benzylamine (26.4 mL, 0.24 mol) in H_2O (60 mL) and the mixture was stirred at rt for 16 h. The precipitate was removed by filtration and recrystallized from CH_3OH. Colorless solid, mp 91 °C, yield 37.6 g (92 %). $C_{20}H_{25}N_3O_2$ (339.4). R_f = 0.65 (CH_3OH). ^1H NMR ($CDCl_3$): δ (ppm) = 1.10 (qd broad, ^2J = ^3J = 12.5 Hz, ^3J = 3.6 Hz, 2H, 4-H_{ax}, 6-H_{ax}), 1.29 (qt, ^2J = ^3J = 13.4 Hz, ^3J = 3.6 Hz, 1H, 5-H_{ax}), 1.77 (dquint, ^2J = 13.8 Hz, ^3J = 3.5 Hz, 1H, 5-H_{eq}), 2.16 (dq, ^2J = 13.7 Hz, ^3J = 3.7 Hz, 2H, 4-H_{eq}, 6-H_{eq}), 3.06 (td, ^3J = 11.0 Hz, ^3J = 4.0 Hz, 2H, 1-H, 3-H), 3.70 (d, ^2J = 13.1 Hz, 2H, 2× Ph-CH_2), 3.85 (d, ^2J = 13.1 Hz, 2H, 2× Ph-CH_2), 4.20 (t, ^3J = 10.3 Hz, 1H, 2-H), 7.22–7.32 (m, 10H, Ph-H). Signals for the NH-protons are not visible in the ^1H NMR spectrum. IR: $\tilde{\nu}$ (cm^{-1}) = 3336 (s, ν (N-H)), 1545 (s, ν (NO_2)), 1338 (s, ν (CNO_2)), 743 (s, out-of-plane (Ar-H)), 701 (s, out-of-plane (Ar-H)). MS (EI): m/z (%) = 304 ($(M-H_2O-OH)^+$, 6), 199 ($(M-H_2O-OH-C_7H_7)^+$, 25), 91 ($C_7H_7^+$, 100).

5.1.2.3. (2*r*)-N^1,N^3-Bis(4-methoxybenzyl)-2-nitrocyclohexane-1,3-diamine (7b). The nitrodiol **6** (102 mg, 0.64 mmol) was added to a solution of 4-methoxybenzylamine (0.18 g, 1.3 mmol) in H_2O (5 mL) and the mixture was stirred at rt for 3 d. the resulting precipitate was removed by filtration and dried. Colorless solid, mp 94 °C, yield 226 mg (87%). $C_{22}H_{29}N_3O_4$ (399.5). R_f = 0.71 (CH_3OH). ^1H NMR ($CDCl_3$): δ (ppm) = 1.09 (qd broad, ^2J = ^3J = 12.3 Hz, ^3J = 3.6 Hz, 2H, 4-H_a, 6-H_a), 1.28 (qt, ^2J = ^3J = 13.4 Hz, ^3J = 3.5 Hz, 1H, 5-H_a), 1.77 (dquint, ^2J = 13.7 Hz, ^3J = 3.3 Hz, 1H, 5-H_e), 2.14 (dq, ^2J = 13.5 Hz, ^3J = 3.4 Hz, 2H, 4-H_e, 6-H_e), 3.03 (td, ^3J = 11.0 Hz, ^3J = 4.3 Hz, 2H, 1-H, 3-H), 3.64 (d, ^2J = 12.8 Hz, 2H, Ph-CH_2), 3.78 (d, ^2J = 12.9 Hz, 2H, Ph-CH_2), 3.79 (s, 6H, OCH_3), 4.18 (t, ^3J = 10.3 Hz, 1H, 2-H), 6.80–6.89 (m, 4H, *ortho*-Ph-H), 7.13–7.27 (m, 4H, *meta*-Ph-H). Signals for the NH-protons are not visible in the ^1H NMR spectrum. IR: $\tilde{\nu}$ (cm^{-1}) = 3313 (s, ν (N-H)), 1553 (s, ν (NO_2)), 1353 (s, ν (NO_2)), 1031 (s, ν (C-O)), 1011 (s, ν (C-O)), 819 (s, out-of-plane (Ar-H)), 807 (s, out-of-plane (Ar-H)). MS (EI): m/z (%) = 399 (M^+, 1), 354 ($(M-NO_2)^+$, 2), 136 ($CH_3OC_7H_6NH^+$, 100), 121 ($CH_3OC_7H_6^+$, 78).

5.1.2.4. (2*r*)-N^1,N^3-Bis(4-chlorobenzyl)-2-nitrocyclohexane-1,3-diamine (7c). 4-Chlorobenzylamine (0.90 g, 6.3 mmol) was added to a solution of nitrodiol **6** (509 mg, 3.2 mmol) in H_2O (15 mL) and the suspension was stirred at rt for 4 d. The resulting precipitate was collected by filtration and dried under high vacuum. Colorless solid, mp 101 °C, yield 636 mg (49%). $C_{20}H_{23}Cl_2N_3O_2$ (408.3). R_f = 0.72 (CH_3OH).^1H NMR ($CDCl_3$): δ (ppm) = 1.08 (qd broad, ^2J = ^3J = 12.5 Hz, ^3J = 3.7 Hz, 2H, 4-H_a, 6-H_a), 1.27 (qt, ^2J = ^3J = 13.3 Hz, ^3J = 3.5 Hz, 1H, 5-H_a), 1.79 (dquint, ^2J = 13.9 Hz, ^3J = 3.6 Hz, 1H, 5-H_e), 2.15 (dq, ^2J = 13.1 Hz, ^3J = 3.8 Hz, 2H, 4-H_e, 6-H_e), 3.00 (td, ^3J = 11.0 Hz, ^3J = 4.1 Hz, 2H, 1-H, 3-H), 3.65 (d, ^2J = 13.4 Hz, 2H, Ph-CH_2), 3.81 (d, ^2J = 13.5 Hz, 2H, Ph-CH_2), 4.14 (t, ^3J = 10.5 Hz, 1H, 2-H), 7.16–7.20 (m, 4H, *meta*-Ph-H), 7.25–7.28 (m, 4 H, *ortho*-Ph-H). Signals for the NH-protons are not visible in the ^1H NMR spectrum. IR: $\tilde{\nu}$ (cm^{-1}) = 3322 (m, ν (N-H)), 1548 (s, ν (NO_2)), 1363 (s, ν (NO_2)), 810 (m, out-of-plane (Ar-H)). MS (EI): m/z (%) = 408 (M^+, <1), 127 ($C_7H_6Cl^+$, 33), 125 ($C_7H_6Cl^+$, 100).

5.1.2.5. (2*r*)-N^1,N^3-Bis(3,4-dichlorobenzyl)-2-nitrocyclohexane-1,3-diamine (7d). 3,4-Dichlorobenzylamine (221 mg, 1.3 mmol) was added to a solution of nitrodiol **6** (101 mg, 0.6 mmol) in H_2O (5 mL) and the suspension was stirred overnight at rt. A few drops of CH_3OH were added and the mixture was stirred at rt for another 24 h. The mixture was cooled to −6 °C for a few days. The resulting precipitate was collected by filtration and dried. Colorless solid, mp 92 °C, yield 258 mg (86%). $C_{20}H_{21}Cl_4N_3O_2$ (477.2). R_f = 0.78 (CH_3OH). ^1H NMR ($CDCl_3$): δ (ppm) = 1.08 (qd broad, ^2J = ^3J = 12.6 Hz, ^3J = 3.6 Hz, 2H, 4-H_a, 6-H_a), 1.29 (qt, ^2J = ^3J = 13.2 Hz, ^3J = 3.2 Hz, 1H, 5-H_a), 1.81 (dquint, ^2J = 13.8 Hz, ^3J = 3.3 Hz, 1H, 5-H_e), 2.16 (dq, ^2J = 13.1 Hz, ^3J = 3.4 Hz, 2H, 4-H_e, 6-H_e), 3.00 (td, ^3J = 11.1 Hz, ^3J = 4.1 Hz, 2H, 1-H, 3-H), 3.65 (d, ^2J = 13.7 Hz, 2H, Ph-CH_2), 3.81 (d, ^2J = 13.7 Hz, 2H, Ph-CH_2), 4.17 (t, ^3J = 10.4 Hz, 1H, 2-H), 7.08–7.12 (m, 2H, Ph-H), 7.33–7.38 (m, 4H, Ph-H). Signals for the NH-protons are not visible in the ^1H NMR spectrum. IR: $\tilde{\nu}$ (cm^{-1}) = 3329 (m, ν (N-H)), 1557 (s, ν (NO_2)), 1359 (m, ν (NO_2)), 820 (m, out-of-plane (Ar-H)). MS (EI): m/z (%) = 477 (M^+, <1), 174 ($C_7H_5Cl_2NH^+$, 56), 159 ($C_7H_5Cl_2^+$, 100).

5.1.2.6. (2*r*)-1,3-Di(pyrrolidin-1-yl)-2-nitrocyclohexane (7e). Pyrrolidine (2.02 g, 29 mmol) was added dropwise to a solution of nitrodiol **6** (2.09 g, 13 mmol) in H_2O (21 mL) and the mixture was stirred at rt overnight. The resulting precipitate was collected by filtration and dried. Colorless solid, mp 98 °C, yield 3.23 g (93%), $C_{14}H_{25}N_3O_2$ (267.4). R_f = 0.68 (CH_3OH). ^1H NMR ($CDCl_3$): δ (ppm) = 1.19–1.28 (m, 3H, 4-H_a, 5-H_a, 6-H_a), 1.63–1.71 (m, 8H, $N(CH_2CH_2)_2$), 1.85–1.94 (m, 3H, 4-H_e, 5-H_e, 6-H_e), 2.53–2.60 (m, 4H, $N(CH_2CH_2)_2$), 2.67–2.73 (m, 4H, $N(CH_2CH_2)_2$), 3.26 (td, ^3J = 10.9 Hz, ^3J = 3.2 Hz, 2H, 1-H, 3-H), 4.49 (t, ^3J = 10.8 Hz, 1H, 2-H). IR: $\tilde{\nu}$ (cm^{-1}) = 1555 (s, ν (NO_2)), 1373 (m, ν (NO_2)). MS (EI): m/z (%) = 268 (MH^+, 4), 197 ($(M-C_4H_8N)^+$, 27), 150 ($(M-C_4H_8N-HNO_2)^+$, 100).

5.1.2.7. (2*r*)-N^1,N^3-Dibenzylcyclohexane-1,2,3-triamine (8a)[12,13]. Raney Ni (1.6 mL suspension, ca. 0.96 g Raney Ni, Sigma–Aldrich) was added to a solution of **7a** (0.34 g, 1.0 mmol) in CH_3OH (2.5 mL) and the suspension, was stirred at rt under H_2 (1 bar) for 3 h. The suspension was filtered and the filtrate was concentrated in vacuo. Pale yellow oil, yield 0.25 g (81%). $C_{20}H_{27}N_3$ (309.5). R_f = 0.21 (CH_3OH). ^1H NMR (CD_3OD): δ (ppm) = 1.12 (qd broad, ^2J = ^3J = 11.6 Hz, ^3J = 3.2 Hz, 2H, 4-H_a, 6-H_a), 1.22 (qt, ^2J = ^3J = 12.9 Hz, ^3J = 2.9 Hz, 1H, 5-H_a), 1.77 (dquint, ^2J = 12.8 Hz, ^3J = 3.2 Hz, 1H, 5-H_e), 2.11 (dq, ^2J = 12.5 Hz, ^3J = 3.1 Hz, 2H, 4-H_e, 6-H_e), 2.21 (td, ^3J = 10.1 Hz, ^3J = 2.1 Hz, 2H, 1-H, 3-H), 2.25 (t, ^3J = 9.0 Hz, 1H, 2-H), 3.64 (d, ^2J = 12.9 Hz, 2H, Ph-CH_2), 3.88 (d, ^2J = 12.9 Hz, 2H, Ph-CH_2), 7.20–7.36 (m, 10H, Ph-H). Signals for the NH-protons are not visible in the ^1H NMR spectrum. IR: $\tilde{\nu}$ (cm^{-1}) = 3303 (s, ν (N-H)) 730 (s, out-of-plane (Ar-H)), 696 (s, out-of-plane (Ar-H)).

5.1.2.8. (2*r*)-1,3-Di(pyrrolidin-1-yl)cyclohexan-2-amine (8e). Raney Ni (ca. 9.0 g. Sigma–Aldrich) was added to a solution of **7e** (3.23 g, 12 mmol) in CH_3OH (24 mL) and the mixture was stirred at rt under H_2 (1 bar) for 3 h. The suspension was filtered and the solvent was removed in vacuo. Colorless oil, yield 2.33 g (81%). $C_{14}H_{27}N_3$ (237.4 g/mol). R_f = 0.16 (CH_3OH). ^1H NMR (CD_3OD): δ (ppm) = 1.21–1.38 (m, 3H, 4-H_a, 5-H_a, 6-H_a), 1.74–1.82 (m, 8H, $N(CH_2CH_2)_2$), 1.86–1.95 (m, 1H, 5-H_e), 2.10–2.14 (m, 4H, 1-H, 3-H, 4-H_e, 6-H_e), 2.63–2.70 (m, 4H, $N(CH_2CH_2)_2$), 2.70–2.78 (m, 4H, $N(CH_2CH_2)_2$), 2.89 (t, ^3J = 10.8 Hz, 1H, 2-H). A signal for the protons of the NH_2 group is not visible in the ^1H NMR spectrum. IR: $\tilde{\nu}$ (cm^{-1}) = 3356 (w, ν (N-H)), 3263 (w, ν (N-H)). MS (EI): m/z

(%) = 238 (MH^+, 2), 167 ($C_4H_8NC_6H_9NH_2^+$, 16), 150 ($C_4H_8NC_6H_8$, 79), 98 ($C_6H_9NH_3^+$, 30).

5.1.2.9. (4a*RS*,5*SR*,8a*RS*)-1-Benzyl-5-(benzylamino)perhydroquinoxaline-2,3-dione (9a). A solution of triamine **8** (100 mg, 0.32 mmol) and dimethyl oxalate (38 mg, 0.32 mmol) in CH_3OH (2.0 mL) was heated to reflux for 24 h. The solvent was evaporated in vacuo and the residue was recrystallized from ethyl acetate. Colorless solid, mp 194 °C, yield 75 mg (64%). $C_{22}H_{25}N_3O_2$ (363.4). R_f = 0.61 (CH_3OH). ^1H NMR ($CDCl_3$): δ (ppm) = 1.05 (qd, ^2J = ^3J = 12.9 Hz, ^3J = 3.3 Hz, 1H, 6-H_a), 1.21 (qt, ^2J = ^3J = 13.4 Hz, ^3J = 3.4 Hz, 1H, 7-H_a), 1.34 (qd, ^2J = ^3J = 12.4 Hz, ^3J = 3.4 Hz, 1H, 8-H_a), 1.85 (dquint, ^2J = 13.7 Hz, ^3J = 3.1 Hz, 1H, 7-H_e), 2.11 (dq, ^2J = 12.1 Hz, ^3J = 3.2 Hz, 1H, 8-H_e), 2.26 (dq, ^2J = 12.8 Hz, ^3J = 3.0 Hz, 1H, 6-H_e), 2.44 (td, ^3J = 10.8 Hz, ^3J = 3.6 Hz, 1H, 5-H), 3.13 (t, ^3J = 10.5 Hz, 1H, 4a-H), 3.42 (td, ^3J = 11.6 Hz, ^3J = 3.9 Hz, 1H, 8a-H), 3.67 (d, ^2J = 12.7 Hz, 1H, Ph-C*H*$_2$-NH), 3.94 (d, ^2J = 12.7 Hz, 1H, Ph-C*H*$_2$-NH), 4.39 (d, ^2J = 15.6 Hz, 1H, N1-CH_2-Ph), 5.21 (d, ^2J = 15.6 Hz, 1H, N1-CH_2-Ph), 7.16–7.36 (m, 10H, Ph-H). Signals for the NH-protons are not visible in the ^1H NMR spectrum. ^{13}C NMR ($CDCl_3$): δ (ppm) = 22.5 (C-7), 28.2 (C-8), 30.2 (C-6), 46.0 (N1-C), 50.7 (Ph-CH_2), 57.7 (C-5), 58.2 (C-8a), 58.4 (C-4a), 127.3 (2 C, Ph-C), 127.7 (Ph-C), 127.8 (Ph-C), 128.5 (2 C, Ph-C), 128.9 (2 C, Ph-C), 129.1 (2 C, Ph-C), 136.7 (2 C, quart. Ph-C), 157.5 (C-3), 159.5 (C-2). IR: $\tilde{\nu}$ (cm^{-1}) = 3338 (m, ν (N-H)), 3283 (m, ν (N-H)), 1704 (s, ν (C=O), tert. amide), 1663 (s, ν (C=O), sec. amide), 748 (s, out-of-plane (Ar-H)), 701 (s, out-of-plane (Ar-H)).MS (EI): *m*/*z* (%) = 363 (M^+, 46), 335 ($(M-CO)^+$, 11), 272 ($(M-C_7H_7)^+$, 81), 258 ($(M-C_7H_7NH)^+$, 95), 106 ($C_7H_7NH^+$, 71), 91 ($C_7H_7^+$, 100). Anal. Calcd C, 72.70 H 6.93; N 11.56. Found C, 72.55; H, 6.79; N, 11.77. HPLC (method 1): purity 99.6%, t_R = 15.5 min. HPLC (method 2): purity 98.6%, t_R = 13.5 min.

5.1.2.10. (4a*RS*,5*SR*,8a*RS*)-1-(4-Methoxybenzyl)-5-(4-methoxybenzylamino)-perhydro-quinoxaline-2,3-dione (9b). Raney Ni (360 mg, Sigma–Aldrich) was added to a solution of **7b** (1.16 g, 2.91 mmol) in CH_3OH (2 mL) and ethyl acetate (20 mL) and the mixture was stirred at rt under H_2 (1 bar) for 20 h. The suspension was filtered and the solvent was removed in vacuo. Pale yellow oil (**8b**), yield 928 mg (86%). $C_{24}H_{29}N_3O_4$ (423.5 g / mol). R_f = 0.26 (CH_3OH). IR: $\tilde{\nu}$ (cm^{-1}) = 3350–3100 (w, ν (N–H)), 1242 (s, ν (C–O–C)), 1031 (s, ν (C–O–C)), 813 (s, out-of-plane (Ar-H)).

The crude product **8b** (0.49 g, 1.33 mmol) and dimethyl oxalate (0.31 g, 2.66 mmol) were dissolved in CH_3OH (20 mL) and the mixture was heated to reflux for 48 h. The solvent was evaporated in vacuo and the residue was recrystallized from ethyl acetate. Colorless solid, mp 155 °C, yield 0.19 g (33%). $C_{24}H_{29}N_3O_4$ (423.5). R_f = 0.62 (CH_3OH). ^1H NMR ($CDCl_3$): δ (ppm) = 1.02–1.15 (m, 1H, 6-H_a), 1.15–1.28 (m, 1H, 7-H_a), 1.35 (qd, ^2J = ^3J = 12.3 Hz, ^3J = 2.8 Hz, 1H, 8-H_a), 1.86 (dquint, ^2J = 13.4 Hz, ^3J = 3.0 Hz, 1H, 7-H_e), 2.11–2.19 (m, 1H, 8-H_e), 2.21–2.29 (m, 1H, 6-H_e), 2.39–2.50 (m, 1H, 5-H), 3.11–3.20 (m, 1H, 4a-H), 3.39 (td, ^3J = 10.7 Hz, ^3J = 2.8 Hz, 1H, 8a-H), 3.62 (d, ^2J = 12.6 Hz, 1H, Ph-C*H*$_2$-NH), 3.78 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 3.89 (d, ^2J = 12.5 Hz, 1H, Ph-C*H*$_2$-NH), 4.29 (d, ^2J = 15.3 Hz, 1H, N1-CH_2-Ph), 5.19 (d, ^2J = 15.3 Hz, 1H, N1-CH_2-Ph), 6.82 (d, ^3J = 8.7 Hz, 2H, *ortho*-Ph-H), 6.85 (d, ^3J = 8.7 Hz, 2H, *ortho*-Ph-H), 7.13 (d, ^3J = 8.6 Hz, 2H, *meta*-Ph-H), 7.23 (d, ^3J = 8.2 Hz, 2H, *meta*-Ph-H). Signals for the NH-protons are not visible in the ^1H NMR spectrum. IR: $\tilde{\nu}$ (cm^{-1}) = 3345 (m, ν (N–H)), 3282 (m, ν (N–H)), 1694 (s, ν (C=O), *tert* amide), 1674 (s, ν (C=O), sec. amide), 811 (s, out-of-plane (Ar-H)). MS (EI): *m*/*z* (%) = 423 (M^+, 18), 302 ($(M-H_3CO-C_7H_6)^+$, 100), 136 ($H_3CO-C_7H_6NH^+$, 61), 121 ($CH_3O-C_7H_6^+$, 77), 91 ($C_7H_7^+$, 10). HPLC (method 1): purity 97.8%, t_R = 16.2 min. HPLC (method 2): purity 97.1%, t_R = 14.0 min.

5.1.2.11. (4a*RS*,5*SR*,8a*RS*)-1-(4-Chlorobenzyl)-5-(4-chlorobenzylamino)perhydroquinoxaline-2,3-dione (9c). Raney Ni (180 mg, Sigma–Aldrich) was added to a solution of **7c** (636 mg, 1.6 mmol) in CH_3OH (2 mL) and ethyl acetate (20 mL) and the mixture was stirred at rt under H_2 (1 bar) for 20 h. The suspension was filtered and the solvent was removed in vacuo. Pale yellow oil (**8c**), yield 505 mg (83%). $C_{22}H_{23}Cl_2N_3O_2$ (432.3 g/mol). R_f = 0.27 (CH_3OH). IR**:** $\tilde{\nu}$ (cm^{-1}) = 3350–3100 (w, ν (N-H)), 799 (m, out-of-plane (Ar-H)).

The crude product **8c** (493 mg, 1.30 mmol) and dimethyl oxalate (154 mg, 1.30 mmol) were dissolved in CH_3OH (20 mL) and the mixture was heated to reflux for 18 h. The solvent was evaporated in vacuo and the residue was recrystallized from ethyl acetate. Colorless solid, mp 194 °C, yield 262 mg (47%). $C_{22}H_{23}Cl_2N_3O_2$ (432.3). R_f = 0.66 (CH_3OH). ^1H NMR ($CDCl_3$): δ (ppm) = 1.07(q broad, ^2J = ^3J = 12.1 Hz, ^3J = Hz, 1H, 6-H_a), 1.17–1.39 (m, 2H, 7-H_a, 8-H_a), 1.88 (dquint, ^2J = 13.5 Hz, ^3J = 2.9 Hz, 1H, 7-H_e), 2.03–2.10 (m, 1H, 8-H_e), 2.21–2.28 (m, 1H, 6-H_e), 2.44 (t, ^3J = 10.2 Hz, 1H, 5-H), 3.16 (t, ^3J = 10.3 Hz, 1H, 4a-H), 3.42 (td, ^3J = 11.4 Hz, ^3J = 3.8 Hz, 1H, 8a-H), 3.65 (d, ^2J = 13.0 Hz, 1H, Ph-C*H*$_2$-NH), 3.92 (d, ^2J = 13.0 Hz, 1H, Ph-C*H*$_2$-NH), 4.41 (d, ^2J = 15.7 Hz, 1H, N1-CH_2-Ph), 5.10 (d, ^2J = 15.7 Hz, 1H, N1-CH_2-Ph), 7.14 (d, ^3J = 8.4 Hz, 2H, *meta*-Ph-H), 7.23–7.31 (m, 6H, Ph-H). Signals for the NH-protons are not visible in the ^1H NMR spectrum. IR: $\tilde{\nu}$ (cm^{-1}) = 3350 (m, ν (N-H)), 1700 (s, ν (C=O), *tert* amide), 1675 (s, ν (C=O), sec. amide), 796 (m, out-of-plane (Ar-H)). MS (EI): *m*/*z* (%) = 431 (M^+, 7), 308 ($(M-C_7H_6Cl)^+$, 15), 306 ($(M-C_7H_6Cl)^+$, 50), 142 ($C_7H_6ClNH^+$, 15), 140 ($C_7H_6ClNH^+$, 46), 127 ($C_7H_6Cl^+$, 31), 125 ($C_7H_6Cl^+$, 100). HPLC (method 1): purity 97.9%, t_R = 18.1 min. HPLC (method 2): purity 95.8%, t_R = 15.7 min.

5.1.2.12. (4a*RS*,5*SR*,8a*RS*)-5-Amino-1-benzylperhydroquinoxaline-2,3-dione (10). A mixture of benzylamine **9a** (1.19 g, 3.28 mmol), NH_4HCO_2 (2.07 g, 32.8 mmol) and Pd/C (120 mg) in CH_3OH (40 mL) was heated to reflux for 2 h. The suspension was filtered, the filtrate was concentrated in vacuo, the residue was dissolved in CH_2Cl_2 and the solution was washed with 0.1 M NaOH (3×). The organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo. Colorless solid, mp 69 °C, yield 0.89 g (97%). $C_{15}H_{19}N_3O_2$ (273.3). R_f = 0.20 (CH_3OH); 0.16 (CH_2Cl_2/MeOH/NH_3 = 9:1:0.1). ^1H NMR (CD_3OD): δ (ppm) = 1.15–1.40 (m, 3H, 6-H_a, 7-H_a, 8-H_a), 1.72–1.79 (m, 1H, 7-H_e), 1.84–1.91 (m, 1H, 6-H_e), 2.08–2.15 (m, 1H, 8-H_e), 2.61 (td, ^3J = 10.0 Hz, ^3J = 3.3 Hz, 1H, 5-H), 3.20 (t, ^3J = 10.1 Hz, 1H, 4a-H), 3.56 (td, ^3J = 11.4 Hz, ^3J = 3.9 Hz, 1H, 8a-H), 4.61 (d, ^2J = 15.8 Hz, 1H, Ph-CH_2), 5.05 (d, ^2J = 15.8 Hz, 1H, Ph-CH_2), 7.21–7.28 (m, 3H, *ortho*-Ph-H, *para*-Ph-H), 7.30–7.36 (m, 2H, *meta*-Ph-H). Signals for the NH-protons are not visible in the ^1H NMR spectrum. ^{13}C NMR ($CDCl_3$): δ (ppm) = 22.7 (C-7), 28.3 (C-8), 36.0 (C-6), 46.1 (N^1-*C*H_2-Ph), 52.6 (C-5), 58.1 (C-8a), 60.0 (C-4a), 127.3 (2 C, *ortho*-Ph-C), 127.7 (*para*-Ph-C), 129.1 (2 C, *meta*-Ph-C), 136.8 (quart. Ph-C), 158.0 (C-3), 159.6 (C-2). IR: $\tilde{\nu}$ (cm^{-1}) = 3300–3100 (m, ν (N–H)), 1704 (s, ν (C=O), *tert* amide), 1663 (s, ν (C=O), sec. amide). MS (EI): *m*/*z* (%) = 273 (M^+, 55), 255 ($(M-NH_4)^+$, 24), 106 ($C_7H_7NH^+$, 71), 91 ($C_7H_7^+$, 100). HPLC (method 1): purity 98.4%, t_R = 12.4 min. HPLC (method 2): purity 99.1%, t_R = 10.9 min.

5.1.2.13. (4a*RS*,5*SR*,8a*RS*)-1-Benzyl-5-(pyrrolidin-1-yl)perhydroquinoxaline-2,3-dione (11). A mixture of primary amine **10** (3.06 g, 11.2 mmol), 1,4-diiodobutane (13.9 g, 44.8 mmol, 5.9 mL), $NaHCO_3$ (6.4 g, 76.2 mmol) and CH_3CN (300 mL) was heated to reflux for 18 h. $NaHCO_3$ was filtered off and the solvent was removed in vacuo. The residue was dissolved in CH_2Cl_2 and the solution was extracted with 1 M HCl (3×). After addition of 2 M NaOH until pH 8, the aqueous layer was extracted with CH_2Cl_2

(3×). The organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo. Colorless solid, mp 104 °C, yield 2.80 g (76%). $C_{19}H_{25}N_3O_2$ (327.4). R_f = 0.55 (CH_3OH). ^1H NMR ($CDCl_3$): δ (ppm) = 1.14–1.36 (m, 3H, 6-H_a, 7-H_a, 8-H_a), 1.68–1.82 (m, 5H, N(CH_2CH_2)$_2$ (4H), 6-H_e), 1.84–1.92 (m, 1H, 7-H_e), 2.06–2.14 (m, 1H, 8-H_e), 2.49–2.57 (m, 2H, N(CH_2CH_2)$_2$), 2.57–2.64 (m, 2H, N(CH_2CH_2)$_2$), 2.68 (td, ^3J = 11.2 Hz, ^3J = 3.1 Hz, 1H, 5-H), 3.27 (t, ^3J = 10.6 Hz, 1H, 4a-H), 3.46 (td, ^3J = 11.3 Hz, ^3J = 3.7 Hz, 1H, 8a-H), 4.42 (d, ^2J = 15.6 Hz, 1H, Ph-CH_2), 5.18 (d, ^2J = 15.6 Hz, 1H, Ph-CH_2), 7.17–7.33 (m, 5H, Ph-H). A signal for the NH-proton is not visible in the ^1H NMR spectrum. ^{13}C NMR ($CDCl_3$): δ (ppm) = 20.5 (C-6), 22.4 (C-7), 23.8 (2C, N(CH_2CH_2)$_2$), 28.3 (C-8), 46.0 (Ph-CH_2), 47.2 (2C, N(CH_2CH_2)$_2$), 56.2 (C-4a), 58.7 (C-8a), 59.6 (C-5), 127.3 (2 C, Ph-C), 127.7 (Ph-C), 129.0 (2 C, Ph-C), 136.9 (quart. Ph-C), 157.6 (C-3), 159.7 (C-2). IR: $\tilde{\nu}$ (cm^{-1}) = 3186 (m, ν (N–H)), 1695 (s, ν (C=O), *tert* amide), 1667 (s, ν (C=O), sec. amide). MS (EI): m/z (%) = 327 (M^+, 100), 258 ((M−C_4H_7N)$^+$, 29), 236 ((M−C_7H_7)$^+$, 98), 167 ((M−C_7H_7−C_4H_7N)$^+$, 16), 91 ($C_7H_7^+$, 23). Anal. Calcd C, 69.70; H, 7.70; N, 12.83. Found C, 69.5; H, 7.66; N, 12.78. HPLC (method 1): purity 99.6%, t_R = 13.5 min. HPLC (method 2): purity 98.8%, t_R = 12.0 min.

5.1.2.14. (4a*RS*,5*SR*,8a*RS*)-1-Benzyl-5-(pyrrolidin-1-yl)perhydroquinoxaline (12). Under N_2, dry $AlCl_3$ (45 mg, 0.33 mmol) was dissolved in absolute THF (2.5 mL) and the solution was cooled to 0 °C. A solution of $LiAlH_4$ (1.0 M in THF, 1.0 mL, 1.00 mmol) was added dropwise. The suspension was warmed to rt and stirred for 20 min. A solution of diamide **11** (59 mg, 0.18 mmol) in THF (3 mL) was added to the freshly prepared solution of AlH_3 (1.33.M AlH_3) at 0 °C. The mixture was stirred at 0 °C for 45 min and at rt for 20 min. Then 2 M NaOH (2 mL) was added dropwise under cooling and the mixture was extracted with CH_2Cl_2 (5 × 15 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo. Pale yellow solid, mp 54 °C, yield 52 mg (96%). $C_{19}H_{29}N_3$ (299.4). R_f = 0.24 (CH_3OH); 0.71 (CH_2Cl_2/MeOH/NH_3 = 9:1:0.1). ^1H NMR ($CDCl_3$): δ (ppm) = 1.16 (qd, 1H, 8-H_a), 1.23–1.37 (m, 2H, 6-H_a, 7-H_a), 1.65–1.72 (m, 4H, N(CH_2CH_2)$_2$), 1.72–1.78 (m, 1H, 6-H_e), 1.82–1.89 (m, 1H, 7-H_e), 2.04 (td, ^3J = 8.7 Hz, ^3J = 3.7 Hz, 1H, 8a-H), 2.15–2.24 (m, 2H, 2-H_a, 8-H_e), 2.36 (t, ^3J = 10.2 Hz, 1H, 4a-H), 2.53–2.62 (m, 4H, N(CH_2CH_2)$_2$), 2.62–2.66 (m, 1H, 5-H), 2.69 (dt, ^2J = 11.2 Hz, ^3J = 2.5 Hz, 1H, 2-H_e), 2.81 (td, ^2J = ^3J = 11.3 Hz, ^3J = 2.8 Hz, 1H, 3-H_a), 2.92 (dt, ^2J = 10.9 Hz, ^3J = 2.4 Hz, 1H, 3-H_e), 3.13 (d, ^2J = 13.2 Hz, 1H, Ph-CH_2), 4.13 (d, ^2J = 13.3 Hz, 1H, Ph-CH_2), 7.18–7.33 (m, 5H, Ph-H). A signal for the NH-proton is not visible in the ^1H NMR spectrum. ^{13}C NMR ($CDCl_3$): δ (ppm) = 20.9 (C-6), 22.9 (C-7), 23.9 (2 C, N(CH_2CH_2)$_2$), 28.9 (C-8), 46.1 (C-3), 47.3 (2 C, N(CH_2CH_2)$_2$), 53.1 (C-2), 57.9 (Ph-CH_2), 60.7 (C-5), 62.8 (C-4a), 65.9 (C-8a), 127.0 (Ph-C), 128.4 (2 C, Ph-C), 129.4 (2 C, Ph-C) 139.4 (quart. Ph-C). IR: $\tilde{\nu}$ (cm^{-1}) = 3329 (w, ν (N-H)). MS (EI): m/z (%) = 299 (M^+, 78), 229 ((M−C_4H_8N)$^+$, 26), 208 ((M−C_7H_7)$^+$, 12), 91 ($C_7H_7^+$, 29). Anal. Calcd C, 76.21; H, 9.76; N, 14.03. Found C, 75.20; H, 9.89; N, 13.71. HPLC (method 1): purity 96.9%, t_R = 9.5 min. HPLC (method 2): purity 97.4%, t_R = 3.2 min.

5.1.2.15. 1-[(4a*RS*,8*SR*,8a*RS*)-4-Benzyl-8-(pyrrolidin-1-yl)perhydroquinoxalin-1-yl]-2-phenylethan-1-one (13a). Phenylacetyl chloride (160 mg, 1.0 mmol) was added dropwise to a solution of **12** (257 mg, 0.86 mmol) in CH_2Cl_2 (30 mL) and the mixture was stirred overnight at rt. Then 2 M NaOH (30 mL) was added and the mixture was stirred for 2 h at rt. The aqueous layer was separated, and the organic layer was extracted with 1 M HCl (3×). Then 2 M NaOH was added to the combined aqueous layers until pH 8. The aqueous layer was extracted with CH_2Cl_2 (3×) the organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo. Pale yellow oil, yield 325 mg (91%). $C_{27}H_{25}N_3O$ (417.6). R_f = 0.14 (CH_3OH); 0.76 (CH_2Cl_2/MeOH/NH_3 = 9:1:0.1). ^1H NMR (toluene-d_8, 100 °C): δ (ppm) = 0.87–0.96 (m, 1H, 7-H_a), 1.02 (qd, ^2J = ^3J = 14.4 Hz, ^3J = 3.3 Hz, 1H, 5-H_a), 1.11 (qt, ^2J = ^3J = 13.1 Hz, ^3J = 3.1 Hz, 1H, 6-H_a), 1.49–1.56 (m, 5H, 6-H_e, N(CH_2CH_2)$_2$), 1.64–1.71 (m, 1H, 5-H_e), 1.76–1.83 (m, 1H, 7-H_e), 1.83–1.91 (m, 1H, 3-H_e), 2.34 (td, ^3J = 11.0 Hz, ^3J = 4.0 Hz, 1H, 8-H), 2.41–2.48 (m, 1H, 3-H), 2.50–2.59 (m, 4H, N(CH_2CH_2)$_2$), 2.87 (d, ^2J = 13.5 Hz, 1H, Ph-CH_2-N), 3.02–3.11 (m, 3H, 2-H_a, 2-H_e, 8a-H), 3.48 (d, ^2J = 15.2 Hz, 1H, Ph-CH_2-C=O), 3.54 (d, ^2J = 15.2 Hz, 1H, Ph-CH_2-C=O), 3.60–3.66 (m, 1H, 4a-H), 3.68 (d, ^2J = 13.9 Hz, 1H, Ph-CH_2-N), 6.89–7.11 (m, 8H, Ph-H), 7.19–7.23 (m, 2H, Ph-H).IR: $\tilde{\nu}$ (cm^{-1}) = 1645 (s, ν (C=O)), 729 (s, out-of-plane (Ar-H)), 696 (s, out-of-plane (Ar-H)). MS (ESI): m/z (%) = 418 (MH^+, 100). HPLC (method 1): purity 98.3 %, t_R = 15.5 min. HPLC (method 2): purity 96.0 %, t_R = 11.8 min.

5.1.2.16. 1-[(4a*RS*,8*SR*,8a*RS*)-4-Benzyl-8-(pyrrolidin-1-yl)perhydroquinoxalin-1-yl]-2-(3,4-dichlorophenyl)ethan-1-one (13b). 2-(3,4-Dichlorophenyl)acetyl chloride (291 mg, 1.3 mmol) was added dropwise to a solution of quinoxaline **12** (325 mg, 1.09 mmol) in CH_2Cl_2 (35 mL) and the mixture was stirred at rt for 30 min. Then 2 M NaOH (35 mL) was added and the mixture was stirred for 2 h at rt. The aqueous layer was separated and the organic layer was extracted with 1 M HCl (3x). Then the pH value of the aqueous layer was adjusted to pH 8 by addition of 2 M NaOH. The aqueous layer was extracted with CH_2Cl_2 (3×), the organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo. Pale yellow solid, mp 73 °C, yield 458 mg (86%). $C_{27}H_{33}Cl_2N_3O$ (486.5). R_f = 0.11 (CH_3OH); 0.77 (CH_2Cl_2/MeOH/NH_3 = 9:1:0.1). ^1H NMR (toluene-d_8, 100 °C): δ (ppm) = 0.91 (qd, ^2J = ^3J = 12.1 Hz, ^3J = 4.1 Hz, 1H, 7-H_a), 0.99 (qd, ^2J = ^3J = 12.5 Hz, ^3J = 3.3 Hz, 1H, 5-H_a), 1.09 (qt, ^2J = ^3J = 13.1 Hz, ^3J = 3.4 Hz, 1H, 6-H_a), 1.48–1.54 (m, 5H, 6-H_e, N(CH_2CH_2)$_2$ (4H)), 1.62–1.68 (m, 1H, 5-H_e), 1.77–1.83 (m, 1H, 7-H_e), 1.83–1.89 (m, 1H, 3-H), 2.31 (td, ^3J = 10.7 Hz, ^3J = 3.6 Hz, 1H, 8-H), 2.44–2.54 (m, 5H, 3-H, N(CH_2CH_2)$_2$ (4H)), 2.89 (d, ^2J = 13.7 Hz, 1H, Ph-CH_2-N), 2.91–2.97 (m, 1H, 2-H), 2.97–3.05 (m, 2H, 2-H, 8a-H), 3.30 (s, 2H, Ph-CH_2-C=O), 3.47–3.57 (m, 1H, 4a-H), 3.69 (d, ^2J = 13.5 Hz, 1H, Ph-CH_2-N), 6.86–6.92 (m, 2H, *para*-Ph-H, Ph-6-H), 6.98–7.04 (m, 3H, *ortho*-Ph-H, Ph-5-H), 7.08–7.12 (m, 2H, *meta*-Ph-H), 7.24 (d, ^4J = 1.9 Hz, 1H, Ph-2-H). ^{13}C NMR (toluene-d_8, 100 °C): δ (ppm) = 22.8 (C-6), 23.8 (2 C, N(CH_2CH_2)$_2$), 24.9 (C-5), 30.5 (C-7), 40.9 (Ph-CH_2-C=O), 44.8 (C-2), 47.9 (2 C, N(CH_2CH_2)$_2$), 52.7 (C-3), 56.7 (Ph-CH_2-N), 58.0 (C-4a), 61.5 (C-8), 66.4 (C-8a), 126.5 (2 C, Ph-C), 128.3 (2 C, Ph-C), 129.7 (2 C, Ph-C), 130.4 (Ph-C), 130.8 (2 C, Ph-C), 132.1 (Ph-C), 136.8 (quart. Ph-C), 139.7 (quart. Ph-C), 169.5 (C=O). IR: $\tilde{\nu}$ (cm^{-1}) = 1646 (s, ν (C=O)), 875 (w, out-of-plane (Ar-H)), 814 (w, out-of-plane (Ar-H)). MS (ESI): m/z (%) = 486 (MH^+, 2·^{35}Cl, 100), 488 (MH^+, ^{35}Cl/^{37}Cl, 61), 490 (MH^+, 2 ^{37}Cl, 9). Anal. Calcd C, 66.66; H, 6.84; N, 8.64. Found C, 66.15; H, 6.81; N, 8.61. HPLC (method 1): purity 99.8 %, t_R = 17.5 min. HPLC (method 2): purity 99.1%, t_R = 14.2 min.

5.1.2.17. 2-Phenyl-1-[(4a*RS*,8*SR*,8*aSR*)-8-(pyrrolidin-1-yl)perhydroquinoxalin-1-yl]ethan-1-one (14a). Pd/C (10%, 52 mg) was added to a solution of **13a** (111 mg, 0.27 mmol) in THF/H_2O (1:1, 30 mL) and conc. HCl (3 mL) and the reaction mixture was stirred at rt under H_2 (1 bar) for 30 min. The suspension was filtered, the organic solvent was removed in vacuo, the aqueous layer was adjusted to pH 8 by addition of 2 M NaOH and extracted with CH_2Cl_2 (5×). The organic layer was dried (Na_2SO_4), filtered, concentrated in vacuo and the residue was purified by fc (2 cm, CH_2Cl_2/MeOH/NH_3 = 9:1:0.1, 16 cm, 5 mL). Pale yellow resin, yield 78 mg (90%). $C_{20}H_{29}N_3O$ (327.5). R_f = 0.02 (CH_3OH); 0.27 (CH_2Cl_2/MeOH/NH_3 9:1:0.1). ^1H NMR (toluene-d_8, 100 °C): δ (ppm) = 0.71–0.81 (m, 1H, 7-H_a), 0.94–1.03 (m, 1H, 5-H_a), 1.03–1.16 (m, 1H, 6-H_a), 1.41–1.48 (m, 2H, 6-H_e, 7-H_e), 1.48–1.55 (m, 4H, N(CH_2CH_2)$_2$), 1.63–1.70 (m, 1H, 5-H_e), 2.35–2.45 (m, 3H, 3-H_a, 3-H_e, 8-H),

2.48–2.58 (m, 4H, N(CH_2CH_2)$_2$), 2.77 (t, ^3J = 9.8 Hz, 1H, 8a-H), 2.97–3.05 (m, 1H, 2-H), 3.07–3.15 (m, 1H, 2-H), 3.51–3.55 (m, 3H, Ph-CH_2-C=O (2H), 4a-H), 6.89–7.08 (m, 3H, *ortho*-Ph-H, *para*-Ph-H), 7.19–7.23 (m, 2H, *meta*-Ph-H). A signal for the NH-proton is not visible in the ^{1}H NMR spectrum. IR: $\tilde{\nu}$ (cm^{-1}) = 3288 (w, ν (N–H)), 1637 (s, ν (C=O)), 729 (s, out-of-plane (Ar-H)), 697 (s, out-of-plane (Ar-H)). MS (ESI): m/z (%) = 328 (MH^+, 100). HPLC (method 1): purity 99.9%, t_R = 12.7 min.

5.1.2.18. 2-(3,4-Dichlorophenyl)-1-[(*4aRS,8SR,8aRS*)-8-(pyrrolidin-1-yl)-perhydroquinoxalin-1-yl]ethan-1-one (14b). A mixture of **13b** (244 mg, 0.50 mmol), concd HCl (5 mL), Pd/C (98.4 mg) and THF/H_2O (1:1, 50 mL) was stirred at rt under H_2 (1 bar) for 30 min. The suspension was filtered and the solvent was removed in vacuo. The pH value of the aqueous layer was adjusted to pH 8 by addition of 2 M NaOH. The aqueous layer was extracted with CH_2Cl_2 (5×), the combined organic layers were dried (Na_2SO_4), filtered, concentrated in vacuo and the residue was purified by fc (3 cm, 17 cm, CH_2Cl_2/MeOH/NH_3 = 9:1:0.1, 10 mL, R_f = 0.28). Pale yellow resin, yield 186 mg (94%). $C_{20}H_{27}Cl_2N_3O$ (396.4). R_f = 0.02 (CH_3OH); 0.28 (CH_2Cl_2/MeOH/NH_3 = 9:1:0.1). ^{1}H NMR (toluene-d_8, 100 °C): δ (ppm) = 0.80 (qd, ^2J = 11.2 Hz, ^3J = 4.2 Hz, 1H, 7-H_a), 0.96 (qd, ^2J = 11.8 Hz, ^3J = 3.4 Hz, 1H, 5-H_a), 1.10 (qt, ^2J = ^3J = 13.1 Hz, ^3J = 3.6 Hz, 1H, 6-H_a), 1.43–1.49 (m, 2H, 6-H_e, 7-H_e), 1.49–1.55 (m, 4H, N(CH_2CH_2)$_2$), 1.65 (dq, ^2J = 12.6 Hz, ^3J = 1.8 Hz, 1H, 5-H_e), 2.37–2.45 (m, 2H, 3-H_a, 8-H), 2.46–2.53 (m, 5H, 3-H_e, N(CH_2CH_2)$_2$ (4H)), 2.71 (t, ^3J = 10.1 Hz, 1H, 8a-H), 2.92–3.04 (m, 2H, 2-H_a, 2-H_e), 3.29 (d, ^2J = 15.4 Hz, 1H, Ph-CH_2-C=O), 3.37 (d, ^2J = 15.4 Hz, 1H, Ph-CH_2-C=O), 3.45–3.55 (m, 1H, 4a-H), 6.87 (dd, ^3J = 7.9 Hz, ^4J = 2.1 Hz, 1H, Ph-6-H), 7.03 (d, ^3J = 8.2 Hz, 1H, Ph-5-H), 7.24 (d, ^4J = 1.9 Hz, 1H, Ph-2-H). A signal for the NH-proton is not visible in the ^{1}H NMR spectrum. ^{13}C NMR (toluene-d_8, 100 °C): δ (ppm) = 23.5 (C-6), 24.6 (2C, N(CH_2CH_2)$_2$), 26.2 (C-5), 33.6 (C-7), 41.7 (Ph-CH_2-C=O), 46.7 (2C, C-2, C-3), 48.7 (2C, N(CH_2CH_2)$_2$), 56.8 (C-8), 58.9 (C-4a), 69.7 (C-8a), 128.4 (Ph-C), 129.1 (Ph-C), 129.3 (Ph-C), 130.5 (Ph-C), 131.6 (Ph-C), 137.7 (quart. Ph-C), 170.4 (C=O). IR: $\tilde{\nu}$ (cm^{-1}) = 3301 (w, ν (N–H)), 1636 (s, ν (C=O)), 873 (w, out-of-plane (Ar-H)), 814 (w, out-of-plane (Ar-H)). MS (ESI): m/z (%) = 396 (MH^+, 2·^{35}Cl, 100), 398 (MH^+, ^{35}Cl/^{37}Cl, 63), 400 (MH^+, 2·^{37}Cl, 10). HPLC (method 1): purity 99.8%, t_R = 14.7 min. HPLC (method 2): purity 96.0%, t_R = 11.9 min.

5.1.2.19. Methyl (4a*RS*,8*SR*,8a*RS*)-1-[2-(3,4-dichlorophenyl) acetyl]-8-(pyrrolidin-1-yl)-perhydroquinoxaline-4-carboxylate (15). Under N_2, methyl chloroformate (28.9 mg, 0.31 mmol) was added to a solution of **14b** (100.9 mg, 0.25 mmol) in CH_2Cl_2 (13 mL) and the mixture was stirred at rt for 2 h. After evaporation of the solvent in vacuo, the residue was purified by fc (2 cm, 16 cm, CH_2Cl_2/MeOH/NH_3 = 9.5:0.5:0.05, 5 mL, R_f = 0.76). Pale yellow resin, yield 67 mg (59%). $C_{22}H_{29}Cl_2N_3O_3$ (454.4). R_f = 0.17 (CH_3OH); 0.76 (CH_2Cl_2/MeOH/NH_3 9:1:0.1). ^{1}H NMR (CD_2Cl_2, −40 °C): δ (ppm) = 0.74–0.85 (m, 1H, 5-H_a), 1.10–1.40 (m, 4H, 5-H_e, 6-H_a, 7-H_a, 7-H_e), 1.55–1.62 (m, 1H, 6-H_e), 1.63–1.75 (m, 4H, N(CH_2CH_2)$_2$), 1.82–1.92 (m, 2H, 2-H, 3-H), 2.00–2.07 (m, 1H, 8a-H), 2.56–2.71 (m, 4H, N(CH_2CH_2)$_2$), 3.00–3.07 (m, 1H, 2-H), 3.44–3.54 (m, 3H, $COOCH_3$), 3.55–3.61 (m, 2H, Ph-CH_2-C=O), 3.76–3.82 (m, 1H, 8-H), 3.89–3.97 (m, 1H, 4a-H), 4.00–4.12 (m, 1H, 3-H), 6.98–7.03 (m, 1H, Ph-6-H), 7.28–7.32 (m, 1H, Ph-2-H), 7.33–7.38 (m, 1H, Ph-5-H). IR: $\tilde{\nu}$ (cm^{-1}) = 1700 (s, ν (N—C=O—O)), 1646 (s, ν (N—C=O)), 873 (w, out-of-plane (Ar-H)). MS (ESI): m/z (%) = 454 (MH^+, 2·^{35}Cl, 100), 456 (MH^+, ^{35}Cl/^{37}Cl, 66), 458 (MH^+, 2·^{37}Cl, 10). HPLC (method 1): purity 98.4%, t_R = 18.6 min. HPLC (method 2): purity 97.3%, t_R = 15.4 min.

5.2. Receptor binding studies

5.2.1. Materials and general procedures

The guinea pig brains, rat liver and rat brains for the σ_1,σ_2, and κ-opioid receptor binding assays were commercially available (Harlan-Winkelmann, Borchen, Germany). Homogenizer: Elvehjem Potter (B. Braun Biotech International, Melsungen, Germany) and Soniprep 150, MSE, London, UK). Centrifuges: Cooling centrifuge model Rotina 35R (Hettich, Tuttlingen, Germany) and High-speed cooling centrifuge model Sorvall RC-5C plus (Thermo Fisher Scientific, Langenselbold, Germany). Multiplates: standard 96-well multiplates (Diagonal, Muenster, Germany). Shaker: self-made device with adjustable temperature and tumbling speed (scientific workshop of the institute). Vortexer: Vortex Genie 2 (Thermo Fisher Scientific, Langenselbold, Germany). Harvester: MicroBeta FilterMate-96 Harvester. Filter: Printed Filtermat Typ A and B. Scintillator: Meltilex (Typ A or B) solid state scintillator. Scintillation analyzer: MicroBeta Trilux (all Perkin Elmer LAS, Rodgau-Jügesheim, Germany). Chemicals and reagents were purchased from different commercial sources and of analytical grade.

The test compound solutions were prepared by dissolving approximately 10 μmol (usually 2–4 mg) of test compound in DMSO so that a 10 mM stock solution was obtained. To obtain the required test solutions for the assay, the DMSO stock solution was diluted with the respective assay buffer. The filtermats were presoaked in 0.5% aqueous polyethylenimine solution for 2 h at room temperature before use. All binding experiments were carried out in duplicates in 96-well multiplates. The concentrations given are the final concentrations in the assay. Generally, the assays were performed by addition of 50 μL of the respective assay buffer, 50 μL test compound solution in various concentrations (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol/L), 50 μL of corresponding radioligand solution and 50 μL of the respective receptor preparation into each well of the multiplate (total volume 200 μL). The receptor preparation was always added last. During the incubation, the multiplates were shaken at a speed of 500–600 rpm at the specified temperature. Unless otherwise noted, the assays were terminated after 120 min by rapid filtration using the harvester. During the filtration each well was washed five times with 300 μL of water. Subsequently, the filtermats were dried at 95 °C. The solid scintillator was melted on the dried filtermats at a temperature of 95 °C for 5 min. After solidifying of the scintillator at room temperature, the trapped radioactivity in the filtermats was measured with the scintillation analyzer. Each position on the filtermat corresponding to one well of the multiplate was measured for 5 min with the [^{3}H]-counting protocol. The overall counting efficiency was 20%. The IC_{50}-values were calculated with the program GraphPad Prism® 3.0 (GraphPad Software, San Diego, CA, USA) by non-linear regression analysis. Subsequently, the IC_{50} values were transformed into K_i-values using the equation of Cheng and Prusoff.[24] The K_i-values are given as mean value ± SEM from three independent experiments.

5.2.2. Protein determination

The protein concentration was determined by the method of Bradford,[25] modified by Stoscheck.[26] The Bradford solution was prepared by dissolving 5 mg of Coomassie Brilliant Blue G 250 in 2.5 mL of EtOH (95%, v/v). 10 mL deionized H_2O and 5 mL phosphoric acid (85%, m/v) were added to this solution, the mixture was stirred and filled to a total volume of 50.0 mL with deionized water. The calibration was carried out using bovine serum albumin as a standard in 9 concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 and 4.0 mg/mL). In a 96-well standard multiplate, 10 μL of the calibration solution or 10 μL of the membrane receptor preparation

were mixed with 190 μL of the Bradford solution, respectively. After 5 min, the UV absorption of the protein-dye complex at λ = 595 nm was measured with a platereader (Tecan Genios, Tecan, Crailsheim, Germany).

5.2.3. Preparation of membrane homogenates from guinea pig brain

5 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 and frozen (−80 °C) in 1.5 mL portions containing about 1.5 mg protein/mL.

5.2.4. κ Receptor affinity using guinea pig brain (modified according to Refs. 17,18)

The assay was performed with the radioligand [^{3}H]-U-69,593 (55 Ci/mmol, Amersham, Little Chalfont, UK). The thawed guinea pig brain membrane preparation (about 100 μg of the protein) was incubated with various concentrations of test compounds, 1 nM [^{3}H]-U-69,593, and TRIS-$MgCl_2$-Puffer (50 mM, 8 mM $MgCl_2$, pH 7.4) at 37 °C. The non-specific binding was determined with 10 μM unlabeled U-69,593. The K_d-value of U-69,593 is 0.69 nM.

5.2.5. κ, μ and δ Receptor affinity using cell lines expressing human receptors

The affinity towards κ, μ and δ receptors was determined as described in Ref. 18.

5.2.6. σ_1 and σ_2 receptor affinity

The affinity towards σ_1 and σ_2 receptors was determined as described in Refs. 19,20.

5.2.7. Affinity towards the phencyclidine binding site of the NMDA receptor

The affinity towards the phencyclidine binding site of the NMDA was determined as described in Refs. 21,22.

5.3. [^{35}S]GTPγS binding assay

The [^{35}S]-guanosine-5′-3-*O*-(thio)triphosphate (GTPγS) assay was carried out as an homogeneous scintillation proximity assay using 1.5 mg of WGA-coated SPA-beads (Amersham, Cardiff, UK) in microtiter luminescence plates (Costar, Cambridge MA, USA). In order to test the agonist activity of test compounds on human recombinant κ-opioid receptors cell membranes from HEK-293-cells expressing human κ-opioid receptors (PerkinElmer Life Sciences), 10 μg membrane proteins per assay, were incubated with 0.4 nmol/L [^{35}S]GTPγS and series concentrations of agonists in buffer containing 20 mmol/L HEPES pH 7.4, 100 mmol/L NaCl, 10 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, and 10 μmol/L GDP for 45 min at rt. The microtiter plates were thereafter centrifuged for 10 min at 2,100 rpm in a GS6 microtiter plate centrifuge (Beckman Coulter, Krefeld, Germany) to sediment the SPA beads. The bound radioactivity was determined after a delay of 15 min by means of a 1450 Microbeta Trilux (Wallac, Freiburg, Germany). The enhancement of [^{35}S]GTPγS binding above the basal activity was used to determine the potency (EC_{50}) and the relative efficacy (in % of maximal efficacy) of test compounds versus the reference compound U-69,593, which was set as 100%. EC_{50} values were calculated by means of nonlinear regression analysis with the software 'GraphPad Prism 4 for Windows' (Version 4.03, GrapPad Software, San Diego, USA).

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References and notes

1. Vanderah, T. W. *Clin. J. Pain* **2010**, *26*, S10.
2. Scopes, D. I. C. *Drugs Future* **1993**, *18*, 933.
3. Holzgrabe, U.; Nachtsheim, C.; Siener, T.; Drohsin, S.; Brandt, W. *Pharmazie* **1997**, *52*, 4.
4. Riviére, P. J.-M. *Br. J. Pharmcol.* **2004**, *141*, 1331.
5. Vanderath, T. W. *Clin. J. Pain* **2010**, *26*, S10.
6. Togashi, Y.; Umeuchi, H.; Okano, K.; Ando, N.; Yoshizawa, Y.; Honda, T.; Kawamura, K.; Endoh, T.; Utsumi, J.; Kamei, J.; Tanaka, T.; Nagase, H. *Eur. J. Pharmacol.* **2002**, *435*, 259.
7. Horwell, D. C. *Drugs Future* **1988**, *13*, 1061.
8. Roth, B. L.; Baner, K.; Westkaemper, R.; Siebert, D.; Rice, K. C.; Steinberg, S.; Ernsberger, P.; Rothman, R. B. *PNAS* **2002**, *99*, 11934.
9. Siebert, D. *J. Ethnopharmacol.* **1994**, *43*, 53.
10. de Costa, B. R.; Bowen, W. D.; Hellewell, S. B.; George, C.; Rothman, R. B.; Reid, A. A.; Walker, J. M.; Jacobson, A. E.; Rice, K. C. *J. Med. Chem.* **1989**, *32*, 1996.
11. Soukara, S.; Maier, C. A.; Predoiu, U.; Ehret, A.; Jackisch, R.; Wünsch, B. *J. Med. Chem.* **2001**, *44*, 2814.
12. Lichtenthaler, F. W.; Nakagawa, T.; El-Scherbiney, A. *Angew. Chem.* **1967**, *79*, 530.
13. Lichtenthaler, F. W.; Nakagawa, T.; El-Scherbiney, A. *Chem. Ber.* **1968**, *101*, 1837.
14. Eberle, M.; Missbach, M.; Seebach, D. *Org. Synth. Coll.* **2003**, *8*, 332.
15. Ram, S.; Spicer, L. D. *Tetrahedron Lett.* **1987**, *28*, 515.
16. Enders, D.; Schaumann, E. Science of Synthesis In *Reduction of Carbonic and Carboxylic Acid Derivatives*; Wünsch, B., Geiger, C., Eds., 2009; 40a, pp 23–64.
17. Kracht, D.; Rack, E.; Schepmann, D.; Fröhlich, R.; Wünsch, B. *Org. Biomol. Chem.* **2010**, *8*, 212.
18. Geiger, C.; Zelenka, C.; Lehmkuhl, K.; Schepmann, D.; Englberger, W.; Wünsch, B. *J. Med. Chem.* **2010**, *53*, 4212.
19. Maier, C. A.; Wünsch, B. *J. Med. Chem.* **2002**, *45*, 4923.
20. Meyer, C.; Neue, B.; Schepmann, D.; Yanagisawa, S.; Yamaguchi, J.; Würthwein, E.-U.; Itami, K.; Wünsch, B. *Bioorg. Med. Chem.* **2013**, *21*, 1844.
21. Köhler, J.; Bergander, K.; Fabian, J.; Schepmann, D.; Wünsch, B. *J. Med. Chem.* **2012**, *55*, 8953.
22. Banerjee, A.; Schepmann, D.; Köhler, J.; Würthwein, E.-U.; Wünsch, B. *Bioorg. Med. Chem.* **2010**, *18*, 7855.
23. Strange, P. G. *Br. J. Pharmacol.* **2010**, *16*, 1238.
24. Cheng, Y.; Prusoff, H. W. *Biochem. Pharmacol.* **1973**, *22*, 3099.
25. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
26. Stoscheck, C. *Methods Enzymol.* **1990**, *182*, 50.